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6th European Zebrafish
Genetics and Development Meeting
Rome 15th - 19th July 2009

Program & Abstracts

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6th European Zebrafish

Genetics and Development Meeting

Rome 15th - 19th July 2009

Program & Abstracts

Contents

POSTER

Under the auspices of
The Ministry of Foreign Affairs

Organizing committee

Marina Mione
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Temasek Life Sciences Laboratory, Singapore

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Registration

REGISTRATION FEES (VAT 20% INCLUDED)

	Before 30/03/2009	After 30/03/2009
Students /post docs	€ 420,00	€ 550,00
University Faculty	€ 490,00	€ 630,00
Industry	€ 600,00	€ 750,00

The registration fee includes:
- Attendance to all scientific sessions
- Entrance to the exhibition area
- Program and abstracts book
- Badge and congress Welcome kit
- Welcome reception on Wednesday 15th, 2009
- Coffee breaks and Lunches that will be held at the Congress Venue.

REGISTRATION

Participants who have not registered will be able to do so at the Secretariat and Reception Desk at the Congress Venue.

PAYMENT CONDITIONS

Payments accepted “on site” are cash and credit card.

Meeting Information

Congress Venue
Palazzo dei Congressi
Piazzale John Kennedy, 1 - 00144 Roma - Italy

Organizing Secretariat
Adria Congrex and Meetitaly Group
Via Sassonia, 30 - 47900 Rimini (RN) - Italy - Tel. +39.0541.305863 - Fax +39.0541.305842
e-mail: secretariat@zebrafish2009.org - www.adriacongrex.it

The Organizing Secretariat will be available at the Congress Venue during the meeting within the following times:

Opening hours:
- Wednesday July 15th, 2009 13.00 - 20.00
- Thursday July 16th, 2009 9.00 - 18.00
- Friday July 17th, 2009 9.00 - 19.30
- Saturday July 18th, 2009 9.00 - 18.00
- Sunday July 19th, 2009 9.00 - 13.00

Exhibition Area
Exhibition area is placed in the Salone della Cultura and activities will follow the scientific sessions' timetable.

Badges
All participants will receive a personal badge upon registration. You are kindly requested to wear your name badge when attending any scientific session or social gathering. Only participants who are wearing their name badge will be admitted to the meeting rooms. You should also wear your badge in the exhibition area.

Certificate of Attendance
Certificate of Attendance will be requested at the Reception Desk and sent by mail after the Meeting.

Internet Point
An Internet Point is available in the Funi Room.

Cloakcroom and Luggage
The cloackroom is located in the Lounge.

Official Language
The official language of the Meeting is English.

Insurance
Responsibility for personal accidents, loss or damages to private properties of participants and exhibitors can not be accepted. Participants and exhibitors are advised to make their own arrangements if they consider it necessary.

Coffee breaks and Lunches
Coffee breaks and lunches (included in the registration fee) will be served in the Exhibition Area in the Salone della Cultura.

Parking at the Congress Venue
Public Parking is available in the area.

Changes in Program
For any scientific and/or technical reason, the Organizing Committee and the Organizing Secretariat reserve the right to make changes in the Meeting program.

Electricity supply
Electricity supplied is 220V - 50Hz.

SOCIAL PROGRAM

WELCOME RECEPTION
Date: Wednesday 15th July 2009
Time: 18.00
Venue: Palazzo dei Congressi - Exhibition area
Price: free for all registered delegates.

SOCIAL DINNER
Date: 18th July 2009
Time: 20.00
Venue: Centrale Ristotheatre

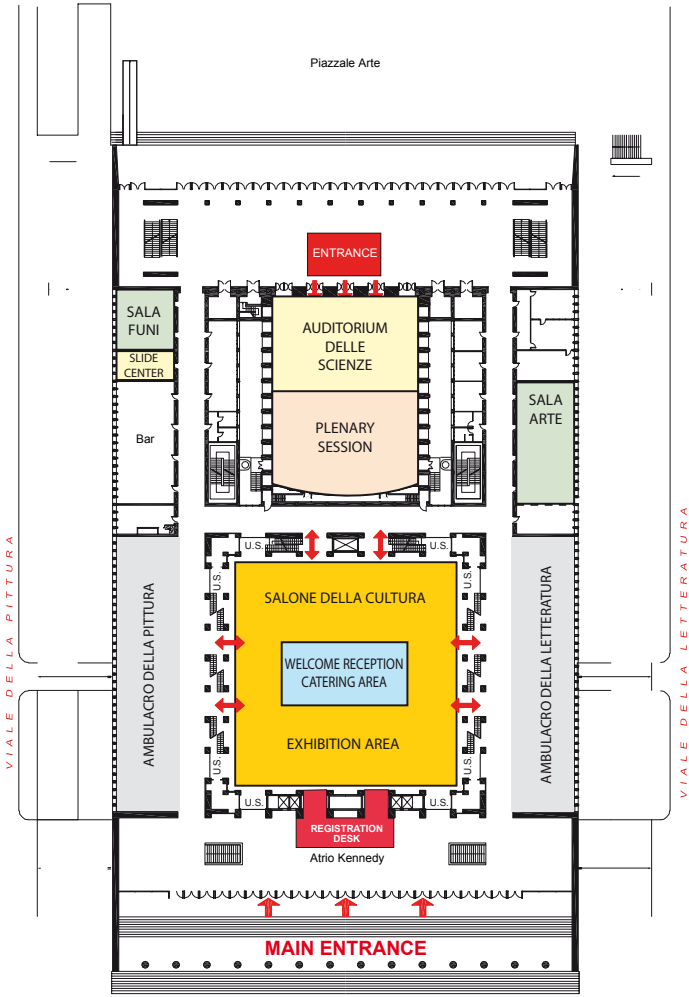
Rates:

Student/Post Docs	€ 60,00 Vat included
University Faculty and Industry	€ 70,00 Vat included

Rates include: Buffet, drinks and Live Music.

It will be possible to register for the Social Dinner also during the Meeting at the Secretary Desk.
Payments accepted are cash and credit card.

Congress Map



SALA FUNI:	SANGER CENTER WORKSHOP - INTERNET POINT
AUDITORIUM DELLE SCIENZE:	TALK SESSIONS AND KEYNOTE LECTURES
SALA ARTE:	ZIRC, ZHA and UCL HUSBANDRY WORKSHOP
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VIEW POINT

ZIRC
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ZFIN
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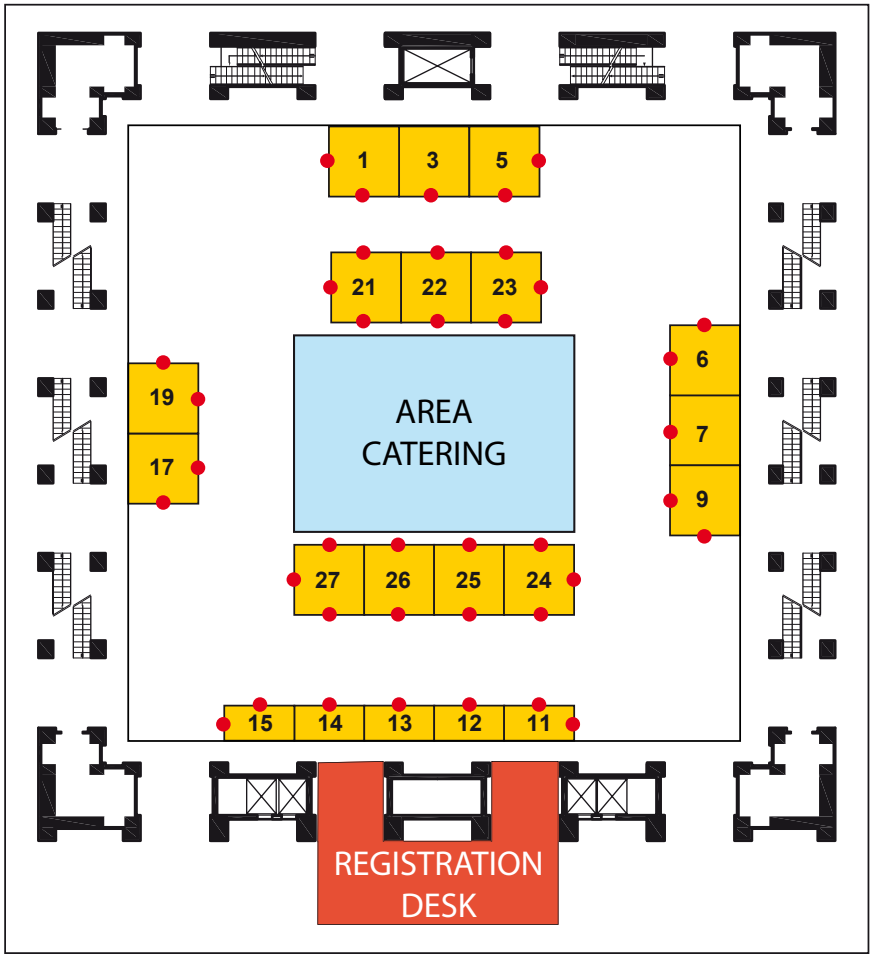
Exhibition Area



● OPENED EDGE

Company	Booth	Company	Booth
Aquaneering	N.17	NIBB - National Institute for Basic Biology	N. 27
Aquarien-Bau Schwarz	N. 6 – 7	Noldus Information Technology	N. 11
Aquatic Habitats	N. 1	Sanger Institute	N. 26
Eppendorf AG	N. 14	Tecniplast	N. 21 – 22 – 23
Gene Tools	N. 3	Union Biometrica	N. 12
Intavis Bioanalytical Instruments AG	N. 15	ViewPoint	N. 13
Leica Microsystems	N. 5	ZFIN Zebrafish Information Network	N. 24
Mole Genetics	N. 9	ZIRC Zebrafish International Resource Center	N. 25
Müller & Pfleger GmbH & Co.KG	N. 19		

Information for Authors

Poster Sessions

Poster will be on display in the Poster Area from July 15th to July 19th, 2009. Poster board dimensions are 70 cm. wide x 100 cm high. The poster can be mounted from July 15th at 13.00 in the corresponding numbered panel. It has to be removed by Sunday, July 19th, 2009 before 11.00. If posters are not removed, they will be removed and destroyed by the Organizing Secretariat.

Assistance and material for mounting posters will be available from set-up time, at the Secretariat Desk. Poster authors are requested to be present at their poster during the poster session as indicated in the program (July 17th: odd numbers, July 18th: even numbers).

Oral presentations

Oral presentations are 10 minutes long plus 5 minutes discussion. All conference rooms for oral presentations have laptop computers and videoprojectors.

All presentations should be prepared as PowerPoint files (or equivalent) in a 1024 x 768 pixels resolutionWhen movies are used during the presentation they should be embedded. If movies do not work they will be converted to a format that PowerPoint accepts through the EO video program. A slide center will be available during the conference. Please deliver your presentator on a pen drive or cd – ROM at the Slide Center at least 2 hours before your session starts. If you are using your laptop, please check that everything works at the Slide Center at least 2 hours before your session starts.

Acknowledgements

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Program at a Glance

WEDNESDAY, JULY 15th, 2009				
	AUDITORIUM DELLE SCIENZE	SALA FUNI	SALA ARTE	AMBULACRO DELLA PITTURA E AMBULACRO DELLA LETTERATURA
09:15 - 10:45		SANGER INSTITUTE WORKSHOP		POSTERS DISPLAYED
10:45 - 11:00	COFFEE BREAK AT SALA FUNI			
11:00 - 12:45		SANGER INSTITUTE WORKSHOP		
12:45 - 13:30	LUNCH BREAK			
13:30 - 16:30		SANGER INSTITUTE WORKSHOP		
14:00 - 16:00			ZIRC, ZHA, AND UCL HUSBANDRY WORKSHOP	
17:00 - 18:00	**KEYNOTE LECTURE I** TGFb signaling in development and cancer **Stefano Piccolo**			
18:00 - 20:00	WELCOME RECEPTION AT SALONE DELLA CULTURA			

THURSDAY JULY 16th, 2009		
	AUDITORIUM DELLE SCIENZE	AMBULACRO DELLA PITTURA E AMBULACRO DELLA LETTERATURA
09:00 - 10:30	**SESSION I** - Early development, gastrulation and segmentation	POSTERS DISPLAYED
10:30 - 11:00	COFFEE BREAK AT SALONE DELLA CULTURA	
11:00 - 12:30	**SESSION II** - Signaling	
12:30 - 14:00	LUNCH AT SALONE DELLA CULTURA	
14:30 - 16:00	**SESSION III** - Gene regulation	
16:00 - 16:30	COFFEE BREAK AT SALONE DELLA CULTURA	
16:30 - 18:00	**SESSION IV** - Organogenesis	

Program at a Glance

FRIDAY JULY 17th, 2009		
	AUDITORIUM DELLE SCIENZE	AMBULACRO DELLA PITTURA E AMBULACRO DELLA LETTERATURA
09:00 - 10:30	**SESSION V** - Emerging technologies	**POSTER DISPLAYED**
10:30 - 11:00	COFFEE BREAK AT SALONE DELLA CULTURA	
11:00 - 12:30	**SESSION VI** - Genomic workshop	
12:30 - 14:00	LUNCH AT SALONE DELLA CULTURA	
14:30 - 16:00		**POSTERS PRESENTATION** ODD NUMBERS
16:00 - 17:30	**SESSION VII** - Cancer	**POSTER DISPLAYED**
17:30 - 18:00	COFFEE BREAK AT SALONE DELLA CULTURA	
18:00 - 19:30	**KEYNOTE LECTURE II** Enhancing mammalian regeneration - **Nadia Rosenthal**	

SATURDAY JULY 18th, 2009		
	AUDITORIUM DELLE SCIENZE	AMBULACRO DELLA PITTURA E AMBULACRO DELLA LETTERATURA
09:00 - 10:30	**SESSION VIII** - Disease models	**POSTER DISPLAYED**
10:30 - 11:00	COFFEE BREAK AT SALONE DELLA CULTURA	
11:00 - 12:30	**SESSION IX** - Cardiovascular system	
12:30 - 14:00	LUNCH AT SALONE DELLA CULTURA	
14:30 - 16:00		**POSTERS PRESENTATION** EVEN NUMBERS
16:00 - 17:30	**SESSION X** - Hematopoiesis amd immune system	**POSTER DISPLAYED**
17:30 - 18:00	**COMMUNITY MEETING - Marina Mione**	
20:00 - 02.00	**SOCIAL DINNER**	

SUNDAY JULY 19th, 2009		
	AUDITORIUM DELLE SCIENZE	AMBULACRO DELLA PITTURA E AMBULACRO DELLA LETTERATURA
09:30 - 11:00	**SESSION XI** - Neurobiology I: patterning and behaviour	**POSTER DISPLAYED**
11:00 - 11:30	COFFEE BREAK AT SALONE DELLA CULTURA	
11:30 - 13:00	**SESSION XII** - Neurobiology II: sensory organs and regeneration	
13:00	CONCLUDING REMARKS AND END OF THE MEETING	

Scientific Program

Wednesday July 15th, 2009

SALA FUNI

09:15 - 16:30 **Sanger Institute Workshop**
Working with Zebrafish Genome Resources
Only for pre-registered participants

09:15 - 09:30 Introduction

09:30 - 10:45 Databases and de novo analysis of sequence

10:45 - 11:00 Coffee Break

11:00 - 12:45 Genome Browsing

12:45 - 13:30 Lunch Break

13:30 - 14:30 Exploring protein function and disease

14:30 - 15:45 Comparative Sequence Analysis

15:45 - 16:30 Working with ZFIN

REGISTRATION DESK – ATRIO KENNEDY

13:00 - 17:00 **Participants' Registration**

Scientific Program

Wednesday July 15th, 2009

SALA ARTE AQUANEERING

14:00 - 16:00 **ZIRC, ZHA, and UCL Husbandry Workshop**
Raising Fish, Nursery Operations, and Animal Use regulations in the US and EU

Chairs: Carole Wilson, London, UK
Zoltan M. Varga, Eugene, USA

73 Fish Breeding in Milan
Federica Pezzimenti, Milan, Italy

74 Zebrafish Larval Rearing at UCL
Jenna Lea Hakkesteeg, London, UK

75 The ZIRC Nursery and Grow-Out Section
Carrie Barton, Eugene, USA

Discussion

EU Legislation for Fish Husbandry and Shipping
Carole Wilson
UCL Fish Facility, London, UK

US Legislation for Fish Husbandry and Shipping
Zoltan M. Varga, Eugene, USA

Scientific Program

Wednesday July 15th, 2009

AUDITORIUM DELLE SCIENZE

17:00 - 18:00 **Keynote lecture I**
Chair: Francesco Argenton, Padua, Italy

TGFb signaling in development and cancer
Stefano Piccolo, Padua, Italy

SALONE DELLA CULTURA - EXHIBITION AREA

18:00 - 20:00 **Welcome Reception**

Scientific Program

Thursday July 16th, 2009

AUDITORIUM DELLE SCIENZE

09:00 - 10:30 **Session I**
Early Development, gastrulation, and segmentation
Session Chairs: Jacek Topczewski, Chicago, USA
Masazumi Tada, London, UK

1 A VEGETAL NETWORK OF TRANSCRIPTION FACTORS REGULATES DORSAL-VENTRAL PATTERNING AND GERM LAYER SPECIFICATION IN ZEBRAFISH
B. Feldman, Bethesda, USA

2 GLOBAL CYTOSKELETAL DYNAMICS DURING TISSUE MOVEMENT IN THE ZEBRAFISH EMBRYO
M. Köppen, Oeiras, Portugal

3 FILOPODIA CHARACTERIZATION IN THE ZEBRAFISH EMBRYOS
L. Caneparo, Pasadena, USA

4 NOVEL FACTORS REGULATING EARLY DORSOVENTRAL PATTERNING IN ZEBRAFISH EMBRYOS
Y-Y. Chen, Tübingen, Germany

5 TIME-RESOLVED ANALYSIS OF Pou5f1/Oct4 DOWNSTREAM GENE REGULATORY NETWORKS IN EARLY ZEBRAFISH EMBRYO
D. Onichtchouk, Freiburg, Germany

6 AN ASYMMETRIC HER GENE REGULATORY NETWORK IN THE SEGMENTATION CLOCK
C. Schroeter, Dresden, Germany

SALONE DELLA CULTURA - EXHIBITION AREA

10:30 - 11:00 Coffee break

Scientific Program

Thursday July 16th, 2009

AUDITORIUM DELLE SCIENZE

11:00 - 12:30 **Session II**
Signaling
Session Chairs: Roland Dosch, Génève, Switzerland
Dirk Meyer, Innsbruck, Austria

7 BUCKY BALL ORGANIZES GERM PLASM ASSEMBLY
F. Bontems, Génève, Switzerland

8 CONTROL OF CELL SHAPE AND CELL MIGRATION BY THE RNA BINDING PROTEIN DEAD END
T.U. Banish, Münster, Germany

9 IDENTIFICATION OF ZEBRAFISH squint RNA BINDING FACTOR 1, Srbf1, WHICH BINDS TO THE DORSAL LOCALIZATION ELEMENT IN squint mRNA
K. Pooja, Singapore

10 CALCIUM SIGNALING IN ZEBRAFISH LEFT/RIGHT PATTERNING
D. Gunther, Utah, USA

11 INTERPRETATION OF FGF8 MORPHOGEN GRADIENT IS REGULATED BY RECEPTOR UBIQUITINATION
M. Nowak, Dresden, Germany

12 ZEBRAFISH OVO GENES REGULATE RAB-MEDIATED INTRACELLULAR TRAFFICKING TO LOCALIZE N-CADHERIN IN MIGRATING NEURAL CREST CELLS
S. Piloto, Irvine, USA

SALONE DELLA CULTURA - EXHIBITION AREA

12:30 - 14:00 Lunch

Scientific Program

Thursday July 16th, 2009

AUDITORIUM DELLE SCIENZE

14:30 - 16:00 **Session III**
Gene regulation
Session Chairs: Gilbert Weidinger, Dresden, Germany
Andrew Oates, Dresden, Germany

13 THE MOLECULAR MECHANISM UNDERLYING LIGHT-REGULATED PERIOD2 EXPRESSION
G. Vatine, Tel Aviv, Israel

14 CONDITIONAL GENE INACTIVATION REVEALS A CRUCIAL ROLE FOR zgc:112094 IN ZEBRAFISH DEVELOPMENT
B. Zhang, Beijing, P. R. CHINA

15 TRANSCRIPTIONAL CONTROL OF BONE FORMATION THROUGH THE REGULATORY GENE RUNX2
S. Fisher, Philadelphia, USA

16 A PHASE-ORDERED MICROARRAY SCREEN FOR CYCLIC GENES IN ZEBRAFISH REVEALS HER GENES AS THE CONSERVED CORE OF THE SOMITOGENESIS CLOCK
D. Roellig, Dresden, Germany

17 A ROLE FOR ASPP2A IN ENDODERM DEVELOPMENT IN ZEBRAFISH
R. Hoffmans, Boston, USA

18 CRUMBS COMPLEX COORDINATELY REGULATES NEUROGENESIS AND NEUROEPITHELIAL POLARITY THROUGH CANONICAL AND NON-CANONICAL NOTCH PATHWAY
S. Ohata, Kobe, Japan

SALONE DELLA CULTURA - EXHIBITION AREA

16:00 - 16:30 Coffee break

Scientific Program

Thursday July 16th, 2009

AUDITORIUM DELLE SCIENZE

16:30 - 18:00 **Session IV**
Organogenesis
Session Chairs: Elke Ober, London, UK
Tom Schilling, Irvine, USA

19 JAGGED-NOTCH SIGNALING PATTERNS THE FACIAL SKELETON
F. Stellabotte, Los Angeles, USA

20 POSITIVE AND NEGATIVE FEEDBACK FROM PROTEOGLYCANS DURING CHONDROCYTE DIFFERENTIATION
B.F. Eames, Eugene, USA

21 MUTATIONS IN COMPONENTS OF DIVERSE ESSENTIAL CELLULAR PATHWAYS DISRUPT ZEBRAFISH INTESTINAL DEVELOPMENT
J.K. Heath, Parkville, Australia

22 CDX GENES PROMOTE HEPATIC FATE AND REPRESS PANCREATIC FATE WITHIN BIPOTENTIAL HEPATOPANCREATIC PROGENITOR CELL POPULATION IN ZEBRAFISH
A. Faro, Utrecht, The Netherlands

23 ZEBRAFISH ANTERIOR CHAMBER DEVELOPMENT AND MAINTENANCE
M. Takamiya, Karlsruhe, Germany

24 RENAL STEM CELLS GIVE RISE TO NEW NEPHRONS DURING ZEBRAFISH KIDNEY REGENERATION
C.Q. Diep, Boston, USA

Scientific Program

Friday July 17th, 2009

AUDITORIUM DELLE SCIENZE

09:00 - 10:30 **Session V**
Emerging technologies
Session Chairs: Reinhard Köster, Neuherberg, Germany
Antonio Giraldez, New Haven, USA

25 IMPROVED GENETIC LINEAGE TRACING USING THE CONDITIONAL RED-TO-GREEN REPORTER Tg (hsp70: loxP-DsRed2-loxP-EGFP)
S. Hans, Dresden, Germany

26 THE ZEBRAFISH AS A MODEL SYSTEM FOR IN VIVO SINGLE-MOLECULE MICROSCOPY
J.M. Schaaf, Leiden, The Netherlands

27 TRANSPOSON-BASED INSERTIONAL MUTAGENESIS PLATFORM
D. Balciunas, Philadelphia, USA

28 OPTIMIZED GAL4 GENETICS FOR PERMANENT GENE EXPRESSION MAPPING IN ZEBRAFISH
R. Köster, Neuherberg, Germany

29 TRANSCRIPTIONAL TARGET DISCOVERY THROUGH CAGED MORPHOLINO TECHNOLOGIES
J.K. Chen, Stanford, USA

30 CHROMOSOME PAINTING OF ZEBRAFISH CHROMOSOMES FOR THE IDENTIFICATION OF INTER-CHROMOSOMAL REARRANGEMENTS AND MARKER CHROMOSOMES
K.H. Brown, Boston, USA

SALONE DELLA CULTURA - EXHIBITION AREA

10:30 - 11:00 Coffee break

Scientific Program

Friday July 17th, 2009

AUDITORIUM DELLE SCIENZE

11:00 - 12:30 **Session VI**
Genomic workshop
Session Chairs: Ferenc Mueller, Birmingham, UK
Shawn M. Burgess, Bethesda, USA

31 A MESODERMAL GENE REGULATORY NETWORK DIRECTED BY T-DOMAIN TRANSCRIPTION FACTORS
F.C. Wardle, Cambridge, UK

32 AUTOMATED HIGH THROUGHPUT MAPPING OF PROMOTER-ENHANCER INTERACTIONS IN ZEBRAFISH EMBRYOS
J. Gehrig, Birmingham, UK

33 IDENTIFYING THE GENE NETWORK INVOLVED IN INNER EAR HAIR CELL REGENERATION IN THE ADULT ZEBRAFISH
S.M. Burgess, Bethesda, USA

34 WHOLE-GENOME SEQUENCING OF A WILDTYPE STRAIN OF ZEBRAFISH FOR VARIANT DISCOVERY
S. Sivasubbu, Delhi, India

35 GENETIC DIVERSITY BETWEEN TÜBINGEN AND AB STRAINS, AND A NEW ULTRA-DENSE GENETIC MAP
M. Clark, Hinxton, UK

36 HIGH THROUGHPUT SCREENING OF INNATE IMMUNE RESPONSES IN ZEBRAFISH AND CARP EMBRYOS
H. Spaink, Leiden, The Netherlands

36 bis AMPLIFY THE IMPACT OF YOUR RESEARCH: ENSURE THAT YOUR DATA CAN BE INTEGRATED INTO THE ELECTRONIC DATA STREAM
M. Haendel, Eugene, USA

SALONE DELLA CULTURA - EXHIBITION AREA

12:30 - 14:00 Lunch

POSTER AREA
AMBULACRO DELLA PITTURA AND AMBULACRO DELLA LETTERATURA

14:30 - 16:00 Poster Presentation ODD NUMBERS

AUDITORIUM DELLE SCIENZE

16:00 - 17:30 **Session VII**
Cancer
Session Chairs: James Amatruda, Dallas, USA
Nikolaus Trede, Salt Lake City, USA

37 ZEBRAFISH AS AN EFFICIENT MODEL SYSTEM TO STUDY THE REGULATION OF MITOSIS AND CHROMOSOME INSTABILITY
H. Lee, Seoul, Korea

38 A MUTATION IN ALK6B CAUSES GERM CELL TUMORS IN ZEBRAFISH
J. Neumann, Dallas, USA

39 NEW ZEBRAFISH MODELS OF T CELL CANCER: IMPORTANT RESOURCES FOR GENE DISCOVERY
N. Meeker, Salt Lake City, USA

40 IDENTIFICATION OF THE SETDB1 HISTONE METHYLTRANSFERASE AS A NEW ONCOGENE IN MELANOMA
L.Z. Zon, Boston, USA

41 GENETIC BACKGROUND DEPENDENT PIGMENT CELL TUMOR FORMATION IN A TRANSGENIC MEDAKA MELANOMA MODEL
M. Schartl, Wuerzburg, Germany

42 RETINOIC ACID RECEPTOR (RAR) ANTAGONISTS INHIBIT MIR-10A EXPRESSION AND BLOCK METASTATIC BEHAVIOUR OF PANCREATIC CANCER
C.P. Bagowski, Leiden, The Netherlands

Scientific Program

Friday July 17th, 2009

SALONE DELLA CULTURA - EXHIBITION AREA

17:30 - 18:00 Coffee Break

AUDITORIUM DELLE SCIENZE

18:00 - 19:30 **Keynote lecture II**
Chair: Karuna Sampath, Singapore

Enhancing mammalian regeneration
Nadia Rosenthal, Rome, Italy

Scientific Program

Saturday July 18th, 2009

AUDITORIUM DELLE SCIENZE

09:00 - 10:30 **Session VIII**
Disease models
Session Chairs: Ela Knapik, Nashville, USA
Derek Stemple, Cambridge, UK

43 CHEMICAL COLITIS MODELS IN ZEBRAFISH LARVAE
S. Oehlers, Auckland, New Zealand

44 DISRUPTION OF THE TROPONIN COMPLEX LEADS TO LOSS OF SARCOMERIC INTEGRITY IN SKELETAL MUSCLE
M.I. Ferrante, Cambridge, UK

45 GENETIC ANALYSIS REVEALS A ZEBRAFISH MODEL FOR FRASER SYNDROME AND IDENTIFIES POTENTIAL NOVEL DISEASE GENES
T.J. Carney, Singapore

46 CEYLON: A ZEBRAFISH MUTANT WITH SHWACHMAN-DIAMOND SYNDROME-LIKE BONE MARROW FAILURE
N.S. Trede, Salt Lake City, USA

47 CELLULAR SENESCENCE AND DNA DAMAGE IN A ZEBRAFISH MODEL OF COSTELLO SYNDROME IS LINKED TO UBIQUITIN-MEDIATED PROTEOSOMAL DEGRADATION OF ONCOGENIC HRAS
M. Mione, Milan, Italy

48 A MODIFIED ACETYLCHOLINE RECEPTOR DELTA-SUBUNIT ENABLES A NULL MUTANT TO SURVIVE BEYOND SEXUAL MATURATION
J.M. Urban, Bethesda, USA

SALONE DELLA CULTURA - EXHIBITION AREA

10:30 - 11:00 Coffee break

Scientific Program

Saturday July 18th, 2009

AUDITORIUM DELLE SCIENZE

11:00 - 12:30 **Session IX**
Cardiovascular system
Session Chairs: Massimo M. Santoro, Turin, Italy
Stefan Schulte-Merker, Utrecht, The Netherlands

49 ROLE OF NOTCH SIGNALING IN EARLY ZEBRAFISH CARDIOGENESIS
K.A. Nembhard, Durham, USA

50 BUNGEE - A NOVEL REGULATOR OF CARDIAC VALVE FORMATION IN ZEBRAFISH
I.M. Berger, Heidelberg, Germany

51 CHEMOKINE SIGNALING CONTRIBUTES TO REGIONAL PATTERNING OF THE FIRST EMBRYONIC ARTERY
A.F. Siekmann, Muenster, Germany

52 MiR-1 REGULATES ANGIOGENESIS BY MODULATING VEGF SIGNALING
A.J. Giraldez, New Haven, USA

53 THE ZEBRAFISH FULL-OF-FLUID MUTANT IDENTIFIES A SECRETED PROTEIN ESSENTIAL FOR LYMPHANGIOGENESIS IN ZEBRAFISH AND HUMANS
B.M. Hogan, Utrecht, The Netherlands

54 CHARACTERIZATION OF VASCULAR MURAL CELLS DURING ZEBRAFISH DEVELOPMENT
M.M. Santoro, Turin, Italy

SALONE DELLA CULTURA - EXHIBITION AREA

12:30 -14:00 Lunch

Scientific Program

Saturday July 18th, 2009

POSTER AREA
AMBULACRO DELLA PITTURA AND AMBULACRO DELLA LETTERATURA

14:30 - 16:00 Poster Presentation EVEN NUMBERS

AUDITORIUM DELLE SCIENZE

16:00 - 17:30 **Session X**
Hematopoiesis and immune system
Session Chairs: Graham Lieschke, Parkville, Australia
Maria Vega Flores, Auckland, New Zealand

55 BOTH PRIMITIVE AND DEFINITIVE HEMATOPOIESIS ARISE FROM HEMOGENIC ENDOTHELIAL CELLS IN THE ZEBRAFISH EMBRYO
J.Y. Bertrand, La Jolla, USA

56 SDF-1 EXPRESSING CELLS ESTABLISH THE HEMATOPOIETIC STEM CELL (HSC) NICHE AND PLAY A ROLE IN HSC HOMING FOLLOWING ADOPTIVE CELL TRANSFER IN A ZEBRAFISH MODEL OF BONE MARROW TRANSPLANT
T.C. Lund, Minneapolis, USA

57 LIVE IMAGING REVEALS THAT DEFINITIVE HAEMATOPOIETIC STEM CELLS EMERGE DIRECTLY FROM HAEMOGENIC ENDOTHELIAL CELLS IN ZEBRAFISH EMBRYOS
M.V. Flores, Auckland, New Zealand

58 DEVELOPMENT OF AN IMMUNE-MATCHED TRANSPLANTATION MODEL TO DETECT HEMATOPOIETIC STEM CELL ACTIVITY IN ZEBRAFISH
J.L.O. de Jong, Boston, USA

59 THYMUS COLONIZATION IS UNDER THE CONTROL OF CHEMOKINES AND CHEMOKINE RECEPTORS
B. Bajoghli, Freiburg, Germany

60 A ZEBRAFISH TRANSGENIC MODEL TO STUDY ANTIGEN-PRESENTING CELLS: B LYMPHOCYTES, MACROPHAGES AND DENDRITIC CELLS
V. Wittamer, San Diego, USA

Scientific Program

Saturday July 18th, 2009

AUDITORIUM DELLE SCIENZE

17:30 - 18:00 **Community Meeting**
Chair: Marina Mione, Milan, Italy

CENTRALE RISTOTHEATRE

20:00 - 02:00 Social Dinner

Scientific Program

Sunday July 19th, 2009

AUDITORIUM DELLE SCIENZE

09:30 - 11:00 **Session XI**
Neurobiology I: patterning and behaviour
Session Chairs: Corinne Houart, London, UK
Marnie Halpern, Baltimore, USA

61 NEGATIVE REGULATION OF NEUROGENESIS BY AN FGF FROM NEURONS
R. Gonzalez-Quevedo, London, UK

62 SEGREGATED PROCESSING OF VISUAL INPUTIN IN THE OPTIC TECTUM BY INTERLAMINAR INHIBITION REVEALED BY CALCIUM IMAGING
F. Del Bene, San Francisco, USA

63 SARA1, a TGFbeta SIGNALING ADAPTOR, REGULATES PROLIFERATION OF THE NEURAL PRECURSORS
C. Campos, Génevè, Switzerland

64 FUNCTIONAL ANALYSIS OF THE HABENULA IN CONTROL OF FEAR
H. Okamoto, Saitama, Japan

65 BEHAVIOURAL CONSEQUENCES OF ZEBRAFISH EPITHALAMIC ASYMMETRY
M.E. Halpern, Baltimore, USA

66 ATLASTIN CONTROLS ZEBRAFISH SPINAL MOTOR AXON ARCHITECTURE VIA INHIBITION OF THE BMP PATHWAY
J. Hazan, Paris, France

SALONE DELLA CULTURA - EXHIBITION AREA

11:00 - 11:30 Coffee break

Scientific Program

Sunday July 19th, 2009

AUDITORIUM DELLE SCIENZE

11:30 - 13:00 **Session XII**
Neurobiology II: Sensory organs and regeneration
Session Chairs: Tanya Whitfield, Sheffield, UK
Catherina Becker, Edinburgh, UK

67 EARLY REQUIREMENTS FOR PREPLACODAL ECTODERM AND SENSORY ORGAN DEVELOPMENT
B.B. Riley, College Station, USA

68 REPRESSION OF HEDGEHOG SIGNALLING IS REQUIRED FOR THE ACQUISITION OF DORSOLATERAL CELL FATES IN THE ZEBRAFISH OTIC VESICLE
K.L. Hammond, Sheffield, UK

69 REGULATING ADULT NEURONAL STEM CELL PROLIFERATION DURING REGENERATION OF THE LIGHT-DAMAGED ZEBRAFISH RETINA
D.R. Hyde, Notre Dame, USA

70 AFFERENT INNERVATION DURING HAIR-CELL REGENERATION IN THE ZEBRAFISH
H. Lopez-Schier, Barcelona, Spain

71 MOLECULAR EVENTS DURING REGENERATION IN THE ZEBRAFISH LATERAL LINE AFTER COPPER TREATMENT
M. Behra, Bethesda, USA

72 DEVELOPMENT AND REGENERATION OF THE ZEBRAFISH MAXILLARY BARBEL (ZMB): A NOVEL STUDY SYSTEM FOR ADULT VERTEBRATE TISSUE GROWTH AND REPAIR
E.E. LeClair, Chicago, USA

AUDITORIUM DELLE SCIENZE

13:00 Concluding remarks and end of the Meeting

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Abstracts

TALKS

A vegetal network of transcription factors regulates dorsal-ventral patterning and germ layer specification in zebrafish

B. Feldman, S-K.Hong, C.Levin, and J.Brown
Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, USA

Two central features of early embryogenesis are the differentiation of germ layers and formation of the dorsal axis. Studies in *Xenopus laevis* indicate that maternal determinants in the yolky vegetal cells have a critical role for inducing mesoderm as well as the dorsal organizer. In zebrafish, factors in the yolk and yolk syncytial layer (YSL) are thought to play roles analogous to maternal determinants in *Xenopus*. We are exploring the functions and interactions of four transcription factors expressed in the YSL or vegetal-most blastomeres prior to germ layer differentiation or axis formation: Hnf4a, Mxtx2, Boz and Irx3a. We find that *hnf4a* mRNA overexpression reduces dorsal markers and recapitulates the *boz* loss-of-function phenotype. Consistent with this, Hnf4a depletion with morpholinos increases expression of *boz* and other dorsal markers. *Mxtx2* overexpression similarly causes dorsal marker reduction, but also strongly induces endoderm. Intriguingly, we also observe that *irx3a* is expressed maternally and that its overexpression quenches the expression of *mxtx2* and *hnf4a* alike. Thus, we are uncovering a vegetal transcriptional regulatory network involving *irx3a*, *hnf4a*, *mxtx2* and *boz* that modulates dorsal-ventral patterning and germ-layer induction.

Global cytoskeletal dynamics during tissue movement in the zebrafish embryo
M. Köppen, I. Cristo, P. Almada
Tissue Morphogenesis, Instituto Gulbenkian de Ciýncia, Oeiras, Portugal

During the first few hours of development, the zebrafish embryo undergoes a dramatic reorganization through a sequence of morphogenetic tissue movements. One such movement is the spreading of the outer epithelium (enveloping layer, EVL) around the spherical yolk cell during epiboly. Our previous findings indicate that EVL movement is driven by a contractile actin band within the yolk syncytial layer (YSL). Our current work suggests that this actin structure derives both from the YSL cortex and a pool of actin originating from the vegetal pole of the embryo. Using live confocal imaging, we found that YSL formation initiates during early cell division stages, as marginal blastomeres fuse with the yolk through disassembly of their partial cell boundaries. The newly formed, actin-rich syncytium then expands through an ordered sequence of nuclear divisions. Simultaneously, actin from the vegetal pole streams towards the YSL. Together, this generates an actin-rich mesh adjacent to the margin of the EVL. During subsequent epiboly, EVL cells progress towards the vegetal pole while the actin mesh markedly condenses. Local contraction of this mesh then progressively shortens the EVL margin, which ultimately seals the embryo at the vegetal pole. What mediates the transport of actin towards the YSL? A good candidate is the microtubule network spanning the vegetal half of the yolk. We are currently testing this hypothesis using imaging tools and functional analysis. Together, our work highlights the importance of global cytoskeletal rearrangements during morphogenetic movements of the embryo.

Filopodia characterization in the zebrafish embryos
L. Caneparo, P. Pantazis, S.E. Fraser
Biology Imaging Center, California Institute of Technology, Pasadena, USA

During vertebrate gastrulation, a series of well conserved signaling events and morphogenetic movements are required, to set up the correct formation of the body plan and to guarantee future cells diversity. In zebrafish, the optical clarity of the embryo, allows the *in vivo* visualization of the gastrula movement and the availability of multiplex fluorescent tags consent to highlight the gastrula cytoarchitecture, with single cell resolution. This approach allowed *in vivo* visualization of cell shape and morphology, often, indicators of changes in the cells function. Cellular protrusions, for example, are characteristic of exploratory behavior and used for cell-cell communication, these mechanisms are ubiquitous and share among animals. Well studied examples, of short range membrane cell protrusions are microvilli, lamellipodia and ruffles.
We describe for the first time the presence of filopodia reaching up to 250 μm in length in the developing zebrafish embryo during gastrulation. We characterized their growth and we topologically mapped the filopodia distribution in the embryos *in vivo*; we quantitatively measured length and persistence of these extensions and we analyzed the cell membrane dynamic along the projection during gastrulation *in vivo*.
Cell protrusion extending over distance more than 50μm such as filopodia, cytonemes and nanotunneling have been previously described respectively in sea urchin, during gastrulation, in *Drosophila*, in the imaginal disc and in different cells types in the vertebrate immune system. During vertebrate development only short filopodia have been previously reported in the mouse blastocystes, with range and extension only up to 20μm and not comparable to what we described in zebrafish or to what it has been reported in invertebrates.

Novel factors regulating early dorsoventral patterning in zebrafish embryos
Y-Y. Chen, M. Sonawane, M. Harris, and C. Nüsslein-Volhard
Max-Planck Institute for Developmental Biology, Department of Genetics, Tübingen, Germany

Virtually all animals have left-right, anterior-posterior, and dorsoventral polarity. The dorsoventral axis is established very early during vertebrate embryonic development by the antagonizing activities of Chordin and BMP signaling. The epidermis is formed from embryonic ectoderm under the influence of BMP signaling. In zebrafish, the embryonic and larval epidermis is bi-layered consisting of outer peridermal and inner basal epidermal cells. While the basal epidermis develops from embryonic ectoderm, the peridermal cells are derived from the enveloping layer (EVL), the outermost protective cell layer. The EVL differentiates at the 256-cell stage, much before the formation of the embryonic shield, which is the zebrafish equivalent of Spemann's organizer. It is not clear whether this cell layer exhibits dorsoventral patterning at all and whether the known dorsoventral patterning cues are involved in its patterning. To address the issue we used a minCrestin::GFP transgenic line which fortuitously labels dorsal EVL cells at the early sphere stage. Intriguingly, the domain of GFP expressing cells did not change when we perturbed Chordin-BMP signaling using morpholinos. This indicates that there is an early pathway that regulates the dorso-ventral patterning of EVL. To isolate these novel components, we performed a yeast-one-hybrid screen and identified members of the Sox family and Myc-associated factor-X (Max) as minCrestin promoter interacting proteins. We show that perturbations in *sox19b* and *max* expression levels, either by over-expression or morpholino knockdown, alter the dorso-ventral polarity of the EVL. Furthermore, over-expression of *sox 19b* and knockdown of *Max* dorsalise the embryos indicating that they have a function in embryonic patterning in addition to the patterning of the EVL. Our analyses have revealed the roles of maternally expressed Sox19b and Max in early embryonic patterning and suggest a novel mechanism whereby the dorsoventral axis of the EVL is specified independently of known dorsoventral signals in early development.

Time-resolved analysis of Pou5f1/Oct4 downstream gene regulatory networks in early zebrafish embryo

D. Onichtchouk[1], F. Geier[2], B. Polok[1], R. Moessner[1], D. Messerschmidt[3], V. Taylor[3], J. Timmer[2], W. Driever[1]

[1]Developmental biology, University of Freiburg, Freiburg, Germany; [2]Institute of Physics, University of Freiburg, Freiburg, Germany; [3]Molecular Embryology, MPI for Immunobiology, Freiburg, Germany

The Pou5f1/Oct4 transcription factor controls pluripotency in mammalian embryonic stem cells, however, evolutionary conservation of this role Pou5f1/Oct4 role in the development of lower vertebrates remains controversial. In zebrafish, a genetic model for Pou5f1 function has been established: MZspg mutants, which are devoid of both maternal and zygotic expression of Pou5f1. The MZspg mutant phenotype includes several independent defects described so far: absence of endoderm, epiboly defect and BMP pathway deficiency. To further understand the evolution and function of the transcriptional networks downstream of Pou5f1/Oct4 we have generated time series data of maternal and early zygotic gene expression profiles (from unfertilised egg to 75% epiboly) in zebrafish wild type and Oct4/Pou5f1 deficient embryos in 1 hr intervals using Agilent microarrays. A combined analysis of time series data identified groups of Oct4/Pou5f1 targets that were not expressed or showed either advanced, delayed or deregulated expression in Oct4/Pou5f1 mutants. Analysis of Gene Ontology function and developmental expression revealed strong enrichment for transcription factors and other genes involved in multicellular organism development. Direct Oct4/Pou5f1 targets were distinguished in additional experiments by the suppression of translation of primary target gene mRNAs, results validates by in-situ hybridisation or real time RT-PCR. We show, that in contrast to mammalian embryos, where Pou5f1 controls pluripotency in the homogenous stem cell population of the inner cell mass, in zebrafish Pou5f1 seems to operate upstream of several spatially separated subnetworks. Transcriptional targets of zebrafish Pou5f1 conserved across vertebrates include Klf4, Sox2 and FOXD3 , each of them expressed in unique spatial patterns at midgastrula stage (ectoderm, neuroectoderm, mesendoderm, respectively). We foccused our efforts in characterising the neuroectodermal part Pou5f1 – Sox2 (SoxB1) dependent transcriptional network and performed additional microarray experiments to distinguish direct Sox2 transcriptional targets. We combined time series, overexpression experiments and in-situ hybridisation data to build a predictive mathematical gene-regulatory network model, which operates during the segregation of totipotent blastomers to neural progenitor cells on the one side, and differentiating cells on the other. The model allows us to link the timing of differentiation in the neural tissue to the dynamic properties of certain network motifs. We suggest, that the elements of the evolutionary ancient Oct4/Pou5f1 embryonic pluripotency control subnetworks were reimplicated as modules of Oct4/Pou5f1 totipotency control in ES cells in mammals. Since the Pou5f1 downstream genes involved in our model are conserved across vertebrates, we envision that the knowledge gained from zebrafish development contributes to the effort of producing defined cell fates from pluripotent stem cells.

An Asymmetric *her* Gene Regulatory Network in the Segmentation Clock

C. Schroter[1], M. Gajewski[2], A. C. Oates[1]

[1]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany; [2]Institut fuer Genetik, Universitaet Koeln, Germany

The segmentation clock, a transcriptional oscillator operating in the unsegmented presomitic mesoderm (PSM), controls the sequential subdivision of the paraxial mesoderm into somites. Members of the *hairy and enhancer of split related (her)* family of transcriptional repressors display transcriptional oscillations in the PSM and are important components of the clock. Current models of the core oscillator mechanism propose a symmetric network of multiple interacting *her* genes that generate oscillations through a delayed negative autoregulatory feedback loop. These models predict that (1) somitogenesis period changes upon mutation of any gene in the network and imply that (2) mutation of different genes should lead to qualitatively similar segmentation phenotypes. However, previous morpholino-knockdown studies of different *her* genes are inconsistent with (2), prompting us to reinvestigate the interaction map of these oscillator components.

We use multiple time-lapse microscopy to measure somitogenesis period in three novel mutants for the *her1*, *her7* and *hes6* genes. Unexpectedly, only mutation of *hes6* leads to an increase in somitogenesis period, while the other two mutants segment at wild type pace. In the *her1* and *hes6* single mutants, oscillations persist throughout segmentation stages whereas they decay in embryos mutant for the *her7* gene. Combined loss of *her1* and *her7* or *her1* and *hes6* completely abrogates oscillations. Strikingly, crossing the *hes6* mutation into the *her7* mutant background rescues the decay of the oscillations seen upon mutation of the *her7* gene alone. Taken together, these findings indicate distinct functions of the *her* genes in the segmentation clock and point towards asymmetric interactions between them.

We develop a minimal mathematical model of the segmentation clock's genetic regulatory network that builds on asymmetric interactions to explain the different mutant segmentation phenotypes. Furthermore we can explain the oscillator period change in the *hes6* mutant, which defines a novel segmentation phenotype. Our model does not rely on her7 homodimers or her1/her7 heterodimers, but proposes that her1/hes6 and her7/hes6 heterodimers have important and opposing functions. This genetic network model makes testable predictions of biochemical interactions in the clockwork and should broaden our understanding of the generation of developmental rhythms.

Bucky ball organizes germ plasm assembly

F. Bontems[1], A. Stein[1], F. Marlow[2], J. Lyautey[1], T. Gupta[2], M. C. Mullins[2] , R. Dosch[1]
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Zebrafish specifies the formation of gametes during embryonic development through the localization of maternal determinants termed germ plasm. Germ plasm assembles during the first stage of oogenesis forming a distinct cytoplasmic structure, the Balbiani body, which has been found in oocytes of many animals including humans. Although germ plasm is critical for the fertility of the future embryo, the key regulators of Balbiani body assembly in vertebrates are unknown.

We show that in *bucky ball* (*buc*) mutant females the formation of the Balbiani body is disrupted demonstrating that Buc is essential for germ plasm assembly. To molecularly identify the *buc* gene, we positionally cloned the *buc* mutation. *Buc* encodes a novel protein and its mRNA is exclusively expressed in the ovary displaying a novel dynamic localization pattern initiating in the Balbiani body. To characterize the function of Buc, we developed an injection and culture assay in zebrafish stage I oocytes. Using this assay we show that Buc protein activity is necessary to aggregate germ plasm in the oocyte . Consistent with this result, we discovered that an injected Buc-eGFP fusion localizes to the Balbiani body and in the germ plasm of the early embryo after fertilization. Interestingly, overexpression of *buc* generates additional germ cells in the zebrafish embryo providing evidence that *buc* is sufficient to recruit germ plasm components. Since we discovered *buc* homologs in many genomes including mammals our results suggest that Buc represents the first regulator of germ plasm formation in vertebrates.

To better understand the role of Buc in Balbiani body localization, we established a transgenic Buc-eGFP line. This line rescues the mutant phenotype indicating that the transgene reflects most of the endogenous Buc activity. In addition the eGFP-fluorescence reports Buc protein localization *in vivo*, which we discover in the Balbiani body and later during oogenesis to the cortex of the oocyte. At the early embryonic stages Buc-eGFP is localized to the germ plasm at the first cleavage furrows and later in the four primordial germ cells until oblong stage. This line not only reports germ plasm localization in vivo, but also will be a good tool to study the biochemical function and cell biological role of Buc during Balbiani body formation.

Control of cell shape and cell migration by the RNA binding protein Dead end
T. U. Banisch, M. Goudarzi, E. Raz
Institute of Cell Biology, ZMBE, Münster, Germany

Specification of zebrafish primordial germ cells (PGCs) depends on maternally provided determinants. These determinants, collectively termed germ-plasm direct cells to enter the germ line and acquire proper cell behavior.
An important such maternally-provided germ-plasm component in zebrafish is encoded by the *dead end* (*dnd*) gene. The Dnd protein contains an RNA binding domain and is specifically expressed in PGCs, where it is localized to the perinuclear granules. Knockdown of *dead end* results in severe defects in cell behavior (e.g. the acquisition of motility), cell fate maintenance and survival.
We found that Dnd functions by protecting specific RNAs (e.g. *tdrd7* and *nanos1*) from micro RNA–mediated translational inhibition and RNA degradation in the germ cells, a process by which the same RNAs are inhibited in somatic cells.
To determine the molecular basis for Dnd function we have identified proteins that interact with Dnd as well as RNAs that are potentially regulated by the Dnd complex.
The identification and functional analysis of such RNAs that are involved in controlling cell shape and motility will be described.

Identification of Zebrafish squint RNA Binding Factor 1, Srbf1, which binds to the dorsal localization element in squint mRNA

K. Pooja[1], P. C. Gilligan[1], R. Philp[2], S. Winkler[3], K. Sampath[1]
[1]Temasek Life Sciences Laboratory, Singapore; [2]Bioprocessing Technology Institute, Biopolis, Singapore; [3]Max Planck Institute of Cell Biology and Genetics, Dresden, Germany

We are investigating specification of the embryonic axis in zebrafish embryos. We previously showed that maternal mRNA encoding the Nodal-related factor Squint (Sqt) is asymmetrically localized to 2 cells by the 4-cell stage, and predicts the embryonic dorsal. This suggests that asymmetric localization of sqt RNA, and possibly other factors, is an early step in dorsal specification (Gore *et al*., *Nature*, 2005). The sqt 3′ untranslated region (UTR) is necessary and sufficient for dorsal localization and the dorsal localization element lies within the first 50 nt of the sqt 3′ UTR. Phylogenetic footprinting of the *sqt* 3′UTR from ~30 cyprinid species revealed multiple conserved blocks, and a potential conserved secondary structure. RNA gel-shift and UV cross-linking assays show multiple RNA-binding factors which bind specifically to distinct regions within the sqt 3′UTR. One of these, sqt-RNA binding factor 1 (Srbf1), binds to the sqt dorsal localization element. We have purified Srbf1 from oocyte extracts by chromatography, and determined its peptide sequence. Srbf1 is a nucleic acid-binding protein. Analysis of deletions and point mutations in Srbf1 shows that the N-terminus of Srbf1 is necessary and sufficient for binding to sqt RNA. We have also identified key residues in Srbf1 that are essential for binding to sqt RNA. An ENU-induced mutation in the *srbf1* locus has been recently obtained by TILLING, and characterization is in progress. Interestingly, over-expression of mutant but not wild type Srbf1, in embryos leads to embryonic defects. Current efforts are aimed at purifying the other factors that bind to sqt RNA, and understanding the mechanisms by which Srbf1 binds and localizes sqt RNA to dorsal.

Calcium Signaling in Zebrafish Left/Right Patterning

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[1]Departments of Human Genetics and [2]Department of Neurobiology & Anatomy, University of Utah, USA

Directional fluid flow in Kupffer's Vesicle (KV) is required for the initiation of the left side-specific cascade of gene expression that leads to left-right asymmetries in zebrafish. We still have little understanding of how the information of fluid flow is transmitted from the KV to the lateral plate mesoderm, where left side-specific gene expression arises. Here we show that left-side-specific mobilization of intracellular calcium is dependent on normal fluid flow in KV and is required for asymmetric gene expression and subsequent left-right patterning.
Ryanodine Receptors (RyRs) are one class of intracellular calcium release channels that are best known for their role in excitation contraction coupling in vertebrate cardiac and skeletal muscle. However RyRs are widely expressed in many non-muscle cell types suggesting they are involved in other processes as well. Here we show that the entire cascade of left-right asymmetric gene expression and organ morphogenesis requires left-side-specific elevation of intracellular calcium dependent on RyR function. At the 5-8 somite stage of zebrafish embryos, free intracellular calcium levels are elevated specifically on the left side of Kupffer's Vesicle. The mobilization of calcium depends on RyR function: When RyR expression or function is inhibited widely, or specifically in the KV, left-side-specific mobilization of calcium is blocked, along with left-side-restricted patterns of gene expression and subsequent organ morphogenesis. Calcium release appears to be part of the signaling pathway between fluid flow and asymmetric gene expression: First, pharmacologic inhibitor studies indicate that RyR function in left-right patterning is required during mid-somitogenesis, at the time of fluid flow. Second, left-side-specific calcium mobilization depends on fluid flow in KV. Third, inhibition of RyR expression or function does not affect KV structure, ciliogenesis or fluid flow. Together these experiments indicate that intracellular calcium mobilization on the left side of the KV is a required step in normal left-right patterning. In additional experiments, we show that elevation of intracellular free calcium levels on the right side of KV is sufficient to drive left-side specific gene expression on the right side of the axis. These experiments place the requirement for calcium mobilization downstream of fluid flow in KV and upstream of the asymmetric gene expression cascade in the establishment of the left/right axis.

Interpretation of Fgf8 morphogen gradient is regulated by receptor ubiquitination
M. Nowak, R. S. Yu, A. Machate and M. Brand
Biotechnology Center and Center for Regenerative Therapies, CRTD; TU Dresden, Germany

During embryonic development and organogenesis cell fate decisions are largely determined by interpretation of morphogen gradients. Recently, much insight has been gained into how morphogen gradients are formed and maintained, however, which cellular mechanisms govern their interpretation within target tissues remains elusive. By interfering with the function of the ubiquitin ligase Cbl we show that reduced ubiquitination of the Fgf receptors leads to a broadening of Fgf target gene expression and a delay of Fgf8 endocytosis and lysosomal transport in zebrafish embryos. Using Fluorescence Correlation Spectroscopy we show that impaired Fgf receptor ubiquitination has no effect on the extracellular Fgf8 morphogen gradient indicating that the observed signaling changes are due to altered gradient interpretation. We propose that recruitment of Fgf8 to the cell surface and early endosomal compartments is sufficient for its clearance from the extracellular space and thus gradient formation. In contrast, regulation of Fgf endocytosis and sorting determines the residence time of receptor-ligand complexes in signaling competent compartments and thus is an important means to uncouple morphogen gradient formation and interpretation.

Zebrafish ovo genes regulate rab-mediated intracellular trafficking to localize n-cadherin in migrating neural crest cells

S. Piloto, T. Schilling
University of California-Irvine, USA

A fundamental issue in cell and developmental biology is how migratory behaviors of cells are controlled by dynamically regulated cell adhesion. Vertebrate neural crest (NC) cells initiate their highly migratory behaviors by regulating the expression of cadherins on their surfaces, downregulating both N- and E-cadherin and upregulating cadherin-6/7. Experimental evidence has suggested that secreted Wnts induce these changes in adhesion in NC, leading to migration, but also play a role in the specification of NC-derived pigment cells.
Here we show that a Wnt target gene Ovo and its close relative are required for migration of pigment progenitors, in part by regulating the secretion of N-cadherin. Ovo interacts genetically with Ncad and depletion causes a decrease in Ncad at the cell membranes. Ovo genes typically act as transcriptional repressors, and we found that some of the most highly upregulated genes in Ovo morphants were members of the Rab family involved in intracellular trafficking. Surprisingly, several of these Rab genes were expressed specifically in NC cells in the early embryo and chemical inhibitors of their function rescued development in Ovo morphants. Taken together these results suggest that Ovo proteins regulate Rab-mediated intracellular trafficking to localize Ncad in migrating NC cells.

The molecular mechanism underlying light-regulated period2 expression

G. Vatine[1], D. Vallone[2], N. S. Foulkes[2], Y. Gothilf[1]
[1]Department of Neurobiology, Faculty of Life Sciences, Tel Aviv University, Israel; [2]Institut für Toxikologie und Genetik, Forschungszentrum Karlsruhe, Germany

Daily rhythms of the melatonin hormonal signal and of gene expression in the pineal gland are the earliest detected circadian rhythms in zebrafish, appearing as early as days 2-3 of development. Light exposure is mandatory for the onset of the pineal circadian clock that drives these rhythms. Light induces the expression of *period2* (*per2*) which is, in turn, important for the onset of the pineal circadian clock. Interestingly, in zebrafish, light exposure induces *per2* expression not only in the pineal gland but also throughout the body and in cell lines, by an unknown mechanism. In order to understand the mechanism underlying light-induced *per2* expression, functional analysis of the *per2* promoter was performed by transient and transgenic expression of *per2* promoter-reporter constructs in zebrafish embryos and by means of stable transfection of PAC-2 zebrafish cells which are known to contain directly light-entrainable circadian clocks. This analysis has revealed a light-responsive module (LRM) in the *per2* promoter which is both necessary and sufficient for light induction. Interestingly, the LRM sequence is highly conserved throughout evolution and the human LRM can substitute for its zebrafish counterpart to confer direct light regulation of gene expression in zebrafish cells. Functional analyses revealed that within the LRM, a D box enhancer is critical for its light-induced expression, while an E-box element is responsible for its clock-regulated expression. Using *in vivo* approaches complemented by *in vitro* techniques, transcription factors that bind to these elements were identified, characterized and functionally tested. The findings of these studies suggest a hierarchic mechanism in which light enables clock regulation of *per2*. This study extends the understanding of the mechanisms underlying light-entrainment of the circadian clock and contributes to a general understanding of how clock gene regulation has evolved in vertebrates as well as of general photic cellular responses.

Conditional Gene Inactivation Reveals a Crucial Role For *zgc:112094* in Zebrafish Development

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We have performed a pilot screen for gene trap in zebrafish using a *Tol2* based construct that contains unidirectional Cre/loxP system coupled with a splice acceptor (SA) and poly-adenylation signal to stop transcription. When the insertion hits a gene of interest in one of its introns with the orientation of the SA sequence being opposite to the gene (R-SA), Cre recombinase can be applied to invert the gene trap cassette to disrupt the endogenous gene expression. Similarly, if the insertion is in the same direction of transcription (F-SA), causing abnormal splicing, Cre recombinase can be used to rescue the phenotype by reversing the insertion. From mapping 94 unique insertions, we obtained 7 in exons and 15 in introns. Among the R-SA intron insertions, one was identified as a conditional mutant with embryonic lethal phenotype only being apparent by reversing the insertion with *Cre* mRNA injection. RT-PCR and mRNA rescue experiment confirmed that the mutant phenotype was caused by the insertion of the conditional gene trap construct in the first intron of the gene *zgc:112094*, which encodes an ASB-5 homologous protein of E3 ligase. Expression of this gene was maternally detectable and became restricted to heart and somites after 22hpf. RNA *in situ* hybridization analysis with markers for different tissues, including *flk1*, *etsrp* and *gata1*, showed that blood vessel formation in the mutant embryos was severely affected. Angioblasts were reduced and failed to migrate to midline although they were present within the lateral mesoderm, while blood cell development remained normal. These data suggest that *zgc:112094* is required for vasculogenesis. To our knowledge, this is the first Cre/loxP mediated conditional mutation in zebrafish and the method could be scaled up for genome wide screen of mutations. The potential role of *zgc:112094* in cell migration and/or proliferation may be related to cancer and human vascular diseases.

Transcriptional control of bone formation through the regulatory gene *Runx2*

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The transcription factor Runx2 is required for bone formation in mouse, and its expression precedes that of other known markers of osteoblast differentiation. The conserved expression pattern of the zebrafish orthologues in early bone formation suggests that the critical functional role of Runx2 will be conserved as well. Despite its importance in skeletogenesis, the specific mechanisms of transcriptional regulation of the *Runx2* gene itself are not known. We have identified an enhancer element from the human *RUNX2* gene that regulates osteoblast–specific reporter gene expression in the zebrafish. In conjunction with a heterologous minimal promoter, the enhancer drives GFP expression before 3 days post fertilization in the earliest forming bones of the craniofacial skeleton, and continues to be expressed transiently in newly forming bones for several weeks. In contrast to mouse *Runx2*, which shows limited expression in cartilage, the zebrafish genes are expressed prominently during cartilage development. However, this enhancer element does not regulate detectable cartilage expression, and is specific to bone. Through analysis of the expression pattern regulated by the enhancer, we are gaining a detailed and dynamic picture of ongoing bone formation throughout embryogenesis and later growth. We have analyzed the sequence of the human enhancer in comparison to orthologous sequences from multiple vertebrates, including one associated with a zebrafish *runx2* gene. We find multiple conserved short sequences, which we hypothesize represent transcription factor binding sites mediating bone–specific regulation of *Runx2*. Based on this information, we are testing specific predictions about what factors regulate the enhancer, and by extension what signals regulate the earliest stages of bone formation in the embryo. Given the conserved function of the human enhancer in zebrafish, we anticipate that the regulatory factors acting upon it will also be conserved across species. Thus our identification of this enhancer, and the transgenic fish carrying it, afford us the opportunity to examine regulation of *Runx2* in detail.

A phase-ordered microarray screen for cyclic genes in zebrafish reveals her genes as the conserved core of the somitogenesis clock

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Somitogenesis is the rhythmic and sequential segmentation of the vertebrate embryonic body axis from unsegmented presomitic mesoderm (PSM) into somites. Oscillating feedback loops of cyclic genes form a segmentation clock in the PSM that defines timing and position of each newly forming somite boundary. Despite its importance for the vertebrate body plan, the evolution and mechanism of this oscillating network are not well understood.

In mouse, there are more than thirty cyclic genes associated with Delta/Notch, Wnt and FGF signalling (Dequeant et al., 2006). To date, cyclic genes in zebrafish are restricted to the Delta/Notch signalling pathway and downstream target genes of the HES/her family of transcriptional repressors. To assess the conservation of this genetic network, we aimed to find cyclic genes in zebrafish using a phase-ordered microarray screen. We identifed the known cyclic genes, and discovered two new components, *her2* and *her4*. Strikingly, no cyclic members of FGF or Wnt pathways were found, suggesting that the zebrafish segmentation clock may be simpler than its mammalian counterpart. Thus across all vertebrates examined, only HES/her genes show conserved cyclic expression, suggesting that HES genes were the core of the ancestral segmentation clock, and today form the minimal unit of the complex clockworks of different species.

Nevertheless, the role of HES/her genes in somitogenesis remains unclear. Eleven her genes oscillate in zebrafish posterior PSM, in contrast to four known in mouse. This large number raises the question whether they function redundantly or play independent roles in different aspects of somitogenesis. To answer this we characterized *her2,* one of nine cyclic Hes5 sub-family genes, and compared it to known core components of the zebrafish segmentation clock, *her1* and *her7*. In contrast to *her1* and *her7*, cyclic expression of *her2* is restricted to the posterior PSM, similar to the other Hes5 sub-family genes. Whereas over-expression of *her1* or *her7* causes weak disturbances, *her2* over-expression results in strong disruption of somitogenesis. Loss of function of *her1* or *her7* yield distinct somitogenesis phenotypes, but somitogenesis is not affected in a *her2* mutant, suggesting that other Hes5 family members compensate. This hypothesis is tested by knockdown of all cyclic Hes5 genes. In conclusion, these data indicate a role for *her2* in somitogenesis that is distinct from known her genes. We propose a model in which the duplicated Hes5 sub-family members form an oscillatory loop conferring robustness to the fast segmentation clock of the zebrafish, a function not required in slower clocks such as the mouse.

A role for Aspp2a in endoderm development in zebrafish

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ASPP (Apoptosis Stimulating Protein of p53) is best known for its role as a transcriptional co-activator and binding partner of p53, a tumor suppressor gene that is mutated in half of all human tumors. Here we used the zebrafish to study the role of *aspp* in development. In zebrafish there are five *aspp* family members. In this work we focused on *aspp2a*, which is ubiquitously expressed both maternally and zygotically. *aspp2a* morpholino injected embryos show at 1 dpf tail defects that are not seen in control morpholino injected embryos. To characterize the function of *aspp2a* in the formation of the three germ layers the expression of marker genes during gastrulation were analyzed. Markers for presumptive mesoderm (*ntl*), ventral mesoderm/ectoderm (*bmp2b*) and neuroectoderm (*sox2*) are unaffected in the *aspp2a* morphants compared to uninjected controls. However, *sox17* expression, which marks endodermal cells and forerunner cells, is severely reduced in *aspp2a* injected embryos. The liver and pancreas are endodermal derived organs and can be marked by *hhex* and *ins*. The expression of both *hhex* and *ins* was reduced in the *aspp2a* morpholino injected embryos compared to control injected embryos. This suggests that *aspp2a* loss affects endoderm formation, which in turn results in defects in liver and pancreas development.
The TGFbeta/Nodal pathway is the major determinant in endoderm formation in vertebrates. Nodal signaling results in phosphorylation and activation of Smad2, which controls the expression of the transcription factors *gata5*, *bon* and *mezzo*. Bon and Gata5 form a complex together with Eomes and bind the *cas* promoter and induce *cas* expression. Mezzo functions redundantly with Bon and can by itself induce *cas* expression. The transcription factor *cas* regulates the expression of *sox17* and *foxa2*. In *aspp2a* morphants Smad2 phosphorylation is unaffected as is the expression of *bon* and *mezzo*. However the number of *foxa2* and *cas* expressing cells is severely reduced in *aspp2a* injected embryos and there is also a slight reduction in *gata5* expressing cells. Epistasis analysis showed that *aspp2a* is epistatic to *gata5* and *eomes* but not to *mezzo* and *cas*. These results indicate that *aspp2a* functions together with *gata5* and *eomes* to induce *cas* expression. Current efforts focus on testing if Aspp2a binds directly to Gata5, Eomes or Bon and if Aspp2a can be found on the promoter of *cas*.

Crumbs Complex Coordinately Regulates Neurogenesis and Neuroepithelial Polarity through Canonical and Non-canonical Notch Pathway

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In the neural development, neuroepithelial cells, which are highly polarized neural stem cells, control various aspects of neural development, such as neurogenesis, guidance of neural migration, and axon guidance. They have very unique morphology with apical and basal processes that extend to the ventricular and pial surfaces, respectively. At the tip of apical processes, cellular junctions such as adherens and tight junctions are formed. For the establishment of a functional nervous system, the cooperative control of neuroepithelial functions, and morphology are crucially important. To understand the molecular mechanisms by which neuroepithelial cells coordinately regulate their functions such as neurogenesis and their morphology such as apicobasal polarity, we analyzed the zebrafish *holm* mutant that has a mis-sense mutation in the neuroepithelial polarity gene, *epb4.1l5* (erythrocyte protein band 4.1-like 5, formerly known as *mosaic eyes*), which encodes a putative adaptor protein.

Neuroepithelial morphology such as apicobasal polarity, adherens junctions, tight junctions, and the extension of apical processes to ventricular surface were disrupted in the *holm* mutant. In addition, neuroepithelial functions such as neurogenesis and guidance for tangential migration of vagus motor neurons were also affected in the holm mutant. These results suggest that *epb4.1l5* is required for the cooperative control of neuroepithelial functions and morphology. To reveal how Epb4.1l5 cooperatively regulate neuroepithelial functions and morphology in the molecular level, we next analyzed potential molecules acting downstream of *epb4.1l5*. Crumbs family proteins had been reported to be negatively regulated by Epb4.1l5 and negatively regulate the Notch activator -Secretase in fruit fly. The *holm* mutant phenotype was partially phenocopied by over-expression of Crumbs2. Interestingly, Notch activity was significantly reduced in the *holm* mutant, and over-expression of the Notch intra-cellular domain (NICD) rescued the *holm* mutant. In addition, over-expression of constitutive active form of R-Ras, which had been reported to act downstream of NICD and regulate cellular polarity and adhesion, partially rescued the *holm* mutant, and knocking down of *r-ras* canceled the recovery of the *holm* mutant phenotypes by NICD. These results may suggest that Epb4.1l5 cooperatively regulates neuroepithelial functions and morphology through the regulation of Notch activity.

Jagged-Notch signaling patterns the facial skeleton
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The facial skeleton in all vertebrates arises from complex cellular interactions that occur between the endoderm, ectoderm, and neural crest cells of the well-conserved pharyngeal arches. Our goal is to identify the signals that are involved in patterning the facial skeleton along the dorsoventral axis. Several genes have been identified that are required for a ventral skeletal fate; however, genes responsible for dorsal identity are less clear. A ventral to dorsal gradient of the Endothelin1 (Edn1) morphogen specifies lower versus upper jaw identity by activating specifier genes, such as *dlx3b*, *dlx5a*, and *dlx6a* in ventral skeletal precursors. Recently, we discovered a mutation in *jagged1b* (*jag1b*^{b1105}), which results in dorsal skeletal elements acquiring a ventral morphology. We show *jag1b* specifies upper jaw identity by inhibiting the expression of ventrally expressed genes. Since Jag1b is a Notch signaling ligand, we next examined which Notch receptor is involved in patterning the facial skeleton along the dorsoventral axis. Whereas *jag1b* is expressed in the dorsal domain of the first two pharyngeal arches, we find that *notch2* is expressed in a complementary pattern in the ventral domain. In addition, reduction of Notch2 function with a morpholino (MO) resulted in dorsal-specific skeletal defects similar to that seen in *jag1b*^{b1105} mutants. Since Jagged-Notch inhibition resulted in dorsal-specific skeletal defects, we predicted that activation of Notch signaling would show the opposite phenotype. In fact, when we activated Notch signaling by misexpressing the Notch intracellular domain (NICD) during skeletal patterning stages, we saw a loss of ventral-specific gene expression and a reduction of the more ventral jaw skeleton. Since the skeletons of NICD embryos resembled the skeletons of larvae with reduced Edn1 function, we wanted to study the relationship between dorsally required Jagged-Notch signaling with the ventrally required Endothelin (Edn1) morphogen. We generated *jag1b*^{b1105} and *edn1*^{tf216b} double mutants in which we find the facial skeleton is rescued. Furthermore, we wanted to identify additional DV-specific genes that control skeletal morphology, we performed fluorescent activated cell sorting (FACS) and microarray analysis of cells from transgenic zebrafish expressing GFP in different DV domains of skeletal precursors. We will present candidate genes that are expressed within discrete domains of the pharyngeal arches during patterning stages. Currently, we are testing whether these candidate genes are also regulated by Jagged-Notch signaling.

Positive and negative feedback from proteoglycans during chondrocyte differentiation
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Despite the fact that skeletogenic cells immerse themselves in abundant extracellular matrix, remarkably little is known about the influence of proteoglycans on skeletal cell differentiation *in vivo*. Here, we reveal the effects of a loss-of-function mutation in *xylosyltransferase1* (*xylt1*) on cartilage and bone development during zebrafish endochondral ossification. Xylosyltransferases initiate glycosaminoglycan side chain addition to core proteins during the formation of proteoglycans, such as *aggrecan*, which is expressed highly in cartilage. Accordingly, *xylt1*$^{-/-}$ zebrafish demonstrate decreased Alcian blue staining in cartilage elements undergoing endochondral ossification, although the patterning of these elements is roughly the same as wild-type siblings. At early stages of overt chondrogenesis in *xylt1*$^{-/-}$ mutants, the cartilage markers *sox9b* and *col2a1b* are expressed in similar domains as wild-type, but at decreased levels. Interestingly, *xylt1*$^{-/-}$ zebrafish also have increased and premature Alizarin red staining in perichondral bone, even though *xylt1* does not appear to be expressed highly in developing perichondrium. Osteoinductive factors, such as Indian hedgehog (Ihh), are expressed in maturing chondrocytes and are known to signal to osteoblast precursors in the overlying perichondrium. In support of a hypothesis that mutant cartilages prematurely initiate chondrocyte maturation, *ihh* genes are expressed in *xylt1*$^{-/-}$ cartilage earlier than in wild type. In total, these data suggest that proteoglycans play a positive role during initial stages of overt chondrocyte differentiation and a negative role during chondrocyte maturation.

Mutations in components of diverse essential cellular pathways disrupt zebrafish intestinal development

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Our aim is to identify zebrafish genes with functions in vertebrate intestinal development and to explore their potential role in the development and progression of colorectal cancer. To fulfill this aim, we have positionally cloned seven intestinal mutants identified in the Liver^{plus} screen at UCSF. This screen, which was carried out on the *Tg*(*gutGFP*)^{s854} background, yielded approximately 100 mutants with defects in intestinal, liver and pancreas development at 4dpf. Interestingly, six out of seven of our cloned genes play roles in a variety of essential cellular processes, while the seventh is a novel gene. The growth of the intestinal epithelium in two of the mutants, *flotte lotte*$^{ti262c/s871}$ and *trinculo*^{s451}, becomes arrested at 3dpf and shortly thereafter large numbers of intestinal epithelial cells undergo apoptosis. The molecular lesion in *flotte lotte* is a premature stop codon in the *AT hook containing transcription factor 1* (*Ahctf1*) */ elys* gene, which encodes a nuclear pore component that is essential for the assembly of nuclear pores at the end of each round of mitosis. In *trinculo*^{s451}, the responsible mutation resides in a gene encoding a subunit of vacuolar (H+) ATPase (V-ATPase), a well-characterized proton pump that contributes to numerous cellular processes, including trafficking of proteins through the endosome-lysosome degradative pathway. We have shown, using confocal and electron microscopy, that endosomes and lysosomes in *trinculo* intestinal epithelial cells are abnormal in both size and morphology. Meanwhile the intestinal epithelium in *titania*$^{s450/s927,}$ *sycorax*^{s845} and *setebos*^{s453} is dysplastic, disorganized and relatively unfolded compared to wildtype. Apoptotic cells are rare. Here, mutations in three different genes all lead to abnormal ribosomal RNA processing and disrupted ribosome biogenesis, a critical process in proliferating cells, including cancer cells. Lastly, the positional cloning of *caliban*^{s846} has identified a nucleotide variation in the gene encoding a component of the U12-dependent minor spliceosome, a highly conserved molecular complex that processes a small fraction of introns (less than 1%) with distinctive consensus splice sites. The conserved and non-random distribution of minor class introns throughout the genome indicate an important role of minor class splicing in gene expression and regulation. Together our studies have identified a number of important cellular genes as being essential for the growth and survival of intestinal epithelial cells. The organ-specific phenotypes we observe in our mutants, though unexpected in view of the critical nature of the defective genes, correlate well with the expression patterns of these genes during the rapid growth phase of digestive organ formation. We are currently determining whether the deregulation of the human orthologues of these genes contributes to the growth and survival of colorectal cancer cells.

CDX genes promote hepatic fate and repress pancreatic fate within bipotential hepatopancreatic progenitor cell population in zebrafish

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Embryonic patterning requires a precise temporal and spatial orchestration of gene expression. The Caudal-related (Cdx) genes are homeobox genes, members of the parahox cluster. Cdx genes are highly conserved among vertebrates and are known to function critically during antero-posterior patterning and development of the gut tube. Liver and pancreas progenitors develop from endoderm cells in the embryonic foregut and previous studies suggest that these two endoderm-derived tissues arise from a subset of common bipotential progenitors. We found that Cdx4 promotes anterior-most fates in hepatopancreatic precursors in zebrafish and depletion of this gene leads to a severe expansion of the endocrine pancreas fate in detriment of both liver and exocrine pancreas fates. Furthermore we demonstrate that depletion of *cdx4* modifies the cross talk between progenitors in the endoderm and signaling environment, influencing the exposure of naïve progenitor cells to Fgf, Retinoic Acid and BMP signals. Combined depletion of *cdx1a*, *cdx1b* and *cdx4* revealed that all *cdx* genes act redundantly during endoderm organogenesis. Thus c*dx* genes are responsible for providing the right positional cues along the anterior-posterior axis, placing the bipotential hepatopancreatic progenitor cells in the right embryonic environment to potentiate proper differentiation into liver and pancreas.

Zebrafish anterior chamber development and maintenance
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Anterior chamber of the eye is a clear space filled with aqueous humour between the lens and cornea which guides the light focused on the retina, hence crucial for a normal vision. To establish the basis for understanding the origin and development of the anterior chamber at the molecular level, we focused on the cornea, a structure of neural crest origin. We established the *sox10::eosFP* transgenic line to follow neural crests, but also to visualize *de novo sox10* transcription activity by using a unique feature of EosFP, a photo-convertible fluorescence protein; photo-conversion prior to the time lapse imaging allows one to discriminate earlier reporter expression from the later. This "pulse-chase" imaging cancelled out *sox10*-inactive cells to reveal a *sox10*-active cluster of non-ectomesenchymal neural crests in the pharyngeal arch at 18-20 hpf as a major source that contribute the anterior chamber. To gain insights into the signalling pathways involved in the anterior chamber formation, the effects of chemical inhibitors on the cornea morphology were analysed by time-lapse imaging and electron microscopy: 1) EGF and FGF signallings are required for the corneal endothelium formation; 2) planar polarity of the corneal epithelium was regulated by multiple pathways of EGF, Notch and Hh/PKA.
To identify genes involved in the maintenance of the anterior chamber, we analysed gene expression profile of the adult cornea using the skin as a reference tissue, which is initially continuous with the cornea during embryonic stages and shares high anatomical similarities at the ultra-structure level. Microarray analysis presented ~165 cornea genes that are enriched in the adult cornea in comparison to the skin ($P<0.01$). Further RT-qPCR quantification established relative amount of the cornea genes over the skin and confirmed the results from the microarray analysis. We mapped spatial distribution of the cornea genes in the adult ocular tissues by *in situ* hybridisation. All together, we provide comprehensive set of gene profiling analyses, which contribute further functional analysis to examine the roles in the anterior chamber maintenance.

Renal stem cells give rise to new nephrons during zebrafish kidney regeneration

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Zebrafish possess tremendous regenerative capacities and can regrow a range of organs, including the kidney. Here, we present evidence supporting the existence of a renal stem cell population in the zebrafish kidney that is involved in regenerating new nephrons (the functional unit of the kidney) in response to injury. Following gentamicin-induced kidney damage, adult fish kidneys showed increased expression of the renal transcription factor *lhx1a* in single mesenchymal-like cells within the kidney marrow. These cells appear to expand and coalesce into basophilic aggregates that express the transcription factor *wt1b* and then epithelialize into primitive nephron structures that fuse with existing nephrons. To further characterize the presumptive renal stem cell population in adult fish, we carried out transplantation experiments. Donor cells genetically tagged with a kidney-specific green fluorescence protein (GFP) transgene were injected into the circulation of unlabeled wild-type or kidney-injured recipient fish. Following transplantation we found functional donor-derived GFP^+ nephrons in 47% of the recipient fish that were undergoing kidney regeneration, and in 9% of wild-type recipient fish. These findings suggest that the adult zebrafish kidney possesses a renal stem/progenitor cell population that can home back to the kidney from circulation and form new nephrons. To determine the origin of the renal stem cells, we examined the formation of the adult kidney during larval development. Single cells expressing *lhx1a* appeared just posterior to the swim bladder at around 10 days post-fertilization. From *in vivo* timecourse analyses of double transgenic larvae, these *lhx1a*$^+$ cells were found to expand, initiate expression of *wt1b*, and differentiate into adult nephrons that fuse with the embryonic kidney. Taken together, we propose that the adult zebrafish kidney is initially formed from *lhx1a*-expressing renal stem cells, and these cells persist in the kidney marrow to serve as a source for *de novo* nephron formation in response to renal injury.

Improved genetic lineage tracing using the conditional red-to-green reporter *Tg(hsp70*: loxP-DsRed2-loxP-EGFP)

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Conventional use of the site-specific recombinase Cre is a powerful technology in mouse, but almost absent in other vertebrate model organisms. In zebrafish, Cre-mediated recombination efficiency was previously very low but using transposon mediated transgenesis, we recently showed that Cre is in fact highly efficient in this organism. Furthermore, temporal control of recombination can be achieved by using the ligand-inducible $CreER^{T2}$ [1].
In order to allow genetic fate mapping we previously generated a red-to-green reporter line *Tg(EF1α loxP-DsRed2-loxP-EGFP*). This line expresses DsRed2 under the control of the ubiquitous *Xenopus Elongation Factor 1 alpha* (*EF1*) promoter in the absence of Cre activity, but changes to EGFP after a successful recombination event. However, although we see strong expression of DsRed2 for up to 7 days, *in situ* analysis reveals strong ubiquitous expression only during gastrulation and mid-somitogenesis stages which limits the use of this line. To establish a faithful red-to-green reporter line we thus turned towards the zebrafish temperature-inducible *hsp70* promoter which has been shown to express in a strong and ubiquitous fashion [2]. We will show that this conditional red-to-green reporter line *Tg(hsp70: loxP-DsRed2-loxP-EGFP)* indeed allows genetic fate mapping at all stages examined. Furthermore, in contrast to a constitutive reporter that always labels the entire lineage of a Cre expression domain, the conditional reporter line can be activated at a certain stage when the Cre expression domain has not reached its final expansion. Consequently, only a portion of the lineage will be labeled and might reveal that a certain structure is formed by the early Cre expressing cells. Hence, the conditional reporter line will facilitate fate mapping studies in the early zebrafish embryo because it takes advantage of the rapid zebrafish development that is much faster than the degradation of EGFP.

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The zebrafish as a model system for *in vivo* single-molecule microscopy

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Studying the dynamics of individual proteins by single-molecule microscopy has been available for several years, using laser excitation of fluorescently tagged proteins and detection by a sensitive CCD camera. However, until now this technology has only been applied to individual cells in culture. In the present study, the zebrafish was used as a model system to extend single-molecule microscopy to the level of a living organism. As a molecule of interest we used yellow fluorescent protein (YFP) fused to the human H-Ras membrane anchor, which has been shown to serve as a model for proteins anchored in the plasma membrane. In order to achieve this, a three-step approach was used. First, individual molecules were visualized in cultured (ZF4) cells using a wide-field fluorescence microscopy setup. Second, zebrafish embryos were injected with RNA encoding the fluorescently tagged protein and the injected embryos were dissociated at the sphere stage. Subsequently, imaging was performed using the dissociated primary cells. Third, imaging in living organisms was performed in epidermal cells in the skin of two-day-old zebrafish embryos, previously injected with RNA encoding the fluorescently tagged protein. A total internal reflection (TIRF) microscopy setup was used for this approach. The results of our experiments are in line with previous findings in mammalian cells and demonstrate that two populations of the YFP-tagged membrane anchor exist, which differ in the type of diffusion behavior they display. Furthermore, we demonstrate the occurrence of membrane microdomains confining the diffusion of membrane proteins. This membrane organization differs significantly between the different cell systems used in our study, illustrating the relevance of performing single-molecule microscopy *in vivo*.

Transposon-based insertional mutagenesis platform
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Two key characteristics of any gene are loss of function phenotype and expression pattern. It has been extremely challenging to develop experimental approaches that simultaneously address these two characteristics in any model system. In Drosophila, the most successful and widely used approach is enhancer trap. It reveals the expression pattern of the affected gene, but is inherently non-mutagenic. In the mouse, several consortia are undertaking genome-wide insertional mutagenesis using gene traps. Due to limitations of mouse as a model system these experiments are carried out in ES cells. Thus, neither the expression pattern nor the mutant phenotype is revealed until a transgenic mouse is generated from these ES cells.
We are developing transposon-based tools to simultaneously address these two characteristics (mutant phenotype and expression pattern) in the zebrafish. Our vectors are built from gene breaking transposon components known to disrupt gene expression upon integration. Two key advances were made to achieve mutagenicity and specificity of gene breaking transposons. To increase mutagenicity, fish-derived splice acceptor and transcriptional termination/polyadenylation sequences were used. To increase specificity, the translation initiation codon was removed from the reporter. Combination of these two advances enables stringent selection for integration into genes and efficient disruption of gene expression.
Our initial vectors used mRFP as the reporter for gene trapping events. One potential drawback of a fluorescent reporter is that integrations into genes expressed at very low levels may be impossible to detect. This and other considerations prompted us to replace the mRFP reporter by Gal4-VP16. To detect Gal4-VP16 expression we also added UAS:eGFP, thus making a self-reporting gene trap vector. To date, we screened 70 fish raised from embryos injected with this transposon and isolated 12 *bona fide* gene trap lines. Gene trap expression patterns range from very bright ubiquitous to weak mosaic to highly specific, such as the nervous system, pancreas, vasculature and blood. Candidate affected genes have been identified for each of the gene trap lines.
To complement this insertional mutagenesis approach, we are generating lines with tissue-specific expression of Cre recombinase for reversion of mutant phenotypes and are investigating the possibility to use I-SceI meganuclease for generation of local deletions involving the transposon integration site.

Optimized Gal4 genetics for permanent gene expression mapping in zebrafish

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Combinatorial genetics for conditional transgene activation is a powerful technology to study gene function with temporal and tissue specific control. The development of the combinatorial Gal4-UAS system has immensely expanded the possibilities for sophisticated genetic studies in *Drosophila*. Recently this system was adapted for zebrafish research and promising applications have been introduced. Here we report a systematic optimization of Gal4-UAS genetics in zebrafish establishing an optimized Gal4-activator (KalTA4) for in vivo use in zebrafish. In addition, we offer quantitative data for KalTA4-mediated transgene activation dependent on UAS copy numbers to allow for studying dosage effects of transgene expression. With the help of a *Tol2* transposon-mediated KalTA4 enhancer trap screen biased for central nervous system expression, we provide a collection of self-reporting red fluorescent KalTA4 activator strains. These strains reliably transactivate UAS-dependent transgenes and can be rendered homozygous. Furthermore, we have characterized the transactivation kinetics of tissue-specific KalTA4 activation in detail. These studies lead to the development of a self-maintaining effector strain "Kaloop", which relates transient KalTA4 expression during embryogenesis via a KalTA4-mediated autoregulatory mechanism to live adult structures. We demonstrate its use by showing that the secondary octaval nucleus in the adult hindbrain is likely derived from *egr2b*-expressing cells in rhombomere 5 during stages of early embryogenesis. These data demonstrate prolonged and maintained expression by Kalooping, a technique that can be used for permanent spatio-temporal genetic fate mapping and targeted transgene expression in zebrafish.

Transcriptional target discovery through caged morpholino technologies
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Deciphering the molecular mechanisms that regulate vertebrate development and physiology requires an ability to alter these genetic programs with spatial and temporal precision. Caged morpholino (cMO) oligonucleotides enable such experimental manipulations in zebrafish and other optically transparent organisms, and we report here the application of cMOs to discover transcriptional targets of the T-box gene *no tail* (*ntl*). In contrast to previous studies that conducted whole-embryo analyses of the *ntl* mutant/morphant transcriptome, we have identified *ntl* targets in a tissue-specific manner by combining cMOs, photoactivatable fluorophores, fluorescence-activated cell sorting, and microarray gene expression analysis. Using this strategy, we have discovered approximately 70 *ntl*-dependent genes that contribute to notochord formation, including proteins associated with developmental signaling pathways, cell adhesion, and extracellular matrix production. To facilitate the application of this approach to other transcription factors, we have also optimized our cMO synthetic procedure and investigated the relationship between cMO structure, *in vitro* thermodynamics, and *in vivo* activity. Through these studies, we have established basic principles for cMO design and successfully applied them to develop cMOs targeting other patterning genes. Our findings establish the generality of cMO technologies and illustrate the potential of these photoactivatable reagents in functional genomics research.

Chromosome painting of zebrafish chromosomes for the identification of inter-chromosomal rearrangements and marker chromosomes

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Despite the importance of zebrafish as a model organism for studying vertebrate development, cell biology and cancer, the establishment of molecular cytogenetic probes to characterize the genomes of mutants and visualize specific chromosomal alterations is still lacking. Recently, we developed fluorescence in situ hybridization (FISH) probes for near-telomeric and near-centromeric regions of all zebrafish chromosomes (Freeman et al., 2007) which has proven to be useful for identifying gross chromosomal rearrangements, but the scale at which they can identify chromosomal rearrangements, amplifications and translocations is limited. To bridge this gap, we have now developed chromosome painting probes containing combinations of DNA probes that "coat" entire chromosomes with a given fluorescent color. The development of these DNA probes permits rapid identification of inter-chromosomal rearrangements and ambiguous supernumerary marker chromosomes. We have developed chromosome-specific paint probes using pools of chromosome-specific bacterial artificial chromosomes (BACs) for each zebrafish chromosomes chosen at approximately 1 Mb intervals. Additionally, we have also identified a chromosomal translocation using two-color FISH. The establishment of this resource will enable the rapid identification of suspected chromosomal translocations in mutant strains. Future efforts to label individual chromosome-specific BACs within a specific chromosome, using a multi-color labeling approach, will eventually lead to a multi-color chromosome probe set that would enable detection of intra-chromosomal events including chromosomal inversions, deletions and duplications.

A mesodermal gene regulatory network directed by T-domain transcription factors

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Using chromatin immunoprecipitation combined with genomic microarrays and massively parallel sequencing we have identified targets of No tail a (Ntla), a zebrafish Brachyury ortholog that plays a central role in mesoderm formation. We have found that Ntla regulates a downstream network of other transcription factors and we identify an in vivo Ntla binding site that resembles the consensus T-box binding site (TBS) previously identified by in vitro studies. Using similar methods we have begun to identify targets of other T-domain factors, and using these data we describe a preliminary Gene Regulatory Network for mesoderm formation and patterning in the early zebrafish embryo.

Automated high throughput mapping of promoter-enhancer interactions in zebrafish embryos

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Zebrafish embryos offer a unique combination of high throughput capabilities and the organismal complexity of the vertebrate animal for a variety of phenotype screening applications. However, there is need for automation of imaging technologies to exploit the potential provided by the transparent embryo. Here we report the development and application of a high throughput pipeline for registering domain specificity of reporter expression in zebrafish embryos with the aim of mapping the interaction specificities between cis-regulatory modules and core promoters. Automated intelligent microscopy and custom built embryo detection and segmentation software allowed the registration of reporter activity in almost 18 thousand microinjected zebrafish embryos. Domain specificity of Venus reporter expression was evaluated by warping of individual embryo images onto a virtual two dimensional reference shape. Using the embryo imaging pipeline we demonstrate a diversity of interaction specificities between 203 enhancer-core promoter combinations. These interaction specificities underscore the importance of the core promoter sequence in cis-regulatory interactions and provide a valuable promoter resource for transgenic reporter studies such as enhancer traps. The technology described here is also suitable for the high throughput analysis of spatial distribution of a variety of fluorescence readouts in zebrafish embryos for genetic, pharmaceutical or toxicological screens. To this end we are exploring the utility of our image analysis tool in detecting gene expression phenotypes in embryos in which signalling pathways have been perturbed.

Identifying the gene network involved in inner ear cell regeneration in the adult zebrafish

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Sensory hair cells of the inner ear are the mechanotransductory units in the neuroepithelia. In mammals, lost hair cells are not replaced, resulting in various permanent deficiencies in vestibuloauditory sensation. In contrast, zebrafish can replace lost hair cells with new ones throughout adulthood. Our ultimate goal is to understand the gene network requiref for inner ear hair cell regeneration in adult vertebrates. As an initial step, we identified the transcriptional response used for hair cell regeneration in zebrafish using next generation sequencing techniques for gene expression profiling.

We modified a previous noise-exposure protocol, which enabled us to induce hair cell loss in the saccular epithelium of the adult zebrafish and characterize the subsequent regeneration process. We determined the inner-ear gene expression profiles at different time points during the regeneration, focusing particularly on those genes with significant increase in expression at the various time points. Here we used a new technique, Tag Profiling (Digital Gene Expression), to generate the gene expression profiles in an in-depth and high-throughput manner. Similar to SAGE in nature, the technique generates 3 million or more gene "tags" from the mRNA pool, giving us a very deep view of gene expression at each time point. We have developed and/or utilized various bioinformatic tools for gene assignment, ontological analysis, and pathway analysis. Our analysis of the Tag Profiling data suggested new candidate genes as well as new candidate pathways involved in the hair cell regeneration.

We are now working on associating the new candidate genes/pathways with various cellular events (e.g. cell division and differentiation) during hair cell regeneration to get a better understanding of the functions of those genes/pathways in the regeneration process.

Whole-genome sequencing of a Wildtype Strain of Zebrafish for variant discovery

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Information encoded by DNA sequences is valuable for understanding genetics and allows discoveries of important biological processes. The existence of a reference sequence for the Zebrafish genome permits application of fast re-sequencing technologies, whereby short reads are compared to a reference to identify genome-wide sequence variations. We have sequenced the genome of a Wildtype Strain of Zebrafish using the Massive Parallel Signature Sequencing (MPSS) technology of Illumina Genome Analyzer. This method allows massively parallel sequencing of millions of genomic fragments ranging from 36 to 75 base pairs, which are then mapped back to the reference genome.

Till date, the main genomics work has been focused on lab grown Zebrafish strains that functionally represent genetic clones; little or no true genetic diversity was captured in these initial genome studies. To address questions related to genomic variations, we have sequenced a Wildtype Strain of Zebrafish caught directly from the water bodies in India. This will facilitate strain-to-reference comparison for genome-wide sequence variant discovery. We collected over 110 GB of sequence data from 11 runs on the Illumina Genome analyzer. We aligned the short reads onto Sanger Zebrafish reference genome, resulting in average 25X sequence coverage of the Zebrafish reference genome. Updates regarding single-nucleotide polymorphism, small indels and other structural variants in the Wildtype strain of Zebrafish will be presented and discussed.

Genetic diversity between Tübingen and AB strains, and a new ultra-dense genetic map

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The zebrafish genome sequencing project is based on the Tübingen strain. Many laboratories use other strains, especially AB. Yet we have limited knowledge of the genetic variation even between these two common lab strains. Using Illumina next generation sequencing we have sequenced to 25x coverage a doubled haploid (DH) male AB fish, and mapped these reads to the reference genome sequence. By so doing we identified over 10 million single nucleotide polymorphisms (SNPs) between the DHAB fish and Tuebingen reference genome. In addition to identifying SNPs we can also identify small insertions and deletions (indels) in the genome based on changes in read sequences, and larger indels (including copy number variantss - CNVs), and inversions based on read pair mapping anomalies.

It is possible to fit over 200,000 SNPs (1 SNP every 7.5kb) onto a single chip, and then to genetically map these at a resolution finer than that of a BAC clone - genetically anchoring the genome assembly along it's length. Furthermore, this would provide researchers with a very dense genetic map and rapid transition from a mutant mapping cross to the genome sequence. By crossing the sequenced DHAB male to a doubled haploid Tuebingen female (currently sequencing) we have generated a set of F1s which have one each AB and one Tuebingen chromosome, and from which we are assembling a 500 F2 panel which would give ~0.1cM resolution (~65kb). Using our sequence data we are currently selecting over 200,000 SNPs polymorphic between the two DH fish for high resolution mapping on a custom SNP array.

Finally in any genome assembly there are regions that are not covered by well assembled sequence. This is true even in the human genome, often due to technical difficulties such as unclonability of certain sequences e.g. long TA stretches, telomeric and centromeric regions or assembly failures. From our 100s of millions of reads - generated without cloning steps - we are able to identify sequence that is not in the current Zv8, much of which is supported by at least one whole genome shotgun read. We have about 40Mb of such sequence, and hope to use this to improve the genome coverage.

Thus we anticipate that the next release, Zv9, will be very densely covered with genetic markers, be the most complete assembly and that it is confirmed not just by sequence and clone overlaps but by extensive genetic mapping data.

High throughput screening of innate immune responses in zebrafish and carp embryos

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In recent years the zebrafish has been shown to be an excellent model for studying the mechanisms of the innate immune defense against pathogens. We have shown that transcriptome responses towards pathogens such as *Mycobacterium marinum* and *Salmonella typhimurium* are very similar to responses in mammalian systems. Using combinations of transcriptomic deep sequencing, morpholino knockdown and transgenic reporter fish technologies we have obtained new insights in the functions of key players of the innate immune system. These results are not only relevant to infectious diseases but also to the study of immune responses to cancer cells, for instance using xeno-transplantation assays. In this presentation we will show that such studies can also be extended to a high throughput level. For this we have applied the Copas XL biosorter (Union Biometrica) for screening disease symptoms using flow-through laser scanning profiling coupled with embryo sorting. We show that it can be highly profitable in this approach to also employ close relatives of the zebrafish for high throughput assays. For instance the common carp that can yield hundreds of thousands of embryos for each fish after in vitro fertilization can overcome limiting quantities of embryos for high throughput screening. We have obtained a shotgun sequence of the carp in order to compare results obtained with zebrafish showing that the hallmarks of innate immune responses in both fish species are extremely similar. Our results show that it is now possible to undertake chemical compound screens in carp fish the results of which can be further analyzed in transgenic zebrafish models.

Amplify the impact of your research: Ensure that your data can be integrated into the electronic data stream

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Publishing a manuscript was once the final step in communicating data. Now, publication is a preliminary step in a larger process that involves public databases.
Public databases, such as the Zebrafish Information Network (zfin.org), integrate and link your data with other data, amplifying the impact of your work on the wider research community. Public databases help researchers refine hypotheses before going to the bench, saving time and resources. Databases are only as good as their data, and those data come largely from publications. However, the quality, amount and type of information that can be captured are critically dependent upon the precision of the information available in each publication. Biocurators are trained professionals who carefully read publications and extract the most salient data for entry into a database. Authors can facilitate the curation process and ensure that their data are represented correctly and completely. Namely, authors should provide accession numbers and sequences whenever possible, check model organism databases for current gene and mutant nomenclature, and consult nomenclature committees before naming new genes. Ambiguity is the enemy - be specific! Which "shh" gene? Which *fgf3* allele? Which *pax2a* morpholino? Which developmental stage? References to prior publications are helpful, but may not eliminate all ambiguity. As a reviewer, one can identify ambiguities and missing information before publication. The choice of journal and format is also important. Some journals do not give permission to reproduce figures in public databases, and the articles themselves may not be readily available. These issues limit the accessibility of data in public databases and should be considered when choosing a journal. Many journals now provide an open-access publication option. This is the friendliest format for a public database. Authors can help by working together with journals and biocurators to facilitate accurate data exchange and reporting. This interaction completes a new and important data feedback among authors, electronic data systems like ZFIN, and journals.

Zebrafish as an efficient model system to study the regulation of mitosis and chromosome instability

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Aggressive cancers often exhibit Chromosome instability (CIN), characterized by aberrant chromosome structures and numbers. Unregulated mitosis is implicated in aneuploidy and CIN. Therefore, understanding the regulation of mitosis is not only a fundamental question in basic biology but is central to understanding cancer. Recently, anti-cancer drug development has focused on CIN and the mitotic kinases. Because established cancer cell lines harbor mutations in cell cycle control genes, development of inhibitors of mitotic kinases requires validation in an alternative *in vivo* system.

We present data showing that zebrafish is a valuable system for studying mitosis. We propose based on this data that zebrafish can be adopted to validate the efficacy of inhibitors of mitotic kinases as anti-cancer drugs. We have specifically focused on two mitotic kinases, Plk1 and Aurora A. While Plk1 and Aurora A are both implicated in centrosome maturation and microtubule outgrowth, they have distinct roles. In zebrafish embryogenesis, loss of Plk1 or Aurora A using morpholinos resulted in growth defects followed by apoptosis. Loss of Plk1 resulted in defective microtubule outgrowth, aberrant centrosome numbers, and unstable kinetochore-microtubule attachments. Interestingly, zebrafish embryogenic cells underwent one or two more divisions before death with the noted mitotic defects, exhibiting aneuploidy. These results indicate that Plk1 is essential for progression through mitosis in multiple stages and is crucial in the genetic integrity in developing embryos. Morpholino knockdown of Aurora A displayed similar results with some distinct patterns compared with those of Plk1 morphants.

A great advantage to zebrafish is the ability to analyze single cells in a living organism. Using live-cell imaging in a H2B-GFP transgenic fish we developed, we found that the loss of Plk1 resulted in a delay in mitosis for hours while normal zebrafish embryos divide in 12-17 minutes. The mitotic arrest accompanied unsegregated sister chromosome pairs, chromosome bridges, micro nuclei, and defects in centrosome numbers and maturation, all of which are hallmarks of CIN in cancer. As a result, loss of Plk1 resulted in the activation of spindle assemblyc checkpoint (SAC). Moreover, we were able to monitor the effects of Aurora A inhibitors at the single cell level with live-imaging. Taken together, we conclude that zebrafish is a valuable tool in studying the molecular mechanisms of mitosis and mitotic kinases, and zebrafish embryos will serve as an efficient model system for validating anti-cancer effects of mitotic kinase inhibitors.

A Mutation in Alk6b Causes Germ Cell Tumors in Zebrafish

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Germ cell tumors (GCTs) affect infants, children and young adults and are increasing in incidence worldwide. GCTs arise from pluripotent germ cells and can exhibit differentiated and undifferentiated histologies, which vary in their malignant potential and response to treatment. The molecular origins of GCTs and the pathways that determine tumor cell differentiation are not known, impeding the development of new therapies. For these reasons, the treatment of GCTs has remained static since the introduction 30 years ago of cisplatin which, while effective, causes severe side effects including hearing loss, infertility and kidney damage. Currently there are no animal models of GCT, which poses a further obstacle to our understanding of the disease.

We identified a zebrafish mutant line with a high incidence of GCT during a forward genetic screen to identify cancer susceptibility loci. Homozygous adult males develop tumors consisting of undifferentiated spermatogonia by 4 months of age while heterozygous males develop tumors around 7 to 9 months of age. Pedigree analysis demonstrated that the mutation is dominantly inherited. We used interval haplotype analysis of 14 affected individuals to localize the mutation to zebrafish chromosome 10. We carried out further high-resolution recombinational mapping to narrow the critical interval to a 0.6 cM interval containing three genes. We identified a premature termination codon in the type IB Bone Morphogenetic Protein Receptor, Alk6b (Activin Receptor-like Kinase 6b) in the mutant animals.

Alk6b is a member of the TGF-beta/BMP superfamily of receptors. BMP signaling has diverse roles including regulation of cell proliferation, differentiation, embryonic development, germ cell specification and gonadogenesis. Misregulation of the BMP signaling pathway has been implicated in various human cancers. In agreement with a critical role for Alk6b in controlling germ cell differentiation, we find evidence of impaired BMP signal transduction in the zebrafish GCTs, as well as evidence of alterations in the expression level of BMP target genes.

We are conducting experiments to further characterize the role of Alk6b in germ cell proliferation and differentiation and to determine the precise mechanisms of tumor development in the mutant zebrafish line. We have also examined activity of the BMP signaling pathway in a series of 40 clinically-annotated human GCTs of diverse histologic subtypes. In agreement with the predictions made from our zebrafish model, we find that undifferentiated GCTs such as dysgerminomas lack BMP signaling activity, whereas signaling is maintained in the differentiated subtype of Yolk Sac Tumors. These results confirm the relevance of the zebrafish model for understanding germ cell tumorigenesis, and will foster the development of improved, targeted therapy of human GCTs.

New Zebrafish Models of T Cell Cancer: Important Resources for Gene Discovery

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The rapidly evolving field of cancer genetics is revolutionizing contemporary therapies for human malignancies. To identify new genes involved in the pathogenesis of T cell cancer, we performed a phenotypic mutagenesis screen and identified three new zebrafish models of T cell acute lymphoblastic leukemia (T-ALL). Conceptually, T-ALL genes can be grouped into three overlapping categories: 1) those predisposing to cancer; 2) those directly causing cancer; and 3) those modulating disease severity. Our new mutants are valuable resources for gene discovery in all three categories.

T-ALL penetrance in the lines is incomplete, indicating that the mutated gene in each case confers a predisposition to develop disease. To identify the genetic lesions, we carried out genome-wide scans in each line using restriction site associated DNA (RAD) markers and high-throughput Solexa sequencing. Linked regions were rapidly identified using relatively small mapping populations. The search for candidate genes is ongoing.

Regarding the second category, cancer-causing genes, we hypothesize that the moderate T-ALL incidence in each line represents a requirement for somatic "second hits" occurring in proto-oncogenes or tumor suppressors. To identify these genes, we are using a combination of complementary technologies including the following: micro-array transcriptome analysis to identify dysregulated genes, aCGH to identify amplified or deleted loci, and MeDIP to identify changes in DNA methylation patterns. These studies have uncovered several candidate oncogenic loci, and current progress will be presented.

Finally, regarding cancer-modulating genes, serial transplantation of malignant T cells into irradiated recipients leads to a more aggressive disease with accelerated progression to death. In addition, affected zebrafish from each line show a marked reduction in tumor burden in response to irradiation treatment, but typically relapse with rapid progression to death. We hypothesize that the aggressive disease seen in both situations results from somatic mutations, either newly acquired, or already present in a subclone now evident through selective pressure. Comparison of the final and initial T-ALL clones should reveal genetic, epigenetic and genomic differences that are responsible for the more severe phenotypes. Using the technologies cited, these salient differences are currently being identified.

Identification of the SETDB1 histone methyltransferase as a new oncogene in melanoma
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Cancer is initiated and maintained by successive genetic alterations that lead to enhanced tumor development, coupled with invasion and metastasis. In a variety of solid tumors, genomic copy number is a major mechanism of such modulation. Human melanoma is associated with copy number gain at specific genomic loci, and yet most of the genes in these intervals that participate in melanoma have not been molecularly defined. Here, we have developed a novel strategy to test large numbers of genes within these intervals for their effect on melanoma progression. This approach utilized a zebrafish strain that reliably forms melanomas due to expression of *BRAF^{V600E}*, an oncogenic variant found in roughly half of all human melanomas, coupled with a loss-of-function allele of *p53*. When the *BRAF^{V600E}* transgene and *p53* mutation are put into a melanocyte-deficient *mitfa* mutant background, melanoma formation is completely suppressed. Injection of a rescuing *mitfa* minigene into *BRAF^{V600E};p53;mitfa* mutants leads to mosaic rescue of melanocytes and subsequent melanoma. Tumors arise at 3 to 4 months of age at a reproducible rate. We developed a plasmid vector called miniCoopR that harbors the *mitfa* minigene and a cassette with the *mitfa* promoter driving cDNAs of interest. This vector allows for the testing of candidate genes in regions of genome amplification in human melanoma. We examined all genes in the 1q21 region that are recurrently amplified and overexpressed in human melanoma. Our screen identified *SETDB1*, an H3K9 histone methyltransferase, as the sole gene in the 1q21.3 interval that cooperates with *BRAF^{V600E};p53$^{-/-}$* to increase the rate of tumor formation. Human melanoma lines with amplified *SETDB1* have tri-methylation of H3K9 on Western blot analysis, but lines with the lowest level of *SETDB1* lacks H3K9 tri-methylation. This suggests that SETDB1 is the major H3K9 histone methyltransferase for tri-methylation in melanoma cells. Knockdown of SETDB1 in human melanoma cells decreases cell proliferation. This study demonstrates that screening candidate oncogenes using this whole animal vertebrate zebrafish system is robust, scalable and offers a unique approach to discovering oncogenes in human cancer.

Genetic background dependent pigment cell tumor formation in a transgenic medaka melanoma model

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Melanoma is a tumor with a very low cure rate once metastasized. Although many genes important for melanoma induction, transformation and metastasis have been identified, the process of melanomagenesis is only partly understood. The analysis of melanoma mediators is easiest to investigate in cell culture models, but animal models are clearly required to evaluate their importance in the context of the whole organism. The Xiphophorus melanoma model has been useful to study the molecular mechanisms that lead to pigment cell transformation and tumor development. The melanoma-inducing oncogene encodes Xmrk, a constitutively active mutant version of the epidermal growth factor receptor. Overexpression of Xmrk leads to malignant transformation of pigment cells. The critical intracellular signaling events that are responsible for establishing the neoplastic phenotype including stimulation of proliferation, suppression of pigment cell differentiation, antiapoptosis, survival at ectopic sites and tumor cell migration have been identified. To further analyze Xmrk function in a transgenic fish, stable transgenic lines of medaka were produced that express the *xmrk* cDNA under control of the pigment cell specific mitf promoter. Several lines were established that develop pigmentary disorders ranging from benign hyperpigmentation to highly malignant pigment cell tumors beginning at larval stages and with 100% penetrance. Depending on the genetic background, invasive melanoma, uveal melanoma or exophytic and less aggressive pigment cell tumors, derived from xanthoerythrophores, occurred. These pigment cell tumors were shown to perfectly recapitulate the oncogenic molecular changes known from the Xiphophorus system. The model was used for a first monitoring of the in-vivo relevance of several apoptosis and differentiation genes and for the induction of melanoma-relevant signal transduction pathways. We found that Stat5 activation and Mitf- and Bcl-2 levels correlated with a more aggressive stage of the malignancy. Interestingly, on p53 negative background the expression of *xmrk* led to the appearance of giant focal pigment cell tumors, while tumor onset was unchanged compared to p53 wildtype medaka. Double transgenic lines are produced to study the effect of putative tumor modifiers.

Retinoic acid receptor (RAR) antagonists inhibit miR-10 a expression and block metastatic behaviour of pancreatic cancer

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Background & Aims: The infiltrating ductal adenocarcinoma of the pancreas is among the most lethal of all solid malignancies, largely due to a high frequency of early metastasis. We transplant here for the first time human tumour fragments into zebrafish embryos and identify microRNA-10a (miR-10a) as an important mediator of metastasis formation in pancreatic tumours. We further investigate the upstream and downstream regulatory mechanisms of miR-10a expression and function.
Methods: Northern blot analysis revealed elevated expression levels of miR-10a in metastatic pancreatic adenocarcinoma. The role of miR-10a was analysed by Morpholino and siRNA transfection of pancreatic carcinoma cell lines and resected specimen of human pancreatic carcinoma. Metastatic behaviour of primary pancreatic tumours and cancer cell lines was tested in xenotransplantation experiments in zebrafish embryos.
Results: We show that miR-10a expression promotes metastatic behaviour of pancreatic tumour cells and that repression of miR-10a is sufficient to inhibit invasion and metastasis formation. We further demonstrate that miR-10a is a retinoid acid target and that retinoic acid receptor (RAR) antagonists effectively repress miR-10a expression and completely block metastasis. This anti-metastatic activity can be prevented by specific knock down of HOX genes, HOXB1 and HOXB3. Interestingly, suppression of HOXB1 and HOXB3 in pancreatic cancer cells is sufficient to promote metastasis formation.
Conclusions: These findings demonstrate that miR-10a is a key mediator of metastatic behaviour in pancreatic cancer which regulates metastasis via suppression of HOXB1 and HOXB3. Inhibition of miR-10a expression (with RAR antagonists) or function (with specific inhibitors) is a promising starting point for anti-metastatic therapies.

Chemical colitis models in zebrafish larvae

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Inflammatory bowel disease (IBD) is characterised by aberrant host-microbe interactions. Epithelial dysfunction, most commonly studied *in vivo* using chemically-induced colitis, is necessary and possibly sufficient to trigger the escalating inflammation characteristic of IBD. The relative contributions of genetic and environmental factors to the aetiology of colitis have, to date, mainly been studied separately. Zebrafish provide a system where these interacting factors can be investigated together *in vivo*.

To establish doses for inducing colitis in zebrafish, larvae were exposed to standard chemicals used in murine experiments. An acute zebrafish colitis model was generated with key characteristics shared with murine models of gut inflammation. The development of inflammation was co-dependent on microbiota. There was a requirement for the Toll-like receptor adaptor molecule Myd88 for survival. Alteration of microbial diversity was also noted, a feature typical of colitis.

Analysis of gene expression following chemical exposure demonstrated both the induction of an inflammatory state within the larval gut and changes to regional markers of intestinal epithelial cell differentiation. Increased pro-inflammatory cytokine transcription was prevented by the administration of broad-spectrum antibiotics or a non-steroidal anti-inflammatory drug.

Studies of intestinal function during inflammation revealed impaired lipid metabolism and reduced intestinal vascularisation. These functional phenotypes were associated with increased intestinal cell proliferation suggesting an active damage repair response during inflammation.

This study has delineated similarities between chemically-induced colitis in zebrafish larvae and human IBD. The responsiveness of the phenotypes to pharmacological modulation further demonstrates the applicability of the zebrafish to model complex disease processes.

Disruption of the Troponin Complex Leads to Loss of Sarcomeric Integrity in Skeletal Muscle
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In myofibrils contractile filaments are organised in a highly regular fashion and are associated with several other proteins required for regulation of contraction and response to physiological stimuli. The basic unit of contraction, the sarcomere, is composed of thin filaments (Actin, Tropomyosin, Troponin, Nebulin, etc) intercalated with thick filaments (mainly Myosin), attached to the Z-disks and M-lines respectively. In striated muscle, regulation of contraction is mediated by the Troponin complex, which confers calcium dependence to the actin-myosin interactions. The three subunits of the Troponin complex are Troponin-C, which is required to bind calcium ions; Troponin-I, which is required to inhibit interaction of myosin heads with actin; and Troponin-T, which anchors the complex to the thin filament by binding to Tropomyosin. All of the subunits of the Troponin complex are implicated in human genetic diseases of the muscle, including forms of cardiomyopathy, nemaline myopathy and distal arthrogryposis. Despite the medical relevance there are few animal models available to study mechanisms underlying the different pathologies. In zebrafish, because of the ancestral teleost genome duplication, *troponin* genes are found in multiple copies. Genes in the same family have diversified, and in some cases show partially overlapping patterns of expression, displaying specificity to cardiac, skeletal slow or skeletal fast muscle.

We have undertaken a systematic analysis of the function of sarcomeric proteins in sarcomere assembly using loss of function strategies in the zebrafish. Muscle in zebrafish larvae is easily accessible and terminal differentiation of muscle cells in the trunk is completed by 48 hours post fertilisation (hpf). To understand the role of the Troponin complex during sarcomere assembly, we have focussed on skeletal muscle isoforms of the T and I subunits.

We have used antisense morpholino oligonucleotides to disrupt expression of the previously reported *tnnt1*, *tnnt3a* and *tnnt3b*, as well as other genes of the same family that have emerged with improved annotation of the zebrafish genome. We have also identified and characterised a mutation in the *tnni2* gene, which is the first animal model of a fast-twitch muscle specific, Troponin complex subunit. We find that in the absence of functional components of the Troponin complex sarcomere assembly is impaired. Specifically, we find that disruption of the slow twitch subunit T1 prevents sarcomere formation, whereas disruption of the fast-twitch components T3 or I2 affects maintenance of sarcomere structure.

Genetic analysis reveals a zebrafish model for fraser syndrome and identifies potential novel disease genes

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Fraser Syndrome is a congenital condition characterized by syndactyly and chryptophthalmos. Approximately 50% of investigated causes are due to mutations in one of two large extracellular matrix protein encoding genes, *FRAS1* and *FREM2*, whereas the causative genetic lesions in the other half of Fraser syndrome patients are unknown. Analysis of mouse Fras mutants suggest that embryonic blistering of the apical ectodermal ridge of the limbs underlies the syndactyly seen in Fraser patients. Here we describe the positional cloning of a number of zebrafish embryonic fin mutants, which can be grouped into two phenotypic classes. Whilst mutants of the first class display embryonic fin degeneration, the mutants of the second class display blistering of the epidermis of the fin fold. Electron microscopy demonstrates that fin blisters occur at the level of the sublamina densa of the basement membrane, as seen in mice mutant for components of the Fraser complex. Indeed, two of mutants are due to mutations in zebrafish orthologues of *FRAS1* and *FREM2*. Critically, we identified that another fin blistering mutant is caused by mutations in the gene encoding the zebrafish orthologue of Hemicentin1. This is the first description of a role for this extracellular matrix protein in vertebrates and, along with genetic interaction data, suggests a potential involvement in the Fraser complex. Furthermore, we present biochemical and genetic interaction data demonstrating a role for the proprotein convertase FurinA in membrane shedding of Frem2 and Fras1 proteins, and identify Fibrillin2 as an essential interacting partner of Hemicentin1. Thus through identification of the genes mutated in a number of zebrafish fin mutants, we have demonstrated that zebrafish is a relevant model for human distal limb anomalies, and can serve as a useful tool for the identification of novel disease genes.

Ceylon: a zebrafish mutant with Shwachman-Diamond syndrome-like bone marrow failure

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Shwachman-Diamond syndrome (SDS) is a bone marrow failure syndrome that frequently results in leukemia. SDS is also characterized by exocrine pancreas insufficiency and skeletal abnormalities. Eighty percent of patients with SDS have mutations in the *SBDS* gene. The SBDS protein product has been implicated in RNA metabolism but its function has not been fully elucidated. As twenty percent of SDS patients do not have mutations in the *SBDS* gene, mutations in other genes can result in a clinical SDS phenotype. However, to date no other genetic lesions have been identified that cause SDS.

Here, we describe the zebrafish mutant *ceylon* (*cey*) that was originally identified in a screen for immunodeficiency. *cey* has an SDS-like phenotype characterized by skeletal abnormalities and exocrine pancreas insufficiency. Furthermore, primitive and early definitive hematopoietic stem cells (HSCs) are normal, while late HSCs are severely reduced, similar to the bone marrow failure in SDS patients. Thus, the *cey* lesion may represent a mutation that leads to an SDS-like syndrome in humans.

Similar to *cey*, *sbds* morpholino-injected embryos exhibit defective exocrine pancreas development and dysregulated definitive hematopoiesis. However, skeletal abnormalities have not been observed in Sbds knockdown embryos. We have mapped the *cey* lesion to chromosome 3 and have identified candidate genes. In contrast, the zebrafish *sbds* gene maps to chromosome 15, and is therefore distinct from *cey*. We are in the process of determining if the *cey* gene product and Sbds interact genetically or if the *cey* lesion represents a new pathway that leads to SDS-like disease when mutated.

Identification of the *cey* lesion and its potential interaction with *sbds* could be useful for prenatal diagnosis and reveal possible therapeutic strategies for SDS patients without a mutation in the *SBDS* gene.

Cellular senescence and DNA damage in a zebrafish model of Costello syndrome is linked to ubiquitin-mediated proteosomal degradation of oncogenic HRAS

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Some tissues are more affected than others by the expression of oncogenic Ras. This is confirmed by the preferential association of Ras mutations with certain types of cancers. We have developed several zebrafish models where oncogenic HRAS can be expressed at different levels and in different cell types, using promoters like hsp70, UAS in combination with different GAL4 driver lines. Moreover we have generated a model where HRAS is expressed in a ubiquitous and constitutive fashion.
Expression of oncogenic HRAS in our models does not necessarily lead to cellular transformation, even if the oncogene is highly expressed. We therefore examined the reasons for this cell type specific "resistance" to oncogenic transformation in our model of constitutive HRAS expression (which mimics Costello syndrome, Santoriello et al., 2009). The model shows brain and heart cellular senescence, while liver and kidney are hyperproliferating. Analysis of oncogenic H-RAS post-translational modifications (PTMs) shows that in heart and brain, oncogenic HRAS is ubiquitinated and targeted for degradation. In parallel we checked HRAS ubiquitination in senescence and proliferating human fibroblasts, which have been transduced with oncogenic HRAS. Our results show that in senescent cells oncogenic HRAS is ubiquitinated, whereas in proliferating cells it is not significantly bound to ubiquitin.
These data establish a link between a novel PTM of oncogenic HRAS (which presumably reduces its ability to activate downstream targets and transform) and oncogene-induced cellular senescence (a mechanism that allows cells to escape RAS induced transformation).
We are now investigating the machinery involved in ras ubiquitination. Microarray data obtained in our zebrafish model of Costello syndrome show an increase of the expression of a number of ubiquitin conjugating enzymes. These are candidates for processing mutant HRAS into a less active oncogene and target it for degradation. We are investigating whether blocking proteosomal degradation restores HRAS ability to activate ERK and AKT, which is lost in zebrafish larvae constitutively expressing oncogenic ras and in our zebrafish model of Costello syndrome.

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A modified acetylcholine receptor d-subunit enables a null mutant to survive beyond sexual maturation

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The contraction of skeletal muscle is dependent upon synaptic transmission through acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ). The lack of an AChR subunit causes a fetal akinesia in humans, leading to death in the first trimester and characteristic features of Fetal Akinesia Deformation Sequences (FADS). A corresponding null mutation of the d-subunit in zebrafish (*sofa potato; sop*$^{-/-}$) leads to the death of embryos around 5 days post-fertilization (dpf). In *sop*$^{-/-}$ mutants, we expressed modified d-subunits, with one (d1YFP) or two yellow fluorescent protein (d2YFP) molecules fused at the intracellular loop, under the control of an a-actin promoter. AChRs containing these fusion proteins are fluorescent, assemble on the plasma membrane, make clusters under motor neuron endings, and generate synaptic current. We screened for germ-line transmission of the transgene and established a line of *sop*$^{-/-}$ fish stably expressing the d2YFP. These d2YFP/*sop*$^{-/-}$ embryos can mount escape behavior close to that of their wild type siblings. Synaptic currents in these embryos had a smaller amplitude, slower rise time, and slower decay when compared to wild type fish. Remarkably, these embryos grow to adulthood and display complex behaviors such as feeding and breeding.
To the best of our knowledge, this is the first case of a mutant animal corresponding to first trimester lethality in human that has been rescued by a transgene and survived to adulthood. In the rescued fish, a foreign promoter drove the transgene expression and the NMJ had altered synaptic strength. The survival of the transgenic animal delineates requirements for gene therapies of NMJ.

Role of notch signaling in early zebrafish cardiogenesis
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Congenital heart defects account for at least 50% of deaths amongst sufferers of Alagille syndrome, a disease usually associated with a haplodeficiency of the *Jagged1* or the *Notch2* gene, and these genes encode members of the Notch signaling pathway. This pathway has been shown to establish subpopulations within the cardiogenic mesoderm in *Drosophila melongaster* and *Xenopus laevis*. During early mouse and chick embryogenesis, the cardiogenic mesoderm is segregated into two distinct cell populations. The first heart field is the first population to differentiate into myocardium and form the initial, non-proliferating myocardial heart tube. The second heart field is maintained in an undifferentiated state as a progenitor cell population that gradually differentiates into myocardium and smooth muscle and adds to the heart tube. Understanding how and when these two populations are formed, and how their differentiation is coordinated, are fundamental to understanding heart morphogenesis. I propose that the Notch pathway establishes these subpopulations of the cardiogenic field by delaying the contribution of the second population to heart morphogenesis until after initial myocardial tube formation. Having chosen zebrafish for its genetic tractability, an RNA *in situ* hybridization screen of the Notch pathway genes led to selecting *zfjagged1b* and *zfnotch2,* the zebrafish orthologues of the Notch pathway members associated with Alagille syndrome as candidates for controlling early cardiogenesis. Knocking down these genes in the zebrafish embryo via morpholinos demonstrated that both regulate the myocardial cell number in the initial myocardial tube but do so by two separate mechanisms. The timing and extent of the increase in myocardial cell number in *zfjagged1b* morphants suggests premature differentiation of the second heart field whereas that by *zfnotch2* suggests an increase in the proliferative index of the initial myocardial tube. These results show a perfect example of how multifaceted Notch signaling is in a small time window of organ development.

Bungee – A novel regulator of cardiac valve formation in zebrafish

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Congenital heart defects are the leading cause of death amongst newborns. Although within the group of cardiovascular malformations a high percentage accounts for defects of heart valves, so far little is known about disease causing genes and their respective function. To characterize new signaling pathways involved in cardiac valve formation, the zebrafish has become an excellent model organism.

In a forward genetic screen of zebrafish mutants with defects in cardiac valve formation, we isolated the recessive, embryonic lethal mutant *bungee* (*bng*^{IH177}). While first steps of cardiac development including chamber formation proceed normally in *bng* mutant embryos, around 72 hours post fertilization (hpf) *bng* mutant hearts dilate and blood regurgitates from the ventricle into the atrium leading to a progressive loss of blood circulation. Histological analysis and confocal microscopy of transgenic *fli*:GFP zebrafish revealed that *bng* mutant hearts have a constricted bulbus arteriosus and widened atrio-ventricular canal (AVC), missing endocardial cushions, the precursors of heart valve leaflets. Altered expression of important endocardial (*notch1b* and *has2*) and myocardial (*versican* and *bmp4*) markers for heart valve development suggests that the failure of heart valve formation in *bng* mutant embryos is due to perturbed signalling between endocardial and myocardial cells within the early formation of the AVC.

By positional cloning we revealed that the *bungee* phenotype is caused by a point mutation within a gene encoding a zebrafish Serine/Threonine protein kinase, leading to a substitution of a Thyrosine to an Asparagine within the highly conserved kinase domain. Injection of mRNA in *bng* mutant embryos restores the wild-type phenotype, while injection of morpholino-modified antisense oligonucleotides directed against a splice-donor site inside the kinase-domain phenocopies the *bng* mutant phenotype. By *in situ* hybridisation we demonstrate that expression of the *bungee* protein kinase within the embryonic heart is restricted to the endocardial cell layer of the atrio-ventricular canal, suggesting an important role for proper AVC and heart valve development.

Serine/Threonine kinases are known to be involved in multiple signaling pathways. Here we show for the first time that the *bungee* kinase plays an essential role during heart valve formation.

Chemokine signaling contributes to regional patterning of the first embryonic artery

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The aorta traverses the length of the vertebrate body to deliver oxygenated blood to the systemic arterial circulation. In zebrafish embryos, this artery is "Y"-shaped, consisting of two lateral dorsal aortae (LDA) in anterior regions of the embryo and a single dorsal aorta (DA) in the trunk. Much progress has been made in elucidating the mechanisms leading to DA formation, which has been shown to develop via vasculogenesis, when angioblasts from the lateral plate mesoderm migrate towards the midline. In contrast to this, the mechanisms and signaling molecules contributing to the formation of the branched LDA have remained elusive. In this study we show that the LDA form in a bidirectional manner from endothelial cells originating in the anterior and posterior lateral plate mesoderm. Furthermore, we show that these endothelial cells are genetically distinct. In particular, we find that expression of the *chemokine (C-X-C motif) receptor 4a (cxcr4a)* specifically labels endothelial cells of the anterior LDA. Accordingly, targeted inactivation of *cxcr4a* leads to a specific defect in anterior LDA formation. We further show that *cxcl12b*, a ligand for *cxcr4a*, is expressed in endoderm adjacent to the anterior branches of the LDA and that loss of *cxcl12b* phenocopies *cxcr4a* deficiency. Thus, our results suggest that an endoderm-derived chemokine specifically guides the development of the branched region of the aorta, while the single DA in the trunk remains unaffected. Taken together, our studies reveal an unexpected molecular diversity within arterial endothelial cells and show that this differentiation is necessary to facilitate regional patterning of the developing aorta by local extrinsic cues.

MiR-1 regulates angiogenesis by modulating VEGF signaling
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Cell signaling between different tissues ensures coordinated development during organogenesis. A fundamental question in the field is to understand how these signals are modulated in vivo. MicroRNAs have recently emerged as important modulators gene expression that repress mRNA translation and accelerate target mRNA decay. However, it remains a challenge to identify their targets and dissect the functional relevance of individual miRNA-target interactions in vivo. Our previous study identified a large set of target mRNAs regulated by miRNAs during muscle development. Analysis of these targets revealed that a fraction of them were also expressed in the vasculature. Conversely, genes expressed in the vasculature were enriched for the muscle miR-1 target site in their 3'UTRs compared to a control set of genes. These observations suggested the hypothesis that the muscle miR-1 might regulate the cross talk between the muscle and the vasculature during development. To test this hypothesis, we analyzed whether loss of miR-1 function affected vasculature development. Inhibition of miR-1 with two non-overlapping MOs caused an increased in the area of intersomitic vessels and the number of cells from 3 cells in wild type to 6-9 cells in miR-1 morphants. These results are reminiscent of an increase in VEGF signaling or a loss of Notch signaling during intersomitic vessel formation. To distinguish between these possibilities we performed clonal analysis and epistasis experiments. First, wild type cells labeled with the vascular marker fli1-GFP transplanted into miR-1 MO embryos show the vasculature overgrowth phenotype, suggesting that the effect of miR-1 is non-autonomous. Second, epistasis experiments using a kdrl mutant embryos and miR-1 loss of function revealed that the intersomitic overgrowth phenotype require a functional receptor. Thus the effect of miR-1 LOF is likely to act upstream of the receptor. Third, we searched the components of the VEGF pathway for miR-1 target sites. Sequence analysis and reporter studies revealed that VEGFa includes three functionally conserved miR-1 target sites in their 3'UTR able to regulate expression of a reporter mRNA. Taken together these results suggested that miR-1 regulates VEGF expression levels in the muscle thus controlling angiogenesis during intersomitic vessel formation. To test this hypothesis we blocked miR-1 function in wild type and VEGFa knock down embryos. Reducing the levels of VEGFa suppressed the miR-1 knock down phenotype. We are currently dissecting the regulation of individual target sites in VEGF using target protector MOs (TP). These TPs bind the target sites in the 3'UTR and block miRNA-mediated repression of the target mRNA. These results will be presented at the meeting. Taken together these results support a model where miR-1 regulates the potent ligand VEGFa to regulate the crosstalk between the muscle and the vasculature to modulate angiogenesis during intersomitic vessel formation.

The zebrafish *full-of-fluid* mutant identifies a secreted protein essential for lymphangiogenesis in zebrafish and humans

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The lymphatic vascular system plays pivotal roles in fluid homeostasis, fat resorption, the immune response and cancer metastasis. Only very few factors regulating the initial aspects of lymphangiogenesis are known, such as the transcription factor Prox-1 and VEGFC, a secreted ligand that acts through Vegfr3 to direct lymphangiogenesis during development and disease.
We have recently demonstrated that zebrafish possess a lymphatic system that can be both imaged *in vivo* as well as manipulated genetically. We have performed a forward genetic screen to identify genes required for embryonic lymphangiogenesis and identified three mutants to date. Positional cloning found one mutant, *full-of-fluid*, to encode a novel predicted secreted protein, Ccbe1. Ccbe1 is expressed in the somitic mesoderm along the migration pathway of lymphangioblasts, the earliest precursors of the lymphatic system, and acts non-autonomously during lymphangiogenesis. Using a series of new transgenic lines to visualize the development of the lymphatic vasculature, we found that the loss of either *ccbe1* or Vegfc-Ft4 signalling leads to an identical failure of lymphangiogenesis and hemangiogenesis at the level of endothelial cell sprouting from the posterior cardinal vein.
Significantly, we have recently identified a human patient population that develops lymphedema with genetic linkage to the human *CCBE1* locus. We demonstrate here that these patients have molecular lesions in the human *CCBE1* gene and that the equivalent mutations inhibit the function of zebrafish *ccbe1* in lymphangiogenesis. These studies identify a novel conserved regulator of lymphangiogenesis in the predicted secreted protein Ccbe1 and highlight its critical role in vertebrate development and human disease.

Characterization of vascular mural cells during zebrafish development

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Development and maturation of the nascent cardiovascular system requires the recruitment of mural cells (MCs) around the vascular tree in a process called vascular myogenesis. Understanding the origins of vascular MCs has been hampered by difficulties in observing these cells *in vivo* and in performing defined genetic and experimental manipulations in available model organisms. Here, we investigate the origin of vascular MCs using molecular and genetic tools in zebrafish. We show that vascular MCs are present around the lateral dorsal aortae (LDA), and anterior mesenteric arteries (AMA) of developing animals and that they also contribute to the ventral aorta (VA) and outflow tract of the developing heart. Our genetic data further indicate that the vascular MCs of the trunk vessels do not arise from blood or endothelial progenitors but from other derivatives of the lateral plate mesoderm. Thus, zebrafish vascular MCs share many of the morphological, molecular and functional characteristics of the vascular smooth muscle cells and pericytes found in higher vertebrates, establishing zebrafish as a useful cellular and genetic model to study vascular myogenesis as well as tumor angiogenesis and other MCs-associated diseases.

Both primitive and definitive hematopoiesis arise from hemogenic endothelial cells in the zebrafish embryo

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Development of the blood system is complex, with multiple waves of hematopoietic precursors arising in different embryonic locations. Monopotent, or primitive, precursors first give rise to embryonic macrophages or erythrocytes. Multipotent, or definitive, precursors are subsequently generated to produce the adult-type lineages. In both the zebrafish and mouse, the first definitive precursors are committed erythromyeloid progenitors (EMPs) that lack lymphoid potential. We have previously shown that zebrafish EMPs arise in the posterior blood island independently from hematopoietic stem cells (HSCs) that arise along the ventral side of the aorta. By using the CRE/LOX technology, recent reports have nicely showed that vascular endothelial cells on the ventral side of the aorta were giving rise to HSCs in the mouse embryo.

Here we show similar results in the zebrafish, that HSCs are also born from hemogenic endothelial cells (ECs). We used the Flk1:CRE transgenic line. Unlike in mammals, the zebrafish *flk1* promoter used here is not pan-mesodermic, so we could specifically trace the progeny of ECs in the zebrafish by crossing this CRE line to a switch-reporter line. This latter consists of the b-actin promoter driving the expression of DsRED after a STOP cassette is excised by the CRE recombinase. Double transgenic adults were sacrificed at 3 months, and in all animals (n=30), 100% of adult leukocytes were switched (DsRED+). Moreover, we could show that "switched" cells harbor long-term reconstitution when transplanted into irradiated recipients. Thus, zebrafish HSCs originate from ECs in the embryo. To corroborate these findings, we could isolate cells during their transition from EC to hematopoietic cells using the flk1:RFP and cmyb:GFP reporter lines, and show that, during the transition, the endothelial genetic program is shut down, while hematopoietic genes are turned on.

As stated above, three other hematopoietic lineages arise during embryogenesis: primitive macrophages/erythrocytes and definitive EMPs. Whether or not these other lineages arise from hemogenic ECs is still unknown. To answer that question, we sorted "switched" cells at 27-30 hours post fertilization, when primitive lineages and EMPs, but not HSCs, have been generated, and characterized them. To further investigate this issue, switched adult fish have been crossed to transgenic lines that mark primitive macrophages and EMPs in order to determine if these subsets also come from *flk1*+ vascular cells. Altogether, preliminary data indicate that all waves of hematopoiesis might originate from hemogenic ECs.

These results should give new insights on the generation of blood cells in the vertebrate embryo.

SDF-1 Expressing Cells Establish the Hematopoietic Stem Cell (HSC) Niche and Play a Role in HSC Homing Following Adoptive Cell Transfer in a Zebrafish Model of Bone Marrow Transplant

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The zebrafish, *danio rerio*, is increasingly becoming an important model in unraveling the development of hematopoietic cells. Recent work has also shown that myeloablative hematopoietic stem cell (HSC) transplant can be accomplished in the zebrafish. How the transplanted HSC find their way to the HSC niche and engraft is unknown in the zebrafish and currently the subject of much experimentation in the mammalian systems. In mammals the HSC niche is thought to contain various stromal cells. These cells have been known secrete stromal derived factor-1 (SDF-1), an important mediator that functions to recruit CXCR4 expressing HSCs to a supportive environment essential for fostering long-term hematopoiesis. To identify potential HSC niche-establishing cells in the zebrafish and to understand their role during marrow transplant, we created an SDF1a transgenic zebrafish by fusing the SDF1a proximal promoter to dsRed2. We have found dsRed positive cells in areas where SDF1 has previously been shown to be important in organogenesis including the heart, notochord, fins, myofibers and retinal axons. As the fish mature, we also found dsRed positive expression in the kidney tubular epithelial cells, which was maintained throughout adulthood. These cells showed in increase in numbers when fish were gamma-irradiated which correlated with an increase in SDF1a RNA expression as expected following myeloablative radiation. Finally, marrow from donor fish which was pretreated with AMD3100 (to block CXCR4) failed to home to the kidney region immediately after transplant giving an indication that the SDF1-CXCR4 axis contributes to cellular homing in the zebrafish during the transplant process as it does in mammalian systems. The creation of this tg(sdf1a:dsRed2) zebrafish line will be important in elucidating the mechanisms by which HSC interact with their niche under steady state and stress hematopoiesis conditions. In addition, this line should prove useful in the analysis of the homing process involving organogenesis and regeneration/repair of other organ systems that express SDF-1 during embryogenesis and postnatally.

Live imaging reveals that definitive haematopoietic stem cells emerge directly from haemogenic endothelial cells in zebrafish embryos

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The identity of the cells that give rise to the first haematopoietic stem cells (HSCs) in the developing embryo has long been controversial. HSCs have been proposed to emerge from mesodermal cells, mesenchymal progenitors, a bipotential endothelial-haematopoietic precursor (the haemangioblast) or the haemogenic endothelium. In mammalian embryos, some resolution to this debate has recently come from *in vitro* live imaging technology that shows evidence for the central role of haemogenic endothelial cells in the generation of primitive and definitive blood. Time-lapse microscopy revealed that bipotential haemangioblasts generate primitive blood cells through formation of a transient haemogenic endothelial stage *in vitro*. Similarly, the cell fates of single mouse mesodermal cells were tracked by long-term imaging to show that they can generate haemogenic endothelial cells that directly give rise to blood cells in culture. However, as these studies were performed *in vitro,* they still do not definitively identify the haemogenic endothelium as the origin of HSCs within the organism.
Here, we provide the first demonstration of the direct emergence of definitive HSCs from the haemogenic endothelium in a living embryo. The haematopoietic program is very conserved in vertebrates. In zebrafish, as in mammals, definitive HSCs are believed to have their origins in the haemogenic endothelium. Runx1, a transcription factor essential for the development of HSCs in all vertebrates is also required for the transition from haemogenic endothelium to HSCs in mouse and zebrafish. We have generated a zebrafish Runx1 transgenic line, *Tg(runx1P2:EGFP)* marking definitive HSCs in the aorta-gonad-mesonephros (AGM) that later populate the pronephros and thymus. *Kdr (Flk-1)* is expressed in haemangioblasts and in early endothelial cells. Double *Tg(runx1P2:EGFP/kdrl:nls-mCherry)* transgenic lines express *mCherry* in the nuclei of endothelial cells, while HSCs emerging from the AGM express EGFP. During time-lapse imaging of these compound transgenic embryos over 11 hours (24-33 hours post fertilisation), all of the cells that initiate expression of EGFP, were red fluorescent mCherry-expressing endothelial cells. This demonstrates that haematopoietic cells in the AGM arise from further differentiation of endothelial cells, providing strong *in vivo* evidence for a 'haemogenic endothelium'.

Development of an immune-matched transplantation model to detect hematopoietic stem cell activity in zebrafish

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Transplantation of hematopoietic cells, tumors or any allogeneic tissue has been hindered in the zebrafish due to poor understanding of the functional major histocompatibility complex (MHC) genes and inability to immune-match donors and recipients. Despite this, the zebrafish has been used prominently for the study of hematopoiesis, due to the advantages of large scale forward and reverse screening methodologies leading to the discovery of novel genetic mechanisms. However, methodologies to assess hematopoietic stem cell (HSC) function are largely undeveloped. Here, we created a quantitative long-term hematopoietic reconstitution assay in adult zebrafish. We identified a sublethal radiation dose of 25Gy which was optimal for hematopoietic reconstitution while minimizing the mortality presumably due to radiation damage of other organs. At 3 months post-transplant, primary and secondary recipients showed multi-lineage engraftment, as measured by flow cytometric analysis of GFP-expressing donor cells. Statistical analyses of limiting dilution data suggest that at least 1 out of 65,000 nucleated zebrafish marrow cells contain repopulating activity, consistent with mammalian HSC frequencies. We then defined zebrafish haplotypes at the proposed core MHC locus on chromosome 19, testing the functional significance of these haplotypes by performing matched and mismatched transplants. Utilizing a single family for sibling marrow transplants, we identified the four parental MHC haplotypes by sequencing PCR products amplified for specific genes. MHC-typed recipient fish were transplanted with whole kidney marrow cells from sibling donors, demonstrating that matching the donor and recipient MHC haplotypes at the chromosome 19 locus increases engraftment and percentage of donor chimerism in recipients compared to MHC-mismatched donors and recipients. At 3 months post-transplant, MHC-matched recipients had multilineage engraftment in 13 of 15 fish with mean donor chimerism of 47.86% (range 5.56- 93.44%) for myeloid cells and 10.51% (range 0.92-77.57%) for lymphoid cells. Engrafted animals receiving MHC-mismatched donor marrow (n= 4 of 6) had only 6.45% mean myeloid donor chimerism (range 4.58 to 8.58%) and 1.28% mean lymphoid donor chimerism (range 0.83 to 1.41%). These data represent the first assay allowing long term HSCs to be distinguished from other hematopoietic progenitors, and provide the first functional test of MHC genes between zebrafish haplotypes. This method will facilitate MHC-matched long-term transplantation experiments in zebrafish that have previously not been possible, including competitive transplantation experiments with zebrafish mutants already identified in prior genetic screens, and long-term tumor transplantation assays. By harnessing the unique genetic and screening advantages of the zebrafish model, such experiments may provide critical insight into mammalian transplantation biology.

Thymus colonization is under the control of chemokines and chemokine receptors
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Thymopoiesis is a complex process, involving colonization of the epithelial organ anlage by lymphoid progenitor cells, commitment of these progenitors to the T cell lineage. The transcription factor *Foxn1* has a key role in the establishing a functional thymic niche. Mutations in this gene are associated with a failure of thymopoiesis owing to a non-functional epithelial microenvironment. Given that thymic epithelial cells in *Foxn1*-deficient mice lack the expression of chemokine *ccl25*, we analyzed the expression of chemokines and chemokine receptors during thymus development. Of several chemokine receptors in medaka, only *ccr9a/b* receptors and their ligand *ccl25a* are expressed in the thymic rudiment. Moreover, the chemokine *cxcl12a* is expressed in the cells encapsulating the thymic epithelium, which is consistent with possible functions in attracting lymphocyte progenitors to the thymic primordium. Knock-down of *ccl25a* alone or in combination with *ccr9* genes specifically affected thymus homing, but did not abolish it. However, simultaneous interference with *ccl25a* and *cxcl12a* specifically and completely blocked thymopoiesis, suggesting that thymus colonization requires the cooperation of *ccl25a/ccr9* and *cxcl12a/cxcr4* chemokine/chemokine receptor pairs and might explain why thymus homing is not abolished in mice deficient for either Cxcl12/Cxcr4 or Ccl25/Ccr9.
Additionally, we provide evidence that *ccl25a* is a downstream target gene of *Foxn1*. To identify potential regulators for *ccr9*, we considered transcription factors that are highly expressed in thymocyte populations encompassing thymic immigrants that are known to express high levels of *Ccr9*. This analysis suggested *bcl11b* as a potential candidate. In medaka and zebrafish only one of the two *bcl11b* homologues (*bcl11b.2*) is expressed in the developing thymus. Indeed, our data suggest that *ccr9a/b* are downstream target genes of *Bcl11b*. Taken together, our results identify genetic networks regulating the homing of lymphocyte progenitors to the thymic microenvironment.

A zebrafish transgenic model to study antigen-presenting cells: b lymphocytes, macrophages and dendritic cells

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Host defense against infection requires an integrated response of both the innate and adaptive arms of the immune system. Linking innate and adaptive immunity are antigen-presenting cells (APCs), which play key roles in the orchestration of the various forms of immunity and tolerance to self-antigens. In mammals, the immune system contains three types of APCs: macrophages, dendritic cells (DCs) and B cells, with DCs being the most potent activators of naïve T cells. A major challenge to our understanding of APC biology lies in improving studies of dynamic cell behavior in vivo. Defining how, where and when APCs are produced, and later interact with the different immune cell compartments during the phases of an immune response are important for understanding disease etiology and the design of new treatments. Zebrafish have unique potential for examining specific aspects of immune development and function in ways not possible to other vertebrates. The hematopoietic system of zebrafish is highly conserved and features all major blood cell lineages found in mammals, including erythroid, thrombocytic, myeloid and lymphoid cells. We have previously identified putative DCs in the lymphoid organs of adult zebrafish by cytochemical analyses and electron microscopy. To enable in vivo imaging, prospective isolation, and functional analyses of APCs, we have generated new transgenic zebrafish lines. Because APCs rely upon class II Major Histocompatibility complex (MHCII) genes for antigen presentation, we identified, characterized, and utilized the $MHCII^{DAB}$ promoter to generate fluorescent transgenic animals. Founders were identified in which expression of the transgene is observed in all APC subtypes, including B cells, macrophages and DCs. Consistent with the role of MHCII during T cell development, expression of the transgene was also observed in thymic epithelial cells by 5 days post fertilization. Here we present the characterization of these APC-reporter lines following co-expression studies, FACS analyses, gene profiling and functional assays. The APC-reporter lines were crossed with available T cell-reporter lines, allowing an unprecedented view of APC/T cell interaction in primary (thymus) and secondary (spleen) lymphoid organs. By allowing the study of APCs in their natural environment, $MHCII^{DAB}$ transgenic lines should provide new insights into zebrafish immunity and the complex mechanisms that underlie APC ontogeny and functional behavior.

Negative regulation of neurogenesis by an FGF from neurons

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The precise regulation of neurogenesis is critical to generate the correct number of neurons at the appropriate location in the nervous system. This is achieved in specific regions of the vertebrate nervous system by the formation of distinct neurogenic and non-neurogenic zones. Despite the increasing progress in dissecting the mechanisms that control the temporal and spatial regulation of neurogenesis, little is known about the extrinsic signals involved in the formation of non-neurogenic zones. We have investigated how neuronal differentiation is patterned in the zebrafish hindbrain, in which neurogenesis becomes restricted to zones adjacent to boundaries. Our studies have identified a population of neural progenitors in neurogenic-free areas in segment centres. Using transgenic approaches, we have shown that FGFR signaling is required to restrict neurogenesis in the hindbrain by regulating this progenitor population. Moreover, we have identified an FGF secreted by specific neurons that maintains the non-neurogenic zones. Our findings reveal a novel mechanism in which signaling from neurons underlies the formation of a non-neurogenic zone of neural progenitor cells.

Segregated processing of visual inputin the optic tectum by interlaminar inhibition revealed by calcium imaging

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The optic tectum is the major processing center for visual information in zebrafish. Axons of retinal ganglion cells (RGCs) form synaptic connections with the dendrites of tectal neurons. The retinotectal projection is organized in a topographic fashion, re-establishing a map of visual space in the brain. We have previously discovered that subtypes of RGCs terminate in four distinct layers of the tectum, one of which is further divided into at least three sublayers (Xiao et al., 2005; Xiao & Baier, 2007). Thus, the retinotectal neuropil contains six or more parallel visuotopic maps. Moreover, studies in both the tectum of fish and superior colliculus of higher vertebrates have demonstrated a size selectivity, wherein neurons respond optimally to objects smaller than the receptive field size and decrease their response to larger or multiple objects even within the region that would normally elicit a positive response. We are interested in understanding the functional principles underlying the layered architecture of the tectum. Intriguingly, the main dendritic arbors of the majority of tectal neurons are arranged perpendicularly to the layers and appear to extend through the entire neuropil, stratifying in several of the visual and non-visual layers. This raises the question if the segregation of layered inputs is lost at the level of the tectal dendrites. As a first step toward answering this question, we generated a UAS:GCaMP1.6 transgenic line. GCaMP1.6 is a genetically encoded calcium indicator, which has proven useful in a variety of preparations including mouse brain slices and Drosophila antennal lobes to visualize presynaptic and postsynaptic activity. We used a series of GAL4 driver lines to express GCamP1.6 either in the retinal axons or in several subpopulations of tectal neurons and recorded in vivo light responses by functional imaging of the tectum. We demonstrated that dendritic processing of visual inputs remains confined to the most superficial layers of the tectum when the visual stimulus is broad covering the entire receptive field. This localization is dependent on the function of GABAergic cells located in the neuropil itself – only in experiments in which GABA action was blocked or these neurons were selectively ablated did the calcium responses spread to other layers. On the contrary, these neuropil located inhibitory neurons remain silent when the visual stimulus is localized. In this case we instead observed neuronal activity spreading from the superficial layers of the optic tectum to the deeper ones and to the cell bodies of the preiventricular neurons. Thus, broad visual inputs are segregated and confined to the superficial dendritic compartments of tectal neurons by a local circuit mediating interlaminar inhibition, allowing smaller stimuli to be selectively processed by the tectum. This neuronal circuitry provides an explanation for a central role of the tectum in "higher" visual function such as prey capture, while its activity is dispensable for more basic visual responses like visual background adaptation (VBA), optokinetic response (OKR), optomotor response (OMR).

SARA1, a TGFbeta signaling adaptor, regulates proliferation of the neural precursors
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Upon TGFbeta signaling, the activated TGFbeta receptors phosphorylate Smad transcription factors. These can then shuttle to the nucleus where they regulate gene transcription. Sara (Smad anchor for receptor activation) is an adaptor protein necessary to bring the R-Smad close to the activated receptors and allow the phosphorylation of the transcription factor. Sara contains a FYVE domain that confers it endosomal localization. Studies in our lab have shown that Drosophila SARA not only ensures the correct partition of TGFbeta signaling levels across mitosis in the wing disc (Boekel, Science 2006), but also that in the SOP there is directional Notch trafficking in Sara endosomes, unraveling a new mechanism of directional signaling (Coumailleau, Nature 2009).
We are studying the zebrafish SARA1 to perform a first study on this factor during vertebrate embryogenesis. The zebrafish genome contains two SARA genes: SARA1, containing the FYVE and Smad binding domain (SBD), and SARA2, without the SBD. We are focusing on SARA1, the homologous to the human SARA.
By knocking down the protein function with morpholinos, we have observed that zSARA1 modulates the ratio between the proliferating and differentiated cells during the neural development of the Zebrafish embryo. ZSARA1 can be unequally segregated during mitosis in the neuroepithelium and by tracking the fate of the daughter cells we could associate the inheritance of the zSARA1 endosomes with a proliferative state. We are now characterizing the zSARA1 endosome and analyzing it's cargo to clarify which signaling pathway, TGFbeta or Notch, is using the SARA endosomes to ensure the correct signaling levels across mitosis. We are also interested in knowing if zSARA1 is necessary to maintain the neuronal diversity in the Zebrafish neural tube.

Functional analysis of the habenula in control of fear

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The habenulae are part of an evolutionarily highly conserved conduction pathway within the limbic system that connects telencephalic nuclei to the monoaminergic neurons in the midbrain and hindbrain either directly or indirectly by way of the interpeduncular nucleus (IPN). In zebrafish, we showed a prominent asymmetric habenulo-interpeduncular projection caused by a prominent left-right (LR) difference in the size ratio of the medial and lateral subnuclei of the dorsal habenulae, each of which specifically projects either to the ventral or dorsal IPN targets. Furthermore, we have recently discovered that the neurons in the dorsal IPN specifically send the descending axons along the central gray in the hindbrain, and the neurons in the ventral IPN project to the raphe. Considering that the central gray is involved in instinctive defense behaviors such as freezing and that the raphe and its serotonin neurons are involved in more adaptive behaviors, we are now suspecting that the dorsal and ventral IPN may be involved in the alternative behavioral choice in the face of danger, such as whether to react in a panic or to cope with it in more sedate manners. To further investigate this hypothesis, we have established the transgenic zebrafish line expressing Gal4-VP16 specifically in the dorsal habenular subnuclei. By crossing such lines with other transgenic lines carrying the tetanus toxin gene or the nitroreductase gene under control of the target site of Gal4-VP16, we have succeeded in establishing the lines in which the neural signal transmission by way of the lateral subnucleus of the dorsal habenula is selectively impaired either constitutively or conditionally. In the fear conditioning, the manipulated fish showed extremely enhanced levels of the freezing response to the presentation of the conditioned light stimulus, suggesting the tract connecting the left-dominant lateral subnuclei of the dorsal habenula with the dorsal IPN may normally function to suppress excessive fear response. This result is especially intriguing if we take the previous report into consideration on the preferential right eye use by zebrafish when they are approaching novel objects.

Behavioral consequences of zebrafish epithalamic asymmetry
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We have been studying the relationship between asymmetry in the epithalamus and behaviors in larval and adult zebrafish. In >98% of wild-type zebrafish, the parapineal develops to the left of the pineal anlage and the adjacent left habenular nucleus is larger, has more dense neuropil and shows different gene expression than the right habenula. By disrupting the Nodal-signaling pathway, larvae can be produced that show a left-right (L-R) reversal of these asymmetries. Laser-mediated ablation of the parapineal causes the habenular nuclei to develop more symmetrically, both with properties resembling the right habenula. Because larvae with reversed asymmetry or symmetry in this brain region are viable, we can test their behaviors as well as those of the resulting adult fish. In prior studies, we found that larvae with reversed asymmetry were indistinguishable from their siblings in a variety of tests for motor activity and responses to vibrational or visual stimuli. In the mirror test, a commonly used assay for laterality in eye use, statistically significant differences were also not observed in 8-day wild-type larvae or in larvae with reversed asymmetry. Surprisingly, reversed larvae showed a significant reduction in navigational behavior, covering a shorter distance and a smaller swimming area than controls. As adults, the reversed fish tended to show a similar pattern of behavior. Parapineal-ablated larvae also exhibited reduced swimming activity. We are currently examining whether this altered behavior is mediated by the habenular nuclei and exploring how altered asymmetry might influence other neural pathways in the brain.

Atlastin controls zebrafish spinal motor axon architecture via inhibition of the bmp pathway

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Functional studies of genes responsible for several neurodegenerative disorders have recently unveiled various links between neurodegenerative processes and developmental signalling pathways. Concomitantly, some families of morphogens, such as the Bone Morphogenetic proteins (BMP), Wnt or Hedgehog, have been identified as guidance cues for axonal pathfinding and synaptic growth at later embryonic stages. Among these signalling molecules, the BMPs were shown to be specifically required for proper development of neuromuscular junctions (NMJ) and correct pathfinding of motor neuron axons in invertebrate models. To achieve significant progress in the understanding of spinal motor neuron degeneration, we performed lack- and gain-of-function analyses of proteins involved in hereditary spastic paraplegia (HSP) during development of the zebrafish.
HSP is a heterogeneous group of neurodegenerative disorders mainly characterised by progressive spasticity of the lower limbs due to retrograde degeneration of the cortico-spinal tracts. We have shown that the knockdown of Atlastin, a protein involved in a major early-onset form of HSP, causes a drastic decrease of larval mobility from 72 hours post-fertilisation onwards, which is preceeded by abnormal pathfinding of spinal motor neuron (SMN) axons and subsequent axonal degeneration (microtubule fragmentation), accompanied by a significant up-regulation of the BMP/Smad pathway. We have confirmed that this phenotype was due to the lack of Atlastin by performing rescue experiments with both the zebrafish and human full-length transcripts. Overexpression studies in wild-type and chordino mutant embryos have allowed us to show that Atlastin functions as an inhibitor of the BMP canonical pathway. Interestingly, both the mobility and SMN axon defects observed in Atlastin morphants could be rescued by inhibiting the BMP pathway using either a genetically-modified zebrafish line expressing a dominant-negative version of the BMP receptor I (BMPRI), or a pharmacological reagent, dorsomorphin. Finally, primary culture of zebrafish spinal neurons from prim-5 embryos enabled us to show that Atlastin partially co-localises with BMPRI in endosomal structures distributed all along neurites and enriched in growth cones, which suggests that Atlastin acts in SMN axon development by regulating BMP receptor endocytosis/trafficking.

Early requirements for preplacodal ectoderm and sensory organ development

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Preplacodal ectoderm arises during gastrulation as a narrow band of cells surrounding the anterior neural plate. This domain later resolves into discrete cranial placodes that produce portions of the paired sensory structures of the head. Most current models posit that preplacodal identity is specified by readout of a discrete level of Bmp signaling along a DV gradient. However, our studies in zebrafish support a strikingly different model: Rather than acting as a morphogen, Bmp simply establishes preplacodal competence throughout its signaling range within the nonneural ectoderm. Moreover, we find that four transcription factors co-induced by Bmp prior to gastrulation (Ap2, Ap2 Foxi1 and Gata3) are necessary and sufficient to promote preplacodal competence. Subsequently, Bmp-antagonists and Fgf secreted from dorsal tissue specify preplacodal identity within competent cells abutting the neural plate. Misexpression of Fgf8 and Chordin can activate preplacodal development anywhere within the zone of competence, leading to production of ectopic placodal derivatives on the ventral side of the embryo. Both Bmp-antagonism and Fgf are necessary, as neither signal alone is sufficient to activate preplacodal development. Knockdown of all four competence factors specifically blocks preplacodal specification without disrupting the Bmp gradient or formation of a neural-nonneural interface. Conversely, misexpression of competence factors can convert neurectoderm into preplacodal ectoderm without altering the Bmp gradient. These data support a relatively simple two-step model that traces the origins of preplacodal development to late blastula stage and resolves discrepancies in the literature regarding the role of Bmp signaling.

Repression of hedgehog signalling is required for the acquisition of dorsolateral cell fates in the zebrafish otic vesicle

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In zebrafish, Hedgehog signalling from ventral midline structures is both necessary and sufficient to specify posterior otic identity. A severe or complete loss of Hedgehog signalling leads to mirror symmetric double anterior ears, while severe overactivation of the Hedgehog signalling pathway gives rise to mirror symmetric double posterior ears. In contrast, in the mouse and chick otic vesicle, Hedgehog is required for dorsoventral patterning; anteroposterior effects, if present, are much less obvious. We now show, however, that while in zebrafish a loss of Hedgehog function does not affect dorsoventral and mediolateral otic patterning, an increase in Hedgehog signalling activity causes an expansion of ventromedial otic territories at the expense of dorsolateral domains. In a panel of lines carrying mutations in inhibitors of Hedgehog signalling, Hedgehog pathway activity is variably increased throughout the embryo, and dorsolateral otic structures are lost or reduced. Even a relatively modest increase in Hedgehog signalling has consequences for the ear. In the most severely affected line, the *ptc1-/-; ptc2-/-* double mutants, the inner ear is severely ventralised and medialised as well as displaying the previously reported double posterior character: markers of ventromedial otic domains (*eya1, pax2a*) are expanded at the expense of dorsolateral markers (*dlx3b, tbx1*). Overall these new data suggest that Hedgehog signalling must be kept tightly repressed for the correct acquisition of dorsolateral cell fates in the zebrafish otic vesicle. The role of Hedgehog in zebrafish and amniote inner ear patterning may not be as different as it at first appeared.

Regulating adult neuronal stem cell proliferation during regeneration of the light-damaged zebrafish retina

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We wish to define the mechanisms underlying regeneration of retinal neurons from an adult neuronal stem cell population. We use the light-damaged adult *albino* zebrafish retina, which rapidly undergoes apoptosis of both rod and cone photoreceptors and subsequently regenerates only photoreceptors. The regeneration response initiates with the Müller glia, which represents the adult neuronal stem cell population in the inner nuclear layer (INL), reentering the cell cycle and producing neuronal progenitor cells through an asymmetric cell division. The neuronal progenitors continue to proliferate to generate cell clusters that migrate to the outer nuclear layer (ONL), where they differentiate into both rods and cones. To study the molecular mechanisms regulating the initiation of Müller glial cell proliferation and neuronal progenitor cell amplification, we developed a technique to inject morpholinos into the adult zebrafish eye and then electroporate them into the retina either prior to or during the regeneration response, which knocks down the expression of specific target proteins.
We performed a gene microarray experiment and identified that the *stat3*, *pax6b*, and *pax6a* genes increased in expression at different times during regeneration (16, 31, and 51 hours, respectively). Knockdown of Stat3 expression, which is expressed in the Müller glia, blocked almost all Müller glia from beginning to proliferate, suggesting that Stat3 is required for Müller glia-dependent photoreceptor regeneration. In contrast, knockdown of either Pax6a or Pax6b, which were not detected in the Müller glia until after the asymmetric cell division, failed to disrupt the Müller glial cell division. However, Pax6b was required for the initial cell divisions of the resulting neuronal progenitor cell. In contrast, knockdown of Pax6a yielded proliferating cell clusters that contained approximately half the number neuronal progenitors as wild-type. Furthermore, knockdown of Pax6a, but not Pax6b, after 68 hours of constant light produced proliferating cell clusters that contained an increased number of neuronal progenitors. Thus, Pax6a and Pax6b are required for three critical points in the regeneration process: i) the proliferation of the first neuronal progenitor cell in each cluster (Pax6b), ii) the continued proliferation of the neuronal progenitors to reach the wild-type number per cluster (Pax6a), and iii) the migration of neuronal progenitors to the ONL, which removed them from the proliferative niche in the INL.

Afferent innervation during hair-cell regeneration in the zebrafish
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Hearing loss due to sensory hair-cell death is irreversible because mammals are unable to regenerate cochlear sensory cells after birth. Other vertebrate species, however, can repair damaged neuroepithelia with new hair cells throughout their entire lifetimes. These new hair cell become innervated shortly thereafter, granting the organ full anatomical and functional recovery after damage. The underlying mechanisms that permit hair-cell re-innervation remain unclear, however, because the process has not yet been described. Using live imaging in the zebrafish lateral-line organ, whose neuroepithelium is comparable to that of the mammalian inner ear, we have identified key activity-dependent events that underlie the re-innervation process. This has allowed us to formulate an experimental framework to perform an exhaustive analysis of the cellular and molecular events leading to the formation of neuronal topography and the kinetics of target recognition. We also found that the planar polarization of hair cells in the sensory epithelium influences the neuron's axon arbor complexity.

Molecular events during regeneration in the zebrafish lateral line after copper treatment
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The lateral line in amphibians and fish is a sensory organ that is used to detect water movements. It is comprised of stereotypically distributed neuroepithelial patches called neuromasts. These are similar to the inner ear's neuroepithelia both, in ultrastructure and in the expression of various molecular markers. In the inner ear, the loss or absence of hair cells, the mechanoreceptors, is the leading cause for deafness in all mammals, because they are unable to regenerate them after damage. Birds, amphibians, and fish do have the ability to regenerate damaged hair cells in the inner ear and in the lateral line. Regeneration occurs mainly by dedifferentiation and division of the surrounding supporting cells. Regeneration can be easily triggered in the lateral line by exposure to copper which is destroying in a highly selective manner its hair cells. Their regeneration over the course of 3 days can be observed in live larvae, using vital dyes and/or transgenic lines. By combining morphologic observations of the regeneration events in transgenic and mutant zebrafish lines, we are unraveling the molecular events underlying the regeneration process in the neuromasts. We have previously characterized a mutant line *phoenix* (*pho*), which is deficient in the regeneration process because supporting cells are unable to divide. We are characterizing a transgenic line expressing GFP exclusively in the supporting cells of the lateral line, which we called SCM1 (Supporting Cells Marker 1). Using FACs sorted cells from this transgenic line in the wildtype and in the *phoenix* mutant background, we are looking at transcriptional differences, using microarrays and the Illumina Genome Analyzer platform. This will allow us to define both the genes expressed specifically in the supporting cells of the lateral line and the genes involved in different steps of the regeneration process.

Development and regeneration of the zebrafish maxillary barbel (ZMB): a novel study system for adult vertebrate tissue growth and repair

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Barbels are skin sensory appendages found in fishes, reptiles and amphibians. The zebrafish, *Danio rerio*, develops two sets of barbels– a short nasal barbel and a longer maxillary barbel, hereafter called the ZMB. This elongated appendage contains a small number of ectodermal, mesodermal and neural crest derivatives, including skin cells, glands, taste buds, pigment cells, circulatory vessels and sensory nerves. Small in diameter, simple in structure, and optically clear, the ZMB is a potential system for studying the development and maintenance of these tissue types in an adult vertebrate. Using light and scanning-electron microscopy, histological sectioning and immunohistochemistry, we have described the development of the ZMB from the early bud stage (30-40 days post fertilization) to the adult (3-6 months). Beginning as a small, epithelial bud, the ZMB accumulates thin strands of extracellular matrix that condense to form a central U-shaped rod. The dorsal epithelium is invaded by melanocytes that are regularly spaced, while the ventral epithelium differentiates many tastebuds. As the ZMB elongates, sprouts from the craniofacial nerves and blood supply invade. Using transgenic zebrafish with fluorescently labeled endothelial cells (*fli1*:EGFP), we demonstrate that the ZMB contains not only a capillary loop, but also an adjacent closed-end vessel that we interpret as a large lymphatic. The identity of this vessel was further investigated by live imaging of the barbel circulation, extending recent descriptions of the lymphatic system in zebrafish. Barbel tissue is known to regenerate in other species of cyprinids (*e.g.* catfish) but this capacity has not been tested in zebrafish. We show that the ZMB can regenerate after proximal amputation (n = 350, >95% of barbel length removed), elongating rapidly within 2 weeks of injury. Unlike fin regeneration, which produces a well-integrated copy of the missing structure, ZMB regeneration is imperfect. Although superficially normal, regenerates had shorter lengths, internal scars at the plane of section, disorganized connective tissue rods, and, in some cases, misdirected nerve tracts. These defects were not remodeled even after long periods of healing, up to 6 months after surgery. However, these imperfect ZMBs could still regenerate completely after multiple rounds of amputation, demonstrating a repeatable response to injury. The ZMB also regenerates in senescent zebrafish (~ 2 years old), suggesting that its regenerative capacity is life-long. To investigate the genetic mechanisms of barbel outgrowth and regeneration, we have investigated members of the Wnt pathway, which are known to be expressed in skin differentiation and response to injury. Adult zebrafish mutant for glypican4 *(knypek)* show a complete arrest of barbel development, suggesting a key gene in this process. Reverse-transcription polymerase chain reaction (RT-PCR) analyses of developing and regenerating ZMBs showed several of these genes to be highly expressed in both tissues, including *glypican4*, *Wnt5a*, *Wnt5b*, and *Wnt11*. Our preliminary observations establish a novel, highly accessible organ system for studying appendage outgrowth and regeneration in adult zebrafish. Although the ZMB has no human equivalent, the cell types it contains are highly conserved. Thus "barbology" may be a useful system for studying epithelial-mesenchymal interactions, melanocyte migration, angiogenesis and lymphangiogenesis, wound healing, reinnervation, scar formation, and other key processes in vertebrate physiology.

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264 TIF1gamma REGULATES THE ERYTHROID AND MYELOID LINEAGE OUTPUT FROM HAEMATOPOIETIC PROGENITORS
R. Monteiro, Oxford, UK

265 LIVE IMAGING DEMAND-DRIVEN HAEMATOPOIESIS DURING INFECTION
C. Hall, Auckland, New Zealand

266 NEW INSIGHTS INTO THE EVOLUTION OF LIPOPOLYCCHARIDE (LPS) RECOGNITION AND SIGNALING
M.P. Sepulcre, Murcia, Spain

267 NEGATIVE REGULATION OF TOLL-LIKE RECEPTOR (TLR) SIGNALING: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ZEBRAFISH MD1 AND RP105
M.P. Sepulcre, Murcia, Spain

268 MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ZEBRAFISH IFNg1-1
M.A. Lýpez Muñoz, Murcia, Spain

269 ZEBRAFISH NUMB AND NUMBLIKE ARE INVOLVED IN PRIMITIVE ERYTHROCYTES DIFFERENTIATION
E. Bresciani, Milan, Italy

270 THE HOX COFACTORS PBX AND MEIS1 ACT UPSTREAM OF GATA1 TO REGULATE PRIMITIVE ERYTHROPOIESIS
L.M. Reaume, Edmonton, Canada

271 USING TRANSGENIC ZEBRAFISH TO SCREEN FOR SMALL MOLECULE INDUCERS OF INFLAMMATION RESOLUTION
C.A. Loynes, Sheffield, UK

272 DISCOVERY AND EXPRESSION ANALYSIS OF IMPORTANT MARKERS OF T-CELL SUBSET IN THE ZEBRAFISH (DANIO RERIO)
S. Mitra, Aberdeen, UK

273 ELUCIDATING THE ROLES OF NOTCH LIGANDS, RECEPTORS AND DOWNSTREAM TARGET IN EMBRYONIC HAEMATOPOIESIS AND ANGIOGENESIS
KA. McMahon, Nottingham, UK

274 THE ROLE OF RGS18 IN HEMATOPOIESIS AND MEGAKARYOPOIESIS
S. Louwette, Leuven, Belgium

275 A TISSUE-SCALE GRADIENT OF HYDROGEN PEROXIDE MEDIATERS RAPID WOUND DETECTION IN ZEBRAFISH
C. Grabher, Boston, USA

276 ZEBRAFISH MIR-126 AND MIR-150 COORDINATELY DETERMINE HEMETOPOIETIC CELL FATE THROUGH C-MYB
C. Grabher, Boston, USA

277 GENE/ENHANCER TRAP BASED SCREEN IDENTIFIES NOVEL TRANSGENIC LINES WITH REPORTER GENE EXPRESSION IN HAEMATOPOIETIC AND ENDOTHELIAL CELLS
R. Thambyrajah, Nottingham, UK

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A. Moriyama, Yokohama, Japan

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SPL. Hwang, Keelung, Taiwan

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Z. Zeng, Edinburgh, UK

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Y. Alvarez, Dublin, Ireland

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M. Newman, Adelaide, Australia

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P. van Tijn, Utrecht, The Netherlands

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D. Sheng, Singapore

285 TRNA SPLICING ENDONUCLEAS MUTATION CAUSE PONTOCELEBELLAR HYPOPLASIA
P. Kasher, Amsterdam, The Netherlands

286 DITHIOCARBAMATES ARE TERATOGENIC TO ZEBRAFISH THROUGH INHIBITION OF LYSYL OXIDASES
T. van Boxtel, Amsterdam, Netherlands

287 DEVELOPMENT OF SEMICIRCULAR CANALS IN THE ZEBRAFISH INNER EAR
F. Geng, Sheffield, UK

288 ZEBRAFISH AS A MODEL FOR NEURODEGENERATIVE DISEASES: FUNCTIONAL ANALYSIS OF GIGYF2, A CANDIDATE GENE FOR PARKINSON'S DISEASE
C. Brusegan, Milan, Italy

289 SPLICE FACTOR DEFICIENCY LEADS TO PHOTORECEPTOR DEGENERATION IN A ZEBRAFISH MODEL FOR RETINITIS PIGMENTOSA
J. Brocher, Singapore

290 EXPRESSION OF H-RASV12 IN A ZEBRAFISH MODEL OF COSTELLO SYNDROME CAUSES CELLULAR SENESCENCE IN ADULT PROLIFERATING CELLS
C. Santoriello, Milan, Italy

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D. Paquet, Munich, Germany

292 DISC-1 IS AN ESSENTIAL MODULATOR OF THE WNT PATHWAY
G. De Rienzo, Cambridge, USA

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J. Yin, Singapore, China

294 FISHING FOR NEURORPOTECTANTS FROM CHINESE MEDICINE
Z. Zhang, Macau, China

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M. Poyatos, J.S. Burgos, Granada, Spain

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P. Drapeau, Montreal, Canada

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S.A. Sakowski, Ann Arbor, USA

298 USING ZEBRAFISH TO INVESTIGATE PRESENILIN, -SECRETASE, AND APP FOR ALZHEIMER'S DISEASE RESEARCH
L.Wilson, Adelaide, Australia

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H-C. Peng, Taipei, Taiwan

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J.S. Müller, Newcastle, UK

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L. Araujo-Bazan, Madrid, Spain

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Y. van der Velden, Amsterdam, The Netherlands

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B.J.C. van den Bosch, Maastricht, The Netherlands

304 ANALYSIS OF DISEASE ASSOCIATED SPLICE FACTORS IN A ZEBRAFISH MODEL FOR RETINITIS PIGMENTOSA
H. Dill, Wuerzburg, Germany

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G. Gaudenzi, Milan, Italy

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B. Schmid, Munich, Germany

307 CHARACTERIZATION AND EXPRESSION OF slc2a10, THE ZEBRAFISH ORTHOLOG OF THE HUMAN GENE INVOLVED IN ARTERIAL TORTUOSITY SYNDROME
N. Chiarelli, Brescia, Italy

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F. van Bebber, Munich, Germany

309 DYNAMIN-2 FUNCTION IN EMBRYOGENESIS AND SKELETAL MUSCLE FORMATION
E. Gibbs, Ann Arbor, USA

310 A NOVEL FUNCTIONAL ROLE OF IDURONATE-2-SULFATASE IN ZEBRAFISH EARLY DEVELOPMENT
E. Moro, Padua, Italy

311 ROLE OF COLLAGEN XVIII IN EYE DEVELOPMENT
S. Bretaud, Lyon, France

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C. Lillesaar, Neuherberg, Germany

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M. Moennich, Dunedin, New Zealand

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Y-Y. Lin, Cambridge, UK

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M. Sundvik, Helsinki, Finland

316 A ZEBRAFISH MODEL OF CHARCOT-MARIE-TOOTH 2D
N. Malissovas, Athens, Greece

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C. Anastasaki, Edinburgh, UK

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A. Indrieri, Naples, Italy

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A. Benini, Brescia, Italy

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M. Priyadarshini, Helsinki, Finland

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A. Benini, Brescia, Italy

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J. Berger, Clayton, Australia

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C. Brösamle, Munich, Germany

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E. Kabashi, Montreal, Canada

325 ZEBRAFISH MITOFUSIN-2 KNOCKDOWN: A NEW MODEL FOR CMT2A NEUROPATHY?
G. Bergamin, Padua, Italy

326 NEPHROCYSTIN-4 IS A CILIARY PROTEIN REQUIRED FOR CONTROL OF WNT/ -CATENIN VERSUSWNT/PCP BALANCE AND CILIOGENESIS IN ZEBRAFISH EMBRYOS
C. Burcklý, Paris, France

327 MODELLING MUSCULAR DYSTROPHY AND EXPLORING THERAPEUTIC STRATEGIES IN ZEBRAFISH
T. Hall, Melbourne, Australia

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A. Magnabosco, Padua, Italy

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C. Dooley, Tuebingen, Germany

330 IDENTIFICATION OF SMALL MOLECULES THAT MODULATE INFECTION OF PSEUDOMONAS AERUGINOSA IN ZEBRAFISH EMBRYOS
A. Clatworthy, Boston, USA

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N. Temperley, Edinburgh, UK

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M. Hughes, Manchester, UK

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S. Noble, Ottawa, Canada

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P.A. Walker, Manchester, UK

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S. Choorapoikayil, Utrecht, The Netherlands

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G. De Sena, Brescia, Italy

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J.N. Berman, Halifax, Canada

338 METASTATIC BEHAVIOUR OF PRIMARY HUMAN TUMOURS IN A ZEBRAFISH XENOTRANSPLANTATION MODEL
I.J. Marques, Leiden, The Netherlands

339 A ZEBRAFISH ENGRAFT MODEL REVEALS A ROLE FOR THE PDZ/LIM GENES LMO7, MYSTIQUE AND RIL IN METASTATIC BEHAVIOUR OF HUMANN PANCREATIC CANCER CELLS
A.I. Belo, Leiden, The Netherlands

340 VALIDATING NOVEL CANCER GENES IN A ZEBRAFISH MELANOMA MODEL
V. Anelli, Milan, Italy

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H. Ishizaki, Edinburgh, UK

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J. Holzschuh, Freiburg, Germany

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J. Richardson, Edinburgh, UK

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D. Liedtke, Wuerzburg, Germany

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M. Anchelin, Murcia, Spain

346 ZEBRAFISH PICH PLAYS A CRITICAL ROLE IN SISTER CHROMATID SEPARATION DURING MITOSIS
K-H. Jeong, Seoul, Korea

347 MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF TRIM8 GENES IN ZEBRAFISH
M. Manzoni, Padua, Italy

348 GENERATION AND ANALYSIS OF ZEBRAFISH LIVER CANCER BY OVEREXPRESSING K-RASV12 USING MIFEPRISTONE-INDUCIBLE CRE/LOX SYSTEM
A.T. Nguyen, Singapore

349 ZEBRAFISH BRAIN REPRESENTS A RELIABLE NICHE FOR XENOTRANSPLANTED HUMAN GLIOBLASTOMA DERIVED CELLS
E. Rampazzo, Padua, Italy

350 HERITABLE ZEBRAFISH T CELL CANCER MODELS FROM A MUTAGENESIS SCREEN
J.K. Frazer, Salt Lake City, USA

351 EVALUATION OF SMALL-MOLECULE PLK1 INHIBITORS USING ZEBRAFISH
S. Xin, Shenzhen, China

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L. Zhen, Singapore

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H. Jeon, Seoul, Korea

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354 KIDNEY DAMAGE AND REGENERATION IN ZEBRAFISH LARVAE
C. Cianciolo Cosentino, Pittsburgh, USA

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M. Varga, London, UK

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G. Becker, Edinburgh, UK

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C. Stewart-Swift, Boston, USA

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M. Marai, Milan, Italy

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H. Tanaka, Tokyo, Japan

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T.C. Lund, Minneapolis, USA

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E. Sleep, Barcelona, Spain

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V. Parente, Milan, Italy

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B. Ayari, Paris, France

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B. Grotek, Dresden, Germany

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J-M. Tee, Utrecht, The Netherlands

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A. Ketley, Nottingham, UK

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I. Conte, Naples, Italy

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Y. Takeda, Kobe, Japan

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H. Pendeville, Liège, Belgium

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K. Naruse, Okazaki, Japan

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K. Sampath, Singapore

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G. Laird, Cambridge, UK

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HK. Sehra, Cambridge, UK

375 GENETIC ANALYSIS OF COLONAL CHARACTERISTIC OF MADAKA NUCLEAR TRSNSPLANTS GENERATED FROM THE SOMATIC CELL NUCLEI TRANSFER TO NON-ENUCLEATED DIPLOIDIZED EGGS
T. Adachi, Nagoya, Japan

376 GENOME-WIDE FUNCTIONAL SCREEN FOR THE ENHANCERS IN ZEBRAFISH I. Kondrychyn, Singapore

377 RETINAL PROGENITOR APOTOSIS AND ABERRANT PHOTORECEPTOR MORPHOLOGY CHARACTERISE THE DYING ON EDGE (DYE) MUTANT
L. Shine, Dublin, Ireland

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S. Scholz, Leipzig, Germany

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R. Geisler, Karlsruhe, Germany

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J. Bessa, Seville, Spain

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S. Berger, Clayton, Australia

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S. Sims, Cambridge, UK

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R. Kettleborough, Cambridge, UK

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C. Torroja, Hinxton, UK

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K. Howe, Cambridge, UK

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O. Lee, Exeter, UK

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D. Wilkinson, S.S. Gerety, London, UK

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J. Horne, Pleasantville, USA

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K. Kobayashi, M. Tanaka, Okazaki, Japan

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I. Rothenaigner, Neuherberg, Germany

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H.B.G. Ng Singapore

393 COMBINING BAC RECOMBINEERING & I-SCEI TRANSGENESIS TO ASSESS OSCILLATORY GENE EXPRESSION
AC. Oates, Dresden, Germany

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R. Nourizadeh-Lillabadi, Dresden, Germany

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S. Baxendale, Oslo, Norway

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C. Wyart, Berkeley, USA

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R. Brookfield, Nottingham, UK

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T. Sýker, Aachen, Germany

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L. Ziv, Ramat Gan, Israel

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T.A. Hawkins, London, UK

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M.A. Pardo, Derio, Spain

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GK. Wong, Cambridge, UK

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E. Warp, Berkeley, USA

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D. Schul, Wuerzburg, Germany

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F. Ruf-Zamosjki, Pasadena, Canada

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R. Noche, Boston, USA

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C.L. Crespo, Milan, Italy

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C. Araya, London, UK

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M. Tada, London, UK

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H. Otsuna, Salt Lake City, USA

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A. Sawada, Nashville, USA

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C. Dambly-Chaudière, Montpellier, France

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R. Marco-Ferreres, Madrid, Spain

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L. Beccari, Madrid, Spain

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A. Pistocchi, Milan, Italy

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A. Santos-Ledo, Salamanca, Spain

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I. Della Noce, Modena, Italy

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A. Oswald, Dresden, Germany

420 CONDITIONAL ACTIVATION OF NOTCH SIGNALING DURING ZEBRAFISH SOMITOGENESIS
BK. Liao, Dresden, Germany

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C. Eugster, Dresden, Germany

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B. Rajasekaran, Dresden, Germany

423 ROLE OF TERRA IN VERTEBRATES
R. Lourenço, Lisboa, Portugal

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M. Holder, London, UK

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PR. Jusuf, Cambridge, UK

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R. Madelaine, Toulouse, France

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M.R. Bogo, Alegre, Brazil

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E.H. Barriga, Santiago, Chile

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M. Andersson-Lendahl, Stockholm, Sweden

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A. Archer, Stockholm, Sweden

431 ABNORMAL NEUROGENESIS, ANGIOGENESIS AND HAEMATOPOIESIS IN ZEBRAFISH LACKING GROUP 4 PARALOG OF HOX GENES
A. Anusha Amali, Singapore

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M. Gering, Nottingham, UK

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N. Wittkopp, Tuebingen, Germany

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Zirc and UCL Husbandry Workshop: raising fish, nursery operations, and animal use regulations in the USA and EU

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At the 2008 Zebrafish Husbandry & Facility Management Workshop held during the International Zebrafish Development and Genetics Conference in Madison, a majority of the attending fish caretakers, facility managers, and principal investigators identified the nursery and raising larvae/juveniles as the main area of concern in their daily fish maintenance operation.
To address this concern and help improve nursery performance and survival rates, the Zebrafish International Resource Center (ZIRC) and University College London (UCL) are hosting a workshop to highlight the most crucial needs for a successful fish nursery. There will be two – three brief presentations and ample time for discussion, exchange of information, tips and tricks, and general troubleshooting.
A second part of the workshop will be devoted to address recent developments in animal use regulatory oversight. There will be two brief presentations by the organizers to provide an overview of regulations in the USA and in the EU. This part of the workshop will also be followed with time for sharing concerns and discussion.

Fish breeding at Campus IFOM-IEO
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In this presentation I would like to explain my experience in breeding and maintenance of zebrafish larvae used at the Campus-IFOM-IEO of Milan.
To develop our method of breeding, I tried different types of dry food for larvae and several providers of *Artemia salina*. Initially the larval diet included also paramecia, but I then found out not to be necessary. I have devised a way to clean the nursery tanks from the excess food and set up a schedule for feeding and cleaning that gives a 70-80% survival. Moreover, I have tried to release them in different numbers and feed larvae according to their size and not for their age.
Now in our fish facility the breeding and the maintenance of zebrafish larvae has been standardized to the level that we have 80% survival to adulthood and homogeneous size.

Zebrafish Larval Rearing at UCL
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This presentation is aimed to show how zebrafish larvae are successfully raised at UCL according to standardized protocols. In order to achieve the best survival rates various methods have been implied such as feeding appropriately according to their size and increasing water flow rates accordingly as well as keeping a good paramecium culture and following protocols for the de-capsulation of brine shrimp cysts. From a trial where three different types of popular diets for raising fry - dry, live, and a combination of both - were compared, it was found that the combination diet produced higher survival rates as well as a much faster increase in body mass. Owing to the combination diet and protocols followed, we currently have survival rates of 85%.

The ZIRC Nursery and Grow-Out Section
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Zebrafish International Resource Center, University of Oregon, Eugene, USA

Our goal is to optimize the throughput, survival, growth rate, and health of zebrafish. By adapting optimal and cost-efficient husbandry methods, we improved the efficiency, speed, and success of growing zebrafish at ZIRC. Thus, the nursery and larval grow-out sections contribute significantly to the turnover of fish generations at the Resource Center.
At the ZIRC, we use age- and size-specific powdered dry food as well as live food, such as *paramecia*, and *artemia*. We also incorporated the use of a modified E2 embryo medium in which embryos/larvae are maintained before transfer to the nursery. In the nursery and grow-out sections, we developed feeding schedules, optimized water flow times and flow rates, and we streamlined the general workflow for rearing zebrafish. Because our zebrafish larvae are given an incremental progression of diets and water flow changes as the fish grow, our average survival rate ranges between 80% (wild types) and 70% (some mutant lines). We will discuss ZIRC-specific procedures; however, we will also emphasize the three components that ensure a successful nursery in general: food size, food quantity, and water quality.

The soufflé gene is required for vesicle trafficking during oogenesis

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In egg laying animals yolk provides essential nutrition for the embryo until a functional digestive system has developed. Since yolk proteins are not expressed in the oocyte, they need to be imported by clathrin-mediated endocytosis. Endocytosis has been intensely studied in yeast and tissue culture, which discovered many of the key molecules controlling this cellular process.
We identified a novel regulator of endocytosis called Soufflé (Suf). *Suf* was isolated in a screen for zebrafish mutations causing defects during oogenesis. Unlike wild-type eggs with transparent cytoplasm, *suf* oocytes stay opaque similar to immature oocytes. However, our results show that the nucleus of *suf* oocytes progresses through all steps of oocyte maturation excluding a defect in meiosis. To better understand the role of Soufflé during oogenesis, we performed electron microscopy on mutant oocytes. In contrast to wild-type oocytes, small vesicles accumulate in *suf* mutants and yolk does not form crystalline arrays in yolk globules. During oocyte maturation wild type eggs become transparent, because yolk protein is proteolytically cleaved. In contrast, in *suf* oocytes small vesicles persist and the uncleaved yolk protein causes the egg cytoplasm to stay opaque. Together these results suggest that in mutant oocytes yolk protein never arrives in the hydrolyzing lysosomal compartment and that Soufflé controls vesicle trafficking during endocytosis.
Positional cloning of the mutation shows that *suf* encodes a zinc finger FYVE domain protein. FYVE domains are predicted to localize to endosomes consistent with the vesicle trafficking defect observed in *suf* mutants. Phylogenetic studies discover Suf homologs in invertebrate and vertebrate genomes and detect a vertebrate specific domain deleted in our mutant allele. Interestingly, *suf* is also present in mammalian genomes, which do not deposit yolk into their eggs. In addition, we find *suf* expression outside the zebrafish germline indicating that it controls endocytosis in other tissues. In fact, mutations in the human homolog cause hereditary paraplegia resulting in paralysis of the leg muscles. Although we have not observed swimming defects in *suf* zebrafish, the egg phenotype will an important tool to understand the cellular and molecular basis of the human disorder.

Intraovarian transplantation of stage I-II follicles results in viable zebrafish embryos

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Maternal gene products drive early embryogenesis almost exclusively until the mid blastula transition (MBT) in many animal models including fishes. However, maternal contribution to embryogenesis does not stop at MBT, but continues to be an essential regulator of key developmental processes. The extent to which maternal effects contribute to embryonic and larval development is hard to estimate due the technical difficulty of interfering with maternal gene products by conventional forward and reverse genetic tools. Therefore, novel methods to manipulate maternal factors in oocytes needs to be developed. Here we provide proof of principle protocol for transplanting stage I-II follicles of zebrafish into recipient mothers where donor stage I oocytes can develop to stage IV in 2 weeks and in 3 weeks they develop into mature eggs and produce viable offspring. Moreover, we show that simple microinjection of stage I-II follicles with RNA results in reporter gene expression in early stage oocytes and paves the way to developing tools for interfering with maternal gene activities. The early stage oocyte transplantation protocol provides a means to study cellular and molecular aspects of oocyte development in the zebrafish.

Irxl1 is a novel homeobox gene involved in brain, muscle and pharyngeal arch development in zebrafish

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Homeobox genes are transcription factors that play important roles in the regulation of embryonic development. *Iroquois homeobox-like 1* (*Irxl1*) is a novel member of the TALE superfamily of homeobox genes that is most-closely related to the *Iroquois* class. We have identified the zebrafish *Irxl1* gene and obtained the full-length cDNA of two splicing variants. Zebrafish *Irxl1* gene is located on chromosome 12. It contains 7 exons and encodes a homeodomain 100 % identical to vertebrate orthologs. Transcription of z-*Irxl1* was detected from 18 hpf to 5 dpf. Both isoforms were broadly expressed in adult tissues. Whole-mount *in situ* hybridization analysis revealed *Irxl1* expression mainly in the brain and the first two pharyngeal arches. Antisense morpholino knockdown of *Irxl1* resulted in deformed head and jaw in the larvae, which only survived for 5 to 7 days. The phenotype can be partially rescued by coinjection of *Irxl1* cRNA. The most severe defects were observed in the craniofacial muscles and arch cartilages. This phenotype is reminiscent of a mouse *Twirler* mutation, to which the *Irxl1* gene is linked. The promoter region of the *Irxl1* gene contains several consensus Mef2 binding sites, and co-injection of Mef2ca into zebrafish embryos up-regulates the promoter activity of *Irxl1*. In addition, *Irxl1* expression was decreased in *Mef2ca* mutants and morpholino-knockdown morphants. Meanwhile, *myoD* expression was increased in the somites of *Irxl1*-knockdown embryos. Co-injecting *myoD* promoter-driven luciferase construct with *Irxl1* into zebrafish embryos revealed a dramatic inhibition of *myoD* promoter activity by Irxl1. These results suggest that Irxl1 is an important regulator of brain, muscle and arch morphogenesis, and its function in arch development may be mediated by *Mef2ca*. Moreover, Irxl1 protein may regulate muscle development through myoD-dependent pathways.

Functional studies of muscleblind proteins in early zebrafish development

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Muscleblind (MBNL) is a family of proteins that participate in the regulation of tissue-specific alternative splicing. They bind to RNA through a conserved CCCH zinc finger domain. Three paralogs (Mbnl1, 2, and 3) have been identified in mammalian, each promotes inclusion or exclusion of specific exons on different genes. Misregulation of MBNL activity in human leads to RNA-mediated pathogenesis. To investigate the functions of Mbnl proteins in early vertebrate development, we cloned the *muscleblind* genes in zebrafish (*zmbnl1*, *2* and *3*). Alternative splicing of the three *zmbnl* primary transcripts gives rise to at least 13 protein isoforms. These genes are broadly expressed in most adult tissues. During embryogenesis, *zmbnl1* and *zmbnl2* are both maternally and zygotically expressed. In contrast, *zmbnl3* transcripts are not detected until late pharyngula stage. Whole-mount *in situ* hybridization reveals that *zmbnl1* is expressed in lens, liver, otic vesicle, and muscle, while *zmbnl2* is expressed in lens, olfactory epithelium, branchial arches, midbrain hindbrain boundary and swim bladder. Expression of *zmbnl3* is more ubiquitous rather than specific. Knockdown of *zmbnl1* and *zmbnl2* results in malformation of the brain, eyes, otoliths, pharyngeal arches, heart and swim bladder. In addition, these morphants are defective in hatching and swimming behavior. In consistent with the phenotypes, the splicing patterns of several pre-mRNAs that are misregulated in cells with CUG RNA expansion, are altered in the morphant embryos. Knockdown of *zmbnl3* does not result in overt phenotype. On the contrary, microinjection of *zmbnl3* cRNA into the embryos resulted in defective embryos with crooked body axes and short somites. When introduced into C2C12 cells, *zmbnl3* is able to inhibit cell differentiation, as evidenced by lack of cell fusion, change of splicing pattern and reduction of MHC expression. Dual-luciferase assay further reveals that zmbnl3 down-regulates *myoD* promoter activity in fish embryos. These data indicate that *zmbnl1* and *zmbnl2* are crucial for early fish development and that *zmbnl3* may interfere with muscle differentiation through the MyoD-dependent pathway.

Def6, a novel guanine nucleotide exchange factor, activates Wnt5 in the non-canonical Wnt signalling pathway

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Gastrulation is driven by coordinated cell movements that form the embryo proper into the three germ layers: endoderm, mesoderm and ectoderm. One of the major cell movements taking place is convergence and extension (CE), where cells move toward the dorsal midline and intercalate with one another leading to the mediolateral narrowing (convergence) and anterior-posterior lengthening (extension) of the forming embryonic axis. Vertebrate CE is regulated by the non-canonical Wnt pathway, similar to the planar cell polarity (PCP) pathway that mediates the establishment of cell polarity in the plane of epithelia in *Drosophila*.
In zebrafish, mutations in the wnt11 (Silberblick) and wnt5 (Pipetail) genes lead to defective CE movements without affecting cell fate. The two secreted glycoproteins Wnt11 and Wnt5 signal through their Frizzled receptor, Dishevelled, and Daam1 to activate the small GTPases Rho and Rac leading to the reorganization of the actin cytoskeleton. The Rho family of GTPases including Rho, Rac and Cdc42, act as molecular switches and regulate the cytoskeleton via cycling between inactive GDP-bound and active GTP-bound forms. Rho and Rac require guanine nucleotide exchange factors (GEFs) for their activation. However, the identity of the GEF involved in this pathway remains unknown, as neither Dishevelled nor Daam-1 can directly mediate the GDP-GTP exchange reaction.
Def6 is a novel type of GEF activating Rho GTPases through the exchange of bound GDP for GTP. We have previously shown that Def6 acts upstream of Rho, Rac and Cdc42 and also co-localizes with F-actin in mammalian cells. However, the role Def6 has in regulating cell shape, cell polarity and cell movement remain unknown. Here, we show that knock down of def6 affected CE cell movements, but not cell fate specification. Overexpression of GFP-tagged def6 protein was sufficient to rescue the def6-Morpholino-mediated phenotype indicating that the MO-induced defects were a direct result of specific def6 knockdown. To determine whether def6 functions downstream of Wnt11 and Wnt5 we attempted to rescue the Wnt11 and Wnt5 MO-induced phenotypes with def6 RNA. Although co-injection of def6 and Wnt11 MOs suggested synergism, def6 RNA did not rescue the Wnt11 knockdown. In contrast, the Wnt5-MO-induced phenotype was largely rescued by def6 RNA. Together these results suggest that def6 functions downstream of Wnt5 in the same pathway and synergistically with Wnt11 in either the same or parallel pathways. Since non-canonical Wnt signalling converges on RhoA we further tested whether RhoA acts downstream of def6.Constitutively active RhoA partially rescued the severe def6 knockdown phenotype. Our results so far show that def6 acts downstream of Wnt5 in the non-canonical Wnt signalling pathway and provide a starting point to further elucidate the role it plays in regulating CE movement during gastrulation.

Analysis of STAT3-activity in embryonic stem cells and during early development of Oryzias latipes

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Introduction: An important aspect in stem cell research is the understanding of how the potential for self-renewal is maintained and the prevention of spontaneous differentiation in stem cells is achieved. In murine embryonic stem (mES) cells, one member of the family of signal transducers and activators of transcription proteins, STAT3, is needed to keep mES cells in the „stemness"-status. The latent transcription factor STAT3 is activated by the leukaemia inhibitory factor (LIF) and consequently imported into the nucleus. In contrast to mES-cells, activated nuclear STAT3 is neither necessary nor sufficient to keep human embryonic stem (hES) cells in the undifferentiated status. In this work, we analyzed the localization and thereby the activity of STAT3 in embryonic stem cells of medaka fish (MES) and during early embryonic development of medaka.
Material and Methods: The embryonic stem cell line MESI was obtained from blastulae of medaka fish (*Oryzias latipes*) and remains undifferentiated in cell culture in presence of a complex mix of growth factors. STAT3-activity in MESI-cells and early embryos of medaka fish was investigated via immunofluorescence.
Results: Ectopic expression of an eGFP-tagged STAT3 resulted in dominantly cytoplasmic and thus inactive localization in MESI. Analyses in medaka embryos of blastula-stage demonstrated that STAT3 is inactive in all cells. Investigations on embryos before 1024-cell-stage revealed that STAT3 has a wave-like import into the nucleus between 64-cell-stage and 512-cell-stage with a peak at 64-cell-stage. No increase of nuclear STAT3-levels was detected before 64-cell-stage or after 512-cell-stage.
Discussion and Conclusions: The assays on MESI cells and blastula-staged medaka embryos demonstrated that STAT3 is not activated in medaka embryonic stem cells. Consequently, these data indicate that activated STAT3 is not necessary for stem-cell status in medaka. Hence, the mouse-system is the only vertebrate stem cell system depending on active STAT3.
Furthermore, the import-wave of STAT3 spanning 64-cell-stage to 512-cell-stage medaka embryos argues for a specific process in medaka development which is depending on nuclear presence of activated STAT3.

Seretonergic system and the effects of neurotropic drugs at the early prenervous stages of teleost fish development

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Various neurotransmitter systems were extensively studied over the past 100 years. Most of these studies were focused on the physiological role of neurotransmitters either in cell culture or in adult organisms which contains differentiated cells and complex tissues. However, the serotonergic system, for example, is active from the very early developmental stages. Thus, serotonin (5-HT), enzymes necessary for 5-HT synthesis, transportation and degradation, as well as 5-HT receptors have been found in various animals starting from oocyte stage. It is known that neurotropic drugs that targeted on serotonergic system have an impact on early development. Despite of all existing data, the functions of neurotransmitters in morphogenetic processes are still purely understood.

No data on the serotonergic system at the fish development before appearance of neurons exist. However, physiological and pharmacological properties of the serotonergic system are well known in larval and adult nervous system of zebrafish. Normal development of teleost fishes has been described in great details, and provides an opportunity to use a wide range of cytological, pharmacological and molecular techniques in a developing embryo. Taken altogether this facts makes the zebrafish embryos a favorable model to study the mechanisms of morphogenetic effects of 5-HT.

We used biochemistry and laser scanning confocal microscopy to describe the distribution of 5-HT and serotonin receptors during the early development (from zigote to blastula) of two teleost fishes, zebrafish (Brachydanio rerio) and Eurasian weather loach (Misgurnus fossilis). 5-HT expression started in zigote and continued in the blastoderm. Incubation of dichorioneted eggs with 5-HT precursor increase the concentration of both 5-HT and 5-HIAA, the product of serotonin degradation. Western blot analysis with antibody against human 5HT1a receptor revealed a band with size of 44 kDa which corresponds to the zebrafish 5HT1a receptor. Utilizing the same antibody we detected a distinct punctate signal on the surface of blastomers in wholemount preparations. Pharmacological screening of serotonin-related drugs demonstrated a significant difference in induced developmental defects in two studied species which belongs to the different families from the same order Cypriniformes.

Mutagenesis in Zebrafish by Transposon Gene Trap System
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Zebrafish is a powerful model organism for studying vertebrate genetics and development. To identify genes involved in development and diseases, the strategies of chemical and insertional genetic screens have been carried out. Many genes essential for zebrafish development were identified by mutagenesis screening. The transposon system is an efficient insertional mutagen. We constructed a Tol2-based gene trap vector, TSBGS, which contains the reduced 5' and 3' ends of Tol2, *bcl2* splicing acceptor, GFP reporter gene and the SV40 polyA signal. The transposon vector and the transposase mRNA synthesized in vitro were co-injected into fertilized eggs at one-cell stage. The injected embryos were raised and mated. In the pilot screening, 4900 injected fish were screened and 183 F1 families expressing GFP in spatial and temporal fashion were identified. Four recessive lethal mutations that are tightly linked to the transposon insertions have been identified from 121 F2 families. Beginning at 2 days post fertilization (dpf), mutants YT068 exhibit small eyes, a reduced brain, lack of jaws, branchial arches and swimming bladder, and then die at 5 dpf. YT101 mutants can be recognized at 28 hours post fertilization by the presence of hydrocephalus, edema, reduced pigmentation, reduced yolk sac extension and eyes. We have identified the interrupted genes in these mutant lines and their functions are being studied in detail. Our experience indicates that the Tol2 transposon gene trap system is applicable for mutagenesis in zebrafish though its efficiency is low.

Allosteric Control of Alpha-Catenin in Cell Adhesion and Migration

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The Adherense junctions (AJs) are essential for cell-cell adhesion, and the organization of membrane domains and cell polarity. AJ organization is dynamic as the balance between assembly and disassembly plays important roles in cell migration and epithelial-mesenchymal transition during embryogenesis, wound healing and cancer. The AJs comprise members of the cadherin family, transmembrane cell adhesion proteins that bind through their cytoplasmic domain to p120 and β-catenin; and β-catenin in turn binds to α-catenin (α-cat). The textbook model of the AJ depicts a stable complex in which the AJ is bound to the actin cytoskeleton, with the α-cat providing the molecular link between the actin cytoskeleton and the cadherin/beta-catenin complex. A direct test of this model showed, however, that α-cat cannot form a quaternary complex with cadherin, beta-catenin and actin. Instead α-cat behaves as an allosteric protein in which monomeric α-cat is able to form a ternary complex with cadherin/β-catenin, but not actin, while dimeric α-cat binds to and bundles actin filaments but does not interact with the cadherin/beta-catenin complex (Drees et al, 2005; Yamada et al., 2005). α-cat is in dynamic equilibrium between monomer and dimer pools in the cytoplasm and monomer bound to cadherin at sites of cell-cell adhesion; it has been proposed that a local increase in α-cat concentration during clustering of the cadherin/catenin complex could regulate local formation of α-cat dimers in the cytoplasm. These results point towards a dynamic, rather than static role of α-cat in locally regulating actin and membrane dynamics at sites of cell-cell adhesion. However, the new α-cat's properties were shown by *in vitro* study. We chose the zebrafish gastrulation, a model system for cell adhesion, to verify the new α-cat properties *in vivo*. We are using, together with the morpholino approach, a number of constructs that are able to mislocalized the α-cat interfering with his allosteric regulation. These constructs are able to direct α-cat to the mitochondria or to the plasma membrane (in a AJs independent manner). The rational of this approach is to perturb dynamically the α-cat concentration of the different pools. For example, diverting the α-cat to the mitochondria, we can lower the concentration of the protein available for the cytosol and plasma membrane pools. Our preliminary results shows that the morpholino caused delayed or arrested in epiboly and the embryos do not complete somitogenenis. Inducing mitochondria mislocalization of α-cat causes delay in epiboly, but in contrast to the morpholino injected embryos, these embryos complete somitogenesis. However, they display morphological defects.

Pou5f1/Oct4 contributes to dorsoventral patterning by transcriptional activation of *vox* expression

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Pou5f1/Oct4 in mice is required for embryonic pluripotent cell populations. In zebrafish, *pou5f1/pou2* has both maternal and zygotic expression. The zygotic *pou5f1/pou2* mutant spiel ohne grenzen (*spg*) shows neural plate pattering defects. We rescued the *spg* mutants by *pou5f1* mRNA injection. These fish mate to produce maternal zygotic *spg* (*MZspg*) mutants, indicating that *pou5f1* acts as a transcriptional activator during dorsal-ventral patterning. Overexpression of larger quantities of *Pou5f1-VP16* can ventralize wild-type embryos, while overexpression of a *Pou5f1-En* repressor fusion protein can dorsalize. *caBMPR1a* can rescue dorsalization both in *MZspg* mutants and in wild-type embryos overexpressing *Pou5f1-En*. Thus, we propose a role for *pou5f1* in the *bmp* pathway leading to ventral tissue specification. In support of this idea, via *bmp2b* and *fgf8* expression assays, we see that *MZspg* mutants are dorsalized prior to 30% epiboly, and that *Pou5f1-En* can dorsalize wild-type embryos prior to 30% epiboly. Notably, *MZspg* mutants, like wild-type embryos, can be further dorsalized in response of *fgf8*. Microarray analysis revealed that expression of several other ventral factors, including *vox*, is affected in *MZspg*. Overexpression of *Pou5f1-VP16* strongly activates *vox* expression in wild-type embryos by 40% epiboly, even in the presence of cycloheximide (CHX), while it hardly effects *bmp2b* and *bmp4* expression in the presence of CHX. In silico analysis of the *vox* promoter reveals conserved *Pou5f1* binding sites which could be confirmed by gel shift assay. Taken together, our data provide embryological and molecular evidence for the direct activation of the *vox* by *Pou5f1* in dorsal-ventral patterning.

Cloning, expression pattern and function analysis foxd (1,3,5) from flounder (paralichthys olivaceu)

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Since the first Forkhead Box (Fox) gene was discovered from fruit fly, more than 100 members of this family are identified in a variety of metazoan species. All of them contained a highly conserved DNA binding domain. It had been shown genes of this family play important roles in early embryonic patterning and physiology. As an economically important fish in the Asian region, flounder has been cultured for many years, but there is little information about its skeletal muscle development and maturation. To further clarify the function of regulatory factors that are involved in muscle formation of flounder, *FoxD* family genes were isolated and their expression pattern and function in regulating muscle were analyzed.

The flounder *FoxD1 FoxD3 FoxD5* contain only one exon and a conserved winged helix DNA binding domain. During the early stage of embryogenesis, *FoxD1* was mainly expressed in the somites, kidney progenitor cells, brain and intestine. *FoxD3* was expressed in premigratory neural crest cells, somites, post otic placodes, cranial and trunk neural crest cells, and pineal gland. *FoxD5* was expressed in several tissues including somites, tail bud, forebrain and otic vesicle etc.

The function of Flounder *FoxD1,3,5* on muscle development was conducted through over-expressing mRNA in one cell of the two-cell stage zebrafish by microinjection and analyzing the expression of *Myf5* and *MyoD*. After flounder *FoxD3* overexpressed, the two sides separated by the anterior-posterior axis of zebrafish embryo showed an asymmetrical development, the expression of *MyoD* and *Myf5* in the paraxial mesoderm was inhibited. When Flounder *FoxD1* mRNA was over expressed, *MyoD* expression in the somites on one side was reduced, but the expression in the adaxial cells was not affected; *Myf5* expression in the presomitic mesoderm, adaxial cells and somites on one side was reduced. When *FoxD5* was over expressed *MyoD* expression in the somites on one side was enhanced, but the expression in the adaxial cells was not affected; *Myf5* expression in the somites and presomitic mesoderm on one side was also enhanced. So, *FoxD1,3, 5* might regulate the muscle development by regulating the expression of *MyoD* and *Myf5*. To further understand the regulation mechanism of *FoxD1,3,5*, RT-PCR was performed to analyze the engogenous zebrafish *FoxD1,3,5* expression. RT-PCR showed that the over-expressed Flounder *FoxD3* can inhibit the expression of zebrafish endogenous *FoxD3*,while over-expressed Flounder *FoxD1* or *FoxD5* can not inhibit the expression of zebrafish endogenous *FoxD1* or *FoxD5*.So, the regulation of *FoxD1,3,5* was different from each other.

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The role of jnk in zebrafish early morphogenesis

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The zebrafish embryo is an ideal model system to study the molecular and cellular mechanisms implied in the expansion and fusion of epithelial sheets. At epiboly, the coordinated movement of the diferent cell layers suggests specific mechanism directing both cell polarity and cell migration. The interactions between the enveloping layer (EVL), the deep cells layer (DCs) and the yolk syncitial layer (YSL) could be directed by many signalling mechanisms.We propose JNK signalling pathway as a potential candidate to regulate the processes of epiboly and convergence and extension in the zebrafish embryo. Knock-down of zebrafish JNK leads to an epiboly phenotype, in which migration of the majority of deep cells is affected in the morphant embryos, while epibolic expansion of the superficial EVL and the YSL nuclei seems to progress at a normal rate. This suggests that JNK controls cell adhesion between the different layers. We monitor changes in cell behaviour by confocal videomicroscopy and in fixed sections with markers for both cell adhesion and cytoeskeleton. Embryos that overcome the DCs epiboly phenotype show a convergence and extension phenotype with a shorter and broader body. They also have a curled body and display circling movements. We propose that JNK could be controlling cell shape changes and motility underlying mophogenetic movements in early zebrafish embryogenesis.

Role of the wnt/pcp pathway component trilobite/vangl2 in cell behaviors underlying convergence and extension movements

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The Wnt/Planar Cell Polarity (Wnt/PCP) pathway was discovered in Drosophila where it plays an essential role in the establishment of planar polarity in epithelial tissues. In these epithelia, cells are connected by tight junctions and PCP pathway components exhibit a characteristic asymmetric localization to specific membrane domains. In mesenchymal cells such tissue architecture is absent. However, in vertebrates, PCP components are necessary for polarized cell behaviors of mesenchymal cells during convergence and extension (C&E) movements, which narrow and elongate the nascent embryonic body. But, the mechanism whereby the PCP pathway regulates cell polarity in vertebrates remains a challenging open question. We investigate the role of a core PCP component, Trilobite/Vangl2 (Tri) in this process.

At midgastrulation, lateral mesodermal cells are loosely packed and begin to converge slowly towards the dorsal midline by directed migration. At this stage, in *tri* mutants, the mesodermal cells migration is normal. As gastrulation proceeds, in wild-type embryos, cells become tightly packed and highly mediolaterally elongated, and migrate fast and along straight trajectories towards dorsal. But *tri* mutant cells fail to elongate and their movement is slow and less effective. We characterize this transition in cell morphology and behaviors during C&E movements. We show that endogenous Tri/Vangl2 is recruited to the cell membrane at a specific time during gastrulation, correlating with modification of cell behavior. Recent data showed that Prickle and Dishevelled (Dvl) are asymmetrically localized in mesoderm during gastrulation (Yin et al., 2008). This distribution is lost in *tri* mutants. We asked whether Tri/Vangl2 recruitment at the membrane results in its preferential asymmetric localization. We show that mediolateral cell polarization does not involve asymmetric Tri/Vangl2 localization. Using a Vangl2-GFP construct we perform *in vivo* studies to analyze the dynamic behavior of Tri/Vangl2 protein. We also investigated the relationship between Tri/Vangl2 and polarization of the microtubule cytoskeleton, by analyzing the position of microtubule organizing centers (MTOC) position in cells engaged in C&E. Whereas Dvl and Knypek/Glypican4, appear to be essential for MTOC polarization in cells engaged in C&E at late gastrulation stages, in *MZtri* mutants the MTOCs, are properly polarized. Based on these studies we suggest distinct roles for these PCP components in cell polarization during C&E.

Functional Analysis of the *eve1* Gene During Zebrafish Neural Development

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Eve1 is a zebrafish homologue of the *even-skipped* (*eve*) family of transcriptional repressors. Originally characterised in the fruit fly *Drosophila melanogaster*, where it has a crucial role in A/P polarity, eve homologues have since been identified in all vertebrate species so far studied. In vertebrates, it is expressed in the posterior during early embryonic development, and overexpression experiments have revealed its role in tail development in both fish and frog. Here, we have performed loss-of-function and early marker analyses in wild type and *ichabod* mutant embryos and have uncovered an important role for *eve1* in both trunk and tail development. We found that *eve1* activates the expression of posterior genes, such as *hoxb1b*, via the retinoic acid signal. In addition, we found that *eve1* possesses neural inducing activity and may therefore be important both in posteriorisation as well as in the induction of neural tissue in trunk and tail.

Characterization of the AP-1 µ1-adaptin in zebrafish (Danio rerio)

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Protein transport between the trans-Golgi network and endosomes is mediated by vesicles formed by the adaptor-protein complex AP-1. AP-1 consists of four subunits: γ1, β1, µ1, σ1. In mammalia µ1-adaptin subunit exists as the ubiquitously expressed µ1A and the polarized epithelia-specific µ1B. Mice "knock out" for the subunits γ1 or µ1A revealed that AP-1 complex is essential for mammalian embryonic development. We used zebrafish (*Danio rerio*) as a vertebrate model to explore the function of µ1A and µ1B subunits. In silico database analysis revealed two sequences encoding the tissue-specific µ1B and the ubiquitously expressed µ1A subunit, respectively. The two identified sequences share 83-85% identity to the corrisponding mammalian µ1-adaptins. The genomic structure and the exon-intron boundaries of the zebrafish orthologs were determined by performing BlastN alignments of different AP-1 µ1 cDNAs against the ENSEMBL Genome Browser and then compared to the human and mouse genes. Zebrafish *ap1m1* is located on chromosome 2 while *ap1m2* is not yet located on a specific chromosome. The intron-exon structure of zebrafish *ap1m1* and *ap1m2* is similar to human and mouse genes: the first one is organized in 10 exons and 9 introns and the second one is organized in 11 exons and 10 introns. The RT-PCR analysis on different developmental stages revealed that µ1A and µ1B transcripts are already detectable at the first stages of embryogenesis, whereas high levels were observed from late somitogenesis to 72 hpf. The µ1A transcript was present ubiquitously, while the µ1B transcript was present in organs of endodermal derivation as expected. The whole mount *in situ* hybridization confirmed the results obtained by RT-PCR for both subunits. Histological sections analysis revealead that *ap1m1* is expressed ubiquitously and in particular in the notochord, while *ap1m2* is expressed only in organs of endodermal derivation such as such as gut, pharingeal endoderm, liver and pancreas. When the coding sequences of zebrafish *ap1m1* and *ap1m2* were transfected in µ1-deficient mouse embryonic fibroblasts, both subunits could complement the mouse µ1 deficiency and restore AP-1 vesicle formation. Depletion of µ1B expression by using "translation-blocking morpholinos" (Mos *ap1m2)* gave rise to embryos characterized by defective formation of intestine, liver and tissues of endodermal derivation. Moreover, from 24 hpf onward the morphants showed a reduced mobility, that became more severe during the late stages of development and thereafter development ceased at 7-8 dpf. This is the first demonstration of the essential role played by µ1B-adaptin in organs development of vertebrate.We are currently running "knock down" experiments in order to elucidate also the role played by µ1-A adaptin during zebrafish development.

Identification and functional characterization of a novel γ-adaptin subunits (adaptor complex AP-1) in zebrafish (Danio rerio)

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In eukaryotic cells, transport of proteins between the different membrane compartments through the exocytic and endocytic pathways involves clathrin-mediated vesicles, which formation requires an adaptor protein complex (AP). The AP-1 complex mediates transport from TGN to the endocytic pathway and consists of four subunits: γ, ß, μ and σ. In human and mouse γ1-adaptin subunit is well characterized and recently a novel adaptor related protein, named γ2-adaptin subunit, was cloned. γ1 and γ2-adaptin subunits share high identity and the first one is expressed ubiquitously while the second one is expressed in particular in nervous tissues.
We used zebrafish as animal model to explore the function of the γ2 adaptin subunit during development of a multicellular organism. A search in ESEMBLE Genome Browser using TblastN algorithm revealed in zebrafish a sequence which encodes for a protein of 754 amino acids (ap1γ2). The predicted protein shows 66% identity with human and 67% identity with mouse orthologs respectively and it is similar to mammalian γ2-adaptin not only in the primary structure but also in the core-ear domain organization. The genomic structure and the exon-intron boundaries of the zebrafish ortologue is similar to the human and mouse genes, organized in 21 exons and 20 introns. The zebrafish gene *ap1γ2* is located on chromosome 2.
RT-PCR analysis on total embryo RNA showed that the *ap1γ2* transcript is detected in the embryos from early stages (1-2 cells) to 5 days hpf. The same analysis in adult organs showed transcript expression also in brain and eyes. Spatio-temporal expression analysis by whole-mount in situ hybridization confirmed the results obtained by RT-PCR. We used the translation-blocking morpholino (Mos *ap1g2*) approach to better understand the γ2 function during development and the obtained results showed that the γ2 subunit plays an important role during development of nervous system. In addition the morphants showed severe phenotype immobility since they are not able to leave from the chorion; they maintain this immobility till later stages of development (7-8 dpf) and then they dye.
We performed whole-mount in situ hybridization on ap1γ2 morphants, using *prox1a* probe as a marker of nervous tissues and eyes development. A significant reduction of *prox1a* labelling was detected in ap1γ2 morphants compared to standard controls, further indicating a specific role played by γ-2 adaptin in the development of nervous system. Future rescue experiments will help us to elucidate the function of γ2 subunit during development and its role in the machinery of intracellular vesicular traffic.

A comparison of heterochronic development of germ ring closure, tail bud formation and somitogenesis between fish species with large or small eggs

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In zebrafish and medaka, which have a small egg yolk size, it takes a relatively short time for epiboly to cover the yolk and for germ ring closure. Subsequently the tail bud is formed (bud stage) and somitogenesis starts (somitogenesis stage). However, in other fish species, such as rainbow trout, the size of the egg yolk is large (<5mm) which seems to be associated with a longer time for epiboly to cover the yolk. In rainbow trout embryos, a tail bud-like structure is formed and somitogenesis starts when epiboly only covers half of the yolk. Therefore, epiboly movement, germ ring closure, tail bud formation and initiation of somitogenesis are not sequential in such fish species. Our *in situ* staining study using the mesoderm marker *rt-ntl* revealed that the germ ring, tail bud, notochord and somites all co-exist at the late epiboly stage (around 10-20 somite stage) in rainbow trout embryos and the germ ring finally closes at around the 20 somite stage. A similar pattern of embryogenesis has also been observed in another fish species, the tropical marine tomato clownfish *Amphiprion frenatus* which also has a large egg size. To examine if the initiation of somitogenesis before germ ring closure depends on the yolk size, we conducted two experiments. Firstly, we selected exceptionally small (~2.5 mm diameter) eggs from batches of rainbow trout eggs and observed that such eggs completed germ ring closure at around the 5 to 8 somite stage, which is earlier than trout eggs of normal size. Secondly, zebrafish egg yolks were artificially expanded by injection of chicken egg white at the blastula stage. The injected zebrafish yolk was expanded by 10 to 15% in diameter. In such embryos, the early 3-4 somites were formed before germ ring closure. They develop normally at 24h, forming the head, trunk and tail of normal shape and size.

From these data we concluded that, in the evolution of teleost fish, variable yolk size has altered developmental sequence such that in eggs with large yolks, germ ring closure is prolonged according to the expansion of the yolk. Other developmental processes, such as tail bud formation and somitogenesis, occur without major dependence on yolk size.

Regulation and function of Sox6 in zebrafish skeletal myogenesis

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The first muscle fibres to differentiate in the zebrafish embryo derive from the adaxial cells and are of the slow twitch fibre type, a character imparted on them by the activity of the Prdm1a protein, expression of which is induced by Hedgehog signaling. The remainder of the myogenic paraxial mesoderm does not express Prdm1a and differentiates into fast twitch muscle fibres. The *sox6* gene, previously implicated in fibre type specification in the mouse, has been found to be expressed in these fast muscle progenitors and to be capable of repressing slow muscle-specific genes, such as *Prox1*, *smyhc1* and *stnnc*, implying it to be a key regulator of fibre type identity. Consistent with this, *sox6* is not normally expressed in slow-twitch myoblasts but is de-repressed in the adaxial cells of *prdm1a* mutants.

Using chromatin immunoprecipitation (ChIP) with anti-Prdm1 antibody we have found that Prdm1a binds specifically to sequences near to the *sox6* transcription start site. Using transgenic analysis, we have identified a *sox6* genomic fragment capable of driving EGFP expression specifically in the fast twitch fibres in the trunk and head muscles. Within this fragment a 220bp conserved element was identified to be a muscle enhancer by transient expression analysis. Simultaneous morpholino knock-down of MyoD and Myf5 reveals that activation of *sox6* in fast-twitch progenitors is dependent on these myogenic regulatory factors. To indentify direct targets of Sox6, we have developed an antibody against the zebrafish protein. We will report the preliminary results of ChIP on chip experiments using this antibody.

The tumor suppressor genes *prdm5* and *nucleophosmin1 (NPM1)* cooperate in the regulation of wnt signalling during zebrafish embryogenesis

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Proteins encoded by the *Wnt* family of genes act as signals and have been shown to play essential roles during embryogenesis. The spatial and temporal patterns of expression of the vertebrate members of the *Wnt* family, their receptors and effectors suggest roles for these genes in a variety of developmental processes. In all these processes Wnt signals appear to be modulated by specific sets of regulators that act at different levels of the signalling cascade. Moreover, several pathological states that may arise from altered stem cell function, such as degenerative diseses and cancer, are frequently associated with changes in *Wnt/*β*--catenin* pathway activity.

In previous work, we have shown that the tumor suppressor Prdm5, a transcripion factor belonging to the family of PRDM proteins, is able to modulate, in a negative way, both canonical *Wnt/*β*--catenin* and planar cell polarity (PCP) *Wnt* pathways (Meani et al., 2009). Data obtained from *in-vitro* assays, indicated that human PRDM5 associates with Nucleophosmin1 (NPM1), a nucleolar protein that shuttles between the nucleus and the cytoplasm (Grisendi et al., 2006) and that acts both as proto-oncogen and as tumor suppressor. In zebrafish both genes are strongly expressed during embryogenesis. From mid somitogenesis *npm1* expression localizes to domains in which various members of the *Wnt* family are present, whereas *prdm5* remains ubiquitous. We found that the effects of *prdm5* and *npm1* targeted knock-down are similar and resemble an impairment of morphogenetic movements during gastrulation due to a block of PCP Wnt pathway, accompanied by a reduction of the cephalic region. In agreement with this, the overexpression of both *prdm5* and *npm1* causes a "dorsalized" phenotype. Functional studies using loss and gain of function approaches showed that both *prdm5* and *npm1* regulate *dkk1* expression at early stages of development, in a way that results in repression of canonical *Wnt/*β*-catenin* pathway in addition to the PCP pathway. *npm1* synthetic mRNA is able to rescue the phenotype of *prdm5* morphants, whereas *prdm5* overexpression worsen the phenotype due to *npn1* knock down, suggesting an epistatic relation between the two genes rather than simple convergence of functions. So, our results suggest that Prdm5 and Npm1 co-operate in repressing Wnt signalling during early embryogenesis in zebrafish and may as well exert their tumor suppressor activities through negative modulation of Wnt signalling.

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Transgenic zebrafish Tg(*Nogo-B:GFP*) line recapitulates the *Nogo-B* expression pattern in diverse tissues including the liver and intestine

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In mammals, the Nogo family consists of Nogo-A, Nogo-B, and Nogo-C. Using sequences from mammalian counterparts, we cloned three zebrafish Nogo-related transcripts, termed Nogo-B, Nogo-C2, and Nogo-C1, which are generated by alternative promoter usage and alternative RNA splicing. These sequences are identical to other Nogo ligands, such as Rtn4-l/Nogo-alpha, Rtn4-n/Nogo-beta and Rtn4-m/Nogo-gamma, respectively. Whole-mount in situ hybridization was performed to demonstrate that Nogo-B mRNA was predominantly expressed in the brain, brachial arches, eyes, muscle, liver and intestine. The 4.8-kb promoter region of Nogo-B gene was isolated from a BAC clone and it was demonstrated to be functional in both cultured cells and zebrafish embryos. A transgenic zebrafish Tg(Nogo-B:GFP) line was generated using the same promoter region to drive GFP expression through Tol2-mediated transgenesis. Transgenic zebrafish Tg(Nogo-B:GFP) line recapitulates the endogenous expression pattern of Nogo-B mRNA. This line will be useful for the study of Nogo-B gene regulation during development.

The functional role of the zebrafish glucocorticoid receptor beta-isoform

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Glucocorticoids are steroid hormones, which are released from the adrenal glands to regulate the body's adaptation to stress. Synthetic analogues of these hormones are widely prescribed as anti-inflammatory drugs. They exert their effects via the glucocorticoid receptor (GR). In humans, two splice variants exist: the canonical GR alpha-isoform, which acts as a ligand-activated transcription factor, and the GR beta-isoform, which has a dominant-negative effect on GRalpha. Elevated GRbeta levels have been associated with the occurrence of immue-related diseases like asthma and rheumatoid arthritis and glucocorticoid resistance in patients suffering from these diseases. Until recently, the occurrence of GRbeta had only been demonstrated in humans and therefore an animal model for studies on this receptor isoform was lacking. Surprisingly, we have found that the zebrafish GR (zGR) gene, can give rise to a GRbeta isoform. We have explored the function of the zebrafish GRbeta both in vitro and in vivo. Transient overexpression of zGRbeta in cell lines as well as microinjection of its mRNA in zebrafish embryos has shown a functional role for this isoform in the regulation of the response to glucocorticoids on a subset of GR target genes. Further genetical manipulation, like the use of splice-blocking morpholinos and the construction of a transgenic fish line overexpressing zGRbeta, is undertaken in order to explore the significance and role of the zGRbeta in various physiological conditions.

Notch signaling regulates thyroid development in zebrafish

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The incidence of congenital hypothyroidism (CH) has been reported to reach 1:2400 newborns in the recent years. CH pathogenesis is largely undetermined, even if several findings support the hypothesis of a relevant genetic origin. The vertebrate thyroid gland is mainly composed of endoderm derived precursors cells. The main transcription factors and genes involved in thyroid development and differentiation have been identified, but the mechanisms underlying the early follicular cells specification are still largely unknown. A candidate pathway for thyroid formation and differentiation is represented by Notch signaling, a juxtacrine mechanism involved in several processes of cell fate decision. Mutations of Notch signaling members have been shown to affect the formation of different tissues and organs during vertebrate development. This is observed, for instance, in Alagille syndrome, a human multi-organ developmental disease. As zebrafish represents an excellent animal model to identify genes affecting thyroid gland development, in this study we aim to analyze the role of direct cell-to-cell Notch signaling in follicular cells specification *in vivo*.

To determine whether Notch signaling plays a role in thyroid gland formation, we blocked or over-activated Notch signaling and analyzed the expression of thyroid marker genes (*pax2a*, *nkx2.1a*, *tg* and *slc5a5*) by mRNA *in situ* hybridization. In *mib* mutant embryos, which fail to activate Notch signaling, we observed an expansion in the number of thyroid cells together with a modified morphology. Coherently, embryos treated with DAPT, a gamma secretase inhibitor able to block Notch signaling, showed an increased transcription of genes expressed in the thyroid. Conversely, Notch gain of function leads to the opposite phenotype. The excess of Notch signaling, obtained by intercrossing two transgenic lines: hsp70:gal4 x UAS:Notch1a-intracellular domain (NICD), resulted in a strong reduction of thyroid follicular cells.

Given that the pharyngeal vascular structure is seriously affected by these treatments, the observed thyroid phenotypes could result either from cell-autonomous or non cell-autonomous activities of Notch signaling. In order to define if Notch signaling plays a direct role in thyroid early development, we are currently testing the co-expression of thyroid markers with Notch ligands at various stages of zebrafish development.

Sipx is required for zebrafish dorsoventral patterning by augmenting and balancing TGF-beta and BMP signalling

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In view of important roles of Smad2/3 in early embryonic development, it is valuable to identify their interacting factors. Through yeast two hybrid screening, we identified several zebrafish maternal proteins that interact with Smad2/3. One of the Smad2/3 binding proteins is Sipx. Biochemical experiments proved that Sipx not only interacted with Smad2/3, but also bound to Smad1/5. Over-expression of Sipx in mammalian Hep3B cells enhances both the TGF-beta-induced and Bmp-induced reporter expression. Zebrafish *sipx* is maternally and globally distributed at the early stages of embryonic development. To better understand the functions of *sipx* in vivo, we used mRNA over-expression and morpholino knockdown strategies in zebrafish embryos. Knockdown of *sipx* by antisense morpholinos dorsalized embryos, which implies that *sipx* is required for development of ventral tissues. The fact that *sipx* knockdown inhibited *bmp2b-induced* embryonic ventralization suggests an essential role of *sipx* for Bmp signaling during embryogenesis. On the other hand, over-expression of *sipx* mRNA also caused dorsalization of the embryos. The dorsalized phenotypes induced by *sipx* over-expression could be inhibited by co-injection of *dnsmad3b* mRNA encoding a dominant negative form of zebrafish Smad3b. It is likely that over-expression of *sipx* induces ectopic TGF-beta/Nodal signaling, and as a result, the embryos are dorsalized. Thus, Sipx plays important roles in embryonic dorsoventral patterning probably by directly augmenting and balancing TGF-beta and BMP signaling pathways.

Characterisation of a functional physical interaction between STAT3 and BMP-receptor 1a

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Signaling cascades are often displayed in a rather linear, isolated fashion. There is BMP signaling, SHH signaling, MAPK signaling and so on. In reality, a cell is likely more similar to a communication hub, where inputs from a massive array of antennae are integrated to produce a concerted response.
During a series of experiments aimed at comparing signaling activities of cultured stem cells of medaka, the mouse and their respective embryonic counterparts, we were able to identify a large (>1MDa) protein complex that can be isolated from all studied sources. Within this complex, we always found BMP-receptor 1a (BR1a) and STAT3 – two proteins belonging to thus far unconnected signaling pathways.
Co-immunoprecipitation assays showed that STAT3 physically interacts with BR1a. Furthermore, the activation of STAT3, regulated via phosphorylation at Y705, is actively altered in response to BMP ligand administration. Interestingly, the subcellular localisation of the latent transcription factor STAT3 is influenced in a non-common way through the interaction with BR1a. While theoretical activation of STAT3 by phosphorylation of Y705 occurs, the usually observed consequent nuclear accumulation of STAT3 was not found, but instead nuclear exclusion was observed.
These data suggest that STAT3 – potentially together with other thus far unidentified components of the newly found complex – acts as a signal integrator of at least two signaling pathways. Furthermore, our data demonstrate that the model of STAT3 Y705 phosphorylation and consequent nuclear translocation is oversimplified.
Currently we are trying to untangle the signal integration functionality of STAT3 from its linear transduction role. For this purpose, we have mapped the aminoacids responsible for the interaction between BR1a and STAT3. Next, we will replace the endogenous proteins with variants able to fulfill classical signal transduction roles, but are not able to interact with one-another anymore.

Characterization of expression and function of novel emilin genes in mouse and zebrafish

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Emilins are a family of glycoproteins of the extracellular matrix with common structural organization and containing a characteristic N-terminal cysteine-rich domain. The prototype of this family, Emilin-1, is found in human and murine organs in association with elastic fibers, and other emilins were recently isolated in mammals. To gain insight into these proteins in lower vertebrates, we investigated the expression of emilins in the fish *Danio rerio*. Using sequence similarity tools, we identified eight members of this family in zebrafish. Each emilin gene has two paralogs in zebrafish, showing conserved structure and syntheny with the human ortholog. Whole-mount *in situ* hybridization (ISH) revealed that expression of zebrafish emilin genes is regulated in a spatio-temporal manner during embryonic development, with overlapping and site-specific patterns mostly including mesenchymal structures. Expression of some emilin genes in peculiar areas, such as the central nervous system or the posterior notochord, suggests that they may play a role in certain morphogenetic processes (1). The most distinctive pattern is the one of emilin-3, the only component of the family lacking the C-terminal gC1q domain. While other emilin genes reveal a mesenchymal or cardiovascular expression profile, the two zebrafish genes coding for emilin-3 are abundantly expressed at early stages in notochord and floor plate, suggesting that emilin-3 may play key roles during early development (1). Emilin-3 expression does not appear to be regulated by either Hedgehog, FGF or Notch signaling, as indicated by ISH of embryos in which these molecular pathways were blocked by drug treatments. Additionally, expression of the two Emilin-3 genes appears normal in embryos mutant for Chordin (*dino*) or for BMP2 (*swirl*). We are currently investigating other relevant pathways to check whether they regulate emilin-3 expression during early development. Moreover, we are performing functional studies by microinjection of mRNA or morfolino oligonucleotides in fertilized oocytes. The studies in mice were concentrated on the gene coding for emilin-3. In the past years, targeted Emilin3 gene inactivation in murine embryonic stem (ES) cells was already undertaken in our laboratory. However, generation of *Emilin3* knockout mice was unsuccessful and these previous attempts revealed that, unlike other emilins, targeted inactivation of the Emilin3 gene is particularly difficult. The frequency of homologous recombination of this gene is extremely low and transmission of the inactivated allele to the F1 generation was not reached, probably due to karyotypic instability of targeted ES cell clones. Therefore, we carried out a new Emilin3 gene targeting approach, performed by means of a large-scale experiment. We transfected R1 ES cells with a *Emilin3* targeting construct and, after double positive-negative selection, 1505 clones were isolated; 995 of these individual cell clones were further expanded and investigated for identifying clones in which a correct homologous recombination event had occurred. Six positive ES clones were identified, and after additional karyotypic characterization four of them were used for generation of chimeric mice by microinjection of ES cells into host blastocysts and implant in foster females. Several chimeric mice were obtained from two independent clones, and they were bred to obtain *Emilin3* null mice which are currently under phenotypic analysis.

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Spatiotemporal distribution and developmental role of Cationic Amino acid Transporter 1 (CAT1) in zebrafish (*Danio rerio*)

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CAT1 is a member of the solute carrier 7 family (SLC) of proteins, and is also called SLC7A1. CAT1 is Na^+ independent transporter of cationic amino acids, including the essential amino acids lysine and arginine. It is ubiquitously expressed and has been shown to be essential for cell survival, proliferation and also acts as a receptor for ecotropic retro-virus. In mammals, three additional related proteins, CAT-2, CAT-2A, and CAT-3, have been identified. The aim of this study was to investigate the early expression and developmental role of zebrafish cat1. The cat1 expression was first detected in somite muscles and eyes around 16 h post fertilization (hpf) by whole mount in-situ hybridization. At 24 hpf the expression was widespread but stronger in somite muscles. At 3 days post fertilization (dpf) the expression started appearing in gill arches. The expression levels in gills increased at 5 dpf. Functional characterization of cat1 was done by splice blocking morpholino knock-down. At 24 hpf the morphant embryos showed a bent body axis and reduced yolk absorption. At 3 dpf and 5 dpf the pharyngeal arches of the morphants were not developed properly which was characterized by shortened head to thoracic trunk distance. Cat1 is involved in L-Arginine transport, and L-Arginine homeostasis is involved in the regulation of cellular signaling pathways, mainly because it determines NO production through its availability to Nitrous oxide synthase. Arginase converts L-Arginine to L-ornithine which can further be metabolized into polyamines and l-proline. The polyamines are important in cell proliferation and l-proline is important for the collagen synthesis. Based on this we hypothesize that if there is lack of arginine then the branchial cell proliferation might stall or due to lack of collagen pharyngeal arches might not develop. We will further investigate this hypothesis in accordance with the role of cat1 in the development of the gills in the cat1 zebrafish morphants.

nkx6 genes control endocrine pancreatic cell fate in zebrafish

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The homeodomain proteins Nkx6.1 and Nkx6.2 have been shown to be expressed in the murine pancreas and deficiency for Nkx6.1 results in a specific abrogation of beta-cell neogenesis while the pancreas develops normally in *Nkx6.2* single-mutant mice. In contrast, *Nkx6.1/Nkx6.2* double-mutant embryos display a severe reduction in alpha-cell number.
In zebrafish, *nkx6.1* and *nkx6.2* are expressed at the beginning of the somitogenesis in the endoderm before to be restricted to the pancreas. First expressed in the same cells, their expression domains rapidly segregate, *nkx6.1* being expressed more ventrally. While *nkx6.1* is never expressed in the mature hormone-producing endocrine cells, *nkx6.2* is expressed in beta-cells until at least 24hpf.
The knock-down of *nkx6.1* or of *nkx6.2* leads to a striking decrease of the number of *glucagon*-expressing cells, while the expression of *insulin*, *somatostatin* or *ghrelin* is not affected. In contrast, the double knock-down of the two genes results in a clear decrease of *insulin* expression as well as in a almost complete disappearance of *glucagon* expression.
One hypothesis we are currently investigating is that the global dose of nkx6 proteins present in the embryo is critical for pancreatic cell fate: a high dose of *nkx6* would be necessary to allow alpha-cell differentiation while lower amount would be required to permit beta-cells to differentiate.

An in vitro approach for the study of zebrafish nc mutants

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Whilst in vivo analysis of zebrafish mutants is widespread, the ability to study these phenotypes in vitro has been less keenly pursued. The purpose of this project is to develop culture conditions that allow for the study *in vitro* of the phenotypes of zebrafish neural crest (NC) mutants, as a complement to in vivo studies. The NC is responsible for several cellular lineages such as pigment cells and neurons and glia of the peripheral nervous system and is used as a model system to study developmental processes such as specification and differentiation. The study of individual NCC mutants in cell culture would open up new possibilities for testing of gene function.
In the absence of published zebrafish NC cell cultures, we began with avian and mouse NCC culture conditions. Using these culture conditions, we achieved survival and differentiation of NCCs and many derivatives from a pool of disaggregated zebrafish embryos. However, application of these conditions to single NCCs or to NCCs at clonal density resulted in a failure in attachment/growth, probably due to lack of growth factors in the medium. Likewise, cultures from disaggregated single embryos also failed to thrive, highlighting the need for significant cultured cell numbers for NCC survival and differentiation. In order to solve this problem a strategy was devised where a single embryo with tranhsgenically-labelled NC was cultured in a pool of disaggregated, non-transgenic embryos. We anticipate this approach will allow us to study NCCs derived from a single embryo; in particular, it will be useful for testing the cell-autonomous effects of mutant genes. For initial tests of this approach, we are using lines in which GFP is driven under the control of the *sox10* promoter. This system has the limitation that tracking individual cells and their progeny is difficult since GFP expression is rapidly lost as NCCs differentiate. Consequently, we are modifying the system to use the Cre/Lox system for permanent labelling of NCCs in culture. Initial tests of a *sox10:cre* construct indicate that this system gives Cre expression in a *sox10*-like pattern. We are now attempting to generate stable *sox10:cre* transgenic lines.
Once combined, we expect these systems will allow us to compare NCC development in cultures of NC mutants and their wild-type siblings.

Analysis of the Hedgehog signaling pathway in zebrafish

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Hedgehog (Hh) signaling plays a fundamental role in animal development and in post-embryonic tissue homeostasis. Although mutant analysis in Drosophila and subsequently in mouse has uncovered the core components of the Hh signaling pathway there are still gaps in our understanding of this complex and unusual system. Zebrafish present an interesting model for the analysis of Hedgehog signaling, as they retain the function of pathway components that are absent from both Drosophila (eg Hip) and mammals (eg Fused). We are taking advantage of the Hh pathway mutants in zebrafish and the ease with which transgenic animals can be generated to investigate various aspects of the signaling process.

In an effort to identify the functionally important domains of the Ptc proteins, we have generated Ptc fusion protein whose expression is driven by 13 kb upstream of Ptc1 translation initiation site. The efficiency of rescue of *ptc1* mutants using full length Ptc1 and Ptc1 in which the C-terminus domain is removed suggests that C-terminal domain of the Ptc1 protein regulates its turnover.

In previous studies, we have identified an essential role for the iguana/DZIP1 protein in Hh signaling, implicating it in the control of Gli protein activity. To investigate this function further, we have engineered a genomic BAC clone to encode a GFP tagged form of Gli2 and used this to study the sub cellular distribution of this key transcription factor in wild type and mutant embryos. As previously reported in cultured cells, we find that Gli2 localises to the tip of the primary cilia as well as to the nucleus in wild type embryos. We will present the results of our analysis of Gli2 distribution in iguana mutant embryos.

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Notch signaling is essential for coordinating cell fate, morphogenesis and migration in the lateral line primordium

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The posterior lateral line primordium (pLLp) migrates caudally and periodically deposits neuromasts under the skin in the zebrafish trunk and tail. Each neuromast, formed within the migrating pLLp, has a central atoh1-positive hair cell determined by Notch mediated-lateral inhibition. The generation of new neuromasts and their deposition as the pLLp migrates caudally is coordinated by mutually antagonistic signaling centers; a Wnt signaling center at the leading edge and a FGF signaling center in the adjacent trailing domain, which determines both the morphogenesis of epithelial rosettes and expression of atoh1 in the forming neuromasts. We have now shown that the central atoh1 expressing cell in the neuromast also plays a critical role in regulating FGF signaling. When Notch signaling fails, too many cells express atoh1 and this eventually leads to failure of FGF signaling and unregulated Wnt signaling. This leads to collapse and disorganization of the migrating pLLp.

Unraveling pten function in zebrafish development

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The tumor suppressor PTEN primarily acts as a lipid phosphatase by specifically dephosphorylating of PIP3, a lipid second messenger produced by PI3K. PTEN and PI3K thus have opposing effects on the PIP3 level and as a consequence they counteract each other's actions in the regulation of important cell biological processes, such as proliferation, survival and migration. PTEN was also found to be crucially important for normal embryonic development as homozygous *Pten* null mice die early during embryogenesis. As being an ideal model for studying developmental processes we have used zebrafish to further investigate the function of PTEN in embryonic development. In the zebrafish genome two PTEN genes, *ptena* and *ptenb* were identified, which were shown to have redundant functions during embryogenesis. Whereas *ptena*-/- or *ptenb*-/- mutants did not show embryonic phenotypes, double mutant embryos died at 5 days post fertilization. In the double mutant embryos loss of pten expression also seemed to affect the development of the primordial germ cells (PGCs). Detailed analysis of the offspring resulting from crosses of our pten mutant fish with the transgenic VASA:EGFP line, which specifically express EGFP in their PGCs, will provide more insight into the specific role of pten in the development of the primordial germ cells. In addition, live imaging of the developing zebrafish embryos using fluorescently labeled pten proteins will be used to increase our understanding of the role of pten in embryonic development.

Myofibril organisation in skeletal muscle is regulated by a contraction-dependent signalling mechanism

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The earliest movements (17 - 22 hours post fertilization (hpf)) in zebrafish embryos are characterised by spontaneous muscle contractions induced by acetylcholine-driven Calcium (Ca^{2+}) signals. Mutant lines lacking key components of the excitation-contraction (E-C) pathway, namely the acetylcholine receptor or dihydropyridine receptor, show disrupted skeletal muscle development, specifically shortened sarcomeres and lengthened, misaligned myofibrils [1]. Based on evidence from these mutants we propose that myofibril organisation is controlled downstream of membrane depolarisation. The underlying intracellular signalling mechanisms are currently still unknown.
Ca^{2+}-mediated muscle contractions or Ca^{2+} signals acting independently of contraction could be involved in myofibril organisation downstream of membrane depolarisation. To distinguish between these two mechanisms we employed a pharmacological approach using the myosin II inhibitor blebbistatin to block muscle contraction. Movement was blocked for a period of 7 hours starting at 17 hpf and skeletal muscle morphology was subsequently examined using immunocytochemisty. Slow muscle fibres of blebbistatin-treated embryos (10, 50 µM) displayed misaligned myofibrils similar to the mutants lacking E-C coupling, suggesting that muscle contraction plays a critical regulatory role. However, as blebbistatin inhibits both muscle and non-muscle myosin II, it might affect not only muscle fibre but also neuronal development. Axon outgrowth was similar in control and blebbistatin-treated embryos, but an apparent increase in neurite number and the loss of varicosities was observed during drug treatment. The disruption of neuronal pathfinding might affect E-C coupling upstream of contraction and be the underlying cause of the observed myofibre phenotype. Nonetheless, assessing functional neuromuscular communication by measuring Ca^{2+} signals in muscle fibres revealed that the excitatory component of E-C coupling remained intact. Taken together, our data suggest that a functional contractile apparatus is required for myofibril organisation during muscle fibre development. In conclusion, the late steps of muscle differentiation, which rely upon embryonic movement, appear to be regulated by a novel contraction-dependent mechanism.

1. Brennan, C., et al., *Acetylcholine and calcium signalling regulates muscle fibre formation in the zebrafish embryo.* J Cell Sci, 2005. **118**(Pt 22): p. 5181-90.

Function of the ciliary gene *ftm/rpgrip1l* during zebrafish development

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Cilia project from the surface of eukaryotic cells and have evolved to perform diverse roles in animal development and physiology (reviewed in 1). In the mouse, alterations in cilia formation or function lead to a range of developmental defects, such as polydactyly, renal cysts, randomisation of left-right asymmetry, and defects in dorsoventral patterning of the spinal cord. The *Ftm/Rpgrip1l* gene codes for a protein localised in the basal body of cilia. Inactivation of this gene in the mouse has shown its involvement in Hedgehog (Hh) signalling (2). We recently found mutations in the *RPGRIP1L* gene in two human ciliopathies with different severities, Joubert syndrome type B and Meckel syndrome. The phenotype of the *Ftm* mouse mutant recapitulates the anomalies found in fœtuses with Meckel syndrome, the most severe form of ciliopathy (3).
In order to address in greater detail *Ftm/Rpgrip1L* function in signalling pathways, we cloned the zebrafish orthologue and designed morpholinos for knock-down analysis. Injection of two different morpholinos led to the same phenotype: embryos displayed a shorter tail and an undulated notochord. The phenotype was dose-dependent and could be rescued by injection of the human *RPGRIP1L* RNA, which is not targeted by the morpholinos. This phenotype is reminiscent of a defect in convergence-extension, often found in loss- and gain-of-function of genes involved in the Wnt-planar cell polarity (PCP) pathway. Indeed, we could demonstrate a genetic interaction of *rpgrip1L* with the core PCP gene *vangl2* in double knock-down experiments. We are currently looking for a physical interaction between Rpgrip1l and different PCP proteins, and trying to rescue the *rpgip1l* morphant phenotype by injection of active components of the Wnt-PCP pathway. Because several components of this pathway have been described at the base of the primary cilium, a function of Rpgrip1l could be to regulate their traffic to the cilium or their function at this specific location. To test this hypothesis, we are currently assaying the abundance and the subcellular localization of different components of this pathway in *rpgrip1l* morphants.
Previous studies have involved the primary cilium in the balance between the Wnt- beta-catenin and the Wnt-PCP pathways (reviewed in 1). The present study should allow us to better understand the function of Rpgrip1l and more generally of cilia in this process.

(1) Gerdes et al., 2009. Cell 137, 32-45.
(2) Vierkotten et al., 2007. Development. 134(14): 2569-77
(3) Delous et al., .2007. Nature Genet. 39(7): 875-81

Feedback Modulation of Wnt Signaling by Trophoblast Glycoprotein-like

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Wnt/beta-catenin signaling regulates a plethora of processes during animal development, tissue homeostasis and regeneration, yet for many of its roles few target genes mediating Wnt function are known. We used inducible overexpression of Dkk1, Axin1 and Wnt8 in transgenic zebrafish lines and microarray-based transcriptional profiling to identify Wnt target genes during zebrafish embryogenesis. We found that *trophoblast glycoprotein-like* (*tpbgl)*, a gene encoding a single-pass transmembrane protein with a glycosylated extracellular domain, is regulated by beta-catenin signaling in many tissues and developmental stages. Although *tpbgl* is conserved in vertebrates and is upregulated in a variety of carcinomas, little is known about its function. Interestingly, we found that overexpressed *tpbgl* potently inhibits beta-catenin signaling activated by Wnt ligands both in zebrafish embryos and cultured mammalian cells. *Tpbgl* acts in Wnt receiving cells, since it interferes with signal activation by Wnt-conditioned media, and upstream of the destruction complex, since it cannot interfere with pathway activation by dominant-negative Gsk3b, dominant-negative Axin1, or stabilized beta-catenin. Furthermore, *tpbgl* inhibits signaling induced by forced interaction of Frizzled and LRP6. Thus, *tpbgl* appears to interfere with beta-catenin signaling at the level of the Wnt receptor complex. Knock-down experiments in zebrafish embryos show that endogenous *tpbgl* is a required inhibitor of beta-catenin signaling, counteracting *wnt8* function in brain patterning.

Intriguingly, in contrast to its role as a feedback inhibitor of the beta-catenin dependent Wnt pathway, *tpbgl* enhances beta-catenin independent Wnt signaling in zebrafish embryos. Thus, we suggest that *tpbgl* is an important regulator of Wnt receptor complex formation or activity.

PKCγ is required for the normal development of glutamate synapses on zebrafish Mauthner neurons

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Glutamate AMPA receptors (AMPARs) are major excitatory receptors in the vertebrate CNS. Phosphorylation of AMPARs has been shown to be an important mechanism for controlling the function of these receptors in synaptic transmission and synaptic plasticity. We have previously shown that fast excitatory synaptic transmission in zebrafish Mauthner neurons (M-cells) is mediated predominantly by AMPARs. Furthermore, our recent results indicated that protein kinase C (PKC) is highly expressed in M-cells of developing zebrafish. Therefore, we sought to determine if PKC might be involved in the modulation of AMPARs.

We used whole cell patch clamp electrophysiology to record AMPA miniature excitatory postsynaptic currents (AMPAR-mEPSCs) from M-cells ranging in age from 30 hour post-fertilization (hpf) to 72 hpf. AMPA mEPSCs were recorded in the presence of the PKC activators, DOG and PMA. Both DOG and PMA increased mEPSC mean peak amplitude (~1.5-fold) and frequency (~2.5-fold). Pipette application of the specific PKC inhibitor, bisindolylmaleimide I (BIS 1), blocked the effects of DOG and PMA on mEPSC amplitude but not frequency, indicating that PKC has both a presynaptic and postsynaptic locus of action. We used immunohistochemistry to identify the PKC isoform that might be responsible for the effects of PMA and found that M-cells express relatively high levels of PKCγ. Intracellular application of the active form of PKCγ led to an increase in mEPSC amplitude and the PKCγ inhibitory peptide (γV5-3) was able to block the effects of PMA. To determine if trafficking mechanisms were involved in the increase in mEPSC amplitude, we applied a selective blocker of actin polymerization (latrunculin B), and peptides that inhibit the association of the trafficking proteins NSF and PICK1, with AMPA receptors. All of these agents were capable of blocking the effect of PMA and a high K^+ saline, which increases synaptic activity, suggesting that activating PKCγ leads to the trafficking of AMPA receptors to the synaptic membrane. Injection of morpholinos against PKCγ resulted in fish that were incapable of trafficking AMPA receptors to the cell membrane, and which exhibited overall mEPSC characteristics much like the young embryos at 33 hpf rather than the developmentally mature fish at 48 hpf or 72 hpf. Moreover, fish injected with PKCγ morpholinos exhibited severe deficits in touch responses and were unable to hatch out of the chorion.

Taken together, our results suggest that activation of PKCγ leads to the normal developmental recruitment and insertion of AMPA receptors into excitatory synapses on zebrafish M-cells. In the absence of PKCγ, this normal developmental paradigm does not occur and fish exhibit major deficits in M-cell function.

Anthrax toxin receptor 2A/CMG2 interacts with LRP6 to mediate wnt-dependent convergent extension during gastrulation

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CMG2 (Capillary Morphogenesis Protein 2) is one of the two cell surface receptors that bind directly to Anthrax toxin and supports cellular intoxication. CMG2 was found to be upregulated in an *in vitro* model for human capillary tube formation, and mutations in this gene are associated with juvenile hyaline fibromatosis (JHF) and infantile systemic hyalinosis (ISH), two autosomal recessive syndromes. CMG2 is a type I membrane protein with an extracellular highly related to von Willebrand Factor type A domain that has been proposed to bind extracellular matrix components, and could also potentially mediate cell-cell interactions. However, its normal physiological and/or developmental functions are still unclear.

Genome analysis revealed that CMG2 is only found in vertebrates. Zebrafish contains 6 genes encoding Anthrax Toxin Receptors, being Antr2a the closest homolog of the human CMG2. Analysis of the functions of *antr2a* during zebrafish embryonic development by morpholino (MO)-mediated gene knockdown indicate that Antr2a mediates convergent and extension (CE) movements during gastrulation. Antr2a regulates CE by signalling through the non-canonical Wnt pathway. We found synergistic interactions between the *anthr2a* morphants and mutants/morphants of the non-canonical Wnt signaling pathway, such as *wnt11*, *wnt5a* and *knypek*. Conversely, the CE phenotype resulting from the injection of *antr2a* MO could be rescued by injection of *rhoA* mRNA, a downstream component of the non-canonical Wnt signalling, which has been shown to regulate the cytoskeletal architecture during vertebrate gastrulation.

Pull-down experiments indicate that Antr2a binds to the typical canonical-Wnt signalling pathway receptor LRP6 (Low-density-lipoprotein receptor-related protein 6) in zebrafish. Consistent with this observation, *lrp6* morphants also showed a Wnt11-dependent CE phenotype, which is enhanced in the antr2a/lrp6 double morphants. Both LRP6 and Antr2a localize to the plasma membrane of zebrafish embryonic cells as well as in intracellular Rab5-, and Rab11-positive endosomes. However, while LRP6 localization at the plasma membrane is mostly uniform, Antr2a appears clearly polarized. Antr2a is enriched at the sites of cell-cell contact, suggesting a possible role in regulating cell-cell interaction. Moreover, this polarization is Wnt11-dependent: embryonic cells overexpressing Wnt11 showed an increase of Antr2a at cell-cell contact sites, while in *wnt11* mutant embryos (*slb*), Antr2a is mostly absent from the plasma membrane and accumulates intracellularly. Taken together these results indicate that Antr2a interacts with LRP6 to mediate CE movements during gastrulation presumably by regulating cell-cell interaction.

Positional cloning reveals that *futile cycle* encodes a maternal gene homologous to human *lrmp/jaw1* with essential roles in nuclear-centrosomal attachment and pronuclear congression

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During normal fertilization, a compacted sperm nucleus enters the egg cytoplasm bringing with it a pair of centrioles. As the sperm nucleus expands and the pronuclear envelope begins to form, the sperm-derived centrioles, along with maternally-derived centrosomal components, nucleate a sperm monoaster. The growing monoaster eventually makes contact with the female pronucleus, which then begins a rapid migration toward the male pronucleus. A close association between the sperm pronucleus and the nascent centrosome is maintained throughout pronuclear migration. After pronuclear fusion, duplicated centrosomes align on opposite sides of the newly formed zygotic nucleus in preparation for the first mitotic division.
The recessive maternal-effect mutation *futile cycle* (*fue*) was previously identified as part of a gynogenesis-based screen for maternally-supplied products acting during early developmental stages in zebrafish. Embryos from homozygous mutant *fue* females (*fue* embryos) fail to undergo pronuclear migration and fusion, never form mitotic spindles, and consequently fail to properly segregate chromosomes. Interestingly, in the majority of mutant embryos, cytokinesis proceeds normally during early cell divisions even though DNA is absent from most dividing cells. Here we show that *fue* embryos develop a normal monoaster, but manifest abnormal nuclear membrane dynamics and morphology shortly after fertilization. Additionally, the close apposition of male pronucleus and centrosome appears to be lost shortly after fertilization. Positional cloning allowed us to narrow down the location of the *fue* mutation to a 56 kb region on linkage group four that contains two genes. Both genes were sequenced in their entirely and a single missense mutation was detected in the longest transcript of *lymphoid restricted membrane protein* (*lrmp*). Very little is known about the molecular function of *lrmp* in any system, and the maternal variant found in zebrafish appears to be significantly larger than variants described in adult tissues of other species. Analysis of *lrmp* transcript localization in wild-type embryos by *in situ* hybridization and gamma-tubulin antibody staining showed that *lrmp* mRNA colocalizes with centrosomes as early as 15 mpf, prior to pronuclear fusion. During prophase, *lrmp* is associated with nuclear membranes and centrosomes, and is later found on the spindle during mitosis. This distinct localization pattern is completely lost in *fue* embryos, where the transcript appears to be spread diffusely throughout the blastodisc. Our results suggest that recruitment of *lrmp* transcript to centrosomes and/or the nuclear periphery may be critical for proper function and distribution of these organelles during early development, possibly by providing a highly localized source of Lrmp protein.

Regulation of Chemokine Signaling by the microRNA miR-430

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MicroRNAs shape gene expression by regulating mRNA degradation and translation. To study the role of miRNAs during zebrafish development, we used embryos deficient in the miRNA processing enzyme dicer (maternal and zygotic dicer, MZ*dicer*). These mutants are depleted of mature microRNAs. Our laboratory has shown that one miRNA in particular, miR-430, is ubiquitously expressed in early development and plays important roles in embryo morphogenesis. Analysis of the cell migration phenotype in MZ*dicer* mutants revealed that primordial germ cell migration was affected in the absence of miRNAs. Because chemokine signaling guides germ cell migration, we assayed whether any components of this pathway are regulated by miRNAs. Migrating cells express the receptor CXCR4 and follow a gradient of the secreted ligand SDF1. CXCR7 is believed to be a non-signaling receptor responsible for refining SDF1 protein expression. Our analysis revealed that (i) SDF1, CXCR4 and CXCR7 have putative miR-430 target sites in their 3'UTRs, (ii) loss of dicer function enhances the expression of the GFP-3'UTR reporters for all three genes and (iii) mutation of the putative miR-430 target site abolished GFP-reporter repression in wild type embryos. Taken together, these results indicate that SDF1a, CXCR7a, and CXCR7b mRNAs are likely regulated by miR-430 in vivo. To assess the physiological significance of this regulation for germ cell migration, we used Target Protector morpholinos. These target protectors are complementary to the miRNA target site and have been shown to specifically interfere with the individual miRNA-target interactions. This approach allowed us to investigate the functional relevance of miR-430-mediated regulation of each individual target. Interestingly, protection of SDF1a affected germ cell migration to the germ line. We are currently analyzing the regulation of the antagonist CXCR7, and these results will be presented at the meeting. Our results support a model in which miR-430-mediated repression is acting at the transcript level to modulate and refine the expression patterns of these potent regulatory molecules, thereby promoting precise and accurate migration.

Notum-homologue is an element of the negative feedback loop regulating Wnt/beta-catenin signaling

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Wnts, a large family of secreted proteins, are crucial for numerous processes in the developing embryo. Proper development requires tight regulation of Wnt signaling both intracellularly and extracellularly. Glypicans are a class of heparan sulfate proteoglycans linked to the cell surface via a glycosophosphotidylinositol (gpi) anchor that serve as co-receptors for ligands of several signaling pathways including Wnts, in both invertebrates and vertebrates. A secreted alpha/beta-hydrolase called Notum was found to antagonize the signaling of the prototypical *Drosophila* Wnt, Wingless, by shedding the glypican Dally and Dally-like from the cell-surface. Biochemical work has demonstrated that a mammalian Notum homologue can similarly induce the release of several glypicans by gpi cleavage.
We have identified multiple zebrafish *notum* homologues. We determined that Wnt/beta-catenin signaling regulates the expression of one of these homologues called *notum3*. Overexpression of Notum3 demonstrates that it can function as a potent inhibitor of Wnt/beta-catenin. Specifically, Notum3 antagonizes the effects of Wnt8 overexpression and can synergize with the well-established Wnt/beta-catenin inhibitor Dkk1 to suppress posterior body formation. We have created a stable transgenic line, Tg(*hsp70:notum3*), to allow inducible overexpression of Notum3. Ectopic expression of Notum3 induces stage-dependent perturbations of development consistent with Wnt/beta-catenin inhibition. In particular, we have shown through loss and gain of function studies that Notum3 functions in the patterning of the dorsal neural tube. We propose that Notum3 acts to restrict the spread of Wnts signaling from the roof plate.
Our analysis of Notum3 overexpression phenotypes suggests that Notum3 has a limited set of targets. We have found no evidence that Notum3 inhibits the function of Glypican4 (Knypek) in Wnt/PCP signaling or the activity of other gpi anchored proteins. Together these data suggest that Notum3 functions as part of an autoregulatory loop by which Wnt signaling is restricted and that there is an unexplored specific requirement for glypicans for proper Wnt/beta-catenin activity during vertebrate development.

Role of WNT/β-CATENIN signalling during segmentation of the presomitic mesoderm

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The segmentation clock controls the periodic formation of somites from the presomitic mesoderm (PSM); it involves Notch, Wnt and FGF signaling pathways. Nrarpa (Notch regulated ankyrin repeat protein a) is both a target and an inhibitor of Notch signaling. We propose that a fast turnover of Nrarpa protein limits the duration of the inhibitory effect and sustains the oscillation of Notch activity in the PSM. Furthermore, we provide evidence that Wnt/β-catenin signaling positively regulates *nrarpa* transcription. By transiently decreasing Wnt activity, we established that Wnt determines the area of the PSM that is responsive to Notch signaling. In summary, canonical Wnt signaling is required to maintain the identity of PSM cells; Wnt initiates periodicity of Notch activity by controlling the expression of *nrarpa,* and permits cells to respond to the Notch oscillator. Therefore, Wnt/β-catenin signaling underlies segmentation of the PSM in the zebrafish.

The signaling component Inka links cytoskeletal dynamics to pattern formation

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Complex and linked inductive interactions pattern the early embryo across the animal kingdom, including the zebrafish. At the end of gastrulation in vertebrates, the neural crest (NC) is induced by interactions among neuroectoderm, the epidermis and paraxial mesoderm at the neural plate border. This is accompanied by the expression of numerous transcription factors, among which is the transcriptional activator protein AP2a (TFAP2a). We and others have shown that TFAP2a is a critical element in the induction, migration and differentiation of cranial NC cells into skeletal and other tissues in the embryonic head. To further understand this function we carried out a microarray-based screen for TFAP2a-target genes expressed in NC, and identified several, including a novel adhesion molecule, the protocadherin PCNS, an unconventional myosin MyoX, and a novel protein we named Inka (formerly Inca).

Inka proteins interact physically with p21-activated kinase 4 (PAK4), an effector of Rho-class of small GTPase, regulate cytoskeletal dynamics including stress fibers formation and microtubule modifications, subsequently change cell migration. These effects correlate with the inhibition of PAK4, in both kinase-dependent and kinase-independent manners. There are three *inka* genes in zebrafish: *inka1a, inka1b* and *inka2*. Each has a unique expression pattern. The *inka1a* gene is zygotically expressed predominantly in the early ectoderm and later in neural crest (NC), and loss of function by MO knockdowns showed that it is required for terminal differentiation of cranial NC into head cartilage in both fish and frog embryos. The *inka1b* gene is expressed maternally, which is replaced by the margin expression and ventral localizations at gastrula. Our preliminary results showed that *inka1b* is involved in the early axis patterning of zebrafish embryos. We are current investigating the function of *inka1b*, and the possible links between its cellular functions and the modulation of inductive interactions in the embryo.

Zebrafish mutant showing defects in lens epithelial integrity and fiber differentiation
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Lens placode is specified in ectoderm overlaying the retinal primordium, and invaginates to form the lens vesicle, a sphere of single epithelium, which initially consists of mitotic lens progenitor cells. During lens development, epithelial cells in the posterior half of the sphere differentiate into primary lens fiber cells, while anterior epithelial cells are maintained as mitotic lens progenitor cells. Lens progenitor cells exit from the cell cycle at the equatorial zone of the lens sphere and start to differentiate into secondary lens fiber cells, which subsequently elongate and cover the core of old lens fibers like the layers of an onion. Thus, lens provides a good model in which to study the regulation of cell shape change and epithelial polarity associated with cell differentiation. To elucidate this mechanism, we identified zebrafish mutants showing defects in lens development. *rw341* is one of these mutants and shows a protruding lens phenotype after 4 dpf. We found that lens cell differentiation proceeds normally in the *rw341* mutant until 3 dpf, but later the integrity of anterior lens epithelium is disrupted, resulting in the mislocalization of lens progenitor cells into the entire lens surface. Phallodin labeling revealed that actin fibers were abnormally accumulated within lens cells, suggesting that cytoskeleton regulation is disorganized in *rw341* mutant lens cells. These data suggest that the integrity of lens epithelium and their differentiation into lens fiber cells are compromised in the *rw341* mutant.

Ubiquitin proteasome system is essential for lens fiber differentiation

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Lens precursor region is specified in ectoderm overlying the neural retina and invaginates to form the lens vesicle, a sphere of single epithelium, which initially consists of mitotic lens progenitor cells. During development, lens progenitor cells start to differentiate into lens fiber cells at the equatorial zone of the lens sphere, and subsequently elongate and cover the core of old lens fibers like the layers of an onion. Thus, lens provides a good model to investigate the mechanism that regulates cell shape change associated with cell differentiation processes. Previously, we identified zebrafish mutants showing defects in lens development. Here, we report one of them, *volvox* (*vov*). Differentiating lens cells normally change their cell shape into highly elongated lens fibers and lose their subcellular organelles such as nuclei for their transparency. However, in the *vov* mutant, lens cells fail to fully elongate into lens fibers, and their nuclei do not undergo degradation. These data suggest that lens fiber differentiation is compromised in the *vov* mutant. We found that the *vov* mutant gene encodes a component of proteasome. Poly-ubiquitinated proteins were accumulated in nuclei of wild-type differentiating lens cells, and their level was markedly elevated in the *vov* mutant. Taken together, these data suggest that protein degradation through the ubiquitin proteasome system is important for lens fiber differentiation.

The role of the lysosomal membrane protein LIMP2 in brain and notochord formation during zebrafish development

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On embryos, from all vertebrates, the notochord is a rod like organ that supports the body trunk maintaining the shape of the developing body. It is a transient structure eventually replaced by the bone skeleton. The notochord it is an elongated tube filled with tightly packed and bulky cells, each containing a single swollen vacuole. In zebrafish the notochord keeps the trunk rigid maintaining the larvae characteristic elongated and straight form. Evidently, the notochord, it is also essential for fish larvae swimming behavior as it support somite contraction. We report that in zebrafish larvae mutant limp2a^{hi1463}, that lacks expression of the *limp2a* gene, notochordal vacuoles are not formed properly and in consequence abnormal shaped notochords were found, impairing, at the same time, swimming behavior.
Limp2a is the membrane lysosomal receptor for Beta-glucocerebrosidase. When mutated it causes Action myoclonus-renal failure (AMRF), a human disorder, which combines focal glumerulosclerosis and progressive myoclonus epilepsy. Therefore zebrafish mutant limp2a^{hi1463} is a useful model to understand the underlying molecular problems in the AMRF disorder and at the same time a valuable tool for studying vacuole notochord formation.
Using histology and electron microscopy we analyzed the phenotypic characteristics of the limp2a^{hi1463} mutant. We found abnormal protruding lumps in the hindbrain and midbrain region at 20 – 28 hpf that disappear later on. Apparently this is not caused by neuroectoderm over-proliferation. The notochord phenotype became apparent at 2 dpf it persist till 7 – 9 dpf when the larvae die, even if food is provided. Notochord mutant cells are smaller and with numerous vacuoles, therefore we concluded that Limp2a participate on vesicular fusion necessary to form the big notochord cell vacuole. We are in the process to analyze Limp2a expression pattern and by means of cell transplants determine if the notochord problem is due to the lack of autonomous expression in notochord cells or the cells in the surrounding sheath cells.

Combined knockdown of type 1 and type 2 deiodinases severely disturbs embryonic development in zebrafish

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Thyroid hormones (THs) play an important role in vertebrate development via their stimulatory effect on the expression of a wide variety of TH-responsive genes. In teleosts, early embryos rely on the maternal TH deposit in the egg yolk, which consists predominantly of tetraiodothyronine or T_4. Therefore activation of T_4 to 3,5,3′-triiodothyronine or T_3 by iodothyronine deiodinases (Ds) may be an important factor in determining T_3-dependent development. In zebrafish embryos, the two deiodinases capable of T_3 production, D1 and D2, are already expressed from the earliest stages tested (8 hours post fertilization). We tried to determine their relative importance for zebrafish embryonic development by inhibiting their expression via injection of antisense morpholino oligonucleotides (MOs) against D1, D2, or a combination of both. The impact of these treatments on the speed of embryonic development was estimated via three morphological indices: otic vesicle length, head to trunk angle and pigmentation index. Knockdown of D1 alone did not seem to affect developmental progression. In contrast, D2 knockdown resulted in a clear developmental delay (relative to control MO injected embryos) for all three morphological parameters scored, suggesting that D2 is the major contributor to TH activation in developing zebrafish embryos. Importantly, combined knockdown of D1 and D2 not only delayed development more than D2 knockdown alone, but also resulted in the occurrence of 20-25% morphologically abnormal embryos per batch. At 31 hours post fertilization the observed abnormalities included extremely curved bodies, posterior truncation and the absence of structures of the inner ear. These results show that although D1 does not seem to be very important when TH levels remain within the normal range, it may be crucial under depleted thyroid status as is the case when T_3 production by D2 is inhibited. The present study suggests that zebrafish embryos are dependent on T_4 uptake and its subsequent activation to T_3 and that substantial inhibition of embryonic T_4 to T_3 conversion reduces intracellular T_3 availability below the threshold level necessary for normal development.

Dissecting the role of sox9b in hepato-pancreatic development in zebrafish

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Pancreas and liver development involves specification and differentiation of multipotential progenitors appearing in neighboring endodermal anlagen. In zebrafish like in mammals, the ventral pancreas anlage gives rise to acinar, ductal and endocrine cells while, in the liver primordium, hepatoblasts give birth to hepatocytes and biliary cells. In the mouse embryo, sox9 was shown to be expressed in and required for the maintenance of the pancreatic multipotent progenitor cell pool. In the adult, sox9 expression is restricted to the pancreatic ducts though no role for this gene has been assigned yet in this structure. Here we report the analysis of embryos and larvae loss-of-function for a sox9 zebrafish orthologue, sox9b.

The expression analysis in the hepato-pancreatic region of zebrafish embryos reveals that sox9b is first detected in the dorsal pancreatic bud (exclusively comprised of endocrine cells), followed by the expression in hepatoblasts, and then in the ventral pancreatic bud progenitors. Its expression is after confined to the whole hepato-pancreatic ductal network.

In sox9b mutant embryos, both the ventral pancreas and the liver are correctly specified. However, the ventral pancreas does not grow apparently due to both failed maintenance of the progenitor state and to poor differentiation. In addition, sox9b mutants display an altered hepatic development resulting from similar defective processes to those observed in the mutant pancreas. Surprisingly, the dorsal pancreatic bud appears normal in sox9b mutant embryos and generates endocrine cells. We also present some evidences of the implication of sox9b in hepato-pancreatic ducts formation. The regulation of sox9b by various signals known to be crucial for hepatic and pancreatic development (FGFs, BMPs, Wnt) is currently investigated.

Our data indicate that, as suggested by their closely related embryonic origin, both the liver and the pancreas rely on a common transcription factor, Sox9b, which controls the maintenance of their multipotential progenitors and their further differentiation.

Renal progenitors are sequentially patterned by retinoic acid and the IRX3B transcription factor to generate proximo-distal domains in the zebrafish pronephric nephron

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The nephron is the functional unit of the kidney and is comprised of specialized segments of epithelial cells. The developmental pathways that establish segment identities among renal progenitors during nephron formation remain poorly understood. We have previously shown that the zebrafish embryo is a good model of nephrogenesis, as it forms nephrons with two proximal and two distal segments that are organized in a similar fashion to mammalian nephrons, and that this segment pattern is dependent on retinoic acid (RA) activity. To determine the origin of segment populations, we examined the expression of renal markers in the intermediate mesoderm (IM) from which the nephrons arise. IM kidney progenitors are initially separated into rostral and caudal domains that then get progressively subdivided into distinct populations that presage segment domains of the mature nephron. An examination of renal progenitors in RA-deficient embryos found that RA was requisite for specifying the rostral domain. When RA is absent, the IM renal progenitors adopt a caudal identity and the resulting nephron is comprised solely of distal segment fates. Conversely, in embryos treated with exogenous RA, the IM renal progenitors adopt a rostral identity and the resulting nephron is comprised entirely of proximal segment fates. To delineate the molecular pathways downstream of RA, we are performing morpholino-mediated knockdown of genes unique to the various renal progenitor groups. This strategy has discovered that the Iroquois transcription factor Irx3b is needed to confer the identity of the first distal tubule segment and prevent a corresponding expansion of proximal fates. Interestingly, this phenotype recapitulates a recently reported role for Irx3 in the amphibian pronephros, thus providing evidence that Irx3 function in nephrogenesis is conserved. Furthermore, embryos doubly-deficient for RA and Irx3b develop nephrons which express markers of the second distal segment. This suggests that in the absence of signals from RA and Irx3b, the default fate of renal progenitors is to acquire a distal segment identity. Taken together, our analysis suggests a model whereby the IM is initially patterned into two broad domains by RA, followed by sequential and dynamic refinement into two proximal and two distal tubule segments by later-acting transcription factors like Irx3b. These studies establish a powerful framework to assemble the molecular networks that drive nephron formation during vertebrate embryogenesis, and will likely provide useful insights into both normal and defective kidney development and disease.

Identification and functional analysis of the promoter region in the torafugu myosin heavy chain gene, *MYH*$_{M5}$, involved in cardiac and superficial slow muscle specific expressions

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Myosin heavy chain gene (*MYH*) encodes a large subunit of myosin, the major contractile and structural muscular protein. In vertebrate genome, *MYH* forms multiple gene family, and tissue-specific and developmental stage-specific expressions of each *MYH* lead to the formation of different muscle-fiber types such as slow, fast and cardiac ones. However, the details of molecular mechanisms involved in such complex expression patterns of *MYH*s are still unknown. In this study, we examined transcriptional regulation in the expression of *MYH*$_{M5}$ from torafugu *Takifugu rubripes,* having the smallest genome size among vertebrates, in zebrafish *Danio rerio* where various transgenic approaches are possible.

MYH$_{M5}$ is one of the torafugu *MYHs* identified from the genome database by *in silico* screening (Ikeda et al., 2007). *In situ* hybridization and RT-PCR previously showed its specific expression in torafugu embryos and adult slow and cardiac muscles. In this study, the GFP reporter vector carrying the -4kb sequence from the start codon of *MYH*$_{M5}$ was constructed and injected into zebrafish embryos. The expression of the GFP was observed in cardiac and myotomal skeletal muscles of transgenic embryos at 1-2 days post fertilization. Myotomal skeletal muscle of zebrafish embryos consists of fast muscle, superficial slow muscle and medial fast fibers in addition to muscle pioneer cells. GFP-expressing cells were not stained with anti-engrailed antibody, suggesting that they are not medial fast or muscle pioneer cells. All GFP-expressing cells were stained with F59 antibody specific to slow muscle myosin but not with F310 antibody specific to fast muscle myosin, indicating that these correspond to superficial slow muscle fibers. In the differentiation of superficial slow muscle fibers, hedgehog signaling has been reported to be a key regulatory factor. The GFP expression by *MYH*$_{M5}$ promoter was apparently reduced in embryos treated with cyclopamine, a hedgehog signaling inhibitor, as compared to that in the control. These results indicate that the -4kb sequence from the start codon of *MYH*$_{M5}$ is sufficient for gene expression specific to superficial slow and cardiac muscles, and its regulatory activity is conserved among different fish species.

Smyhc1 encodes an MYH isoform expressed in superficial slow muscle in zebrafish embryos (Elworthy et al., 2008). In this study, -5kb upstream sequence from the start codon of torafugu *MYH*$_{M5}$ and zebrafish *smyhc1* were compared by LAGAN program, yielding one conserved region of 62b. Then we created deletion mutants in the -4kb region of *MYH*$_{M5}$ and examined their functions. Unexpectedly, the reporter construct lacking the conserved region induced GFP expression in slow and cardiac muscles with the same activity as that having the intact -4kb. On the other hand, the deletion of -4k~-2k, -4k~-1.5k and -4k~-1kb decreased progressively the rate of GFP expression, suggesting that not a single sequence but several regions distributed from -1kb to -4kb are involved in the transcriptional regulation of *MYH*$_{M5}$.

Retinoic acid acts upstream of WT1A and FOXC1A to specify podocytes from the intermediate mesoderm

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The nephron is the functional unit of the kidney and filters the blood via a size-selective sieve that contains specialized epithelial cells called podocytes. The developmental pathways governing podocyte formation during renal development are poorly understood. Using zebrafish as a model, with its simple two-nephron pronephric kidney, we have identified a number of key developmental pathways that are involved in podocyte formation. Retinoic acid (RA) was found to be essential during a small window at the end of gastrulation for the induction of podocytes from the intermediate mesoderm. Expression of the transcription factor *Wilms' Tumor Suppressor-1a* (*wt1a*), implicated in podocyte differentiation, is dependent on RA signaling. Knockdown of *wt1a* led to an early reduction in podocyte progenitor number followed by a progressive decline between 24-48 hours post-fertilization. A similar phenotype was observed following knockdown of the forkhead transcription factor *foxc1a*, which is also dependent upon RA signaling for its expression in podocyte progenitors. Double *wt1a/foxc1a* knockdown led to a complete loss of podocyte specification suggesting that these transcription factors act in a partially redundant fashion, downstream of RA, to induce podocyte cell fate from the intermediate mesoderm. Taken together, these data provide a better understanding of how podocytes arise during kidney development and may provide insights into the molecular mechanisms that cause renal birth defects and disease.

Functional analysis of zebrafish connexin41.8 for pattern formation

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One of the most beautiful features seen in nature is skin pattern of animals. We have proposed that pigment cells, melanophore and xanthophore behave like reaction-diffusion wave on fish body, consequently the pigment cells make stripe pattern. However molecular mechanism of the pattern formation remains unclear. *leopard*, a pigment pattern mutant of zebrafish is one of the good models for the pattern study. This mutant has spot pattern in spite of stripe pattern, and this phenotype is caused by the mutation on connexi41.8 gene. Connexin is a component of gap-junctions that mediate intercellular communication. Null mutant of this gene, namely allele leo^{t1}, shows clear spot pattern. Dominant negative mutations in this gene induce variations of pattern in zebrafish. Allele leo^{tq270}, which has I202F amino acid substitution, shows severer phenotype; smaller spots than that of allele leot1 and reduced number of melanophores. On the other hand allele leo^{tw28}, I31F substitution, shows weaker phenotype than that of leo^{tq270}; stripe of leo^{tw28} is waved but the number of melanophore is as same as that of WT. Here we show that overexpression of connexin or its mutants under the control of pigment cell-specific promoter can lead to unique pigment pattern. Our data indicate that connexin is a key factor not only to change the stripe to spot pattern but also change the width of stripe.

Zebrafish Krüppel-like factor 4a functioning like mammalian KLF4 tumor suppressor is negatively regulated by Notch signaling in embryonic intestinal cell differentiation

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Zebrafish *KLF4a* cDNA encoding a zinc finger transcription factor was cloned and deduced amino acid sequence shares 65.8% similarity with mammalian *KLF4*. *KLF4a* starts to be expressed in the intestine at 36 hours post fertilization (hpf) and can be detected in the intestine, liver, and exocrine pancreas from 48 hpf. KLF4a specific antisense morpholino oligonucleotide knockdown was used to analyze its function in morphogenesis of gastrointestinal tract. The numbers of Phospho-Histone H3 stained intestinal cells in 74 hpf *KLF4a* morphant embryos were significantly higher than that in wild type embryos. In addition, ultrastructural analyses by TEM showed the appearance of pseudo stratified intestinal cells in 72 hpf and 102 hpf KLF4a morphants as compared with single layer of columnar intestinal epithelium in wild type embryos. These results indicate that KLF4a inhibits the proliferation of intestinal epithelium. Decreased *IFABP* expression in 75 hpf *KLF4a* morphant embryos indicates KLF4a may mediate the differentiation of enterocytes. Moreover, results from both whole-mount *in situ* hybridization on *agr2,* which is expressed in goblet cells in the intestine, and alcian blue staining that can stain acidic mucins within goblet cells showed that goblet cell numbers were less in *KLF4a* 102 hpf and 120 hpf morphants than in wild-type embryos. Additionally, the expression level of *KLF4a* was higher in embryos which Notch signaling was inhibited by DAPT, a γ-secretase inhibitor, revealing KLF4a was negatively regulated by Notch signaling. Overall, our results demonstrate that KLF4a like its mammalian homologue KLF4 may function as a tumor suppressor to inhibit intestinal cell proliferation and regulate the differentiation of enterocytes and goblet cells which is negatively regulated by Notch signaling.

PGAF regulates the attachment of podocytes to the glomerular basement membrane

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The development of the kidney glomerular filtration barrier requires the proper differentiation of podocytes. These specialized epithelial cells form interdigitated foot processes that adhere to the underlying glomerular basement membrane (GBM) and envelope the glomerular capillaries. The fenestrated endothelial cells of the capillaries, the GBM and the interdigitated foot processes together form the filtration barrier that is important for retaining plasma proteins in circulation but allowing the passage of waste products and small molecules into the filtrate. Foot process detachment is a hallmark of various kidney diseases. Detachment compromises the filtration barrier leading to proteinuria and eventual kidney failure. Zebrafish podocytes show remarkable conservation in structure, function, and gene expression compared to mammals. We have identified a novel coiled-coil protein, PGAF (Podocyte-GBM attachment factor), that is essential for maintaining podocyte attachment to the GBM in zebrafish embryos. PGAF is expressed almost exclusively in podocytes and morpholino knockdown induces edema, a sign of kidney malfunction. Electron microscopy analysis of glomeruli from knockdown embryos reveals the detachment of numerous podocyte foot processes from the underlying GBM. Subsequent analysis found that PGAF is a direct target of Wilms' tumor suppressor-1, a podocyte transcription factor, and interacts with calpain, a calcium-activated cysteine protease. Active calpains promote the detachment of cellular adhesions through the cleavage of proteins that help link the podocyte to the GBM. We hypothesize that in the knockdown embryos, calpain activity is unregulated due to the loss of its interaction with PGAF. This results in the cleavage of proteins involved in adhesion and subsequent detachment of foot processes from the GBM. In support of this idea, treatment of PGAF knockdown embryos with calpain inhibitors partially rescues the edemic phenotype. Taken together, these data have identified a novel pathway used to retain podocyte-GBM attachments and could lead to new therapies to treat glomerular diseases.

Transgenic medaka overexpressing dominant-negative form of myostatin develops increased number of the muscle fibers

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Myostatin (MSTN), a member of the TGF-ß superfamily, is known to act as a negative regulator of skeletal muscle development and growth. Similar to other members of the TGF-ß superfamily, MSTN is synthesized as a pre-pro-peptide that undergoes proteolytic cleavage steps to yield the bioactive, mature C-terminal domain, the dimer of which binds to membrane receptors on target cells. The double-muscle phenotype, which is primarily attributed to an increase in the number of muscle fibers in several domesticated cattle breeds, has been shown to be caused by a series of MSTN mutations. In Piedmontese double-muscle cattle, there is a guanine-to-adenine transition mutation causing a substitution of a critical cysteine with a tyrosine (C313Y) in the signaling portion of the protein. This particular cysteine residue is indispensable for proper folding of mature MSTN and is required for full bioactivity. In mice, the overexpression of C313Y-type MSTN resulted in double-muscling, implying that the C313Y-type MSTN protein exhibits dominant-negative activity.

In this study, we sought to determine whether overexpression of C313Y-type MSTN in medaka (*Oryzias latipes*) causes the same phenotypic trait, an increase in muscle mass as reported previously in mice. The similarity in the mature regions of medaka and cattle MSTN (87 %) allowed us to specify the position of cysteine (C315), the corresponding residue of which is substituted to tyrosine (C313Y) in the Piedmontese breed. In order to obtain a mutant *mstn* cDNA corresponding to the C313Y-type MSTN mutant, we introduced a point mutation (guanine-to-adenine) to produce C313Y-type (C315Y) MSTN in medaka *mstn* cDNA. The transgene was designed to harbor FLAG-tagged C315Y-*mstn* and *hrgfp II* (GFP) cDNAs joined by internal ribosomal entry site (IRES), with expression driven by the medaka ß-actin regulatory regions. We successfully created transgenic medaka overexpressing C315Y-*mstn* exclusively in skeletal muscle. Green fluorescence, which mimicked exogenous MSTN expression and was visualized by anti-FLAG immuno-staining, indicated the occurrence of translational activity of the IRES element located between C315Y-*mstn* and GFP cDNAs in the transgenic medaka. In order to reveal the skeletal muscle formation, we performed whole-mount immuno-staining by using antibody against myosin heavy chain. Transgenic fish showed abnormal alignment of embryonic muscle fibers. Since the external appearance of wild-type and transgenic fish was not remarkably different, we made serial sections of adult fish and analyzed the number and size of muscle fibers. As a result, our histological examinations revealed clear evidence of marked hyperplasia, but not hypertrophy, in the skeletal muscle of transgenic medaka. Each of the muscle fibers in transgenic fish was smaller than that in wild-type, resulting in no apparent alteration of the whole muscle mass.

In conclusion, phenotypic analysis of the transgenic medaka revealed that exogenous expression of a dominant-negative form MSTN (C315Y-MSTN) induces hyperplasia of skeletal muscle, but not hypertrophy. Our results suggest that endogenous MSTN has an inhibitory effect on muscle cell proliferation in medaka.

Characterisation of neural stem cells in medaka fish midbrain

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Very few adult neural stem cells have been described in detail and their diversity thus remains undocumented. Adult neurogenesis in mammals is restricted to the forebrain. Neuronal production in teleost occurs throughout life-span in many regions of the brain. Whether non-mammalian progenitors have similar characteristics to the mammalian neural stem cells remains unknown.

To this aim, we use an ideal model found in the midbrain of the medaka fish *Oryzias latipes*, the optic tectum (OT). We previously demonstratedthat this cortical structure of the dorsal midbrain grows by successive additions of open rings of progenitor cells originating from a population of actively dividing progenitors located at the periphery of the organ.

To characterize further this region, we performed two long pulses of nucleoside analogs (IdU and CldU) at different intervals in juveniles and adult fish. This strategy lead to the identification of a subset of long-term label retaining cells that are likely to be the OT stem cells. They are located in the marginalmost regions of the proliferation zones (PZ) of the adult brain but are not detected in the juvenile brain.

We also analyzed the expression patterns of the pluripotency-associated markers *Sox2*, *Musashi 1* and *Bmi1*.

These genes are expressed in the PZ of the adult OT, indicating that the core machinery for neural pluripotency is conserved throughout vertebrate evolution. Moreover, the adult gene expression patterns differ considerably from those at embryonic stages. This provides evidence that a molecular mechanism specifies an adult stem cell niche. Fish tectum thus represents a new powerful model to study the diversity of neural stem cells.

The molecular structures and expression patterns of zebrafish troponin I genes

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Troponin I (TnnI), a constituent of the troponin complex located on the thin filament, provides a calciumsensitive switch for striated muscle contraction. Cardiac TnnI is, therefore, a highly sensitive and specific marker of myocardial injury in acute coronary syndromes. The TnnI gene, which has been identified in birds and mammals, encodes the isoforms expressed in cardiac muscle, fast skeletal muscle and slow skeletal muscle. However, very little is known about the TnnI gene in lower vertebrates. Here, we cloned and characterized the molecular structures and expression patterns of three types of zebrafish tnni genes: tnni1, tnni2 and tnni-HC (heart and craniofacial). Based on the unrooted radial gene tree analysis of the TnnI gene among vertebrates, the zebrafish TnnI1 and TnnI2 we cloned were homologous of the slow muscle TnnI1 and fast muscle TnnI2 of other vertebrates, respectively. In addition, reverse transcription-polymerase chain reaction (RT-PCR) and whole-mount in situ hybridization demonstrated that zebrafish tnni1 and tnni2 transcripts were not detectable in the somites until 16 h post-fertilization (hpf), after which they were identified as slow- and fast-muscle-specific, respectively. Interestingly, tnni-HC, a novel tnni isoform of zebrafish was expressed exclusively in heart during early cardiogenesis at 16 hpf, but then extended its expression in craniofacial muscle after 48 hpf. Thus, using zebrafish as our system model, it is suggested that the results, as noted above, may provide more insight into the molecular structure and expression patterns of the lower vertebrate TnnI gene.

Preliminary study on the role of sialidase neu3.1 and neu4 in zebrafish development

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Sialidases or neuraminidases are a family of enzymes that catalyze the removal of terminally linked sialic acid residues from glycoproteins and glycolipids. Thus, sialidases represent the initial and essential step for the degradation of sialoglycoconjugates. Four different mammalian sialidases have been described: lysosomal (NEU1), cytoplasmic (NEU2), plasma membrane-associated (NEU3) and intracellular membrane-associated (NEU4) sialidase. Mammalian sialidases are implicated in lysosomal catabolism, as well as in regulation of important cellular events including cell differentiation, growth, adhesion, and apoptosis. We recently cloned and characterized seven different genes of zebrafish (*Danio rerio*) related to human sialidases. Two of them, *neu1* and *neu4*, represent the orthologs of the mammalian sialidases *NEU1* and *NEU4*, respectively. The remaining genes, i.e. *neu3.1*, *neu3.2*, *neu3.3*, *neu3.4* and *neu3.5*, are all more closely related to mammalian sialidase *NEU3*.
Here we focus on *neu3.1* and *neu4*. RT-PCR and *in situ* hybridization experiments demonstrate that *neu3.1* and *neu4* are expressed already at early development stages and show temporal and spatially localized expression areas corresponding to gut and lens, respectively. In order to test the role of *neu3.1* and *neu4* in zebrafish development we performed gene inactivation experiments using splice-inhibiting Morpholinos (MO).
neu3.1 knock-down causes significant phenotypic alteration. Morphants show head and eye development retardation, trunk and tail abnormal curvature and defective somites patterning. We now plan to characterize the morphant phenotype observed by *in situ* hybridization experiments using different markers.
Knock-down of *neu4* by injecting 1 ng of MO results in a spectrum of phenotypes ranging from brain edema, heart edema, shortening of the main body axis, misshaped somites. Moreover, at 24 hpf, a significant impairment of the region where digestive system will develop is observed. Higher injection amounts gave rise to much stronger phenotypic alterations but also caused severe mortality at 24 hpf, suggesting that a complete depletion of *neu4* is lethal. We are currently characterizing the phenotypes observed by whole-mount *in situ* hybridization of injected embryos for the expression of different marker genes. mRNA rescue experiments are presently ongoing in order to confirm the specificity of the phenotypic alterations observed.

Characterization of vsp, an interactor of arms/kidins220 expresses in the ventral region of zebrafish eye

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Looking for genes involved in eye development, we selected *vsp* in the course of a large scale in situ screening. *vsp* encodes a protein containing two PDZ domains and a RING-finger domain and displays specific expression in the developing eye. *vsp* expression initiate in the ventral region of the optic primordia at the 10-somite stage. As eye morphogenesis proceeds, *vsp* expression becomes restricted to the ventral retina and to the initial tract of the optic stalk. Outside the optic regions, *vsp* is expressed in rhombomere 1 and in specific areas of the trunk possibly corresponding to ganglia. We recently found that *vsp* specifically interacts with the protein ARMS/kidins220, a target of neurotrophin and ephrins. These data, together with the absence of *vsp* expression in the ventral eye region of *noi* mutants, suggest that this gene might be involved in controlling axon outgrowth of retinal ganglion cells.

Unc119b Regulates Ciliogenesis and Left-Right Axis Determination

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The Unc-119 protein is required for normal nervous system development in metazoans, although its molecular role remains unclear. *C. elegans* mutants in *unc-119* have abnormalities of dye uptake and the distal tips of cilia are not formed properly. In mammalian systems, as well as work done in *C. elegans* in our lab, Unc-119 protein family members have been shown to interact Arl family members, which are involved in ciliogenesis.

We therefore tested whether Unc119b has a role in ciliogenesis in zebrafish. Morpholino (MO)-mediated knockdown of Unc119b produces animals with a curved body, a phenotype reminiscent to other cilia mutants. Unc119b also displays a cilia phenotype in the pronephric duct similar to an *arl13b* mutant, and genetic epistasis experiments indicate that *unc119b* acts either upstream or parallel of *arl13b*. In addition, Unc119b morphant hearts are unlooped or displayed randomized looping indicating a role in the establishment of left-right asymmetry. The pineal gland, as marked by *crx*, fails to migrate in Unc119b morphants, which is a left-right dependent event. These results are consistent with Unc119b acting as a ciliogenesis gene. Analysis of cilia at Kupffer's vesicle demonstrates shorter and fewer cilia in Unc119b morphants.

In addition to defects in ciliogenesis, Unc119b have hydocephalus and an open neural tube phenotype. Injection of Rhodamine-dextran into the hindbrain reveals that the midbrain-hindbrain boundary does not close in Unc119b morphants. Further analysis reveals that *atonal homologue1a (atoh1a)* is excluded from the midbrain-hindbrain boundary, reminiscent of Fgf mutants. This result coupled with the pineal gland and Kupffer's vesicle phenotypes suggests Unc119b interacts with the Fgf signalling pathway, a hypothesis we are currently testing. Altogether, this work provides a novel role for Unc119b in ciliogenesis and determination of left-right asymmetry.

Modelling arrhythmogenic right ventricular cardiomyopathy in zebrafish

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Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) is a congenital heart disease associated with ventricular arrhythmias and sudden death in young people. Several proteins have been implicated in ARVC including the desmosomal armadillo proteins plakophilin 2 and plakoglobin. These are integral to maintaining tissue integrity and strength, and are particularly important in tissues which undergo routine mechanical stress such as the skin and heart. Loss of plakophilin 2 and plakoglobin in mice leads to cardiac instability and embryonic lethality. We have previously shown that loss of plakoglobin in zebrafish is a model for ARVC (Martin *et al.*, 2009).

Knockdown of zebrafish plakophilin 2 or plakoglobin by morpholino antisense technology has resulted in morphant embryos with specific cardiovascular defects - pericardial oedema, enlarged hearts, reduced heart beat. This is accompanied by stage-dependent anterior neural and tail defects. Structural analysis of plakoglobin morphants using transmission electron microscopy (TEM) showed altered adhesion and cellular morphology in their cardiac valves along with reduced numbers of junctions in the heart as a whole. Initial TEM studies in plakophilin 2 morphants also show a decrease in junction numbers along with altered junctional morphology.

These data show the importance of desmosomal armadillo proteins in zebrafish cardiac development and the usefulness of zebrafish as a model for ARVC.

Martin, E.D., Moriarty, M.A., Byrnes, L., Grealy, M. (2009) 'Plakoglobin has both structural and signaling roles in zebrafish development.' Developmental Biology ***327***

The Transcription Factor Six1a Plays an Essential Role in the Craniofacial Myogenesis of Zebrafish

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Transcription factor Six1a plays important roles in morphogenesis, organogenesis, and cell differentiation. However, the role of Six1a during zebrafish cranial muscle development is still unclear. Here, we demonstrated that Six1a was required for sternohyoideus, medial rectus, inferior rectus, and all pharyngeal arch muscle development. Although Six1a was also necessary for *myod* and *myogenin* expression in head muscles, it did not affect *myf5* expression in cranial muscles that originate from head mesoderm. Overexpression of *myod* enabled embryos to rescue all the defects in cranial muscles induced by injection of *six1a*-morpholino (MO), suggesting that *myod* is directly downstream of *six1a* in controlling craniofacial myogenesis. However, overexpression of *six1a* was unable to rescue arch muscle defects in the *tbx1*- and *myf5*-morphants, suggesting that *six1a* is only involved in myogenic maintenance, not its initiation, during arch muscle myogenesis. Although the craniofacial muscle defects caused by *pax3*-MO phenocopied those induced by *six1a*-MO, injection of *six1a*, *myod* or *myf5* mRNA did not rescue the cranial muscle defects in *pax3* morphants, suggesting that *six1a* and *pax3* do not function in the same regulatory network. Therefore, we proposed four putative regulatory pathways to understand how *six1a* distinctly interacts with either *myf5* or *myod* during zebrafish craniofacial muscle development.

Zebrafish as a model to study of thyroid development

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The thyroid is an endocrine gland composed of follicular cells that develops from a primordium bud derived from the ventral floor of the anterior pharyngeal endoderm. After a migration process, the primordium loses contact with the pharynx, relocates in the hypopharyngeal mesenchyme and adopts its final bilobed shape in the neck area of mice and man or forms an elongated strand of tissue along the ventral aorta in zebrafish. At this stage of development, the thyroid becomes fully differentiated, expresses thyroid specific genes and produces thyroid hormones.
In human, congenital hypothyroidism (CH) is a frequent congenital disease. In 85% of the cases, CH is the result of thyroid dysgenesis which comprises agenesis, hypoplasia and/or ectopy of this gland in newborn humans. To date, only a minor proportion of familial CH cases have been associated with mutations located in transcription factors known to be involved in thyroid development, such as NKX-2.1, PAX8 or FOXE1. This suggests that other, still unknown genes might play an important role during thyroid organogenesis.
To identify novel genes involved in the regulation of thyroid development and differentiation, we combined LongSAGE with Solexa deep sequencing to analyze the transcriptome of thyroid primordium, surrounding mesenchyme and pharyngeal endoderm in E11.5 mouse embryos. This approach proved successful to quantitatively and qualitatively characterize gene expression at an early stage of thyroid development. Subsequent application of various bioinformatical approaches yielded more than 1300 candidates genes potentially involved in early thyroid development.
Previous studies clearly demonstrated the value of zebrafish as a model to study early aspects of thyroid development. We therefore devised a strategy to exploit the unique opportunities of the zebrafish model for a functional characterization of the candidates genes identified in mouse. Using a high throughput protocol for whole-mount *in situ* hybridization, we are screening mouse candidate genes for thyroid-specific expression in zebrafish embryos at five distinct developmental stages. Positive candidates are further characterized by using morpholino knock-down and overexpression approaches. In addition, we established a transgenic zebrafish line that will allow us to monitor thyroid migration and differentiation in living animals. In these transgenic fish, GFP-mCherry is driven by regulatory sequences of the zebrafish thyroglobulin gene. Experiments with embryos and larvae showed that GFP-mCherry is specifically expressed in the thyroid primordium and differentiated thyroid follicles.

Somitogenesis and development of Primary Motor Neurons: the role of the homeobox *uncx4.1*

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The gene *uncx4.1* is a paired-type homeobox transcription factor, expressed during zebrafish embryonic development in branchial arches, somites, CNS and pronephric ducts. In particular, the expression at the somite level is detectable from 5ss stage and becomes progressively posteriorly and ventrally restricted to the presumptive myoblast cells in later stages.
Interestingly, the progressive restriction of *uncx4.1* activity anticipates and accompanies the appearance of the first outgrowing primary motor axons. Indeed, after *uncx4.1* expression is not detected anymore in medial somitic cells, a ventrally projecting CaP motor axons emerge from the ventro-lateral spinal cord.
In relation with muscle differentiation, double hybridizations showed that *uncx4.1* expression precedes *myoD* activation in muscle precursor cells. Moreover, co-localization of *uncx4.1* mRNA and MYOSIN protein (fibroblast marker) reveals a complementary distribution of the two molecules, suggesting that *uncx4.1* activity steps back from maturing muscle cells.
uncx4.1 seems to be negatively regulated by s*hh* pathway, indeed shh overexpression abolishes *uncx4.1* transcription. Analyses of the shh pathway zebrafish mutants (*floating head (flh)*, *cyclops (nodal)*, *sonic you (shh)* and *you-too (gli2))* showed that the expression of the *uncx4.1* gene extends abnormally from dorsal to ventral within somitic compartments, confirming the negative regulation.
Currently, *uncx4.1* functional characterization is performed by a morpholino mediated strategy. Spatio-temporal dynamics of *uncx4.1* expression, along with functional experimental evidences, suggest a double role for the *uncx4.1* gene, in guiding outgrowth of motor axons, and regulating cell cycle in muscle progenitors at the margin of each somite.

The cell cycle regulator Cdc14B is essential for ciliogenesis in zebrafish

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Progression through the cell cycle relies on oscillation of Cyclin-dependent kinase (Cdk) activity. Specifically, exit from mitosis requires the inactivation of Cdk1 and dephosphorylation of its substrates. The Cdc14 family of phosphatases is involved in this latter event and thus contributes to chromosome segregation, mitotic exit and cytokinesis. Although the Cdc14 family is conserved among eukaryotes, the role and regulation of Cdc14 phosphatases has been well studied in budding and fission yeast, but remains poorly understood in vertebrates. Using zebrafish as a model, we aim to further understand the function of Cdc14 phosphatases during vertebrate development.

Our analysis of the zebrafish genome reveals three *cdc14* paralogs: *cdc14A1*, *cdc14A2* and *cdc14B*. Here, we report our results on *cdc14B* during zebrafish embryogenesis. Two *cdc14B* isoforms were identified and are both expressed ubiquitously throughout development. Downregulation of *cdc14B* function during embryonic development using antisense morpholino oligonucleotides, unexpectedly impairs Left-Right asymmetry. *cdc14B* morphant embryos exhibit randomized heart tube shifting, gut looping and pancreas and liver position as well as aberrant expression of *southpaw*, the earliest marker showing asymmetric expression. These phenotypes are suppressed when *cdc14B* RNA is co-injected. In zebrafish, the Kupffer's vesicle (KV) is the organ responsible for establishing Left-Right asymmetry. Although Cdc14B plays a role during the cell cycle, the number of cells in the KV is unaffected in *cdc14B* deficient embryos and so is its morphogenesis. Interestingly however, we find that the length of the cilia in the KV is considerably reduced likely providing a mechanism for the Left-Right asymmetry defect in *cdc14B* morphants. Preliminary results indicate that other types of cilia are also affected in the *cdc14B* morphants, strongly suggesting that we have uncovered a novel function for Cdc14B in the process of ciliogenesis.

Screening for mutations affecting the development of exocrine and endocrine pancreas

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The main research theme of our laboratory is to study the molecular mechanisms controlling pancreas development in zebrafish using two main approaches : i) knock-down of selected candidate genes and ii) screening of mutants displaying pancreatic defects. In order to identify mutations affecting endocrine or exocrine pancreas development, 2 mutagenesis screens were performed: the first mutagenesis was performed on a double transgenic zebrafish line Pax6b:GFP / Insulin:DsRed which allows the direct observation of beta cells, endocrine pancreatic cells and enterodendocrine cells. The second screen was performed on non transgenic mutagenized AB/TL fish (Driever's lab) by in situ hybridization using glucagon and trypsin probes. More than 500 mutagenized genomes were analyzed. While we did not identify mutant lacking endocrine pancreatic cells, several mutants were detected based on a reduction of the pancreatic exocrine tissue or on a decrease of enteroendocrine cells in the gut. We are presently analysing expression of various pancreatic, hepatic and intestinal markers in these mutants. The phenotype of some of these mutants will be presented in details.

Lrp5 and its putative inhibitor Sclerostin are required for development of the zebrafish cranial skeleton

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The major portion of the cranial skeleton is established by cells that originate from the rostral neural crest. In the course of development, these cranial neural crest cells (CNCCs) undergo an epithelial to mesenchymal transition (EMT), delaminate from the neural plate and migrate in separate mesenchymal streams ventrally to invade the respective cranial regions in which they subsequently differentiate to form the cranial cartilage. Each stream is thereby committed to give rise to a distinct cartilage structure. It has been shown earlier that canonical Wnt signaling is essential for various steps during this process. Here we show that the frizzled co-receptor low-density-lipoprotein (LDL) receptor-related protein 5 (Lrp5) and its putative inhibitor Sclerostin (Sost) are involved in these processes. Both genes are expressed in adjacent and highly dynamic patterns in the developing head. Early in development, *lrp5* and *sost* are expressed in the dorsal hindbrain, directly adjacent to the delaminating CNCCs. At later stages, they are expressed in regions of the ventral head, where CNCCs differentiate to form the gill cartilage structures. Morpholino mediated knockdown of either *lrp5* or *sost* prevents formation of the ceratobranchials in a concentration dependent manner. *lrp5* morphants completely lack cartilaginous structures in the region that would form the ceratobranchials. In contrast, knockdown of *sost* results in excess clusters of cartilage cells that subsequently fail to form the gill cartilage elements. This opens the possibility that Sost controlled Lrp5 signalling is involved in two independent processes of cranial skeleton formation: (i) CNCC migration and (ii) establishment of gill cartilage morphology.

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The Zebrafish hmg-box transcription factor sox4b activates pituitary expression of *GATA2* and specification of thyrotrope cells

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The adenohypophysis consists of at least six different cell types: somatotropes, lactotropes, thyrotropes, melanotropes, corticotropes and gonadotropes in mammals, and an additional cell type in fish expressing somatolactin. We investigate the role of Sox4b, a member of the SRY-like HMG-box (SOX) family in pituitary development. We found that *sox4b* is strongly expressed in the pituitary anlagen starting at 24 and 26 hpf and in the entire head region including the pituitary at 48hpf. We show that *sox4b* mRNA colocalizes with the pan-pituitary marker *lim3* at 33 hpf and with *tsh*ß at 48 hpf. *sox4b* knock-down leads to a drastic decrease in *tsh*β and *gsu*α expression and reduced levels of *gh* and *sl*ß mRNA, while other anterior pituitary gland markers including *prl* and *lim3* are not affected. We also used a mutant form of Sox4 deleted of its C-terminal domain (Sox4 ΔCter) that is unable to activate transcription and therefore acts as a dominant-negative mutant on endogenous sox4 proteins. Expression of this mutant also led to a decrease in *tsh*ß, *gsu*ß and *gh*-positive cells. Furthermore, expression of the zinc finger transcription factor *gata2* is downregulated in *sox4b* morphants specifically in the pituitary. We showed that *gata2* expression colocalizes with *tsh*ß, *gsu*ß and *sox4b* mRNAs in the pituitary of 48 hpf embryos. Finally, we could clearly observe that *gata 2* knock-down leads to a decrease in *tsh*ß and *gsu*ß expression at 48hpf. but *prl* expression was not significantly affected; similar to the effects observed in *sox4b* morphants.

In conclusion, Sox4b is expressed in zebrafish during pituitary development and plays a crucial role in the differentiation of thyrotrope and somatotrope cells through induction of *gata2* expression in the developing pituitary.

Regulation of brain ventricle inflation

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The brain ventricular system is a highly conserved set of cavities, containing cerebrospinal fluid (CSF), which is essential for brain function. Although the embryonic brain ventricles contain large amounts of embryonic CSF (eCSF) little is known about how this fluid is secreted or how it functions. In the zebrafish, the Na^+K^+ATPase ion pump is crucial for initial embryonic brain ventricle inflation. This pump includes three subunits - alpha, beta and gamma. We previously showed that the alpha subunit, *atp1a1*, is required for zebrafish brain ventricle inflation (Lowery and Sive 2005). Here we demonstrate that Atp1a1 is necessary for epithelial integrity early in development, and for brain ventricle inflation after neurulation. In addition, we show that the beta subunit *atp1b3a* is strongly expressed in neuroepithelium surrounding the forebrain and midbrain ventricles, and that it synergizes with Atp1a1 during ventricle inflation. Dose response assays show that forebrain brain ventricle area is highly sensitive to the amount of functional Atp1a1. Preliminary data suggest that the gamma subunits, FXYD1, 5 and 6 are essential for brain ventricle inflation. In sum, these data show that the Na^+K^+ATPase plays different roles before and after neurulation, and that the correct balance of subunits is necessary to direct brain ventricle size, results with implications for the understanding the etiology of hydrocephalus.

X-ray imaging of 3D structures of teeth and skeletal elements in zebrafish and medaka, and their motions during respiration and feeding

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X-ray micro tomography at SPring-8 synchrotron was used to analyze three-dimensional structures of skeletal elements in adult and larval zebrafish and medaka. Adult zebrafish has relatively large ~24 pharyngeal teeth only at the ventral side. Right and left ventral teeth were found to face each other towards midline. In contrast, adult medaka has more than 1000 pharyngeal teeth distributed both at ventral and dorsal sides. Dorsal teeth and ventral teeth are facing each other. Medaka also has teeth on upper and lower jaws.

We then performed live X-ray imaging of the skeletal elements to study their motions during respiration and feeding. Each skeletal element was found to move periodically with different phase during respiration.

Functions of pharyngeal teeth in fish have been poorly understood. We hope to elucidate their roles by comparing these two teleosts established as model vertebrates that appear to have teeth with different anatomical characteristics.

Gene expression profiling of zebrafish embryonic hearts identifies molecular networks involved in early cardiac morphogenesis

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The multi-chambered hearts of vertebrates initially arise from two lateral populations of cardiac precursor cells (CPCs), which are subject to morphogenetic rearrangements in the course of cardiogenesis. In zebrafish, heart tube formation involves the asymmetric involution of the right posterior heart field and extensive migratory processes within distal portions of the heart field. Although a couple of signaling pathways have been implicated in these processes based on loss- or gain-of-function phenotypic analyses, the exact genetic and cellular mechanisms that underlie CPC behavior remain to be unraveled.

Here, we make use of whole transcriptome gene expression profiling on highly purified embryonic heart tissue to identify novel and key regulators of heart tube formation. Out of all annotated zebrafish genes expressed in the heart, we find hundreds that are associated with particular morphogenetic or physiological processes. These include extra- intracellular signal transduction, specification or patterning of the heart, ion homeostasis and myogenesis. Further, we are particularly interested in genes that regulate migratory behavior, cell polarity or adhesion, as these genes are likely to be involved in morphogenetic processes. Comparison of myocardial gene expression to a reference sample of whole embryos lacking heart tissue at the stage of cardiac field involution reveals that about one quarter of all genes that fall within those categories are specifically up-regulated in the heart and likely contribute to its unique features. Based on our findings, we have now started to verify prime candidates and to elucidate their function in cardiogenesis. In addition, we now employ transcriptional profiling to assess the impact of known signaling pathways on CPC gene expression. Thereby we hope to elucidate their specific requirements during heart development.

Characterization and functional analysis of gluconeogenesis during zebrafish development
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The steadily rising obesity prevalence in western civilization has lead to a pandemic increase in insulin resistance as part of the Metabolic Syndrome and Type 2 Diabetes Mellitus (T2DM). Insulin resistance in hepatocytes induces elevated glucose output from the liver and contributes to hyperglycemia in T2DM.
In order to characterize zebrafish as a model for studies on the regulation of glucose metabolism and insulin resistance, we carried out *in-situ* hybridization expression studies for three key enzymes of the gluconeogenic pathway: Glucose-6-phospatase (encoded by *g6pc*), fructose-1,6-bisphosphatase (encoded by *fbp1*) and Phosphoenolpyruvate-carboxykinase (encoded by *pck1*).
In the early embryo *pck1* is strongly expressed in the yolk syncytial layer. Considering the higher energy density of lipids compared to glycogen, this early *pck1* expression suggests a conversion from abundant yolk lipids to glucose "on the spot" prior to the functional differentiation of gluconeogenic organs.
At 48 hpf we detect expression of *fbp1* and *g6pc* in the developing liver whereas *pck1* is initiated later in liver and intestine, between 72 and 96 hpf. In addition to the onset of *pck1*, *g6pc* and *fbp1* show a strong increase around this time point when larvae have hatched and start to swim. Energy demand and lactate production of the swimming fish is likely to contribute to the logic of this time course of gene expression. Using pharmacological and genetic studies we want to test this hypothesis and gain insights into the regulation of glucose production.
To study the requirement of gluconeogenesis for the developing embryo, we are conducting Morpholino-mediated gene knock down analyses targeted against *g6pc* and *pck1*. Glucose-6-phosphatase functions at the end point of the gluconeogenic program where it is important for the release of glucose into the blood stream. Preliminary results indicate a largely normal development, but decreased liver size in injected embryos compared to controls. Pck1 is the most upstream enzyme of the pathway that converts oxaloacetate to phosphoenolpyruvate. Since oxaloacetate is derived from lactate, pyruvate or glucogenic amino acids, we expect to see a phenotype that is specific to impaired utilization of those metabolites, but not of the entire gluconeogenic program.

Defects in ribosome biogenesis underpin the morphological characteristics of a zebrafish development mutant, *setebos*, with eye, intestinal and craniofacial abnormalities

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Ribosome biogenesis is the most energy consuming process in growing cells. As yet, the mechanisms that link ribosome biogenesis to the regulation of cell growth and proliferation are incompletely understood. In mammals, disruption of ribosome biogenesis leads to 'nucleolar stress' resulting in apoptosis or cell cycle arrest through activation of the tumour suppressor protein, p53. The ribosomal protein L11 has been shown to be a key player in this process, highlighting the need to understand extra-ribosomal functions of ribosomal proteins.

setebos^{s453} is a zebrafish mutant that was identified in the Liver^{plus} screen designed to identify genes involved in endodermal organ morphogenesis. At 5 days post fertilisation the intestinal epithelium in *setebos* is hypoplastic compared to wildtype embryos. The liver, pancreas and eye are also markedly under-developed and craniofacial cartilage development is severely disrupted.

The responsible mutation in *setebos* is a premature stop codon in *nucleolar protein 8* (*nol8*), a gene that has been shown to be required for ribosomal RNA (rRNA) processing in yeast. We have employed metabolic labeling experiments alongside Northern blot and bioanalyzer analyses to demonstrate defective rRNA processing in *setebos* embryos. This disruption culminates in reduced levels of mature 28S rRNA species. Thus, Nol8 function is conserved from yeast to vertebrates.

In *setebos* embryos, the mRNA level of the N-terminally truncated isoform of p53, Δ113p53, is specifically and markedly upregulated in response to impaired rRNA processing, and the mRNA levels of the cell cycle arrest genes, *p21* and *cyclinG1* are also elevated. We are using *setebos* embryos to dissect the role of p53 and its various isoforms in the cellular response to impaired ribosome biogenesis, and to explore the consequences of compromising this important cellular process on tumour formation.

Nkcc1/Slc12a2 is required for the regulation of endolymph in the otic vesicle and volume of the swim bladder in the zebrafish larva

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Endolymph is the specialised extracellular fluid present inside the inner ear. In mammals, disruptions to endolymph homeostasis can result in either collapse or distension of the endolymphatic compartment in the cochlea, and concomitant hearing loss. The zebrafish *little ears* (*lte*) mutant shows a collapse of the otic vesicle in the larva, apparently due to a loss of endolymphatic fluid in the ear, together with an over-inflation of the swim bladder. Mutant larvae display signs of abnormal vestibular function by circling and swimming upside down. The two available alleles of *lte* are homozygous lethal: mutant larvae fail to thrive beyond 6 days post fertilisation. Patterning of the otic vesicle is apparently normal. However, expression of several genes thought to play a role in endolymph production is downregulated, including the sodium-potassium-chloride cotransporter gene *nkcc1* (*slc12a2*) and several *Na^+/K^+-ATPase* channel subunit genes. We have cloned the *lte* mutations and show that they correspond to lesions in *nkcc1*. Each allele has a point mutation that disrupts splicing, leading to frame shifts in the coding region, and predicting the generation of truncated products. Endolymph collapse in the *lte/nkcc1* mutant shows distinct parallels to that seen in mouse *Nkcc1* mutants, validating the zebrafish as a model for the study of endolymph disorders. The collapse in ear volume can be ameliorated in the *to27d* allele by injection of a morpholino to block splicing at the new site, forcing use of the original site. This exemplifies the use of morpholinos as potential therapeutic agents for genetic disease.

Cellular and genetic interactions underlying choroid fissure formation and closure

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The choroid fissure is a transient opening on the ventral side of the optic cup through which blood vessels enter and retinal axons leave the developing eye. Failure of the choroid fissure to close results in ocular colobomas, a family of common ocular pathogeneses that can cause severe visual impairment. Despite this fact, virtually nothing is known about the genetic mechanisms and cell movements that underlie choroid fissure closure.

Recent studies by us and others indicate that mesenchymal cells outside the retina play a critical role in choroid fissure closure. Although the importance of these periocular mesenchymal cells (POM) is established, we do not know how they function. We hypothesize that there is signaling between these cells and the retina to coordinate the timing of closure. In our ongoing research we are exploring the identity of the POM cells required for choroid fissure fusion, determining when and how they function and elucidating the molecular pathways that mediate fusion.

We are collaborating with the laboratories of William Harris and Brian Link.

***Soul-2*, a heme binding protein-coding gene in kidney development**

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Besides iron is a crucial element for body homeostasis, being mainly incorporated in heme and acting as cofactor in many proteins and enzymes, our knowledge of iron genetics during embryonic development is still fragmentary. During kidney differentiation, abnormal iron metabolism contributes to the development of anemia in chronic kidney disease patients. We are studying the SOUL/p22HBP family of heme binding proteins, generic tetrapyrrole-binding factors with high affinity for porphyrins and heme. SOUL proteins may act as cytosolic buffer against cell intoxication by iron products, or promote necrotic cell death. We identified four zebrafish *Soul* orthologs, as candidate factors for heme biosynthesis and iron homeostasis. We focused our attention on *soul2*, a gene that is expressed in collective ducts and distal segment of the pronephric ducts from eighteen somites stage until 96 hours post fertilization (hpf). Preliminary results by morpholino-driven knockdown support a dual role in patterning and differentiation during pronephric duct development. Kidney of *soul2* morphant embryos displays thickening of the distal tubules, as shown by histology and *in vivo* imaging, as well as altered expression patterns of regionalization and differentiation markers. We are further characterizing additional phenotypic aspects, including a mechanistic link with heme oxygenase 1 (HO-1), a regulator of sodium uptake in the kidney and a rate-limiting enzyme in the degradation of heme, and with Nitric Oxide (NO) signaling, which is required for proper HO-1 activity.

Uncovering new genes involved in zebrafish thyroid development

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The thyroid is an endocrine gland that produces the thyroid hormones involved in many functions in vertebrate and invertebrate organisms. Thyroid organogenesis is a very complex developmental process that, through the combination of migration of thyroid precursor cells, their organization into follicular structures and functional differentiation, leads to the formation of an organ able to produce specific hormones. In mammals, alteration of these mechanisms might be responsible of congenital hypothyroidism (CH), the most frequent inherited endocrine disease. The genetic basis of CH is known only in few cases.

Thyroid formation in fish is comparable to mammals at both ontogenetic and molecular levels: the thyroid develops from the endodermal tissue, at the midline of the pharyngeal floor, and its formation is subdivided into few successive steps. The genetic programme involved in thyroid differentiation, including the activity of transcription factors such as *Nkx2.1a*, *Pax8* and *Hhex*, is conserved with respect to expression patterns and functions.

To gain further insights on the genetic machinery implicated in normal thyroid development, and therefore in CH disease, an LMC (Laser Capture Microdissection)-based oligonucleotide microarray analysis was performed in mouse to identify genes differentially expressed during the gland organogenesis. This approach highlighted a list of genes that are enriched in mouse E10.5 thyroid. We are now using the zebrafish as a simple model to perform functional analysis on the fish counterparts of these genes. Surfing the zebrafish genome for the homologs of 74 murine genes, we found 89 orthologs/paralogs of 56 mouse counterparts, while 18 mouse genes did not show zebrafish orthologous matches. To date, we have analyzed the expression patterns of 20 genes, finding new factors that are expressed in the developing thyroid gland of zebrafish. In addition, some genes show expression in the regions surrounding the gland primordium raising the possibility of contamination from these tissues during the LMC procedure. One of the genes expressed in zebrafish thyroid primordium is an anti-apoptotic factor. We found that this gene is expressed in thyroid from 30hpf. Interestingly its expression disappears early in both *Nkx2.1a* and *Hhex* morphant embryos when the thyroid primordium is still present. These results lend support to a hypothetical implication of anti-apoptotic processes in the conservation of thyroid structure.

In the future we will advance in the characterization of the transcriptional profiles of zebrafish orthologues, with the aim to provide new hypotheses on the genetic and cellular bases of specification and differentiation of thyroid follicles. In this frame, the role of thyroid-expressed genes will be addressed by loss-of-function approaches, whereas knock-down embryos will be characterized by WMIHC (Tg, T4), WMISH (*hhex*, *nk2.1a*, *pax8*, *pax2.1*, *tg*) and TUNEL in wild-type, mutant (*no isthmus*, *hands off*) and transgenic (Tg(*nk2.1a*:YFP) backgrounds.

Sec24d transports extracellular matrix proteins during zebrafish skeletal morphogenesis

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Protein transport from endoplasmic reticulum (ER) to Golgi is primarily conducted by coated vesicular carriers such as COPII. Cargo binding mechanisms have been studied *in vitro* by biochemical and cellular biology approaches using model proteins, however, the cargo specificity and the role of individual transport adaptors during vertebrate development are largely unknown.

Cargo adaptor Sec24D is an integral component of the COPII complexes. We have positionaly cloned zebrafish mutation *bulldog* and shown that Sec24D is essential for secretion of cartilage matrix by mature chondrocytes, whereas transport of extracellular matrix proteins critical for migration of craniofacial primordia and pre-chondrogenic condensations proceeds normally. Consequently, chondroblasts fail to change shape and intercalate, thus resulting in small and malformed cartilages and severe craniofacial dysmorphology. We demonstrate that the arrest in cartilage development stems from matrix deficits, and not a lack of membrane bound receptors since b1-integrin and cell adhesion molecules are transported in Sec24D-independent fashion. Furthermore, Sec24D-deficient cells accumulate proteins in the distended ER, although a subset of ER compartments and Golgi complexes visualized by electron microscopy and NBD_6-ceramide staining appear functional. The backlog of proteins in the ER leads to upregulation of BiP and activation of the ER stress response machinery. We found that knockdown of Sec24C, a close paralog of Sec24D, does not result in craniofacial dysmorphology. However, craniofacial development in double Sec24C/Sec24D-deficient animals is arrested earlier than *bulldog/sec24d*, indicating that Sec24C can compensate for loss of Sec24D at initial stages of chondrogenesis, but Sec24D is indispensable for chondrocyte maturation. This study presents the first developmental prospective on the Sec24D cargo specificity during development. Our findings establish Sec24D as a strong candidate for cartilage maintenance diseases and craniofacial birth defects.

Modulators of calcium signaling induce developmental brain defects and behavioral changes

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Pharmaceuticals that modulate calcium signaling are successfully used for treating high blood pressure, heart arrhythmias, angina pectoris, and migraine. However, along with their widespread use, comes an increased risk for exposure during embryonic development. Human embryos may inadvertently be exposed by maternal use of medicine during early pregnancy or by trace concentrations of pharmaceuticals in the environment and drinking water. These exposures are troubling, as studies in animal model systems have shown that subtle calcium manipulations during embryonic development can induce specific brain defects. The potential risk for human brain development is difficult to evaluate because of the large number of pharmaceuticals that can affect calcium signaling either directly or indirectly, a lack of information on the sensitive developmental times, and the potentially pleiotropic effects on brain development and behavior. We use zebrafish as a model system to examine how modulators of calcium signaling affect brain development and behavior. We found that zebrafish embryos are sensitive to modulation of calcium signaling during gastrulation. Exposures during this time affect the development of laterality in the brain and asymmetry in behavior. The underlying calcium-sensitive mechanisms include convergence extension during gastrulation and the formation of Kupffer's vesicle. Further information on the developmental mechanisms, windows of sensitivity, and dose-response relationship is essential for risk assessment of pharmaceuticals that modulate calcium signaling.

Hypocretin interacts with melatonin in regulating sleep in zebrafish

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In mammals, hypocretin/orexin (HCRT) is strongly wake-promoting and HCRT deficiency causes narcolepsy. This indicates that a major function of the HCRT system is to consolidate wakefulness. In addition to fragmented wake however, narcoleptic mammals also display sleep fragmentation, a less understood phenotype recapitulated in zebrafish HCRT receptor mutant (*hcrtr-/-*). This suggests that HCRT regulates not only wake but also sleep consolidation, and that the later phenotype is conserved in zebrafish. To investigate potential mediators of sleep consolidation in zebrafish, we characterized HCRT neurons in this species. We found that, like in mammals, zebrafish HCRT neurons express vesicular glutamate transporter *vglut2* but not the glutamic acid decarboxylase *gad67* indicating conservation of the glutamatergic phenotype. Visualization of the entire HCRT circuit in zebrafish stably expressing *hcrt:EGFP* revealed clear parallel with established mammalian HCRT neuroanatomy. We also found significant projections to the pineal gland, a brain structure expressing *hcrtr* mRNA. As pineal melatonin is a major sleep promoting hormone in diurnal zebrafish and is released in the dark, we further characterized melatonin response in *hcrtr-/-* larvae and adults. Interestingly, a low dose of melatonin increases sleep amount and sleep consolidation in *hcrtr-/-* fish but not in their wildtype siblings. No difference was found between genotypes using other hypnotic compounds. The presence of HCRT pineal gland projections and the striking hypersensitivity to melatonin, which abrogates the sleep fragmentation phenotype of *hcrtr-/-* fish demonstrate a specific interaction between these two major sleep regulator pathways and suggests a HCRT sleep consolidation role through the melatonin system. As similar HCRT-Pineal projections have been described in both diurnal and nocturnal mammals, the role of this circuit in other species will need further investigation.

Netrin-DCC, Robo-Slit and HSPGs coordinate lateral positioning of longitudinal dopaminergic diencephalospinal axons

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Longitudinal axons provide connectivity between remote areas of the nervous system. While the molecular determinants driving commissural pathway formation have been well characterized, mechanisms specifying the formation of longitudinal axon tracts in the vertebrate nervous system are largely unknown. Here we study axon guidance mechanisms of the longitudinal dopaminergic (DA) diencephalospinal tract. This tract is established by DA neurons located in the ventral diencephalon and is thought to be involved in modulating locomotor activity. Using mutant analysis as well as gain- and loss of function experiments we demonstrate that longitudinal DA axons navigate by integrating long range signaling of midline derived cues. Repulsive Robo2/Slit signaling keeps longitudinal DA axons away from the midline. In the absence of repulsive Robo2/Slit function, DA axons are attracted towards the midline by DCC/Netrin1 signaling. Thus Slit based repulsion counteracts Netrin mediated attraction to specify lateral positions of the DA diencephalospinal tract. We further identified heparan sulfate proteglycans as essential modulators of DA diencephalospinal guidance mechanisms. Our findings provide insight into the complexity of positioning far-projecting longitudinal axons, and allow us to provide a model for DA diencephalospinal pathfinding. Simultaneous integrations of repulsive and attractive long-range cues from the midline act in a concerted fashion to define lateral positions of DA longitudinal axon tracts.

Her6 regulates the Neurogenetic Gradient and Neuronal Identity in the Thalamus

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The onset and progression of neurogenesis are strictly regulated. In the mammalian thalamus neurogenesis proceeds in a spatially regulated manner referred to as the Neurogenetic Gradient, the underlying mechanism of which is unknown. Here we describe the existence of a neurogenetic gradient in the zebrafish thalamus and its regulation by the bHLH gene *her6*. The Hes/Her family has been shown to control neurogenesis by regulating proneural genes, such as Neurogenin and Ascl. Here we show that Her6 determines not only the onset and progression of neurogenesis but also, as important, the subsequent identity of neurons. Loss of Her6 leads to premature Neurogenin1-mediated genesis of glutamatergic, excitatory neurons, whereas forced maintenance of Her6 leads to Ascl1-mediated production of GABAergic, inhibitory neurons. Thus, the presence or absence of a single upstream regulator of proneural gene expression, Her6, leads to the establishment of discrete neuronal domains in the thalamus.

Knock-Down of a Glutaredoxin (Zebrafish Glutaredoxin 2) Leads to Impaired Neuron Development

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Glutaredoxins (Grx) are members of the Thioredox family of proteins and major regulators of the intracellular redox potential. The human mitochondrial hGrx2 coordinates a [2Fe2S] cluster between two monomers and protects cells against oxidative stress induced apoptosis.
Here, we report the first *in vitro* and *in vivo* characterization of a dithiol Grx in zebrafish (zfGrx2), a homologue of human Grx2.
UV/Vis spectra of recombinant zfGrx2 indicated the coordination of an [2Fe2S] cluster as well. In contrast to its human homologue, protein gelfiltration implies [FeS] coordination in monomers.
During zebrafish embryogenesis zfGrx2 transcripts are ubiquitously distributed, whereas in the adult situation zfGrx2 protein was mainly confined to differentiated neurons in the CNS. Morpholino induced knock-down of zfGrx2 results in a neurodegeneration phenotype. It is characterized by apoptosis in the CNS during a distinct developmental time window, from midsomitogenesis to 48 hours post fertilization (hpf). At 24 hpf, apoptotic cells were confined by TUNEL staining and *in vivo* Caspase-3 activity measurements predominantly to the forebrain, hindbrain and spinal cord. *Acetylated tubulin*, *zn5* and *HuC* immunohistochemistry revealed a significant reduction of neurons and axon tracts in the CNS as well as loss of the trigeminal ganglion in zebrafish embryos lacking zfGrx2.
A model of the underlying molecular mechanism explaining the phenotype will be presented.

Early-life Exposure to Estrogen Impacts on Subsequent Reproductive Behaviour Altering Breeding Outcome in Zebrafish (*Danio rerio*) Colonies

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Endocrine disrupting chemicals (EDCs), including environmental oestrogens and anti-androgens, are commonly found in wastewater treatment works effluents and their receiving waters. These chemicals have been shown to affect the physiology of exposed fish, inducing conditions such as inter-sex and malformation of reproductive ducts, and to alter reproductive and courtship behaviours. Using *Danio rerio* (zebrafish) in the laboratory, we are assessing how exposure to EDCs affects reproductive behaviour and the impacts this has on breeding success for individuals and on the breeding dynamic in the colony (through the use of DNA microsatellites). This experimental approach allows us to establish relationships between dominance, spawning effort and reproductive success and probe for effects of EDCs on these interactions and the breeding outcome. Here we report on a recent study where we exposed zebrafish to 3 and 10 ng/L of ethinyloestradiol (EE2) during early life, encompassing the period of sexual differentiation (20 – 60 dpf), allowed them to depurate for 7 months in standard aquarium water, and subsequently observed their courtship behaviour and breeding success as adults in colonies with either two females with one male, or two males competing for one female. We found that exposure during early life had long lasting consequences on subsequent reproductive behaviour and breeding success. Male courtship attempts were significantly reduced with increasing exposure concentration of EE2, and males exposed to the highest EE2 concentration during early life showed a higher level of aggression as adults, to the point of preventing courtship between the remaining pair for conditions with 2 males and 1 female. Reproductive success of the females was modified significantly by EE2 exposure during early life. Whilst unexposed males showed no preference in courting exposed or unexposed females, the unexposed females had significantly higher reproductive success compared with exposed females, and they were dominant in the colonies in most cases. Unexposed females spawned fewer eggs when courted by males exposed to the 10ng/L EE2, compared with those exposed to 3ng/L EE2. These results strongly suggest that exposure to EE2 during early life subsequently impacts on behaviour and social interactions in spawning groups and alters reproductive capability and outcome.

The neurogenic niches in the adult zebrafish telencephalon: proliferation characteristics, cellular composition and regulation by fgf and bmp signaling

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In contrast to mammals, adult neurogenesis is a widespread lifelong process in the central nervous system of non-mammalian vertebrates (Kaslin et al., 2008). We have previously shown that distinct neurogenic niches are present in different brain parts along the whole rostral-caudal axis of the adult zebrafish brain (Grandel et al., 2006, Kaslin et al., 2009). These observations raise several key questions: How are the different neurogenic niches organized, and which cell types and signals regulate neurogenesis in the different neurogenic niches?
By analyzing the cellular and molecular composition of neurogenic niches in the adult zebrafish telencephalon we show that the four different niches in the dorsal and ventral telencephalon display distinct proliferation characteristics, cellular arrangements and glia domains. We demonstrate that, in contrast to the common view, a major part of the progenitor pool in the zebrafish does not display typical radial glia characteristics. Furthermore, by comparing the neurogenic niches between mouse and zebrafish we reveal that the niches in the adult zebrafish telencephalon display differences for example in the spatial arrangement of transit-amplifying cells and the migration characteristics of the neuroblasts in the ventral telencephalon. However, the niches in zebrafish are similar to mouse in their molecular composition. Using gain and loss of function approaches, we demonstrate important functions of the Fgf and Bmp signaling pathways in regulating adult neurogenic niche activity. Surprisingly, we find distinct requirements for Fgf and Bmp signaling, depending on the individual telencephalic proliferation zone considered. These findings highlight the importance of studying adult neural stem cell regulation in the context of their adult anatomical setting *in-vivo*.

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Netrin Signaling is Required for Development of an Identified Zebrafish Motoneuron
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CaP and VaP, identified zebrafish motoneurons, initially form an equivalence pair but later VaP typically dies. CaPs are present in all spinal hemisegments and extend long axons that innervate ventral muscle. VaPs are present in about half of the spinal hemisegments and extend short axons that stop at the muscle pioneers (MPs), identified slow muscle fibers that divide dorsal and ventral muscle. Previous work showed that both CaP and the MPs are required for VaP formation. Here we identify Netrin 1a (Ntn1a) as an MP-derived molecular signal necessary for VaP development. We also identify Deleted in colorectal carcinoma (Dcc) as a Netrin receptor that mediates the ability of Ntn1a to cause VaP axons to stop at the MPs.
Netrin is a secreted ligand with roles in axon guidance and cell survival. Zebrafish *ntn1a* is expressed in MPs at the time of contact by CaP and VaP growth cones. Knocking down Ntn1a using morpholinos (MOs), followed by interval observations of dye-labeled presumptive CaPs and VaPs, revealed that Ntn1a is required for development of VaP but not of CaP. In *ntn1a*-MO injected embryos, presumptive VaP axons extended beyond the MPs and the motoneurons survived longer than wildtype VaPs. Thus, Ntn1a may be the MP-derived signal preventing VaP axons from extending into ventral muscle.
We also examined whether Dcc mediates the ability of Ntn1a to cause VaP axons to stop at the MPs. *dcc* is expressed in CaP and VaP throughout axon outgrowth. MO-mediated Dcc knockdown revealed that, like Ntn1a, Dcc is required for development of VaP but not of CaP. In *dcc*-MO injected embryos, presumptive VaP axons extended beyond the MPs and VaPs survived beyond their typical time of death. Collectively, the *dcc*-MO and *ntn1a*-MO experimental results suggest that Netrin signaling is required for VaP development.

Dissecting the mechanism of action of histone deacetylase 1 in epigenetic control of neural progenitor fate

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The transformation of neural progenitors into differentiated neurons and myelinating glia is under stringent epigenetic control. Our studies have revealed critical functions for Histone Deacetylase 1 (Hdac1) in regulating the pace and pattern of both neurogenesis and oligodendrogenesis. Our results further show that Hdac1 promotes expression of proneural genes throughout the CNS by a mechanism that antagonises the Notch pathway-mediated maintenance of neural progenitors. These observations identify a regulatory role for Hdac1 in promoting commitment of neural progenitors and differentiation of post-mitotic neurons via expression of proneural genes, but the precise molecular mechanisms of Hdac1 action in neurogenesis remain unclear. Consistent with its known in vitro deacetylase activity, we find that Hdac1 regulates steady-state levels of histone acetylation during embryogenesis and specifically promotes histone methylation, which suggests that Hdac1 may repress genes by limiting histone acetylation in the vicinity of target genes. To dissect further the mechanism of action of Hdac1 in neurogenesis, we performed a genome-wide expression analysis of the *hdac1* mutant phenotype and identified several Hdac1-regulated, potential direct targets of Hdac1 in the developing CNS. We are now investigating the binding of Hdac1 to target genes in chromatin, and analysing the functions of Hdac1-regulated genes that are implicated as downstream targets of Hdac1 activity. We also used the Hdac1-regulated genes as markers to carry out a comparative analysis of the phenotypes of *hdac1* mutants and embryos lacking the functions of other chromatin regulators that are likely to interact with Hdac1 in the developing CNS. The results of these experiments indicate that Hdac1 may promote neural development as a component of multiple distinct transcriptional silencing complexes. Taken together, our results also suggest that Hdac1 may regulate genes that control the behaviour of ventricular zone neural progenitors as well as other genes that influence the behaviour of differentiating, post-mitotic cells in the mantle region.

Gdf6a is required for initiation of dorsal-ventral retinal patterning and lens development
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The neural retina is patterned along the dorsal-ventral and nasal-temporal axis, leading to carefully orchestrated retinotectal axon mapping. In chicken embryos, Bmp4 signaling specifies dorsal identity, as evidenced by both knockdown and over-expression experiments. We have investigated dorsal-ventral retinal specification in zebrafish and found that it is profoundly different from the chick model. Knockdown of zebrafish Bmp4 produces no discernable effect on dorsal retinal specification. Rather, our research implicates a related Bmp signaling molecule, Gdf6a, is essential for this process.
In zebrafish, morpholino knockdown of Gdf6a function abolishes Smad 1/5/8 phosphorylation, loss of *tbx5* expression in the dorsal retina, and expansion of *vax2* expression from the ventral retina into the dorsal domain. We clearly show that the earliest markers of dorsal cell fate are not initiated in Gdf6a morphants, and through over-expression analysis, that Gdf6a is sufficient to induce expression of dorsal markers at early stages of eye development. We provide data indicating that Bmp4 is functionally related to Gdf6a, as over-expression of Bmp4 can rescue Gdf6a dependent patterning defects, but also show that Gdf6a can induce dorsal retinal patterning in the absence of Bmp4. Thus, Bmp4 is sufficient to drive dorsal retinal patterning, but is not required for Gdf6a dependent patterning. We also demonstrate that the expansion of ventral markers due to Gdf6a inhibition is independent of Tbx5 function, as knockdown of Tbx5 affects dorsal, but not ventral gene expression.
Finally, we demonstrate a role for Gdf6a in non-neural ocular tissues. Gdf6a is required for Smad phosphorylation in the lens and for the expression of lens-specific genes, leading to mild defects in lens development. These defects are enhanced by addition of Dorsomorphin, a selective Bmp inhibitor. Taken together, these data indicate that Gdf6a functions at the top of the Bmp hierarchy of genes regulating patterning of the neural retina and lens.

Essential requirement for Anosmin-1a in fasciculation and terminal targeting of olfactory sensory neuron axons in zebrafish embryos

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The *KAL-1* gene encodes anosmin-1, an extracellular matrix protein, which mediates cell adhesion, neurite outgrowth, and axon-guidance and -branching activities, *in vitro*. This gene underlies Kallmann syndrome (KS), a human neurological genetic disorder that impairs cell migration, axon elongation, and olfactory bulb (OB) differentiation. Here, we made use of zebrafish (*Danio rerio*) embryos as model to investigate *in vivo* the requirement for this protein in olfactory system (OS) development in vertebrates. We detected anosmin-1a in olfactory sensory neurons (OSNs) from 22 hour post-fertilization onward, *i.e.* prior their pioneer axons reach OB anlagen. We found that anosmin-1a depletion compromises the fasciculation of OSN axons and their terminal targeting within OBs. We also showed that *kal1a* inactivation impairs the differentiation of OB glomeruli and induces a severe decrease in the number of GABAergic and dopaminergic OB neurons. This first investigation of the requirement for anosmin-1 in OS development in vertebrates revealed an evolutionarily conserved role of the protein for proper growth and guidance of OSN axons and OB neuron differentiation. In addition, our data suggest that full differentiation OB glomeruli relies on their proper innervation by OSN axons. However, our results also showed that zebrafish anosmin-1a depletion does not fully block OSN axon elongation or OB differentiation, as it is the case in KS patients, suggesting a distinct requirement for the zebrafish protein in OS development.

Nodal signalling imposes left-right asymmetry upon neurogenesis in the habenular nuclei
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The habenulae are evolutionarily conserved bilateral nuclei in the epithalamus that relay input from the forebrain to the ventral midbrain. In zebrafish, the habenulae display left-right (L/R) asymmetries in gene expression and axonal projections. The elaboration of habenular asymmetries requires the presence of a second asymmetric structure, the parapineal, whose laterality is biased by unilateral Nodal signalling. We have shown recently that neurons are present earlier in the left habenula than in the right and, in contrast to other habenular asymmetry phenotypes, this temporal left-right difference in neurogenesis is not dependent on the parapineal. Removing the left-right bias in Nodal signalling renders habenular neurogenesis symmetric revealing a requirement for this pathway in asymmetrically biasing neurogenesis. Thus, our results provide evidence of a direct requirement for Nodal signalling in promoting an asymmetry *per se* rather than in directing laterality only (Roussigne et al., 2009).
From our data and previous studies, we propose a model in which early Nodal-dependent asymmetry in habenular neurogenesis would bias parapineal migration towards the left. We have shown that parapineal migration is dependent on *fgf8* and L/R differences in the levels of Fgf8 can bias the direction of migration (Regan et al., 2009). Therefore, it is important to determine whether Nodal signalling could impose parapineal laterality by biasing levels of Fgf8 activity; it could do so through promoting a left/right asymmetry in the number of *fgf8* expressing habenular precursors and/or by modulating Fgf pathway activity in habenular and/or parapineal cells. To address this issue, we are currently working on resolving the interactions between Nodal and Fgf signalling pathways in the epithamus.

Mechanisms regulating neuronal apoptosis in the developing zebrafish retina

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Apoptosis is often observed in the developing tissues. However, it remains unclarified how the apoptotic pathway is regulated during development. To elucidate this issue, we identified five zebrafish mutants that show extensive apoptosis in the developing retina. Here we reported one of them, *pinball eye (piy)*, in which most retinal neurons undergo apoptosis during their differentiation. A missense mutation occurred in the small subunit of DNA primase (Prim1) in the *piy* mutant. Prim1 is required for DNA replication, but cell proliferation seems normal in the *piy* mutant, suggesting that this missense mutation does not affect DNA replication. It was reported that RNA synthesis by Prim1 triggers the DNA replication checkpoint, which may activate Ataxia telangiectasia mutated (ATM). ATM activates Checkpoint kinase 2 (Chk2), which subsequently activates a p53-dependent apoptosis. We found that neuronal apoptosis in the *piy* mutant depends on the ATM/Chk2/p53 pathway. We conclude that p53 influences the cell fate choice between cell differentiation and apoptosis in the zebrafish retina. In addition to the *piy* mutant, we examined whether retinal apoptosis depends on p53 in other zebrafish apoptotic mutants: *rw329*, *rw337* and *rw564*. We found that retinal apoptosis also depends on p53 in these mutants. Taken together, these data suggest that the p53-mediated apoptotic regulation is one of monitoring system that ensures retinal neurogenesis in zebrafish.

Developmental retinotectal axon pathfinding in zebrafish: a role for matrix metalloproteinases
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Correct wiring of neuronal circuits in the developing brain relies on the precise spatial positioning of neurons and their axons. The optic circuit, which contains the axons of retinal ganglion cells (RGCs), is a powerful model system to study axon guidance, midline crossing and formation of topographic neuronal maps. Recent loss/gain-of-function studies in different animal models revealed several attractive and repulsive guidance cues that regulate the proper formation of the visual pathway.
Recently matrix metalloproteinases (MMPs) have been shown to regulate migration and survival of neurons, axon guidance and myelination in the developing brain. There is also substantial evidence that MMPs participate in the development of retinotectal projections. However, the nature and working mechanisms of these MMPs/TIMPs (tissue inhibitors of metalloproteinases) in retinotectal pathfinding remain completely unidentified.
We characterized the expression of several MMPs and TIMPs, identified in zebrafish, in the brain of zebrafish embryos at various time points during development of the retinotectal system by *in situ* hybridization. Especially zMMP2, zMMP14a, zMMP14b and zTIMP2 show an extensive expression pattern in the developing brain. Cross-sections of these *in situ* stainings revealed that these MMPs are present in the developing optic tectum / pathway. Knockdown of MMP14a, using a specific MMP14a morpholino, and subsequent immunostainings with the axonal marker anti-acetylated α tubulin, at 5dpf (days post fertilization) showed a significant reduction in the size of the optic tectum ($p<0.05$). Similar results were obtained with the broad-spectrum MMP inhibitor EDTA. They confirm our hypothesis and show that MMPs indeed play a role in retinotectal axonal pathfinding.
Altogether, these novel findings reveal that MMPs are expressed in the zebrafish brain during development of the visual pathways and that axon pathfinding in the retinotectal system of zebrafish can be disrupted by blockade of MMP signaling. Our results might have implications for future strategies interfering with neural circuit development and/or regeneration.

Function of the Lhx genes in thalamic development of the zebrafish

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The thalamus is the major relay station in the forebrain and connects the sensory organs with higher brain parts. In mammals the flow of information is organized in a stereotypic manner: sensory organs project towards specific thalamic nuclei from which subsequently target a specific area in the cortex. Although much simpler, this basic principal can be observed within the non-mammalian lineage. Interestingly, the developmental process of regionalization of the thalamus and formation of its functional units: the nuclei are unknown. Data from mouse and Xenopus show that the LIM homeodomain genes are expressed in the different thalamic compartments. Here, we elucidate the parcellation process in detail by using zebrafish as a model organism. First, we mapped the expression pattern of *lhx2* and *lhx9* relative to well-known markers of the anterior ZLI border and the posterior located pretectum (Scholpp et al., 2006). We find that *lhx2* and *lhx9* mark the pool of thalamic postmitotic projection neurons, whereas the interneuron population does not express *lhx2* and *lhx9*. Furthermore, we could show that *lhx2* and *lhx9* are expressed in distinct but partially overlapping domains within the thalamic projection neurons, suggesting a crucial role during the further parcellation process of this population into the final nuclei. To explore this further we will study the function of these LIM homeodomain factors, which will give us a greater insight into thalamic development in vertebrates.

A Novel Role For Zic Genes In The Developing Forebrain

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Holoprosencephaly (HPE) is the most common congenital malformation of the forebrain in humans. Several genes with essential roles during forebrain development have been identified because they cause HPE when mutated. Among these are genes that encode the secreted growth factor Sonic hedgehog (Shh) and transcription factors Six3 and Zic2. In mouse, Six3 and Shh activate each other's transcription, but a role for Zic2 in this interaction has not been tested. We demonstrate that in zebrafish, as in mouse, Hh signaling activates transcription of *six*3b in the developing forebrain. *zic*2a is also activated by Hh signaling and represses *six*3b outside of its expression domain, likely through limiting Hh signaling. Zic2a repression of *six*3b is essential for the correct formation of the prethalamus. The diencephalon-derived optic stalk (OS) and neural retina are also patterned in response to Hh signaling. We show that zebrafish Zic2a limits transcription of Hh targets *pax*2a and *fgf*8a in the OS and retina. The effects of Zic2a depletion in the forebrain and in the OS/retina are rescued by blocking Hh signaling or by increasing levels of the Hh antagonist Hhip, suggesting that in both tissues Zic2a acts to attenuate the effects of Hh signaling. These data uncover a novel, essential role for Zic2a as a modulator of Hh–activated gene expression in the developing forebrain and advance our understanding of a key gene regulatory network that, when disrupted, causes HPE.

Role of cullin-binding domain of Asb11 in neurogenesis

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Ankyrin repeats and SOCS box proteins (ASB), act as part of an ECS (elonginC–cullin–SOCS-box) type E3 ubiquitin ligase complex regulating the half-life of proteins by targeting them for ubiquitination and degradation. We identified *d-asb11,* in zebrafish, which plays an important role during development as a regulator of the neuronal progenitor compartment size through ubiquitination and degradation of the Notch-ligand DeltaA, positively regulating Notch signaling activation. d-Asb11 lacking the SOCS box domain is not capable of interacting with DeltaA, suggesting that d-Asb11 may mediate ubiquitination as a specific component of the ECS complex. Several studies have demonstrated the role of cullin box to form the ECS complex, albeit its biological function remains unknown. To investigate role of cullin box domain of Asb11 we employed Tilling to generate a zebrafish mutant deleting the cullin box. The expression of Delta/Notch pathway genes in the mutants shows that cullin box is required for correct expression of these genes. Asb11-binding to cullin is involved in Notch signaling activation as demonstrated by upregulation of Notch target genes (*her4, her1, her6*) or downregulation of *her5* in embryos as well as *in vitro* where mutant Asb11 has a reduced capacity to induce Notch reporter Hes1. This is the first genetic evidence that cullin box domain of an ASB-family member is functionally important *in vivo*.

The combinatorial expression of specific transcription factors identifies distinct catecholaminergic groups in zebrafish

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In the developing brain, specific groups of catecholaminergic (CA) neurons form at distinct anatomical locations with distinct cellular morphologies and projection behaviors. Current understanding is that a combination of transcription factor (TF) prepattern in neural precursors and of local signaling events specifies neuronal differentiation. Hence, dopaminergic (DA) or noradrenergic (NA) neurons located in distinct regions of the brain may be specified and differentiate under distinct control by local prepattern of combinatorial expression of TFs. For some CA groups, like the mammalian mesencephalic DA groups A8-A10, the sequential timing of TFs activation during specification and differentiation has been quite well characterized. Diencephalic DA groups are less well understood.

We use zebrafish to investigate the transcription factor code characteristic for each CA neuron group. Zebrafish form DA groups exclusively in the forebrain, while NA neurons are confined to the hindbrain. Diencephalic DA groups can be distinguished based on their position, cell shape, degree of Tyrosine hydroxylase (TH) immunoreactivity, and projection behavior (Rink and Wullimann, 2002).

Based on gene expression data from public databases (www.zfin.org), we selected approximately 60 genes expressed in distinct patterns in the forebrain in territories correlating with CA differentiation. We took advantage of an optimized fluorescent *in situ* hybridization protocol coupled with anti-TH immunohistochemistry and confocal microscopy to conduct co-expression analyses at single cell resolution. The CNS of whole mount zebrafish embryos was analyzed at four developmental stages. Our data enabled us to (1) define a combinatorial code of TFs that identifies each distinct group of CA neurons; (2) determine the location of DA neurons within the longitudinal and transverse subdivisions of the forebrain postulated by the prosomeric model (Puelles and Rubenstein, 2003); (3) establish potential homology between zebrafish and mammalian CA groups based on TF expression codes.

Our data indicate that DA specification may be accomplished by distinct TF codes in distinct regions of the brain, as no TFs have been identified so far as universally expressed in all DA neurons. Further, mechanisms of DA differentiation for some groups are conserved from fish to mammals, e.g. the prethalamic group, which expresses the same TFs in zebrafish and in higher vertebrates. In addition, our analysis calls attention to TFs previously not invoked in CA differentiation, which we considered for further functional analysis.

PACAP in Zebrafish Neurodevelopment

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The pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the glucagon peptide superfamily. The *pacap* gene encodes for a prepropeptide that is cleaved into a PACAP-related peptide (PRP) and PACAP. Two copies of the *pacap* gene, named *pacap1* and *pacap2*, have been found in the genomes of zebrafish and other teleosts. The peptide sequence has been highly conserved during evolution and PACAP is a pleiotropic neuropeptide with various physiological and developmental functions. Besides acting as a hypophysiotropic factor regulating growth-hormone release, PACAP also elicits neurotrophic effects controlling neuron proliferation and survival.
The genetic pathways establishing *pacap* cells are only poorly understood. In a first step towards understanding the differentiation of *pacap* positive cells, we give a detailed description of the expression of *pacap* mRNAs during zebrafish development. By means of *in situ* hybridization expression can be detected from somitogenesis onward. We found *pacap1* to be expressed in the hindbrain, the spinal cord and in the trigeminal ganglion. *pacap2* was present in clusters of the telencephalon, preoptic region, posterior tuberculum, pretectum, tectum, tegmentum, rhombencephalon, spinal cord as well as in the olfactory placode and in cranial ganglia. The co-distribution of *pacap* mRNA with that of several key regulators of neuronal development, suggests a possible function for PACAP during early neuronal differentiation.

Neuromuscular synapse formation: another role for the planar cell polarity pathway
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Formation of neuromuscular connectivity involves proper navigation of motor axons from the spinal cord to their muscle targets as well as the precise alignment of these motor axons with acetylcholine receptors (AChRs) on the muscle surface. One key molecular player in this process is the *mus*cle *s*pecific *k*inase, MuSK, which is expressed only on muscle cells. When activated by the nerve-derived ligand agrin, MuSK triggers the accumulation of AChR clusters in the center of muscle cells. Even before the arrival of motor axons, AChRs accumulate in the center of muscle cells in a MuSK dependent manner and prefigure the sites of the first neuromuscular synapses. Recently, our laboratory has shown that the secreted ligand *wnt11r* binds the zebrafish MuSK ortholog *unplugged*, and that this interaction triggers a *dishevelled*-dependent signaling cascade in muscle cells (Jing et al. 2009). We hypothesize that this signaling cascade establishes a specialized zone in the center of the muscle fiber to which motor axons and AChRs are restricted.
To explore the extent of similarity between the MuSK signaling pathway and the 'classical' frizzled/ planar cell polarity pathway (PCP) pathway, we have begun to analyze whether additional downstream components of the PCP pathway also function in muscle fibers downstream of *unplugged*/MuSK. Using live cell imaging, we are also examining whether the subcellular localization of these PCP components mimics the dynamic relocalization of these components during the activation of the 'classical' frizzled/ PCP pathway.

Role of neurotransmission during neurogenesis in the zebrafish embryo

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One of the first forms of neuronal activity during development is depolarization mediated by chloride ions via glycinergic and GABAergic receptor-channels. This depolarization (excitation) occurs because the intracellular chloride level is initially high and remains so until the delayed expression of the extrusive chloride and potassium ion co-transporter KCC2 during later stages of development. The role of this paradoxical depolarization during early development is unknown. In order to test the role of depolarizing glycinergic transmission during early development, we over-expressed KCC2 and reversed the chloride gradient, rendering glycine hyperpolarizing in all the neurons from the onset of development. We also targeted the glycine receptor directly by either blocking its action using the specific antagonist strychnine or by the specific 'knockdown' of the embryonic alpha2 subunit of glycine receptors. In the three cases (KCC2, strychnine and alpha2 KD), disruption of neuronal activity provoked majors errors in neurogenesis. While examining transgenic lines expressing GFP in specific cells and with the use of several antibodies in confocal microscopy, we observed a major loss of differentiated neurons in several classes of interneurons. This suggests that the glycine receptor and its excitatory activity in the early phases of development are necessary for normal neurogenesis. We now want to determine the expression patterns of the genes regulated by activity in order to define the mechanism by which the activity regulates neurogenesis.

Gene expression profiling of neural progenitor cells in adult zebrafish brain
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Different types of neural progenitors have been identified in various regions of the adult zebrafish brain. Progenitor cells of non-mammalian vertebrate brains show characteristics of both radial glia and neuroepithelial cells [1]. We find that the transgenic line *Tg[her4.1:GFP]* [2] labels cells with ependymal- and radial glia characteristics, including proliferating progenitors. Using fluorescent activated cell sorting we isolated different cell populations from adult *Tg[her4.1:GFP]* zebrafish brains. In addition for selecting GFP-positive cells, we applied a vital DNA dye to isolate specifically GFP positive cells which were in S-/G2-phase of the cell cycle. Using this method, the gene expression profiles of proliferating and non-proliferating glia cells were obtained. In addition, we are interested in the regulatory gene program underlying regeneration in the zebrafish brain which shows a strong proliferative response after lesion. Therefore, we obtained the gene expression profile of glia cells 3 days after a severe lesion of the zebrafish telencephalon. We are currently investigating genes which are likely to play a key role in neurogenesis and regeneration.
In a second approach we use a new transgenic line *Tg[pcna:EGFP]* to obtain the gene expression profiles of progenitor cells independent of their cellular identity.
To do *bona fide* lineage tracing of the progeny of Her4.1 positive cells, we created a transgenic line that enables the temporal controlled activation of Cre recombinase in glia cells: *Tg[her4.1:mCherryT2ACreERT2]*. In combination with a red-to-green reporter line [3] successfully induced recombination can be observed after the administration of tamoxifen. Subsequently the progeny of Her4.1 progenitors carries the EGFP label, revealing the cellular potential of these progenitors.

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A novel zebrafish cyclin dx gene: expression profile, requirement for the development of primordium motor neuron, pmn and characterization of its promoter region

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We identified and characterized a novel zebrafish cyclin Dx/*zccndx* gene. Whole-mount in situ hybridization indicated that the *zccndx* mRNA was expressed in motor neurons of hindbrain and spinal cord during embryogenesis. A 4-kb promoter region of *zccndx* gene could drive GFP expression in the motor neurons of hindbrain and spinal cord, olfactory placode and neuronmast supporting cells in zebrafish lateral line by microinjection into one cell of zebrafish embryos. Thereafter, a transgenic Tg(*zccndx*:GFP) line was established with identical expression pattern. By using this line, morpholino knockdown of zebrafish cyclin Dx resulted in the loss of motor neurons in both hindbrain and spinal cord, while Gli1 knockdown caused the lost of motor neurons only in the hindbrain, but unaffected in the spinal cord. These results suggest that the *zccndx* gene was required for motor neuron proliferation and maintenance in the early development. The transgenic line, Tg(*zccndx*:GFP), displayed robust GFP signal in the motor neurons of zebrafish hindbrain and spinal cord and it will be useful for investigating the differentiation and maintenance of motor neuron progenitors during embryogenesis.

Genetics Tools to Study Neural Circuit Formation in Zebrafish and *Xenopus*
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As yet it is far from understood how neurons acquire specificity, form a given neural network and participate in different chemical and physical stimuli. The simple anatomy of *Danio rerio* and *Xenopus* make them ideal models for the study of neural formation and gene expression, as well as for physiological studies. With this in mind, we aim to develop a series of transgenic lines with specifically labelled cell types in both animal models, focusing on different populations of spinal cord interneurons. These will be used as genetic tools to study the neural circuit formation during development.
As a proof of principle, we made a construct from sequence upstream of *HuC* with a GateWay TOL2 system and successfully generated transgenics that labelled interneurons and Rohon Beard cells in both species. In order to label specific interneuron populations we are using a bioinformatic approach to identify conserved non-coding elements, as a means of isolating putative cis-elements, which are then used to generate reporter constructs. Our analyses of genes such as *evx1, evx2, isl1, chx10* and *lbx1*, have resulted in reporter constructs that are expressed in specific cell types. These will be invaluable tools that will enable us to relate the specific gene expression of spinal cord cells, their particular morphology and function in both species.

Endocytosis and neurogenesis in the zebrafish spinal cord
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For now almost 20 years a new view of signaling has emerged, a view raising the possibility that signal transduction does not exclusively happen at the plasma membrane but also on early endosomes. After this finding for the EGF signaling pathway, during the years, more and more evidence suggested a role for endocytosis in signaling for NGF-, Notch- and also TGFβ-signaling.
In our lab one main focus lies on a TGFβ- signaling adaptor protein called SARA, which stands for **S**mad **a**nchor for **r**eceptor **a**ctivation. Upon ligand binding, the heteromeric receptor complex is internalized into early endosomes and signaling is induced after a phosphorylation event, which propagates the signal to intracellular signaling mediators. This is where SARA comes into play: next to other functional domains it has a FYVE domain by the means of which it binds to the phosphatidylinositol-3-phosphate (PI3P) on the early endosome. An interesting feature of these SARA positive endosomes, studied in our lab mainly in drosophila, is that they either segregate symmetrically in symmetric cell division or asymmetric in asymmetric cell division. Studies in zebrafish SARA show that it can segregate asymmetrically during cell division in the spinal cord and that knock-down by morpholinos leads to a neurogenic phenotype.
My interest lies on exploring the role of trafficking in neurogenesis with SARA being the center of my attention. Several small GTPases, the Rabs, mark the different compartments in the trafficking pathway. I will use live imaging taking advantage of Rab GFP fusion proteins to study their behavior live and during cell division as well as a morpholino knock-down approach to analyze their function in neurogenesis and their influence on the signaling in which SARA is involved. Moreover, drugs and dominant-negative or dominant-active forms of these small GTPases can be used to interfere with trafficking at precise steps of the pathway and to block endocytosis in a more general way.
This study will not only allow us to understand the cellular basis of our phenotype but also understand how cells exploit the traffic machinery to regulate key events such as:
Neurogenesis and directional signaling between cells.

Localization of Urotensin 1 and Urocortin 3 neurons in the embryonic zebrafish nervous system

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The body's response to stress is comprised of a variety of physiological changes and behavioral patterns including alterations in locomotion. Locomotion is dependent on specific neuronal circuits in the brainstem including reticulospinal neurons and the Mauthner cell. These circuits are modulated by the neurotransmitters dopamine, noradrenaline and serotonin in all vertebrates. CRH family peptides are known to interact with these aminergic brain circuits and thereby changing locomotor activity. To achieve a deeper understanding of the connection between the stress system and locomotion, we analyzed the spatial transcript distribution of two CRH family peptides, Urotensin 1 (UTS1) and Urocortin 3 (UCN3) by whole-mount in situ hybridization and immunohistochemistry during zebrafish embryogenesis. We indeed found that UTS1 and UCN3 are expressed in key neuronal sites regulating locomotor behavior, suggesting the interrelation of the stress response and locomotion via these neuropeptides.
With our detailed data on the embryonic expression of UTS1 and UCN3 mRNA, we can now dissect the function of these CRH family peptides in stress induced locomotion.

Homeostatic synaptic plasticity in the developing spinal cord and its behavioural correlates
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Objectives: Behaviors are generated through populations of nerve cells, wired into circuits that form a neural network. There are homeostatic mechanisms in place to modify synaptic strength in response to changes in activity, one of the best documented being synaptic scaling, a uniform increase or decrease in the size of miniature excitatory post-synaptic currents (mEPSCs). There is little understanding of the relationship between synaptic scaling and behaviour, so our goal is to investigate synaptic scaling in the developing *Danio rerio* (zebrafish) spinal cord and its effects on early locomotor behaviours.
Materials and methods: Wild-type embryos were injected between 17 hours post-fertilization (hpf) and 48hpf with either TTX (voltage-gated Na^+ channel blocker), AP-5 (NMDAR blocker), CNQX (AMPAR blocker), TNFα (molecule known to increase mEPSC amplitude during acute application), or vehicle as a control. Whole-cell patch clamp recordings of mEPSCs from spinal neurons and muscle fibres were made at 4dpf. Behavioural analyses of spontaneous coiling (17-28hpf), touch response (30hpf), and swimming (2-4dpf) were also performed.
Results: Blocking activity with TTX and CNQX for 48hrs (2-4dpf) resulted in a significant, multiplicative increase in glutamatergic mEPSC size, scaling mEPSC amplitudes up by ~1.4. Current threshold data shows that increased mEPSC amplitudes in CNQX-treated fish cannot be attributed to changes in intrinsic excitability of neurons. AP-5 treated fish showed no significant changes. Injection of TNFα resulted in significantly scaled down (decreased) glutamatergic mEPSC amplitudes after as little as 24hrs. Other parameters including mEPSC frequency, kinetics, inter-event interval, and AMPA:NMDA ratio are also described. Behaviourally, TTX treatment resulted in immotile embryos, whereas AP-5 and CNQX-injected fish could swim. Further analyses of stereotypical swimming behaviour suggest differences between experimental conditions that may be correlated with synaptic scaling. Muscle recordings show that CNQX-treated embryos have trends towards increased muscle activity, showing a tendency towards longer burst durations in swimming episodes than control.
Conclusions: These results support a role for synaptic scaling in vivo as a means of maintaining homeostasis during development. Glutamatergic mEPSCs scale up multiplicatively in response to activity blockade to strengthen existing synapses. Our observations of muscle fibre and swimming activity following chronic blockade of synaptic activity provide new evidence that synaptic scaling may contribute to compensatory alterations in behaviorally relevant neural networks during embryonic development. Additionally, the chronic effect of TNFα on scaling down mEPSC amplitudes has never been shown before and experiments are outlined to investigate this further.
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SOX-2 Expression in the brain of developing zebrafish

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The proteins encoded by genes of the *Sox* family are related transcription factors, containing a high motility group (HMG box), and which play critical roles in the early stages of development. They are highly phylogenically preserved and have been detected from mammals to nematodes. These molecules are also involved in the development of both central nervous system and sensory organs, and recently has been demonstrated that Sox-2 could be used as presumptive neural stem cell marker. Therefore, we decided to investigate its expression in the brain of zebrafish, in which neurogenesis is not restricted to a limited number of areas but is widely distributed in the brain. This study was aimed to identify the potential neurogenic areas in the brain of zebrafish, and to establish the developmental changes they undergo. The brains of zebrafish from larval to adult stage were assessed by qRT-PCR and immunohistochemistry. Results demonstrated that levels of Sox-2 mRNA expression are maximal at 10dpf (days postfertilization), progressively decrease up to 30 dpf, then increase up to 40 dpf and remain to adult stage (120 dpf). The immunolocalization of Sox-2 revealed that it is restricted to the epithelial cells that lie the brain ventricles during the larval period; by 15 dpf Sox-2 was also detected in scattered cells of the olfactory bulb, the diencephalon and tectum opticum; by 25 and 50 dpf was found in the same localizations and also in epithelial cells of the Rhombencephalic ventricle and cerebellum; finally, in adult animals Sox-2 was restricted to the olfactory bulb fibres and to the ependymal cells and tanycytes of telencephalic ventricle. Taken together present results demonstrate that the neurogenic areas in zebrafish are distributed throughout the brain during development, but concentred around the telencephalic ventricle and olfactory bulb in adults, following a similar localization as in high vertebrates.

Genetic and molecular analysis of a maternal effect allele involved in the development of zebrafish left-right asymmetries

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The vertebrate body plan displays distinct left-right asymmetries in the disposition of visceral organs. This asymmetrical organization extends to the vertebrate brain that is both anatomically and functionally asymmetric. The development of left-right patterning and cerebral lateralization are thought to be regulated by evolutionary conserved genes.
It has been demonstrated that in vertebrates the initiation of left-right patterning depends on the asymmetrical, left-sided activation of a Nodal mediated gene expression cascade (1).
Moreover, studies have found that Nodal-related signals also regulate asymmetric gene expression in the zebrafish forebrain and are involved in early establishment of left-right asymmetries in the epithalamic region of the dorsal diencephalon (2).
In a recent study Facchin et al. (3) showed that the progeny of lines of zebrafish artificially selected for the right eye preference in scrutiny a mirror had a significant increase in the frequency of reversed left-right asymmetry in the epithalamus. In the present study it is proposed that Facchin's selection for behavioral lateralization could have lead to the isolation of a spontaneous mutant allele responsible for the disruption of normal left-right patterning in zebrafish neuroanatomical structures. Based on a preliminary mendelian analysis of this trait, we performed selective crosses on females identified as mutant carriers to test the hypothesis of a recessive maternal effect mutation. The reversed anatomy is expressed in the offspring with a frequency that suggests variable expressivity. The analyses revealed that in most cases these homozygous mutant mothers generate a percentage of progeny with reversed diencephalic asymmetries that progressively increases in the subsequent crosses, suggesting that expressivity of this allele is affected by the age of the mother. We investigated in this strain the expression of members of signaling pathways responsible for the establishment of visceral and diencephalic left-right asymmetries. *In situ* hybridizations analysis of mutant females' progeny showed that at least three genes involved in Nodal signaling pathway are altered in their gene expression pattern (either bilateral or right-sided) in approximately 50% of observed embryos. Current experiments are aim at investigating a possible involvement of this mutation in the generation and maintenance of nodal flow by the cilia of Kupffer's vesicle. We hypothesize that this allele has been positively selected in the *D. rerio* wild populations as an evolutionary stable strategy for predator avoidance.

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Formation of spinal network dependent on domain-specific Pax genes

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During vertebrate development, transcription factors exert a strict control on gene expression, both spatially and temporally. *Pax* genes comprise an important family of transcription factors involved in diverse aspects of development. Using a gene trap technique, we established a stable line of zebrafish in which the red fluorescent protein (RFP) trapped the *pax8* gene. In this line, RFP insertion occurred in the first intron of the *pax8* gene. RFP expression is detected in the midbrain-hindbrain boundary, the otic vesicle, hindbrain, spinal cord, and retina.
Real-time PCR showed that the RFP insertion abolished the *pax8* expression in homozygous embryos. Due to the lack of functional Pax8, otic vesicles in these embryos were smaller than and the expression of *pax2*, a gene closely related to *pax8*, was down regulated.
Pax8 is expressed in the dorsal region of the spinal cord. *Pax2* expression partially overlaps with that of *Pax8*. *Pax2* is expressed in CiAs, while *pax8* is expressed in CoBLs and IpLos. We examined the effect of *pax8* and/or *pax2* knockdown in *pax8*-expressing interneurons. The use of newly identified gene trap line enabled classification of spinal interneurons and revealed roles of transcription factors in the network formation.

The role of amigo1 in the development of zebrafish early brain's catecholaminergic system
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AMIGOs (**am**photerin-**i**nduced **g**ene and **o**rf) form a novel family (AMIGO1, AMIGO2, AMIGO3) of transmembrane proteins that belong both to the LRR (leucine-rich repeat) and Ig superfamilies. Expression of AMIGO1 could be induced by RAGE (receptor for advanced glycation end products) ligation with amphoterin [Juha K.P., 2003]. The expression pattern of AMIGO1 in the mice embryo has been detected by whole mount in situ hybridization and found to be mainly expressed in the central nervous system during early development stage [Shunsaku H, 2009]. But except for its potential roles in the apoptosis of granula neurons and mediating fasciculation of neurites in vitro [Tomio O, 2003], few functions have been so far found. In order to explore AMIGO1 roles during early development stage, we have managed to knock down its expression in the zygote by the introduction of specific morpholino oligos via microinjection. The inhibition has been confirmed both by western-blot and whole mount immunostaining in 3dpf (days past fertilization) and 5dpf injected larvae. AMIGO1 knock-down larval brains usually show development defects at the areas where AMIGO1 is normally expressed. Tyrosine hydroxylase (TH) immunohistochemistry for labeling zebrafish catecholaminergic system shows a prominent decrease and disordered organization in TH-immunoreactive neuronal fiber projections in the hindbrain. TH cell cluster formation and distribution in the morphants' telencephalon, midbrain and eyes are also impaired, which could be detected from the very beginning of development. Furthermore, the swimming behavior test shows a disorganized pattern of swimming in addition to decreased distance in AMIGO1 5dpf morphants, which is an expected consequence of the catecholaminergic system deterioration. The AMIGO1 knock-down morphant phenotypes have been rescued successfully by co-injection of AMIGO1 mRNA together with morpholino oligos. The 5dpf co-injected larvae even show hyperactive effects in the behavioral test. Our study shows an important role of AMIGO1 in the developing zebrafish larval brain and corresponding functions in vertebrate catecholaminergic system development.

Mechanisms controlling polarized dendrite formation of Purkinje cells in the zebrafish cerebellum

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Although neurons display remarkable diversity of dendritic morphology, mechanisms that pattern specific shapes of dendrites are still to be understood. Among processes of dendritic patterning, such as dendritic growth or branching, we focus on the mechanism that controls the number of primary dendrites (dendrites extending directly from a cell body) as the initial process for dendritic patterning. Purkinje cells in the cerebellum give us a good model system for studying this issue since they exhibit characteristic polarized morphology having a single primary dendrite. Taking advantages of zebrafish for live-imaging experiments, we are studying how Purkinje cells acquire the highly polarized morphology and the mechanisms that control the process.

We established transgenic fish that express membrane-targeted Venus specifically in Purkinje cells and observed their morphologies at single cell resolutions. We found that zebrafish Purkinje cells showed different dendritic morphologies according to the somal location along antero-posterior and midio-lateral axes. In spite of the morphological diversity, all Purkinje cells have a single primary dendrite as observed in mammalian cerebellum. Time lapse observations for the morphological changes of Purkinje cells revealed that they initially had multiple neurites and only one of them was finally selected as the future primary dendrite to form the mature polarized morphology.

We found that Golgi apparatus was exclusively localized at the root of primary dendrite in Purkinje cells and Golgi localization preceded morphological polarization, suggesting that localized Golgi regulates the polarized dendrite formation. We also found that Purkinje cells retained multiple primary dendrites in the mutant in which the function of atypical PKC (aPKC) is disrupted. We will further discuss the role of aPKC in the polarized dendrite formation and the Golgi localization in Purkinje cells.

Analysis of Sulfatase1 function in Shh-dependant oligodendrocyte specification in the ventral spinal cord

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Neurons and glial cells (astrocytes and oligodendrocytes) composing the adult central nervous system, arise from neural progenitors during embryonic development. The spinal cord is a simple model to study the emergence of the diverse neural populations. In the ventral region, the Sonic Hedgehog (Shh) morphogenetic gradient, originating from notochord and floor plate, establishes the formation of distinct neural domains generating different neuronal subpopulations. Subsequently to neuronal production, Shh induces the specification of oligodendrocyte precursors (OPC) in the ventral spinal cord, raising the question of the molecular mechanisms underlying this temporal change of neural progenitor response to the same signal.
The sulfatase Sulf1 is potential player involved in these events. Indeed, its expression starts after the main waves of neuronal production in the floorplate and later expands into the ventral neural progenitors just prior to OPC specification in this domain. This spatio-temporal pattern of expression is strongly conserved among vertebrates, including chick and zebrafish (Danesin et al., 2006, present results). Sulf1 encodes for an enzyme hydrolysing sulphate groups at specific position from Heparan Sulfate Proteoglycans (HSPG) at the cell surface. This modification of HSPG sulfation pattern modulates their affinity for extracellular ligands and therefore their activity in regulating signalling pathways.
Studies from Drosophila and Vertebrates have shown that transport and activity of ligands from Hedgehog family depends on HSPG. Gain of function studies in chick indicate that Sulf1 regulates Shh extracellular distribution and signalling (Danesin et al., 2006). We tested *sulf1* function in zebrafish by morpholino loss of function experiments. In *sulf1* morphants, expression of Shh target genes is downregulated in the ventral neural tube. Our preliminary results indicate that production of ventral neurons is not impaired whereas OPC are absent in *sulf1* morphants.
These findings suggest that Sulf1 is a positive regulator of Shh activity required for OPC specification in the ventral spinal cord. We propose that Sulf1 triggers a temporal modulation of Shh activity in the ventral progenitors that induce their switch from producing neuron to oligodendrocyte in the vertebrate spinal cord.

Stem cells in the adult zebrafish cerebellum: initiation and maintenance of a novel stem cell niche

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In the adult CNS, neurogenesis takes place in special niches. It is not understood how these niches are formed during development and how they are maintained. In contrast to mammals, stem cell niches are abundant in zebrafish and also found in other parts of the brain than telencephalon. To understand common characteristics of neural stem cell niches in vertebrates, we studied the origin and architecture of a previously unknown stem cell niche using transgenic lines, *in vivo* imaging and marker analysis. We show that bi-potent stem cells are maintained in a distinct niche in the adult zebrafish cerebellum. Remarkably, the stem cells are not typical glia but instead retain neuroepithelial characteristics. The cerebellar stem cell niche is generated by the coordinated displacement of ventricle and rhombic lip progenitors in a two-step process involving morphogenetic movements and tissue growth. Importantly, the niche and its stem cells still remain in ventricular contact through a previously unknown derivative of the ventricle. Factors propagated in the ventricle are thought to be important regulators of stem cell activity. To test the requirements of one family of important factors, Fibroblast growth factors, we used zebrafish with an inducible dominant-negative Fgf receptor. Inhibition of Fgf signaling leads to significant reduction of stem cell activity. To further understand the molecular mechanism involved in regulating cerebellar stem cells we used fluorescent activated cell sorting of the *Tg(nestin41:egfp)* transgenic line to specifically isolate cerebellar progenitors and analyse their transcriptional profile. In contrast to the predominant view, adult neural stem cells in non-mammalian vertebrates show more neuroepithelial than glial characteristics. Nevertheless, retained epithelial properties such as distinct polarization and ventricular contact are critical common determinants to maintain neural stem cell activity in vertebrates.

A comprehensive analysis of *olig* genes expression, regulation and function in zebrafish CNS development

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The members of the *Olig* gene family encode for bHLH transcription factors involved in neural cell type specification. Three *Olig* genes (*Olig1*, *Olig2* and *Olig3*) have been identified in all known vertebrate models, and a fourth one in anamniotes (*olig4*). We have performed a global analysis of *olig* genes during zebrafish embryonic development and determined which signaling pathways control their induction and regionalization in the CNS. Interestingly, zebrafish *olig3* and *olig4* together establish most of the expression domains corresponding to mammalian *Olig3*. According to our data, *olig1* is specifically confined to the oligodendrocyte lineage, whereas the other members display stratified expression in diencephalon, hindbrain and spinal cord. Notably, we observed differential expression of *olig* genes within specific motoneuron and interneuron domains of the spinal cord. *olig2*, *olig3* and *olig4* expression appears to be regulated by Nodal and FGF signaling during gastrulation and early somitogenesis, by RA signaling in the hindbrain, and by BMP and Hh signals along the dorso-ventral axis of the embryonic CNS. Our findings suggest a role for *olig* genes in CNS patterning, as well as in multiple cell fate decisions during neural differentiation.

To evaluate in detail the role of Olig members in specific neural cell fate decisions, we are currently performing *olig* knock-down/over-expression experiments and characterizing genetic mutants, crossed with transgenic fish lines expressing fluorescent proteins in neuronal or glial districts of the nervous system. In parallel, microarray-based transcriptome analysis and real time PCR on specific neural pathological conditions are helping to assemble a regulatory model of Olig function in neural identity and global shaping of the vertebrate CNS development.

Specification and circuit formation of oxytocin – secreting neurons
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Oxytocinergic (OXT) neurons [also known as oxytocin-like (oxtl) and isotocin (itnp) in fish] constitute an important population of cells of the vertebrate hypothalamus. In mammals, OXT functions as both hormone and neurotransmitter: OXT cells control targets in the periphery through the hypothalamo-hypophyseal system, where it was found to facilitate labor and breast-feeding. In the central nervous system, OXT is involved in social trust, pair bonding, sexual behavior and arousal, anxiety and stress responses. Zebrafish serve as an excellent paradigm to understand the exact molecular and cellular processes underlying the specification of OXT neurons and their circuit formation. Firstly, zebrafish have a relatively simple OXT system – each brain hemisphere contains a single cell cluster of merely 15-20 OT neurons, as compared to larger clusters of cells containing tens of thousand of neurons in mammals. Secondly, studies by others and us indicate that critical factors involved in OT cell fate decisions are conserved between zebrafish and mammals. However, it remains unclear which OXT circuits exist in zebrafish and if so what dictates their formation. To study the mechanisms of differentiation and specification of OXT neurons in zebrafish we have generated a transgenic OXT reporter line with fluorescently labeled OXT neurons. Using two-photon microscopy we could trace the primordial diencephalic area from which OXT precursors are derived and follow the establishment of the hypothalamo-hypophyseal system. The ongoing study employs genetic modulations along with live imaging techniques to investigate OT circuit formation and function.

Characterising the function of transcription factors involved in specifying circumferential ascending spinal interneurons

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CiAs are a multifunctional class of interneurons that provide all of the ipsilateral glycinergic-inhibition in zebrafish spinal cord and are probably functionally homologous to mammalian V1 interneurons. Work from our lab shows that CiAs, like V1 cells, express Pax2. We have FAC sorted GFP-positive cells from Tg(*pax2a*:GFP) embryos where GFP is expressed in CiAs and Tg(*huC*:GFP) embryos where GFP is expressed in most differentiated neurons. We have compared the RNA profiles of these cells to trunk cells at the same stage using Affymetrix's microarrays. Through this comparison we have identified transcription factors that are specifically upregulated in CiAs. Currently we are analysing the functions of these transcription factors in specifying the unique characteristics of CiAs.

Bmp signalling is required to induce cell fates in the marginal neural plate at early and pre-gastrula stages in zebrafish

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The telencephalon evaginates form the anterior forebrain and gives rise to one of the most complex structures in the vertebrate CNS. Although Wnt inhibition has been shown to be an important requirement for the establishment of the rostral forebrain, there is evidence that signalling factors additionally to Wnt antagonists play a vital role in the molecular network inducing telencephalon. In this context we are analysing the impact of the Bone Morphogenetic Proteins (BMPs) which are known to be a key regulators of neural fates. Previous data in *Xenopus* showed that the inhibition of BMP signalling activity is a basic requirement for the induction of anterior neural fates and in zebrafish the neural plate is expanded in *bmp2b/swirl* mutants. However, the telencephalon is absent or strongly reduced in *these* embryos deprived of BMP activity, while diencephalic factors are expanded and radialized. In this project we are assessing the temporal-spatial requirement of BMP signalling activity for the induction and early patterning of the telencephalon in zebrafish. Experiments using dorsomorphin, a competent inhibitor of the BMP type I receptors, at several stages of development revealed that BMP signalling activity is crucial between dome and early gastrula stages for proper expression of telencephalic (*emx3*, *foxg1*) and diencephalic (*flh*) markers. We predict that a radially graded BMP signalling activity across the rostral neural plate is either promoting or inhibiting distinct fate decisions in the process of forebrain regionalisation. Hence, we are currently performing local *bmp2b/bmprI* gain and loss of function experiments in the diencephalic anlage and perspective telencephalon to directly analyse the potential of BMP signalling to induce early telencephalic markers.

The role of Dynein cytoplasmic 1 heavy chain 1 in peripheral nerve myelination

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The formation of myelin sheaths around axons is required for rapid salutatory conduction of action potentials in the central and peripheral nervous systems (CNS, PNS). In the PNS, myelin is formed by Schwann cells, glial cells produced from neural crest that migrate to and ensheath target axons. The failure to form or maintain peripheral myelin can result in muscle weakness or paralysis, motor neuron degeneration, an inability to sense pain or change in temperature and neuropathic pain. To investigate the mechanisms that control the formation of the myelin sheath around axons, our lab analyzes mutations that result in myelin deficits. Surprisingly, we found that larvae homozygous for an insertional mutation in the *dynein cytoplasmic 1 heavy chain 1* gene (*dync1h1*^{hi3684Tg}) have myelin deficits in both the CNSA and PNS. In particular, *dync1h1* mutants have very few cells that express myelin genes. Nevertheless, Schwann cells are p resent at motor roots of mutant embryos and larvae, suggesting that *dync1h1* function is necessary for a late step in myelination. Loss of Dync1h1 function in neurons and disruption of axon transport has previously been linked to peripheral nerve disease in humans and mice. To determine if zebrafish *dync1h1* function is required in axons or Schwann cells for myelination, we initiated a genetic mosaic analysis. Our preliminary data indicate that *dync1h1* function is necessary in Schwann cells for axon wrapping and myelination. Therefore, our data reveal a potential new role for Dynein in peripheral nerve development and maintenance and raise the possibility that disruption of Dynein function in both axons and Schwann cells can contribute to peripheral nerve disorders.

Semaphorin-neuropilin interactions elicit aberrant spinal cord exit by rohon-beard central axons

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Rohon-Beard (RB) sensory neurons reside in the spinal cord and extend two types of axons, central and peripheral, that display distinctly different behaviors. The central axons extend longitudinally within the spinal cord and fasciculate with one another. In contrast, the peripheral axon, which arises as a branch directly off the central axon, exits the spinal cord and extends to the skin. Peripheral axons within the skin repel one another on contact. We are interested in understanding the mechanisms controlling these different behaviors. Perhaps the most notable difference between the RB axons is that the central axons remain in the CNS while the peripheral axons exit to the periphery. Previous work in our lab showed that knockdown of Sema3d, a guidance signal expressed in the spinal cord roof plate, caused a reduction in peripheral RB axons. Furthermore, ectopically expressed Sema3d repelled peripheral axons but did not affect central axons. These results suggest that Sema3d may act to repel the peripheral axons out of the spinal cord.

We are investigating the roles of potential Semaphorin receptors in the guidance of RB axons. We drove expression of a Neuropilin 1a-GFP fusion construct in RB cells, initially to examine its localization. Interestingly, we found that exogenous Nrp1a-GFP leads to errors in central RB axon pathfinding. The most remarkable effect is that some central axons exit the spinal cord and grow into the skin. In other cells, the central axons grow ventrally and take aberrant pathways within the spinal cord. To test whether this effect is caused by a particular Semaphorin, we are examining potential interactions between Nrp1a and Sema3d or three other Class 3 Semaphorins (Sema3h, Sema3aa and Sema3ab) that are expressed in the somites just lateral to the spinal cord. We have used morpholinos to knockdown each Semaphorin in embryos exogenously expressing Nrp1a-GFP in RB cells to ask whether the Nrp1a overexpression effect can occur in the absence of specific Semaphorins. Surprisingly, we found that Sema3h knockdown enhances the phenotype seen with exogenous Nrp1a-GFP expression alone. In embryos injected with Sema3h morpholino and Nrp1a-GFP, 63% of neurons showed central axon errors, compared with 22% in embryos expressing Nrp1a-GFP alone. These results suggest that Sema3h and Nrp-1 may have antagonistic roles in central axon guidance. We are currently investigating potential interactions between Nrp1a and Sema3aa, Sema3ab, or Sema3d.

Function of the histone Macroh2a variant in the embryonic development of the zebrafish

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Embryonic development is a highly complex process that requires a strict regulation of the signaling pathways that participate in the cell differentiation and a basal stage of gene repression, that allows a totipotential zygote express their genome differentially to originate a multicellular organism, without changes in the DNA sequence. The assembly of the DNA into nucleosomes causes a transcriptional massive repression, which can be reversed by different epigenetic mechanisms, such as post-translational modification of the histones and incorporation of histone variants into the chromatin.

Histone MacroH2A variant have two isoforms, named MacroH2A1 and MacroH2A2. The knockout of MacroH2A1 indicates that macroH2A1 is a protein that would be not essential during the embryonic development, because is expressed mainly in adults. Additionally, exist evidence in which macroH2A can regulate the function of specific genes and participates in processes of cell differentiation, which suggests that macroH2A could have a participation during the embryonic development.

In this work we study the expression of Macroh2a during the embryonic development of zebrafish. We have cloned two histone variants, *macroh2a1* and *macroh2a2* from zebrafish. Studies using RT-PCR show that only *macroh2a2* mRNA is expressed during embryonic development. Immunohistochemistry using anti-macroH2A2 from human show that zebrafish Macroh2a2 is expressed ubiquitously at early stages of development. Later in embryonic development Macroh2a2 is expressed in the head region and tail. The fail of function of Macroh2a2 induces defects in the head formation characterized by microcephalia and microphthalmia. Additionally, we have analyzed the effect the knockdown of Macroh2a2 on the expression of *dlx3* and *pax2.1* genes. Our results suggest that Macroh2a2 could be involved in head formation.

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Dissecting genetic components of diencephalic cell-fate specification
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The vertebrate brain arises from a simple sheet of neuroepithelial cells known as the neural plate. It is divided from rostral to caudal into the fore-, mid-, and hindbrain. During early somitogenesis, the forebrain develops through a complex sequence of cell type specifications and morphogenetic behaviours into the telencephalon, eyes, and the diencephalon. The latter can be further subdivided into the ventrally located hypothalamus, the prethalamus, the thalamus, the dorsally positioned epithalamus, and the caudal-most pretectum. A recent fate-mapping study in our lab has helped to reveal the neural plate origins of cells that give raise to these diencephalic subterritories. This study demonstrated that very little mixing occurs between distinct cell populations. Surprisingly, it was also found that cells of the prospective prethalamus are already functionally specified and irreplaceable as early as the bud stage (Staudt and Houart, 2007). In an effort to unravel the genetic components of this specification, we have isolated and RNA-profiled cells of the diencephalic anlage at bud stage and compared their transcriptome to this of the presumptive midbrain. Currently, we are analysing the list of diencephalic enriched transcripts and verifying the data. We have started to allocate gene expression patterns to the cell populations defined by fate-mapping, identify areas of restricted pathway activities and assess gene function for specific candidates.

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Role of the *dlx* genes in GABAergic interneuron and forebrain development of zebrafish

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The expression of the *Dlx* homeobox genes is closely linked with interneurons expressing gamma-aminobutyric acid (GABA) in the forebrain. These neurons provide inhibitory signals to the cortex and hippocampus which are necessary for information processing. GABAergic interneurons are born in the proliferative zones of the ventral telencephalon and will migrate tangentially to the overlying cortex. Single *Dlx* mutant mice show subtle phenotypes if any at all. However, the migration of immature interneurons is blocked in the ventral telencephalon of *Dlx/Dlx2* double mutant mice leading to reduction of GABAergic interneurons in the cortex. Also, *Dlx5/Dlx6* expression is almost entirely absent in the forebrain, due to cross-regulatory mechanisms via their regulatory elements. The forebrain phenotype of *Dlx5/Dlx6* double mutant mice can not be characterized due to exencephaly. Ectopic expression of *Dlx* is sufficient to induce expression of *glutamic acid decarboxylase (Gad)*, the enzyme necessary for GABA production. Therefore, the *Dlx* genes play an important role in the differentiation and migration of GABAergic interneurons of mice. In zebrafish the role of the *dlx* genes in GABAergic interneuron development is unknown. To investigate this issue, we have generated transgenic zebrafish lines with three *dlx* enhancers which are active in the forebrain and tested the role of the *dlx* transcription factors in brain development by Morpholino knockdown. Co-expression of reporter transgenes and *gad* is observed in most cells of the zebrafish forebrain starting at approximately 48 hpf. Morpholino knockdown of the *dlx* genes causes a reduction in the activity of *dlx* regulatory elements and interferes with proper *gad* expression in the zebrafish forebrain. Also, the migration of *gad*-expressing neurons from the ventral telencephalon to the pallium appears *dlx*-dependant. This implies an interconnected *dlx* cascade potentially crucial for the proper expression of *dlx* genes throughout forebrain development, comparable to the one described in the mouse. These results implicate the *dlx* genes in an evolutionarily conserved pathway controlling GABAergic interneuron differentiation and migration in vertebrates.

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Spatial and temporal regulation of robo2 splice variants

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Alternative splicing is actively induced during neuronal development, contributing to proteomic diversity, and has been implicated in cell-fate determination, axon guidance, and tissue patterning (Li et al., 2007). Axon guidance receptors that are known to express different isoforms include Robo1, Robo2, and Robo3. In zebrafish, Robo2 is the only Robo present in zebrafish retinal ganglion cells (Lee et al., 2001), and its binding to Slits guides retinal axon navigation to the tectum. Conceivably, different splice forms of Robo2 could be utilized in different tissues or in the same tissue at different developmental stages. Indeed, it has been reported (Dalkic et al., 2006) that alternative splicing expresses Robo2 exon 27 in most non-neuronal tissues of adult zebrafish, but not in embryos or larvae. Following retinal ganglion cell differentiation, retinal axons encounter many choice points along their pathway to the optic tectum. Different intracellular domains of Robo2 might be used at different choice points. For instance, in mice two forms of Robo3 are required for distinct steps in commissural axon navigation (Chen et al., 2008).

Preliminary results from our lab have identified several Robo2 exons that are preferentially spliced, including exons 7, 19, 23, and 29. Using RT-PCR, we tested the absence or presence of Robo2 exons 7, 19, 23, 29, and 31 in zebrafish retinal versus non-retinal tissue at different developmental stages: 24, 36, 48, and 72 hours post fertilization. We find that several exons are preferentially skipped in eye tissue at later stages of development, during active pathfinding. In contrast, the expression profile in non-retinal tissue does not change significantly over time. We are now using splice-blocking morpholinos to test the functional importance of these alternately spliced exons in retinal ganglion cells, commissural primary ascending neurons (CoPA) and olfactory sensory neurons, whose axon pathfinding is known to require *robo2*. For instance, we are able to effectively force exclusion of *robo2* exon 23 and confirmed the results by RT-PCR and short-probe in situ hybridization. Preliminary results from this morpholino suggest that exon 23 is not crucial for retinal axon pathfinding but may affect the pathfinding of CoPA neurons.

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Investigation of *RUNX3* expression pattern and function in zebrafish peripheral nervous system development

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The Runx (runt-related) family of transcription factors comprises Runx1/AML1/Cbfa2, Runx2/AML2/Cbfa1 and Runx3/AML3/Cbfa3. Gene ablation and gain of function experiments established all three proteins as important regulators of cell fate decisions in early embryonic development and tissue differentiation - blood, neurons, and bone. Runx1 is involved in regulation of hematopoiesis, Runx2 is essential for bone and tooth development and Runx3 is critical for gastric epithelial differentiation, neurogenesis of sensory neurons and T cell differentiation. In zebrafish, a *runx3* orthologue was identified and its embryonic expression partially described. Different splice variants, encoding 2 different protein isoforms, runx3-Shorter and –Longer, were described but no functional studies reported. Recent studies in mice and chick, have indicated that runx3 is expressed in distinct subpopulations of developing dorsal root ganglion (DRG) neurons, but the precise role of Runx3 in zebrafish sensory neurons is unclear and the relative importance of different isoforms unknown.

We have characterised the diversity of *runx3* transcript variants and encoded protein isoforms. This gene contains six exons and encodes multiple variants generated by alternative splicing from dual promoters, P1 and P2. Our characterisation of the *runx3* expression patterns confirms that *runx3* is dynamically expressed in developing embryonic neural tissues, including trigeminal ganglia, Rohon-Beard neurons and the lateral line primordial. *runx3* is also expressed in the base of the developing pectoral fins and cranial sensory ganglia. We are now focusing on investigating the possible roles of Runx3 in peripheral nervous system development, by using morpholino-mediated knockdown of Runx3 function in combination with sensory neuron cell-type specific markers.

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Zebrafish mutations in *GART* and *PAICS* identify critical roles for *DE NOVO* purine synthesis in vertebrate pigmentation and ocular development

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While purines and purinergic signaling are critical for numerous biochemical and cellular processes, their functions during vertebrate embryonic development have not been well characterized. Here we report an analysis of two recessive zebrafish mutations that affect *de novo* purine synthesis, *gart* and *paics*. *gart* encodes phosphoribosylglycinamide formyltransferase,phosphoribosylglycinamidesynthetase,phosphoribosylaminoimidazole synthetase, a tri-functional enzyme that catalyzes steps 2, 3 and 5 of inosine monophosphate (IMP) synthesis, and *paics* encodes phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase, a bi-functional enzyme that catalyzes steps 6 and 7 of this process. Zygotic *gart* and *paics* mutants present with pigmentation defects where xanthophore and iridophore pigmentation are almost completely absent, and melanin-derived pigmentation is significantly decreased, despite the fact that pigment cells are present in normal amounts and distributions in the mutants. Zygotic *gart* and *paics* mutants are also microphthalmic, resulting from defects in cell cycle exit of proliferative retinoblasts within the developing eye. Maternal-zygotic and maternal-effect mutants demonstrate a critical requirement for maternally derived *gart* and *paics*, as these mutants show more severe developmental defects than their zygotic counterparts. Pigmentation and eye growth phenotypes in zygotic *gart* and *paics* mutants can be ascribed to separable biosynthetic pathways: pigmentation defects result from deficiencies in a GTP synthesis pathway, and microphthalmia results from deficiencies in an ATP synthesis pathway. Further analysis of the ATP synthesis pathway reveals that it is required for proliferative retinoblasts to complete S-phase of the cell cycle in a normal duration. In the absence of ATP pathway activity, S-phase is prolonged, cell cycle exit is compromised and this results in microphthalmia. Taken together, these results demonstrate critical maternal and zygotic requirements for *de novo* purine synthesis during vertebrate embryonic development, and they identify independent functions for ATP and GTP pathways in mediating eye growth and pigmentation, respectively.

Glutamate Drives the Touch Response through a Rostral Loop in the Spinal Cord of Zebrafish Embryos

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Characterizing connectivity in the spinal cord of zebrafish embryos is not only prerequisite to understanding the development of locomotion, but is also necessary for maximizing the potential of genetic studies of circuit formation in this model system. During their first day of development, zebrafish embryos show two simple motor behaviors. First, they coil their trunks spontaneously, and a few hours later they start responding to touch with contralateral coils. These behaviors are contemporaneous until spontaneous coils become infrequent by 30 hours. Glutamatergic neurons are distributed throughout the embryonic spinal cord, but their contribution to these early motor behaviors in immature zebrafish is still unclear. We demonstrate that the kinetics of spontaneous coiling and touch-evoked responses show distinct developmental time courses and that the touch response is dependent on AMPA-type glutamate receptor activation. Transection experiments suggest that the circuits required for touch-evoked responses are confined to the spinal cord, and that only the most rostral part of the spinal cord is sufficient for triggering the full response. This rostral sensory connection is presumably established via CoPA interneurons, as they project to the rostral spinal cord. Electrophysiological analysis demonstrates that these neurons receive short latency AMPA-type glutamatergic inputs in response to ipsilateral tactile stimuli. We conclude that touch responses in early embryonic zebrafish arise only after glutamatergic synapses connect sensory neurons and interneurons to the contralateral motor network via a rostral loop. This helps define an elementary circuit that is modified by the addition of sensory inputs, resulting in behavioral transformation.

Interkinetic nuclear migration in the zebrafish retina: actomyosin forces are the prime mover
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As the neurogenerative epithelium of the vertebrate central nervous system expands during embryonic development, nuclei of neural progenitors undergo a process called interkinetic nuclear migration (IKNM). The nuclear movements of IKNM are generally believed to involve gradual migrations from apical to basal and back during the G1 and G2 phases of the cell cycle, respectively. Yet the kinetics of IKNM and the mechanisms that drive it have never been systematically studied.
We use high-resolution time-lapse confocal microscopy to record nuclear movements in zebrafish retinal neuroepithelial cells and show that, rather than being gradual and directed, nuclear movements are mainly stochastic except for brief apical nuclear translocations preceding mitosis.
We also demonstrate that IKNM is driven largely by actomyosin contractions rather than transport along microtubules, as these movements still occur when the microtubule cytoskeleton is destabilized or demolished. However, stochastic as well as rapid apical movement are severely inhibited when Myosin II activity is blocked.
Based on our findings we propose that nuclear movements during IKNM are reminiscent of movements of people at a crowded party held in a room with a bar at one end. People get thirsty and go to the bar to get a drink. Then they move or are pushed away to make room for others at the bar. Between drinks the partygoers jostle around the room, but when one of them gets thirsty again, he returns to the bar in a fast and directed manner.

Establishment and refinement of sensory innervation in the zebrafish lateral-line : afferent neurons as strict selectors of hair cell polarity

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The establishment as well as the continuous refinement of sensory-organ innervation is crucial for behaviour, social interactions and survival. To investigate these processes, we use the zebrafish mechanosensory "lateral line" as a model system. Sensory units of the lateral line, called neuromast, consist of two subpopulations of hair cells, equal in number, whose hair bundles are oriented at 180° relative to each other creating a stimulus-polarity ambiguity. Therefore, it is unknown how these animals resolve the vectorial component of a mechanical stimulus. While each neuromast is innervated by at least two afferent neurons, hair cells within the neuromast can regenerate and be re-innervated very rapidly. This provides a simple model of sensory circuitry whose dynamics can be visualized *in vivo* over long periods and under normal or altered physiological conditions.

To investigate the mechanisms that govern the appropriate innervation of hair cells, we conducted a systematic and comprehensive study of the processes that underlie the elaboration and remodeling of sensory-neuronal architecture in the lateral-line organ. We show that lateral line afferent neurons can recognize the planar polarization of hair cells. Each neuron forms synapses with hair cells of identical orientation to divide the neuromast into functional polarised compartments. We also show that afferent neurons are strict selectors of polarity that can re-establish synapses with identically oriented targets during hair-cell regeneration. Our results provide the anatomical basis for signal-polarity resolution by the lateral line.

We are now investigating the contribution of hair cells and neuron activity on target recognition over the long-term dynamics of sensory innervation. Preliminary results using deaf mutant fish suggest that sensory function could play a role in sensory organ connectivity.

Bigh3 is upregulated in regenerating zebrafish fins

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Teleost fish and urodele amphibians species have the ability to regenerate tissue. Mammals, in the other hand, can only regenerate tips of the digits. Zebrafish is a good model to study regeneration because of the rapidity in which this process occurs and as such can serve to improve our knowledge of the process of regeneration and later on, improving the regenerating capacity of humans.
Zebrafish fin regeneration has already been studied. Signaling factors are the second largest category, regulated during regeneration after regulators of wound healing. Major developmental signaling pathways play a role in that multistep process, like Bmp, Fgf, Notch, retinoic acid, Shh and Wnt. Here we focused on TGF-β signaling and one if its target the TGF-β induced gene (tgfbi).
Bigh3 also called kerathoepithelin, encodes protein first identified as an extracellular matrix protein reported to play a role in cell adhesion, as well as cornea formation and osteogenesis.
The expression of *bigh3* in zebrafish fins was previously reported, here we demonstrate that the induction of expression of *bigh3* correlates with the process of fin regeneration, and also depends on regulation of TGF-β signaling, suggesting a new role for this protein.

Optical control of zebrafish behavior with halorhodopsin

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Expression of halorhodopsin (NpHR), a light-driven microbial chloride pump, allows for optical control of membrane potential and reversible silencing of transfected neurons. Zebrafish are ideal model organisms for optogenetics, since they are optically clear, genetically tractable and display many behaviors within the first two weeks of life. We generated transgenic zebrafish expressing NpHR under control of the Gal4/UAS system. Loose-patch electrophysiological recordings showed that NpHR stimulation effectively suppressed spiking of single neurons *in vivo*. Surface localization and spike suppression was compared across four different zebrafish lines for the original and the enhanced (eNpHR) NpHR. We used Gal4 enhancer trap lines from our laboratory (Scott et al., Nature Methods 2007; L. Mason et al., ZFIN.org direct data submission) to express NpHR broadly or in specific subpopulations of neurons. Applying light through thin optic fibers positioned above the animal's head enabled us to target small groups of cells and to simultaneously test the effect of their silencing on behavior. With this technique, we localized cells in the caudal hindbrain that control forward swimming and determined the kinetics of the swim command in hindbrain and spinal cord by a combination of silencing with NpHR and activation with the light-gated cation channel channelrhodopsin-2 (ChR2). The NpHR-assisted "reversible spinalization" revealed that the central pattern generators in the spinal cord were under tight temporal control of the hindbrain throughout the studied swimming behavior. Furthermore, we used visual behavioral assays (optokinetic response) to identify a cell population in close proximity to the cranial nucleus VI that is required and sufficient for the generation of fast eye movements (saccades) in zebrafish larvae. Bilateral stimulation of NpHR suppressed saccades completely but left slow pursuit eye movements unaffected. Unilateral stimulation blocked saccades of both eyes in the direction of the stimulated side, but not in the other direction. Conversely, ChR2-mediated activation of this neuronal population elicited full-blown saccades in both eyes. In an unrelated forward-genetic screen (Muto et al., PLoS Genet. 2005), our group had identified a sodium-channel mutant, *didy*, with a specific deficit in sustaining saccades. The saccade deficit in mutants could be rescued by locally activating ChR2. Together our studies introduce an optogenetic toolkit for precise loss-of-function and gain-of-function analysis of neural circuits and behavior.

Fgf signaling is necessary for pharyngeal taste bud receptor cell formation
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Taste buds are the vertebrate sensory organs of taste composed of distinct cell types including taste sensory receptor, presynaptic and support cells. They are located in a patterned manner in the vertebrate oropharynx and depending on the species can be harboured in epithelial structures, the papillae. Although much progress has been achieved in understanding the molecular mechanisms underlying papillae formation, little is known about the development of pharyngeal specific taste bud cell types (taste receptor, presynaptic, supportive) per se. To start elucidating the mechanisms underlying specific taste bud cell type formation, we first characterized the zebrafish taste buds and found that are composed of distinct cell types and located in a pattern manner in the oropharyngeal epithelium. Fgf8 and Fgf receptors are expressed in the pharyngeal epithelium and their loss of function in the ace mutant and embryos with pharmacologically compromised Fgf receptor function, respectively, showed that Fgf signaling is necessary for taste bud formation. To dissect the role of Fgf signaling in taste bud cell type formation, we specifically blocked Fgf receptor signaling within the pharyngeal epithelium. We show for the first time that Fgf signaling is critically required within the pharyngeal epithelium for taste receptor cell formation.

Wnt/b-catenin signaling regulates morphogenesis of the lateral line

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The lateral line is an excellent model for studying fundamental developmental mechanisms, such as stem cell regulation, cell migration, cell fate specification and patterning in vivo. The lateral line system develops from a migrating placode (primordium) that sequentially deposits prosensory organs from the trailing edge of the primordium at fairly regular intervals. We have recently described the cell-cell signaling events that occur between cells in the leading and trailing zones of the migrating multicellular primordium. The network is based on localized activation of the Wnt/β-catenin pathway in the leading zone and restriction of Fgf signaling to the trailing zone. This genetic interaction network maintains tissue polarity within the primordium that is important for directed cell migration. Here we demonstrate that Wnt/β-catenin signaling not only controls migration but also periodic neuromast deposition. The mechanism controlling periodic neuromast deposition is, as yet not well understood. Even though lateral line segmentation and somite segmentation superficially resemble each other, we show that lateral line segmentation occurs by a very different mechanism. Our data demonstrates that neuromast deposition is a primordium-autonomous event that is controlled by the rate of cell proliferation in the leading region of the primordium. By manipulating the Fgf and Wnt/β-catenin signaling pathways we show that proliferation and spacing between deposited proneuromasts depends on Wnt/β-catenin signaling. In addition, Wnt/β-catenin signaling stimulates expression of Fgf ligands that lead to proneuromast formation within the primordium. Interestingly, the Wnt/β-catenin activation domain does not oscillate but remains constant, irrespective of the length of the primordium. These results reveal that Wnt/β-catenin signaling coordinates primordium migration, proneuromast formation and proneuromast deposition, ensuring the proper segmentation of the lateral line organ.

Neuronal circuit in the spinal cord of zebrafish before and after lesion
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In contrast to humans, zebrafish regain locomotor ability within six weeks after a spinal cord lesion. We aim to study the main components of the locomotor circuit in unlesioned adult zebrafish and changes thereof after lesion and during recovery. To this aim we use transgenic and immunohistochemical markers specific for different cell types. Recently, we have shown that lost motor neurones are replaced by newly born motor neurones that mature and are integrated into the spinal circuitry after a spinal lesion (Reimer et al., 2008, J. Neurosci. 28, 8510-16). Here we show by BrdU labeling that serotoninergic cells are also newly born after lesion. Combining immunohistochemistry for serotonin and choline acetyl transferase revealed that serotoninergic cells are distinct from motor neurones. Overall numbers of serotoninergic cells were increased 4.5-fold at two weeks post-lesion compared to unlesioned fish and remained at this increased level for up to at least 6 weeks post-lesion, when functional recovery plateaus. In contrast, parvalbuminergic cells do not change in number two weeks after a spinal lesion and are not labelled by BrdU. This demonstrates cell type specific differences in cell death and proliferation in the lesioned spinal cord. We are also analysing changes in other cell types, such as inhibitory interneurones, and in the density of serotoninergic and dopaminergic fibers in the lesioned spinal cord. We hope that these studies will increase our understanding of the adaptive changes in the spinal circuitry that allow adult zebrafish to successfully recover form a lesion.
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Specificity of afferent synapses onto plane-polarized hair cells in the posterior lateral line of the zebrafish

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The proper wiring of the vertebrate brain represents an extraordinary developmental challenge, with 10^{11} neurons forming an estimated 10^{15} synapses in humans. An essential but poorly understood feature of this process is the specification of appropriate neuronal connections. In view of this complexity, simple vertebrate systems provide necessary models for understanding how synaptic specificity arises.

The posterior lateral-line organ of larval zebrafish consists of polarized hair cells organized in discrete clusters known as neuromasts. By labeling single afferent neurons of the posterior lateral line with the fluorescent protein mCherry under the control of a neuronal promoter, we show that each afferent neuron establishes specific contacts with hair cells of the same hair-bundle polarity. Modeling the neuron as a biased selector of hair-cell polarity, we quantify this specificity and find evidence for bias from as early as 2.5 days post-fertilization. More than half of the neurons form contacts on multiple neuromasts, but the innervated organs are spatially consecutive and the polarity preference is consistent. By expressing in neurons HRP-mCherry, a fusion protein comprising horseradish peroxidase and mCherry, we confirm at the electron-microscopic level that these contacts are afferent synapses bearing vesicle-loaded synaptic ribbons. Moreover, afferent neurons reassume their biased innervation pattern after hair-cell ablation and regeneration.

By documenting specificity in the pattern of neuronal connectivity during development and in the context of organ regeneration, our results establish the posterior lateral-line organ as a vertebrate system for the *in vivo* study of synaptic target selection. This system permits a detailed experimental analysis of the role of activity-dependent and -independent mechanisms in governing synaptic specificity during the formation of neural circuits.

touché, a new touch-unresponsive zebrafish mutant

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The process by which light-touch in vertebrates is transformed into an electrical signal within mechanically sensitive neurons is a largely unresolved question in neurobiology. To address this question we undertook a chemical based (ENU) forward mutagenesis screen in zebrafish. Mutated families were screened for progeny exhibiting reduced or absent touch-evoked motor behaviors during the first two days of development. One family, subsequently named *touché* (*tou*^{mi173}), was found to harbor a recessive mutation which results in completely touch-unresponsive offspring. Further examination revealed that *tou*^{mi173} mutants also fail to stop swimming after encountering objects head-first, a behavioral response known to require feedback from mechanosensitive trigeminal neurons in *Xenopus laevis* embryos. To rule out a wholesale disruption of sensory neuron function *tou*^{mi173} mutants were exposed to stimuli which activate the lateral-line system, and noxious stimuli including mustard oil (allyl isothiocyanate) and harsh-touch (pinch). These stimuli were found to evoke motor behaviors in both wild type siblings and *tou*^{mi173} mutants. Finally pair-wise crossings with previously identified touch-unresponsive mutants (*macho* and *touchdown*) complemented the *tou*^{mi173} mutant phenotype. Collectively these findings indicate that *tou*^{mi173} represents a new touch-unresponsive mutant, and suggest that *tou* is required by mechanosensitive neurons for responsiveness to light-touch.

Synaptogenesis *in vivo*: Sequential Recruitment of Components to a Physiologically Relevant Synapse

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Studies of synaptogenesis in primary neuronal cultures have led to the hypothesis that presynaptic specializations are formed by the concomitant arrival of all necessary components. These components are thought to be trafficked together as synaptic precursor transport packets. We are re-examining this issue in vivo at a synapse that forms part of a circuit that underlies an early motor behavior in the zebrafish embryo. This behavior, the touch response, consists in coiling of the trunk of the embryo in response to a tactile stimulus. Rohon-Beard sensory neurons transduce the stimulus and pass the signal on to commissural interneurons (CoPAs) through glutamatergic synapses.

To understand how the synapses between RB cells and CoPA interneurons form during development, we characterized the timeline of recruitment of synaptic components by immunolabeling and live imaging techniques using pre- and postsynaptic markers. We examined synaptic labeling between 16 to 25 hours post fertilization (hpf), a developmental timeframe that straddles the time of initiation of the touch response at 21hpf. Synaptic vesicle precursors labeled with SV2 were equally distributed throughout RB axons at all time points examined and this distribution was confirmed by imaging of fluorescently tagged VAMP2. In contrast, synapsins 1 and 2, proteins that tether synaptic vesicles to the cytoskeleton, localized at contacts with CoPA cells after 21 hpf. On the postsynaptic side, immunoreactivity for the proteins PSD-95, PSD-93, and SAP97 were first seen at 19hpf. This time line of synaptic recruitment reveals a sequential assembly of both pre- and postsynaptic components at a synapse that is critical for a sensory-motor behavior. This is the first description of protein recruitment to a precisely defined population of glutamatergic synapses in an intact vertebrate.

Metabotropic glutamate receptors in the zebrafish retina
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Background: Metabotropic glutamate receptors (mGluRs) have been identified at all synapses of the vertebrate retina, where they likely regulate neurotransmitter release. The only example of an mGluR functioning in direct synaptic transmission is mGluR6, which is expressed on ON bipolar cell dendrites and is found to mediate the ON response in the mammalian retina. As a first step to elucidate the functional role of mGluRs in the teleost retina, we set out to clone all members of this family and determine their respective expression pattern in the developing and adult zebrafish retina.
Results and Conclusion: With its division into three subgroups, the phylogeny of the mGluR family is comparable to humans and mice. For most members (mGluR1, 2, 5, 6, and 8) we detected for each mammalian mGluR ortholog two paralogues. Those likely arose in a teleost specific whole genome duplication event. RNA *in situ* hybridization experiments revealed a unique expression pattern for all *mGluRs* in 5 day old zebrafish, supporting the distinct roles of the different mGluRs in the retina. Different expression patterns between two paralogues, as found for example for *mGluR1a*, and *-1b*, suggest a distinct subfunctionalisation of both gene paralogues. Due to the specific function in the mammalian retina, we focused on the two mGluR6 paralogues. In contrast to mammals, where mGluR6 is expressed in bipolar cells, we located both genes predominantly in retinal ganglion cells. This surprising result was supported by coexpression with *gnao* (coding for Goα proteins), the G-protein which is thought to conduct the mGluR6 signalling pathway. Since *gnao* is also expressed in inner retinal cells, we deem it likely that the *mGluR6* paralogues are expressed below detection level in bipolar cells. We are in the process of generating antibodies to identify the synaptic localization of the mGluR6 paralogues. In the future, knockdown experiments will determine the function of these genes in vision.

A gfp-based genetic screen reveals mutations affect ciliogenesis in photoreceptor cells

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A photoreceptor cell develops a photo-sensitive outer segment on the apical side of the cell body. A cilium with a microtubule axoneme connects between the outer segment and the photoreceptor cell body. Ciliary integrity is important for survival of photoreceptor cells, because large amounts of proteins involved in photo-transduction are transported from the photoreceptor cell body to the outer segments thorough cilia. In human, functional disruption of several ciliary proteins causes photoreceptor cell death and progressive blindness called as retinitis pigmentosa. However, molecular mechanism of ciliary formation and maintenance in photoreceptors has been poorly understood.

To identify mutations that affect the photoreceptor development and maintenance, we performed earl pressure screen using a GFP transgenic line of zebrafish. In this line, GFP is expressed specifically in the rod photoreceptor cells under the control of rhodopsin promoter. We isolated several mutations which affect photoreceptor development at 5 dpf. Here, we show three mutations which affect ciliary development in photoreceptors. We found that these mutations affect in the ciliogenesis of other sensory neurons including olfactory cells in the nasal pit and hair cells in the inner ear. We are currently mapping these loci to discover the underlying cause of these defects.

The zebrafish OL185 mutation affects pharyngeal and taste bud development

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The selection of proper alimentation relies mainly on a chemosensory system, the gustatory system. In vertebrates, this system detects different tastants by the taste sensory organs, the taste buds. The differentiated taste buds are onion-shaped, multi-cellular organs, composed of taste receptor, presynaptic and glial-like cells. In zebrafish, the taste buds arise mainly from the endoderm-derived pharyngeal epithelium after the second day of development. They are regionally patterned on the lips, palate, pharyngeal arches. The molecular and cellular aspects of taste bud formation remain largely unclear. To address this question, we are using primarily a forward genetic approach in zebrafish.
Through an ENU mutagenesis screen, the OL185 mutant was isolated, based on taste receptor cell defects. To characterise this phenotype, we analysed markers specific for the taste bud cell types and the surrounding oropharynx. First, we found that OL185 mutants have a reduced number of taste buds and altered regional distribution. Second, the expression of early and late oro-pharyngeal epithelium markers is not affected in the OL185 mutant supporting the notion that the mutation is not involved in the formation of taste bud tissues of origin. However, ventral cartilaginous structures of the pharyngeal arches are severely altered in OL185 mutants. In particular, the second pharyngeal arch and the five branchial arches are reduced and present an altered conformation. In addition, the branchial muscles (transverse ventral 1 to 5 and the dorsal pharyngeal wall) are severely reduced or missing in OL185 mutants. To give insight to the tissue defects provoking the OL185 phenotype, we are currently performing rescue experiments of the pharyngeal epithelium, muscular and neural crest cells. Introduction of wild type endoderm in to this mutant does not rescue the muscular defects. The mutation is located in chromosome 21 and its mapping is underway.

Differential regulation of neurite outgrowth by reverse signal of ephrin-b1 and ephrin-b3 in zebrafish embryos and in pc-12 cells

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The ephrin-B proteins are transmembrane molecules, which could mediate bidirectional signaling, with a forward signal through the Eph receptors and a reverse signal through the ligand itself. There are four ephrin-B (Efn-B) members in zebrafish, ephrin-B1, B2a, B2b and B3. In order to investigate the ability of each ephrin-B member to regulate neurite outgrowth in zebrafish embryos, we used the neuron-specific HuC promoter to drive the expression of each Efn-B with the HA-tag. When pHuC-GFP was co-injected with each pHuC-zEfn-B-HA into zebrafish embryos at one-cell stage, we found that only zEfn-B2a-HA and zEfn-B2b-HA could induce significant neurite outgrowth from GFP-labeled neurons, but zEfn-B1-HA and zEfn-B3-HA could not. We further deleted the intracellular domain of each zebrafish ephrin-B protein and expressed them under the same HuC promoter followed by the injection into zebrafish embryos at 1-cell stage. Interestingly, zEfn-B1$^{\Delta}$C-HA and zEfn-B3$^{\Delta}$C-HA could induce significant neurite outgrowth from GFP-labeled neurons, suggesting that the reverse signal of zEfn-B1 and zEfn-B3 is inhibitory to the neurite extension in vivo. On the other hand, the forward signal of all four zebrafish ephrin-B proteins enhances neurite outgrowth in zebrafish embryos.
When pCMV-zEfn-B expression constructs were transfected into PC-12 cells and treated with NGF, we found that all zEfn-B-GFP proteins could induce significant neurite outgrowth in PC-12 cells at 48 h after transfection. In contrast, only PC-12 cells transfected with pCMV-zEfn-B1$^{\Delta}$C- HA and pCMV-zEfn-B3$^{\Delta}$C-HA did not show neurite outgrowth, suggesting that the reverse signal of zEfn-B1 and zEfn-B3 has the potential to promote the neurite extension in PC-12 cells. Altogether, zebrafish ephrin-B reverse signal has differential regulation of neurite outgrowth in vivo and in vitro.

TRKB/BDNF expression in the hair cells of developing lateral line system in zebrafish
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The neuromasts are the structural units of the lateral line system in bony and cartilaginous fishes. They consist of sensory hair, supporting and mantle cells, and are supplied by the peripheral processes of bipolar sensory neurons of two cranial ganglia, located anterior or behind to the ear, which innervate the head and trunk-tail neuromasts, respectively. Fishes express both common (BDNF, NT-3, NT-4) or specific (NT-6, NT-7) neurotrophins and their receptors (TRK), which have been detected at the mRNA or protein levels in different fish species, including TRKB and BDNF throughout embryonic development in the zebrafish. As far as we know the developmental related changes in the expression of TRKB/BDNF in zebrafish neuromasts were never investigated. In this study we used immunohistochemistry to investigate the expression of TrkB/BDNF system in neuromast of wild type and transgenic zebrafish (strains ET4, Et (krt4:GFP) sqet4) from 4 days postfertilization (dpf) to 120dpf. Specific TrkB immunoreactivity was regularly detected in the sensory cells of neuromasts through the time examined; moreover TrkB was found in a subpopulation of neurons in the anterior and posterior lateral line ganglia. BDNF undergoes developmental changes. Faint but specific BDNF immunostaining was observed in all hair cells at 4 dpf neuromasts, progressively decreased with development, and it was almost all absent from neuromast of older animals being restricted to a small subpopulation of sensory cells. Present results suggest that the TRKB/BDNF growth factor system is involved in the development of neuromasts acting in a paracrine/autocrine manner; nevertheless, when neuromast reach maturity, become independent although they continue to express TRKB. The functional significance of these results remains still obscure but it could be related to regenerative processes of the sensory cells in the lateral line.

***In vitro* and *ex vivo* models of generation of rod progenitor cells**

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Photoreceptor degeneration is a common cause of blindness in humans, so numerous studies have been made attempting either to delay the death of cones and rods or to replace the cells that are already lost. The use of rod progenitor cells (RPCs) has been revealed as a promising strategy. Transplantation of RPCs into a degenerating retina improves the visual function, as these cells differentiate into mature rods and integrate within the retina (MacLaren *et al*., 2006). The ontogenetic stage of RPCs is directly related to the capacity of these cells to migrate and differentiate within a degenerating retina, although the mechanisms underlying are largely unclear.

Our purpose is the characterization of the RPCs, describing the expression pattern during its migration and differentiation through the retina and studying their proliferative potential *in vitro*.

Teleost retina is an excellent model for the study of RPCs, as these animals have continuous retinal growth and generate new rods throughout adult life. For the detecting of RPCs in the adult retina, we have carried out intraperitoneal injection of a BrdU-containing solution during a week. For the analysis of the expression pattern of retinal markers in the RPCs we developed double immunohistochemistry for BrdU and neural progenitor markers like Nestin, Prox1, Pax6 and Islet1. *In situ* hybridization against Notch1 and NeuroD after BrdU immunohistochemistry was also carried out.

Although we have detected $BrdU^+$ cells in all retinal layers, RPCs are exclusively located in the inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL). Three subtypes of RPCs can be defined according to their location within the retina and proliferative potential: stem cells, migrating progenitors and rod precursors. Whereas the migrating RPCs are positive to almost all the studied markers ($Nestin^+$, $Prox1^+$, $Notch1^+$, $CD133^+$, $NeuroD^+$ and $Islet1^+$), the stem cells ($Pax6^+$) and post-mitotic rod precursors ($Notch1^+$, $NeuroD^+$) have a more restricted expression pattern.

In our *in vitro* characterization assay, we have isolated and grown rod progenitor cells using a proliferative medium. These cells showed a great proliferative potential, confirmed by the formation of primary neurospheres after 5-7 days in culture. The proliferative rate maintains stable after two months in culture.

All the procedures and experimental protocols were in accordance with the guidelines of the European Communities Directives (86/609/EEC and 2003/65/EC), the current Spanish legislation for the use care of animals in research (RD 1201/2005, BOE 252/34367-91, 2005) and conformed to NIH guidelines.

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Characterization of voltage-gated calcium channels within the motor circuit of zebrafish embryos

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How genes, and the proteins they encode, contribute to the formation and function of circuits that underlie behaviour is a central question in biology. To gain insight into this question, we employed a reverse genetic approach to investigate role of voltage-gated calcium channels to motor behaviours in zebrafish. Early motor circuitry results in three characteristic behaviours. The first consists of the apparition of spontaneous coiling, starting around 17 hours post-fertilization (hpf). The frequency of this coiling peaks around 19 hpf, and gradually decreases over the following 10 hours. The second behaviour is the onset of coiling as a response to contralateral tactile stimulation, around 21 hpf. Lastly, around 28 hpf, the embryo develops the ability to swim, with low amplitude and high frequency contractions.
We employed *in situ* hybridization to assess the expression of several calcium channels during development. Thereafter morpholinos were designed to specifically block translation of these calcium channel subunits in order to perturb protein expression. Quantitative behavioural analysis of the three embryonic behaviours was used as an assay for the role of the targeted proteins.
Cav1.3b, although expressed at early stages in V2 interneurons and motoneurons, may be dispensable for motor circuitry, as behaviour of CaV1.3b knockdown embryos appears to be normal. We are currently performing double knockdowns using a morpholino to the duplicated subunit of this gene (CaV1.3a) to check for possible redundancy.
As more data are gathered, we will be able to learn which channel subunits are necessary for the manifestation of early embryonic behaviours and use that information, and the genetic tools we developed, to ask questions about the role of activity in the formation of the spinal cord.

Myelin-specific Claudins in zebrafish: evolutionary and functional characterization
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Myelination is a key step in vertebrate evolution: oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) tightly wrap their cytoplasmic processes around axons. This allows rapid, saltatory nerve impulse conduction while keeping axon diameters small, permitting compact powerful nervous systems at relatively small metabolic cost.
Myelinating glial cells have evolved specific molecular mechanisms and components to form and maintain their myelin sheath. These include members of the Claudin protein family. Claudins are essential components of tight junctions, which form selective permeability barriers across paracellular pathways and function as a fence between apical and basolateral membrane domains in epithelia. In myelin of rodents, Claudins are expressed in autotypic tight junctions of oligodendrocytes (Claudin 11/OSP) and Schwann cells (Claudin 19) and contribute to electrical insulation, presumably by regulating ion flow between interstitium and intramyelinic space. The fence function of Claudins may contribute to compartmentalization of the myelin membrane.
We have identified a teleost specific, novel myelin protein, zebrafish Claudin k (formerly Claudin 31), which is expressed exclusively in Schwann cells and oligodendrocytes. We show that it localizes to the Schmidt-Lantermann incisures, the paranodal loops, and the inner and outer mesaxons – similar to mammalian Claudin 11 and 19. While mammalian Claudin 11 and 19 are restricted to the CNS and PNS respectively, zebrafish possess not only Claudin k but also Claudin 11 and 19, all of which we find to be expressed in both CNS and PNS. That raises the question: are myelin Claudins in teleosts redundant or have they evolved divergent functions? To address this question, we analyzed the phylogenetic relationship of Claudins and are performing knock down experiments in zebrafish larvae. In addition, we are performing co-immunoprecipitation experiments for Claudin k to find interaction partners, which may give insight into the role Claudins play in myelin.
As a tool to further dissect Claudin k function and myelin biology in general, we have cloned a *claudin k* promoter, that drives strong specific expression in myelinating glia – in the CNS as well as in the PNS. Using the flexible Tol2-Gal4-UAS system (Paquet et al., 2009) with independent driver and responder constructs and optional fluorescent reporters, it can be used to express proteins specifically in myelinating glia. Currently, we are generating a transgenic line with membrane bound eGFP. Additionally, we express eGFP / mCherry-Claudin k fusion proteins and are verifying their proper localization to autotypic tight junctions. These lines will allow us to monitor myelination and tight junction formation in vivo and can then serve as a read out for screens affecting myelination and proper tight junction formation.

Impaired Energy Metabolism Leads to Reduced Vision in the Zebrafish *noir* Mutant

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Large-scale mutagenesis screens have been performed in the zebrafish (*Danio rerio)* to identify genes involved in the function of the visual system.
In such a screen the mutant *noir* has been identified due to its dark external appearance and absence of optokinetic behavior. Reduced vision was confirmed by electroretinography (ERG) which suggested a block of signal transduction from primary to secondary neurons starting at 5 days postfertilization (dpf).
Genetic mapping of the mutation revealed that a defect in a subunit of the Pyruvate Dehydrogenase complex underlies the *noir* mutant phenotype. Absence of enzyme activity leads to a decrease of the ERG b-wave amplitude at 5 dpf and to a complete lack of both, a- and b-wave at 7 dpf. At 6 dpf defects in the inner nuclear layer are observed in histological sections of the retina. This defect extends to the outer retina at 7dpf.
Interestingly, cells of the inner nuclear layer, rather than photoreceptors – the most energy-consuming cells of the retina are affected first by the mutation suggesting a more complex pathogenesis than simple energy deprivation.
Strikingly, a ketogenic diet consisting of long-chain fatty acids could rescue the *noir* mutant phenotype. Mutant larvae survived longer and the ERG and histological phenotype were significantly improved. The long-chain fatty acids provide an alternative energy source to restore diminished acetyl-CoA levels caused by the block linking glycolysis and Krebs cycle.
We found that the defect in the inner retina is due to a selective damage to cholinergic amacrine cells, which most likely involves deficient generation of the neurotransmitter acetylcholine.
In conclusion, a mutation in a metabolic key enzyme leads to reduced vision and retinal dystrophy in the zebrafish *noir* mutant. The defect initiates in the inner nuclear layer suggesting a complex pathogenesis.

Defective photoreceptors underlie inherited blindness in the raifteiri mutant

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Purpose: To characterise the basis of the inherited visual function defect in the *Raifteirí* mutant.
Methods: Optokinetic response (OKR) screens were performed on N-ethyl-N-nitrosourea (ENU)-mutagenised zebrafish to identify families with recessive defects in visual function. Retinal differentiation and lamination was characterised by light microscopy, and immunohistochemistry of retinal sections using the cell-specific markers *zpr1*, *zpr3*, *5e11* and *zn-5*. Cell death levels were determined by TUNEL staining. The integrity of the inner retina (hyaloid) and trunk vasculature was analysed by alkaline phosphatase staining. Circadian locomotion and shadow-response behaviours were ascertained using Zebralab (Viewpoint Zebrafish Tracking System).
Results: A novel mutant designated *Raifteirí (raf)* was identified by OKR screens. *Raf* mutants show a significantly reduced or absent OKR and are paler with slightly smaller eyes. The locomotor activity levels of *raf* mutants are ~50% of normal siblings, however, *raf* mutants exhibit circadian behaviour and a shadow reponse. No difference in vasculature morphology or levels of cell death is observed between mutants and siblings. Light microscopy and DAPI staining show *raf* mutants to have normal retinal lamination. *5e11* and *zn-5* staining of the inner retina suggests that amacrine and ganglion cell differentiation is equivalent in siblings and *raf* mutants. In contrast, staining of the outer retina with photoreceptor markers *zpr3* and *zpr1* appears to be reduced overall in *raf* mutants. However, staining of newborn rod photoreceptors in the peripheral retina is normal. Preliminary light microscopy data suggests a morphological defect in *raf* photoreceptors and retinal pigment epithelium (RPE).
Conclusions: We have identified and partially characterised a novel zebrafish model of inherited blindness. Preliminary results suggest that specific defects in *raf* outer retina morphology underlie loss of visual function.

Differentiation and maintenance of zebrafish photoreceptor polarity

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Photoreceptor cells have a unique morphology, essential for their highly specialized function. Polarity in mature photoreceptor cells is particularly evident in their apical surface, which is subdivided into the outer segment, the connecting cilium and the inner segment. In *Drosophila*, the Crumbs protein complex has been shown to control the differentiation of the apical surface in epithelial and photoreceptor cells. The core components are the transmembrane protein Crumbs and the scaffolding proteins Stardust, *D*PATJ and *D*Lin-7. Defects in individual components lead to disintegration of embryonic epithelia, morphogenetic defects in photoreceptor cells and light-dependent retinal degeneration.

The zebrafish genome encodes five *crumbs* genes, a single *stardust* gene and three *lin7* genes. Mutations in one of the *crumbs* orthologues, *oko meduzy*, and in the *stardust* orthologue *nagie oko* affect the polarized structure of the neuroepithelium. In Drosophila, absence of crumbs leads to a reduction in the length of the stalk membrane, a portion of the apical membrane which corresponds to the inner segment of vertebrate photoreceptor cells. In zebrafish, morpholino-induced loss of *crb2b* function has been shown to affect the differentiation of apical characteristics in photoreceptor and kidney epithelial cells.

We are currently performing a detailed analysis of the development of photoreceptor polarity at different stages, both in fixed tissue and by in vivo imaging. In addition, we are screening for mutations in *crb* genes by TILLING. We will report on the results, which can provide insights on the cell biological function of the Crumbs protein complex in the differentiation and maintenance of apico-basal polarity of photoreceptor cells.

Arrestin availability in cone photoreceptors modulates opsin inactivation
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Human vision is governed mainly by cone photoreceptor function. Not only are cones responsible for color vision; they also enable the retina to rapidly signal changes in illumination even in presence of high ambient light levels (photopic vision). However, little is known about the mechanisms shaping the time course of the cone response, avoiding photobleaching and ensuring high temporal resolution.
In contrast, rod photoreceptors are highly sensitive to light and, therefore, responsible for vision at dim light (scotopic) conditions. The initial step in shutting-off rod signal transduction is comprised by the phosphorylation of activated rhodopsin. Subsequent binding of the arrestin protein to phosphorylated rhodopsin is essential for the complete inactivation the photopigment.
Recent studies have shown that phosphorylation of the photopigment opsin is also a crucial step for proper cone response inactivation - suggesting a quenching mechanism similar to the one in rods.
Here, we used the cone-dominated retina of zebrafish larvae to genetically dissect the mechanism of photopigment inactivation in cone photoreceptors, and, in particular, to study the impact of arrestin on quenching cone signal transduction.
We identified two cone arrestins and verified their cell type specificity by mRNA and protein expression analysis. Morpholino-mediated knock down of the arrestins and subsequent electroretinographic (ERG) recordings were performed in order to elucidate functional contributions of cone arrestins to the photopigment inactivation. Paired-flash ERG recordings showed that reduced arrestin protein levels cause a delay in photopigment recovery. Moreover, visual behavior testing revealed that this delay impairs visual performance.
In conclusion, our data demonstrate that cones use a similar mechanism to rods for fast photopigment inactivation and for ensuring a high temporal resolution.

The role of *ush1c* during zebrafish otic sensory patch development
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The inner ear is an organ characteristic of the vertebrates. It rooms specialized mechanosensory cells that are essential for the functions of hearing and balance: the hair cells. In the inner ear, auditory and vestibular stimulation will physically induce a vectorial deflection of the hair bundle, an interconnected actin-rich microvilli structure arranged in a staircase pattern and localized in the apical side of the hair cell. As a result of the deflection, chemical signals are produced by the hair cell and released into the synaptic cleft of the innervating neurons. Responding neurons will generate an action potential that will be driven towards the different interpretation centers in the brain. Thus, the transformation of the mechanical input into a chemical response depends on the deflection of the hair bundle and on its correct integrity.
USH1C gene codes for evolutionary conserved cytoskeleton scaffold proteins that contain 2 or 3 PDZ binding domains in function of the translated isoform. In humans, mutations on the *USH1C* gene cause Usher syndrome type 1 that is characterized by congenital deafness, vestibular areflexia and retinitis pigmentosa. Using the zebrafish as a model and a morpholino knock-down approach, we are currently characterizing the function of *ush1c* during ear sensory patch development. Our results show that most of the 5dpf *ush1c* morphants did not respond to the tapping hearing assay, but that those that did respond, presented balance defects when swimming, thus phenocopying aspects of the human syndrome. In addition, morphological analysis of hair bundle structures using fluorescein coupled phalloidin shows a decrease in number of these structures in distinct sensory patches. However, no statistical differences were found between wild type and *ush1c* morphant larvae in the cell proliferation and cell death assays. Taking these results together, we propose that *ush1c* is required for hair-bundle genesis rather than hair bundle maintenance or hair cell differentiation per se.

Investigating the role of glial cells in the spinal cord using chemical optogenetics

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There is recent evidence that glial cells can modulate neuronal activity in the central nervous system, but the mechanisms are still unknown. We took an optical stimulation approach based on the introduction of light-gated ionotropic glutamate receptor (LiGluR) in the zebrafish larval cells. These genetically engineered channels coupled to a chemical photoswitch are sensitive to specific light wavelengths and allow UV light to depolarize specific cells. We found previously that optical stimulation of specific neurons in the spinal cord led to distinct locomotor behaviors: Rohon Beard neuron activation induced an escape like response, while activation of cerebrospinal fluid neurons led to slow swimming only. We performed an enhancer trap screen in order to select for lines with explicit LiGluR expression in the spinal cord, specifically in glial cells. We conducted light pulse experiments on these lines and found that optical stimulation of glial cells in the spinal cord could induce many different types of locomotion depending on the light pattern. The motion observed could either correspond to a forward swim, a touch escape response or a large struggling motion depending on the spatial patterning of the light. We are currently investigating in vivo the intra-cellular mechanisms by which depolarization of glial cells can modulate neuronal activity.

Early left-right epithalamic asymmetry influences both lateralization and personality in adult zebrafish

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The habenulae and associated parapineal organ are part of a highly conserved conduction system connecting the limbic forebrain areas with midbrain and hindbrain structures of all vertebrates and are implicated in many important functions such as olfactory perception, feeding, mating, avoidance learning, and hormonal response to stress and reward. Asymmetry in these epithalamic structures appears very early in the development of zebrafish and it has been posited that they play a key role in the development of lateralization of cerebral functions and are involved in the genesis of individual differences in coping styles and personality. We sorted zebrafish with left or right parapineal at birth using a *foxD3*:GFP marker and subjected them to four tests of sensory and motor lateralization as adults (mirror, predator inspection, rotational preference and swimming turns in the dark). Fish with opposite parapineal positions differed in all laterality measures. They also showed a different response in some of the measures of personality traits (distance from a predator, open field and social distance). Fish with atypical right parapineal position, maintained a shorter distance from predators, spent more time in the peripheral portion of an open field and covered a shorter distance when released in the dark. Activity in the open field was independent of anatomical asymmetry but correlated with laterality of predator inspection that in turn was influenced by parapineal position. Only measures of social distance were uncorrelated with both anatomical and functional asymmetries while strongly influenced by sex, thus suggesting that other factors, i.e. hormonal, may be implicated in their development.

Taste preferences in Zebrafish juveniles

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The sense of taste helps acquiring and maintaining an appropriate feeding behavior, allowing to select good food and reject deleterious substances. For instance, sweet taste permits the identification of energy rich nutrients, and bitter warns against the intake of potentially poisonous chemicals. Taste impairment (dysgeusia) occurs in several diseases and it can give rise to weight loss through reduced appetite and altered patterns of food intake. On the other hand, obesity in human may be linked to exacerbated appetite for sweet substances, often acquired during childhood. As a consequence of the important role of taste in nutritional behaviour in healthy humans and patients, considerable research work has recently been focused on the mechanisms of development, function and regeneration of the gustatory system.

We have established a behavioural test that will allow us to determine on which principles zebrafish feeding behavior is based and particularly, what can restrict the quantity of food eaten in a given time. Using this assay, we have noticed that taste buds are already functional in 5day-old zebrafish when larvae begin to take food since bitter compounds like denatonium or cycloheximide prevent feeding in larva, consistent with the work of others. This behavioural assay combined with functional data, based on calcium imaging in the sensorial neurons of zebrafish juveniles will help defining developmental aspects of the physiology of taste in vertebrates.

The ontogeny of sleep-wake cycles in zebrafish

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Recently, surprising statistical regularities have been revealed in the structure of bouts of sleep and wakefulness. In adult humans the duration of sleep bouts exhibits an exponential distribution with the rule $P(t) \sim \exp(-t/\tau)$ where t is an individual sleep bout, whereas, wake bouts exhibit a power-law distribution with the rule $P(t) \sim t^{-\alpha}$ where t is an individual wake bout. The wake bouts exhibit a scale-free power law behavior with an exponent, α, that remains constant across mammalian species (humans, cats, rats, and mice). In contrast, sleep bout durations follow an exponential distribution where τ represents a characteristic time scale whose main determinants are body size and metabolic rate. In neonates (rats) both sleep and wake bouts exhibit exponential distribution immediately after birth, with a clear power-law behavior of wake bouts emerging only after the second postnatal week; this occurs in spite of very little change in the overall duration of wake bouts; τ, on the other hand, increases with age. Thus, the power-law exponent α is constant across multiple adult mammalian species, but switches from exponential to power-law behavior during development. In contrast, the sleep-related time constant τ varies across species and age. Importantly, the only information needed to calculate α and τ is sleep and wake durations; one does not need detailed information about the transitions *between* sleep states or information about events within a given state.

In the current experiment we describe an automated behavioral recording system used to measure sleep-wake cycles in zebrafish and, for the first time, describe their sleep-wake cycle development across ontogeny. Preliminary data indicate that the sleep-wake bout structure and development is comparable to that of mammals: First, strong circadian rhythms are found at all ages; second, sleep-wake cycles elongate over development and reach the adult values in the first 3 months; and third, the values α and τ can be used to meaningfully compare sleep in zebrafish and mammals.

The values, α and τ, thus, represent a novel way of thinking about sleep, and offer a novel, simpler method of characterizing sleep states; a method that is based on the stability of behavioral states. Applying this method to the zebrafish offers a way to compare sleep data between fish and mammals - including humans. In contrast to the standard in the field, this method renders sleep comparable across phylogeny and ontogeny and, thus, opens new ways of dissecting sleep at the behavioral, pharmacological, and genetic levels.

Molecular and cellular mechanisms of vascular patterning by PlexinD1 signaling
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The developing vertebrate vascular system, like the vertebrate nervous system, relies on a variety of attractant and repellant ligand-receptor interactions to achieve its highly reproducible anatomical pattern. One such ligand-receptor pair is Semaphorin3 (Sema3)-PlexinD1 (PlxnD1). Paracrine Sema cues from the somites signal to the endothelial-specific PlxnD1 receptor to shape the stereotypic anatomy of the trunk's segmental (Se) vessels. Specifically, PlxnD1 regulates four fundamental aspects of Se vessel angiogenesis: it determines when Se vessels sprout, where they sprout from along the aorta, determines their pathfinding trajectory and limits their branching to specific positions along the dorso-ventral axis. However, the precise biochemical mechanisms and molecules that participate in PlxnD1 signaling remain to be elucidated. Similarly, the endothelial cells that require PlxnD1 activity and the cell autonomy of this requirement have not been uncovered. Here, we describe our ongoing efforts to perform an *in vivo* structure-function analysis of the PlxnD1 receptor using Se vessel patterning as an assay and the results of our cell transplantation experiments between *plxnD1* mutant and wild type embryos.

Investigation of zebrafish homologues to specifically upregulated transcripts with unknown function in cardiomyocytes derived from murine embryonic stem cells

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Heart failure due to the loss of functional cardiomyocytes is one of the most frequent cardiovascular diseases. Understanding the genetic network that leads to functional cardiomyocytes is the first step to develop future therapies. A global transcriptome analysis yielded up-regulated genes in cardiomyocytes, which were derived from murine embryonic stem cells (Doss et al., 2007). We were interested in a fast screen for the functional role of transcripts with unknown function (TUFs) for an intact activity of the heart. Therefore we searched for homologues in the zebrafish genome and performed a morpholino-based knockdown approach. We tested 11 TUFs of the zebrafish and found most of them to be expressed in the cardiovascular system. Morpholino-oligonucleotide injections caused highly specific cardiovascular defects in the majority of them such as altering of heart morphology, vascular defects or accumulation of blood cells to a different extent and penetrance. This pilot approach thus shows the potential of the zebrafish to identify TUFs in the cardiovascular system.

Left/Right Signaling controls Tissue Polarization and Morphogenesis during Zebrafish Heart Tube Formation

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In vertebrates, the heart is the first organ to develop asymmetrically. Recent work in zebrafish has begun to elucidate the link between L/R signaling via a Nodal-BMP signaling cascade and tissue morphogenetic events involved in cardiogenesis. While these factors have been mainly implicated in cell fate specification processes, newly emerging evidence suggests that Nodal and Bmp signaling regulates cell behaviors including cell migration and epithelial tissue morphogenesis. Presently, the challenge is to elucidate the exact roles of each signaling pathway in modulating such cellular responses.

We show that dynamic cellular rearrangements occur during the initial stages of cardiac tube formation. Whereas cardiomyocytes within more lateral positions of the heart field display mesenchymal-like squamous cell shapes and are motile, cardiomycytes in medial positions acquire cuboidal-to-columnar epithelial shapes. Modulation of Nodal-BMP signaling affects cardiomyocyte migration in lateral positions and epithelial maturation within more medial positions of the heart field. Disruption of L/R asymmetric Nodal or BMP expression causes randomized myocardial tissue involution or inhibition of heart tube formation altogether. In functional studies, we show that BMP and Nodal signaling are essential in controlling tissue polarization and morphogenesis.

To further elucidate the control of cardiomyocyte behaviors by Nodal-Bmp signaling, we have performed micro-array experiments comparing myocardial expression of wild-type, Nodal/BMP signaling-deficient, or BMP overexpressing embryos using the Agilent 44K zebrafish array. These genome-wide analyses provide a first glimpse at the potential target genes that mediate the responses of cardiomyocytes to Nodal-BMP signaling during cardiogenesis.

Vascular remodeling is regulated by alki in response to blood flow
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To gain insight into the pathways that lead to development of arteriovenous malformations (AVMs), we are using a zebrafish model for Hereditary Hemorrhagic Telangiectasia type 2. HHT2 is a rare, potentially lethal autosomal dominant vascular disorder caused by heterozygous loss of the endothelial-specific TGF-ß type I receptor, *ALK1*. Patients affected by HHT2 develop telangiectases and AVMs. The zebrafish *alk1* mutant, *violet beauregarde* (*vbg*), displays the hallmarks of the HHT vascular phenotype at the level of a particular set of cranial vessels: enlarged cranial arteries that aberrantly connect to neighboring veins, forming lethal arteriovenous shunts. Interestingly, the conduits that form these arteriovenous shunts are actually vein-derived angiogenic sprouts that contribute to the development of these cranial arteries in both *wt* and *vbg* mutants. Normally these connections are severed once the arterial system forms, but in *vbg* embryos, at least one of these transient connections fails to regress. Detailed analysis of development of this malformation over time demonstrates that the vascular lesion initiates concomitantly with blood flow through the *alk1*-positive cranial vessels, and manifests as an increase in diameter of and cell number within these specific vessels. A further increase occurs later in the development and evidence from embryos that lack heartbeat suggests that later increases in cell number as well as arteriovenous shunts are not genetically determined, but are an adaptive response to increased blood flow. Blood flow plays a critical role not only in manifestation of the *vbg* phenotype, but also in regulation of *alk1* expression. Taken together, these results suggest that *alk1* expression is regulated by blood flow and that Alk1 might function in stabilizing vessel caliber at the onset of flow.

Characterization of the endothelial differentiation gene-1 (*EDG1*) in zebrafish

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The endothelial differentiation gene-1 (*edg1*) is an endothelial cell receptor for sphingosine-1-phosphate (S1P), a blood-borne lysophospholipid with important implications in the modulation of cell growth, survival, and invasion, vascular maturation and angiogenesis in various physiological and pathological conditions, including cancer. In order to assess the role of *edg1* during development in zebrafish, we cloned the *z-edg1* ortholog and we studied its expression at different stages of development by RT-PCR analysis and whole-mount *in situ* hybridization. RT-PCR analysis revealed that *z-edg1* is expressed throughout zebrafish development starting from 15 hours post-fertilization (hpf). In agreement with previous observations, *z-edg1* mRNA is widely present in the central nervous system (CNS) during somitogenesis. At the 20-somite stage, a stripe of *z-edg1*$^+$ cells is apparent in the midline and extends through the trunk and posterior part of the embryo, likely representing the endothelial precursor cells. At 26 hpf, *z-edg1* expression is maintained in the CNS and becomes apparent also in the axial vasculature of the trunk (dorsal aorta and posterior cardinal vein). This vascular pattern of expression is maintained until 30 hpf. Down-regulation of *z-edg1* expression following *z-edg1* morpholino injection results in lack of blood circulation and pericardial edema. Also, intersomitic vessels (ISVs) appeared thinner in diameter even though no delay in ISVs development was observed in tg(*kdr*:EGFP) transgenic embryos or by *in situ* hybridization for the vascular endothelial marker *kdr*. Furthermore, tail blisters were observed in a significant percentage of embryos injected with the highest doses of *z-edg1* morpholino, as already described for the S1P signalling mutants *mil, toh* and *ko157*. At present, experiments are in progress to assess the role of *z-edg1* during tumor vascularization in Zebrafish.

The Rac1 regulator ELMO1 controls vascular morphogenesis in zebrafish

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The small GTPase Rac1 performs essential functions during the regulation of angiogenic processes. To date, several important questions regarding the regulation and function of Rac1 in the embryonic and adult vasculature remain unanswered. First, little is known about which intracellular proteins modulate the activation of Rac1; second, what are the upstream regulators of Rac1 *in vivo*, and third, what are the functions of these regulators in the vasculature *in vivo*? The ELMO1/DOCK180 complex forms a guanine nucleotide exchange factor for Rac1, regulating its activation during cell migration in different biological systems, but its role in the vascular system remains elusive. In situ hybridiziation studies for *elmo1* revealed a temporal and spatial restricted vascular and neuronal expression during early zebrafish development. Loss-off function studies using specific morpholinos against *elmo1* severely impaired in *tg(fli1:EGFP)* embryos the formation of the vasculature, including intersomitic vessels, the dorsal longitudinal anastomotic vessel, the parachordal vessel and the development of the thoracic duct. Moreover, we could identify Netrin-1 and its receptor Unc5B as upstream activators of the ELMO1/DOCK180 complex. Loss-of-function experiments for Netrin-1 and Unc5B display overlapping phenotypes as seen for ELMO1. These functional data were biochemically substantiated by showing that Netrin-1 and Unc5B regulate the functional interaction of the ELMO1/DOCK180 complex, and leading to Rac1 activation in endothelial cells. Thus our data have identified a novel signalling cascade regulating the formation of the vasculature in zebrafish.

Regulation of cardiac development by zSARA1
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The morphogenesis of cardiac valves and septation of the cardiac chamber are biological processes tightly regulated by several signaling pathways. However, valve and cardiac chamber defects are still the most common human congenital abnormalities. In our lab, while performing a study on the TGFbeta signaling adaptor, zSARA1, we serendipitously saw that this protein can affect the atrioventricular valve development.
It has been show in cell culture that SARA is an endosomal protein with a FYVE domain, and acts as a TGFbeta signaling adaptor that brings Smad2 to the activated TGF-beta receptor (Tsukazaki, Cell 1998). Furthermore, studies in our lab have shown that drosophila SARA is required to allow precise transmission of TGFbeta-signaling levels during symmetric cell division in the wing disc (Boekel, Science 2006) and that it labels Notch directional signaling during asymmetric cell division in the SOP (Coumailleau, Nature 2009).
The knock down of zSARA1 function with morpholinos showed us that the morphants had no blood circulation. We have analysed the morphology of blood vessels, the presence of blood cells and the cardiac chambers as well as the cardiac valves. This analysis showed us that in the absence of zSARA1, the atrioventricular (A/V) valve is not properly formed. While BMP4 expression is still present in the A/V valve, Notch1b is missexpressed in this tissue. This Notch1b missexpression might also be the cause for the expansion of the cuboidal endocardium from the A/V valve into the ventricle that we observe. It would be interesting to analyse the expression and distribution of the zSARA1 protein in this tissue and understand the molecular events that lead to this phenotype.

Synergistic role of Neurexins and Neuroligins in vascular embryonic development

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In the last decade the relationships between the vascular and nervous systems have been re-evaluated in light of the discovery of common molecular cues regulating their functions. For example the guidance of axons and endothelial cells is orchestrated by specific signaling molecules in the extracellular environment, which belong to four distinct classes: Netrins-DCCs-Unc5s, Semaphorins-Plexins-Neuropilins, Slits-Robos and Ephrins and their Eph receptors. Despite the high and still growing number of molecules shared by these systems, none of the known key synaptic proteins has been so far shown to participate in blood vessels functions. We have explored this hypothesis and found that specific isoforms of Neurexins and Neuroligins are involved in embryonic vascular development and blood vessel remodeling in zebrafish.

In mammals, Neurexins and Neuroligins constitute two families of synaptic transmembrane proteins involved in synaptogenesis. The knockdown of the expression of the three alpha-Neurexins or Neuroligins 1-3 in the mouse demonstrates that these proteins have a fundamental role in the modulation of synaptic transmission rather than in the early adhesive steps of synapse formation. Previously, we isolated the six zebrafish Neurexins genes and analyzed their phylogenetic relationships and structure in respect to the human counterparts. Like human genes each zebrafish Neurexin gene presents two alternative promoters that drive the synthesis of a long (alpha) and a short (beta) form and contains different sites of alternative splicing (AS) that can give rise to thousands of different transcripts. More recently we isolated the seven zebrafish Neuroligins and here we show that functional knock-down of specific forms of Neuroligin and beta-Neurexin but not alpha-Neurexins induce specific vascular defects in zebrafish. Two different MOs have been designed to respectively block the translation and the splicing of each targeted gene. Each couple of MOs produced very similar vascular phenotypes and the specificity and efficiency of splice-blocking MOs were determined by RT-PCR with primers designed on the exons flanking the MO target sites. Single MOs have been injected in Tg(*fli-1*:*eGFP)* and Tg(*flk1*:*GFP)* fish lines to visualize the development of the vascular system; moreover, to analyze the vascular system integrity of the morphants, we used Tg(*gata-1*:*DsRed*) embryos. While the alpha-Neurexin knock-down impaired locomotor activity of embryos and larvae, without obvious vascular defects, MOs for beta-Neurexin and Neuroligin produced specific vascular phenotypes without any gross morphological effects. The coinjection of low doses of the translation-blocking MOs for beta-Neurexin and Neuroligin in Tg(*fli-1*:*eGFP)* and Tg(*gata-1*:*DsRed*) recapitulates the observed circulatory and vascular abnormalities suggesting a synergistic role between beta-Neurexins and Neuroligins during angiogenesis in vivo. ·

Fgf favours a cardiac fate at the expense of the haemangioblast programme in zebrafish

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The mammalian heart is fashioned from two embryonic heart fields, only one of which appears to be present in zebrafish, possibly explaining its smaller two-chambered heart. Instead zebrafish have a population of anterior haemangioblasts not found in mammalian embryos, possibly representing an evolutionary antecedent of the second heart field. Consistent with such a notion, gain and loss of function experiments have indicated that the haemangioblast programme is inhibitory to the development of the cardiac programme. Here we show that a cardiac regulator also inhibits haemangioblast development, indicating a cross-antagonistic relationship between these two cell fates. We further show that fibroblast growth factor (Fgf) has the characteristics of a signal whose domain of influence could have been extended during evolution leading to the recruitment of the anterior haemangioblasts into the heart field. By temporal application of a small molecule inhibitor of Fgf signalling, we show that Fgf induces the heart programme and concomitantly represses the haemangioblast programme. By knocking down expression of *fgf3* and *fgf8*, separately and together, we demonstrate that they are the key Fgf ligands and that their combined activities are necessary for the full elaboration of the cardiac programme and repression of the blood and endothelial programmes. These observations have implications for the derivation of cardiomyocytes from pluripotential cells and for the potentiality of stem cells in the adult heart.

Fine tuning of heparin sulphate structure by sulfi1 is critical for arterial programming and for functioning of the brain vasculature

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The presence of heparan sulphate (HS) proteoglycans (PGs) on the cell surface and in the extracellular environment is critical to many processes including the growth of new blood vessels from pre-existing vasculature, (angiogenesis). Variation in the sulphation patterns of the complex HS sugar chains affects their binding to protein ligands, including angiogenic growth factors. Our previous biochemical studies have demonstrated that 6-O-sulphation is critical for the binding of the key angiogenic growth factor, vascular endothelial growth factor (VEGF), to HS. It has been shown that HS is important for both gradient formation of VEGF and its interaction with signalling receptors. In zebrafish embryos we found that knockdown of the 6-O-sulphotransferase 2 gene, that 6-O-sulphates HS, reduced VEGF directed vascular branching. This led to our recent focus on the vascular role of HS 6-O-sulphation fine-tuning by the sulf enzymes.
Our results show all three zebrafish sulfs transcripts to be temporally expressed at time points around angiogenesis. Following morpholino knockdown in Tg[fli1EGFP-gataDSred] fish, confocal imaging of the Sulf1c morphants at 26hpf onwards revealed defects in the caudal artery formation, leading to occlusion of the distal aorta. Expression of arterial markers Notch, Delta and EphrinB2 at 26hpf is severely reduced/lost in these areas, with an increase in expression of the Flt4 venous marker, suggesting that sulf1c has a role in arterial programming. There was also reduced circulation and defective formation of the central arteries in the brain that appeared to be due to a reduction in VEGF expression. We are now investigating whether the altered arterial programming reflects sulf1 influence on VEGF activity and if in the brain sulf1 may be acting upstream of VEGF. This work has been funded by the British Heart Foundation.

Function of the Popdc gene family in cardiac conduction system development

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The *Popeye* domain containing (*Popdc*) gene family is predominantly expressed in heart and skeletal muscle. In mice *l*oss of *Popdc1* and *Popdc2* affected sinus node function in the postnatal mouse heart. In this study, we therefore asked whether the requirement of *Popdc* genes for cardiac pacemaking is evolutionary conserved. We addressed this possibility using an antisense morpholino-based approach in zebrafish embryos. Both, *Popdc1* and *Popdc2* morphants developed pericardial edema and aberrant tail muscle development. In *Popdc2* morphants we observed irregular ventricular contractions with 2:1 and 3:1 atrio/ventricular rhythm while sinus rhythm and atrial contractility appeared normal. Recordings of calcium transients using an indicator transgenic line and selective plane illumination microscopy revealed the presence of an AV block in *Popdc2* morphants. Surprisingly, *Popdc1* morphants did not develop an AV block but showed an overall reduced beating rate indicative of a sinus bradycardia. Thus, *Popdc1* and *Popdc2* have overlapping functions, however also differential requirements of these genes in cardiac conduction tissue development became apparent.

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Zebrafish models of cardiac development and disease

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Cardiac valves derive from endocardial cells and function throughout the life of vertebrates to prevent retrograde blood flow. These cells undergo elaborate morphogenesis and several signaling pathways converge to orchestrate valve development. We have published a detailed description of the cellular architecture of the zebrafish heart during stages of atrioventricular valve development, using gfp transgenic lines, immunofluorescence and confocal microscopy. We showed that the differentiation of the atrioventricular canal involves endocardial-myocardial interactions and that it relies on heart function. We also showed that Notch and calcineurin signaling are required for the regulation of the initial stages of zebrafish atrioventricular endocardial cushion formation. In mammals, endocardial cushion development has been linked to the etiology of atrial and ventricular septal defects and of atrioventricular canal defects. Zebrafish offers the possibility of forward genetics and study of gene function at the cellular level. The long-term aim of our efforts is to find out how heart valves form and function throughout the life of vertebrates.

To that effect, we take advantage of the mutants identified during a large-scale forward genetic screen in the UCSF (Stainier and Baier labs). These mutant lines exhibit a retrograde blood flow through the heart as an indication of defective cardiac valve development. In one of these lines (s411), we observed blood regurgitation at the atrioventricular canal as a result of outflow tract stenosis at 72 hours post fertilization (hpf), similar to an aortic stenosis phenotype in humans. Homozygous mutant embryos fail to develop a functional outflow tract and the circulation, normally initiated, stops finally at 55 hours post fertilization. The mutant embryos appear morphologically wild type at 72 hpf and have normal myocardial function, but exhibit pericardial edema due to an outflow tract stenosis. In parallel, we use confocal microscopy to analyze at cellular resolution the valve development in these mutants and we showed that the atrioventricular canal is lined by a single layer of cuboidal endocardial cells. In addition, we have shown that our mutants have, compared to wild type embryos, less elastin-positive cells residing at the outflow tract. We are currently working on identifying and cloning the mutated gene responsible for the phenotype and elucidating how it functions into the developmental pathways that control cardiac valve formation. The mutation maps on linkage group 15 (48cM on MGH).

The latest data on the phenotype, mapping and function of this gene will be presented during the meeting.

Regulation of Sphingosine-1-phosphate (S1P) signaling during zebrafish cardiovascular development

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Sphingosine-1-phosphate (S1P) is a lipid mediator derived from sphingosine and plays an important role in developmental processes including angiogenesis, vascular maturation, heart development and immunity. S1P is a ligand of five G-protein coupled S1P receptors (S1P1-S1P5) which are also known as „Endothelial Differentiation Genes". These receptors are differently expressed and coupled to various G-proteins, so numerous downstream signals can be regulated. The S1P ligand is produced by the phosphorylation of sphingosine, catalyzed by sphingosine kinases (SpK). S1P is degraded by the S1P phosphatases (SPPs) and by the S1P lyase (SPL). The S1P phosphatases dephosphorylate S1P back to the precursor sphingosine which can then be N-acylated to form ceramide that is also a precursor of S1P. SpKs, SPPs and SPL are essential regulators for maintenance of the S1P rheostat inside the cell, which is very important, because ceramide- and sphingosine lead to growth arrest and cell death whereas S1P is needed for growth and cell proliferation. The S1P lyase degrades S1P to phosphoethanolamine and hexadecanal.
Tight control of local levels of S1P is important for heart morphogenesis (Wendler and Rivkees, 2006). In addition SPL knock-out mice displayed histopathologic lesions in the heart by increased interstitial cellularity and vacuolation in the myocardium. S1P level, were elevated in SPL knock-out mice (Vogel et al., 2009). To investigate the role of SPL in zebrafish heart morphogenesis we analyze its expression pattern by *in-situ* hybridization and its function by morpholino mediated knock-down. In addition we determine lipid concentrations of ceramide, sphingosine and S1P by mass spectrometry. Morpholino knock-down led to a phenotype in the heart, which was reproducible and dose dependent.

Vogel P, Donoviel MS, Read R, Hansen GM, Hazlewood J, et al. (2009) Incomplete inhibition of sphingosine 1-phosphate lyase modulates immune system function yet prevents early lethality and non-lymphoid lesions. PLoS ONE 4(1):e4112
Wendler C.C., Rivkees S.A. (2006) Sphingosine-1-phosphate inhibits cell migration and endothelial to mesenchymal cell transformation during cardiac development. Developmental Biol. 291, 264-277

The role of atp binding domain protein 4 in heart development of zebrafish

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The embryonic development of the heart is a highly complex process. Starting from a linear heart tube very early in development the heart undergoes many changes. The mature heart is a result of ballooning, looping, and the formation of the atrio-ventricular and the sinoatrial junctions. It is known that specific patterns of proliferation and differentiation as well as specific changes in cell shape and cell configuration are essential for the normal function of the heart. Understanding the genetic control of these cellular processes during heart development could help in better understanding congenital heart diseases.

In a genetic screen for heart morphogenesis mutants (Beis *et al*, 2005) a recessive lethal mutation *s272* was identified that leads to a collapsed ventricle, blood regurgitation between the atrium and sinus venosus, and edema in the heart region. On a cellular level, mutant embryos show an abnormal number, shape, and configuration of myocardial cells in the atrium and ventricle as well as at the atrio-ventricular junction.

Positional cloning of the mutant maps the mutation to a region on linkage group17 containing a single gene. This gene encodes the ATP binding domain protein 4 (ATPBD4), a novel protein with unknown function. The sequence of this protein, which is highly similar to the human orthologue, suggests that it might bind ATP. So far we have not identified the molecular lesion of the *s272* mutation in this gene but its morpholino knock-down leads to a phenocopy of the mutant. This phenocopy can be rescued by injection of the ATPBD4-mRNA. We are interested in the molecular function of ATPDB4. Therefore we are investigating the gene expression as well as the sub-cellular distribution of the protein. Furthermore we are searching for possible interaction partners of our protein of interest to identify the molecular processes this protein is involved in.

Beis et al (2005) Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. Development 132: 4193-4204.

A cell-autonomous requirement for Vegf signalling in angioblast development

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In the gastrulating mouse embryo, expression of the receptor for the vascular endothelial growth factor (Vegf), Flk1, is found in cells that leave the posterior primitive streak and contribute to the formation of blood and endothelial cells in the extra-embryonic yolk sac and the intra-embryonic vasculature. Targeted deletion of Flk1 leads to early embryonic lethality due to a complete lack of blood and endothelial development. Studies in mouse chimaeras have shown that $Flk1^{-/-}$ cells are unable to contribute to blood and endothelial cells, suggesting a cell autonomous role for Vegf in those cells, possibly at the level of the haemangioblast, the common progenitor for blood and endothelial cells. In zebrafish, Flk1 expression is more restricted and is confined to endothelial cells. Previous studies have shown that Vegf signalling in the zebrafish embryo is not needed for endothelial cell differentiation, but is required for; a) arterial specification of the dorsal aorta (DA), b) sprouting of intersomitic vessels from the DA, and c) specification of haematopoietic stem cells (HSCs).
We took the opportunity to perform blastula stage cell transplantations to test whether the requirement for Vegf signalling is cell autonomous in the above processes. Surprisingly, our results demonstrate that cells which are unable to receive a Vegf signal fail to contribute to the trunk vasculature when in competition with wild-type cells. Our results demonstrate an early role for Vegf in endothelial cell specification/differentiation in zebrafish embryos that is not apparent in zebrafish mutants and morphants of the Vegf signalling pathway. Thus, our findings suggest that Vegf signalling has a cell autonomous role in endothelial cell specification and development downstream of the haemangioblast stage during vertebrate embryogenesis.

Sox18 regulates lymphatic development in zebrafish

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Sox7, -17 and -18 belong to the SoxF-group of Sry-related HMG-box transcription factors. Mutations in *SOX18* have been associated with dominant and recessive forms of Hypotrichosis-Lymphedema-Telangiectasia (HLT), combining defects in hair, blood vessel and lymphatic development. The murine conterpart of the disease is represented by the ragged phenotype, due to spontaneous mutations in *Sox18* and presenting signs of lymphatic dysfunction. The ragged-type SOX18 proteins lack a functional transactivation domain and act in a dominant negative way by preventing wild-type SOXF proteins from binding to target genes. Recently, *Sox18-/-* null mice were shown to display great subcutaneous edema and fetal lethality in a pure B6 background. Moreover, mouse *Sox18* is expressed in a subset of cardinal-vein cells before *Prox1*, a lymphatic hallmark gene, and Sox18 directly activates Prox1 transcription.
We have shown that zebrafish *sox7* and *sox18* are co-expressed in the endothelial cells of developing blood vessels. Noteworthy, *sox7* transcripts are detectable earlier than *sox18* mRNAs in endothelial cell precursors, and they fade away earlier than *sox18* mRNAs in the axial vein. Sox7 and Sox18 play redundant roles in vascular development: their partial double knockdown interferes with the acquisition of a correct arterio-venous identity. In particular, venous endothelial cell differentiation appears more affected than arterial. Given the venous origin of lymphatic cells, this prompted us to investigate whether *sox18* and *sox7* play a role also in zebrafish lymphatic development. The thoracic duct (TD) is the first perfused lymph vessel that forms in zebrafish between the dorsal aorta (DA) and the posterior cardinal vein (PCV) from 3 to 5 dpf. Our data indicate that the single knockdown of *sox18*, but not of *sox7*, greatly impairs TD formation in the tg(*fli1*:EGFP)^{y1} line. The different impact of the two genes in TD formation is probably linked to their different expression levels in the PCV during lymphatic endothelial cell differentiation. Our results confirm the pivotal and evolutionary conserved role of Sox18 in lymphatic development.

Human CD34⁺ Cell Differentiation toward the Endothelial Lineage in the Zebrafish Embryo

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Background: The zebrafish embryo (*Danio rerio*) provides a number of advantages for *in vivo* study of vertebrate vasculogenesis and angiogenesis, including the possibility to observe in real-time the development of the embryonic vasculature as, for example, by confocal *in vivo* imaging of endothelial cells sprouting (Lawson *et al*., 2002). Furthermore, cell transplantation into the zebrafish embryo is a novel and interesting model system for the study of stem cells differentiation and plasticity. (Traver *et al.*, 2003; Traver *et al.*, 2004).

Objective: The objective of the present study was to determine whether human CD34⁺ progenitor (hCD34⁺) cells differentiate toward the endothelial lineage and contribute to new blood vessels development after transplantation into the zebrafish blastula and embryo.

Methods and Results: hCD34⁺ cells were isolated from cord blood and subsequently labelled with Orange Cell Tracker™ dye (Invitrogen). hCD34⁺ cells (400-500) were injected into the zebrafish blastula and hCD14⁻ cells were used as controls. Flow cytometry (FACS) analysis of injected embryos dissociated 24- and 44-hours after transplantation (28hpf and 48hpf stage) showed endothelial differentiation of hCD34⁺ cells, as demonstrated by increased expression levels of endothelial markers (KDR, CD105, CD146), whereas hematopoietic lineage markers (CD3, CD38, CD45 and CD48) were not affected. Further, immunofluorescence analysis showed KDR⁺ and VE-cadherin⁺ human cells localized in the dorsal artery and in the cardinal vein. The above changes were not observed in hCD14⁻ cell – injected zebrafish.

In other experiments hCD34⁺ and hCD14⁻ cells (500-1000) were injected into the sinus venosus of developing Tg(*fli1*:EGFP) transgenic embryos (48hpf), prior to immune system development. Time-lapse confocal analysis performed 2 hours after transplantation showed circulating hCD34⁺ cells in developing vessels and no evidence of vascular occlusions. Further, at 12 hours after injection , some hCD34⁺ cells had incorporated into the vessel wall. One day after transplantation, hCD34⁺- injected embryos exhibited altered blood vessels sprouting in the growing tail vasculature as well as enhanced and ectopic angiogenesis at the level of the subintestinal vein; these results raised the possibility that hCD34⁺ cells may synthesize angiogenic factors. In agreement with this hypothesis it was found that hCD34⁺ cell injection into the zebrafish blastula rescued the vascular phenotype caused by *Vegfc* knock-down. On the contrary, hCD14⁻ cells injected into the embryo do not seem to affect vascular development.

Conclusions: The present study demonstrates the evolutionary conservation of hCD34⁺ cells differentiation mechanisms toward the endothelial lineage and their angiogenic properties in the zebrafish embryo.

Chemical Inhibitors of Developmental Angiogenesis in the Eye

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Retinal neovascularisation is a pathological hallmark of debilitating forms of blindness including diabetic retinopathy and retinopathy of prematurity. In order to identify lead drugs that inhibit ocular neovascularisation, we performed a chemical screen for inhibitors of developmental angiogenesis of the hyaloid vasculature in zebrafish larvae.

Data will be presented on the screening, validation and characterisation of some of the lead drugs uncovered. Primary drug hits are identified as those that induce defects in developmental angiogenesis of the hyaloid at 5 dpf without inducing gross developmental abnormalities. Validation involves confirming a dose- and time-dependent response on hyaloid angiogenesis. Drugs that pass these stages are then screened for their ability to inhibit ectopic angiogenesis in a mutant model and regenerative angiogenesis in adult fin clips. Tertiary characterisation looks at the effect of the selected drugs on visual behaviour and retinal morphology. To date we have screened over 1500 drugs and identified 2 that inhibit angiogenesis in the eye without affecting visual morphology or function.

Dll4 suppresses Vegfc/Flt4 signalling in the developing zebrafish arterial system

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Hemangiogenesis and lymphangiogenesis, the development of blood or lymphatic vessels from pre-existing vessels, are intimately linked processes that share many common molecular regulators. To orchestrate the development of these two distinct vascular systems in the vertebrate embryo, the function of many reiteratively acting molecular pathways is expected to be regulated with exquisite precision.

We performed a forward genetic screen in zebrafish to identify mutants that fail to form embryonic lymphatic vessels and isolated the mutant *expando*. Positional cloning identified *expando* as a *flt4* signalling deficient mutant but arterial hemangiogenesis occurred normally in these mutants. Recent data have demonstrated that Flt4 signalling is required for hemangiogenesis and that Dll4 limits angiogenic potential by limiting Flt4 function in developing vessels. To test whether *flt4* acts downstream of *dll4* in zebrafish, we knocked down *dll4* in the *flt4* mutant background and found that the hyper-branching phenotype observed upon *dll4* knockdown was absent in *flt4* signalling mutants. Furthermore, we found that the injection of *vegfc* targeting morpholinos or soluble inhibitory Ig domain encoding mRNA for *Flt4* were both capable of rescuing the *dll4* hyper-branching phenotype. To test whether loss of *dll4* sensitises intersegmental arteries to *vegfc/flt4* signalling, we overexpressed *vegfc* by mRNA injection in *dll4* morpholino-injected animals. We found that increasing *vegfc* in the context of depleted *dll4* led to a synergistic induction of ectopic sprouting of intersegmental arteries.

Taken together, these data demonstrate that Dll4 suppresses the ability of developing intersegmental arteries to respond to Vegfc driven Flt4 signalling in zebrafish. We propose that this mechanism provides for the differential endothelial response to a constant source of Vegfc in the embryo during arterial and venous hemangiogenesis and lymphangiogenesis.

Generation and analysis of an epicardial-specific transgenic line in zebrafish

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The epicardium is an epithelial layer enclosing the myocardium, which exerts important roles during heart development as a source of progenitor cells and by promoting cardiomyocyte proliferation. The epicardium derives from the proepicardium, a protrusion of splanchnic mesoderm origin allocated at the base of the sinus venosus. Proepicardial cells delaminate and adhere to the surface of the myocardium, forming an epithelial layer that covers it. In the chick some epicardial cells undergo epithelial-mesenchymal transition and invade the myocardium, giving rise to endothelial and smooth muscle cells of the coronary vessels, blood cells and fibroblasts. Recent experiments performed in the mouse partially confirm the results obtained in chick, whereby an additional contribution to the cardiomyocyte lineage has been reported, which is currently under debate. Data on the fate of epicardial derived cell s (EPDCs) in other species might therefore contribute to the understanding of their pluripotency. One of the earliest genes expressed in the epicardium is *Wilms tumour 1* (*WT1*). Its expression is turned on in the proepicardium and switched off upon EPDC differentiation. Other sites of *WT1* expression include the pronephros, pancreas, gonads, central nervous system and limb buds. *Wt1* null mutants display defective epicardium formation.

Recently, the expression and function of *wt1* has been shown to be conserved in the zebrafish. In this species, the epicardium has been suggested to play a role during cardiac homeostasis, since DiI experiments show that epicardial cells contribute to the growing heart in adults. A role during regeneration is supported by the finding that ventricular apex resection leads to re-expression of epicardial marker genes.

We are interested in using the advantages of the zebrafish animal model to study processes of epicardial development. We are establishing the tools to analyze epicardial formation in vivo, to analyze the effect of epicardial ablation on heart development and to study the progeny of epicardial cells. For this, we are generating an epicardial-specific transgenic line. Conserved noncoding elements of the *WT1* genomic region are being tested for their ability to drive epicardial-specific gene expression in zebrafish larvae. Recent progress on the generation and analysis of transgenic lines will be presented.

The gene trap approach reveals involvement of *mekk3b* in the functional blood vessel formation

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The vascular system forms complex networks that are essential for transport of fluids, gases, macromolecules and cells. In the developing zebrafish trunk, primary angiogenic sprouts emerges from the dorsal aorta, then secondary angiogenic sprouts emerge from the posterior cardinal vein. The secondary sprouts make connections to the primary vessels, and then blood flow begins in the intersegmental vessels. The molecular mechanism of functional blood vessel formation has not been fully elucidated.

Previously, we developed the *Tol2*-mediated gene trap method in zebrafish. In the course of the gene trap screen, we identified a SAGFF27C line. In the SAGFF27C;UAS:GFP line, GFP is expressed in the heart, posterior cardinal vein and intersegmental vessels. The gene trap construct is integrated into an intron of the *mekk3b* gene and traps the transcript of *mekk3b*. The *mekk3* gene is a serine-threonine kinase that belongs to the MAP3K family. It has been reported that the knockout of mouse *Mekk3* causes defects in the brain, heart, trunk and placenta.

To determine whether *mekk3b* plays an important role in the blood vessels, we injected *mekk3b* ATG-morpholino oligonucleotides (MO) into fertilized eggs. Blood flow of the *mekk3b* morphant was abolished in major axial vessels of the trunk (dorsal aorta and posterior cardinal vein). In contrast, injection of SD-*mekk3b* MO that blocks splicing did not show defects in dorsal aorta and posterior cardinal vein, but caused defects in the segmental vessels. Thus, we think that maternal *mekk3b* may contribute to functional dorsal aorta and posterior cardinal vein formation, and that zygotic *mekk3b* may contribute to functional intersegmental vessel formation.

Funtional interaction between Hdac9 and Hif-1alfa in heart development of zebrafish

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Heart is one of the first organs formed during the embryonic development, this process is regulated spatial and temporally by the expression of different genes, however the mechanism of this regulation is still unknown. One underline of the regulation mechanism is the action of histone deacetylase (HDAC) enzyme that remove an acetyl group from the histones condensing the chromatin avoiding the binding of transcriptional factors to the DNA. Here we focus our interesting in the action of Hdac9 in the cardiogenesis of zebrafish. First by *in situ* hybridization we found that hdac9 is express from 0 hpf through 72 hpf, and we have detected expression of the mRNA in the heart, this result were confirmed by RT-PCR. Previous result from our laboratory show that the fail of function of Hdac9 generate cardiac malformation by down regulation of differentiation gene expression as *vmhc* and over expression of cardiac gene such as *gata5, hand2*, and *mef2c,* this over expression is similar to the over expression observed when embryos are exposed to hypoxia. We propose that the knockdown of Hdac9 allows a transcriptional factor to bind to the DNA. One potential candidate to be regulated by Hdac9 is the transcription factor induced by hypoxia-1alpha (Hif-1alfa), a transcriptional factor that is active under low oxygen tension, supporting evidence for this is that the lost of function of this factor generated a down regulation of cardiac gene expression, the opposite effect of the knockdown of Hdac9. To answer if Hdac9 regulated the action of Hif-1alfa to activated cardiac genes we did a double knockdown to both proteins. The effect was evaluated by *in situ* hybridization, showing that double knockdown induces a down-regulation of cardiac gene, like results obtained by the single knockdown of Hif-1alfa. These results suggest that Hdac9 may have a functional interaction with Hif-1alfa. We propose that Hdac9 induces chromatin compactization avoiding the binding of Hif-1alfa to the DNA, for the contrary the absence of Hdac9 facilitate the binding of Hif-1alfa to the DNA, activating the cardiac genes.

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A Single Serine in the Carboxyl Terminus of Cardiac Essential Myosin Light Chain-1 Controls Cardiomyocyte Contractility in Vivo

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Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disorder, accounting for over half of the cases of sudden cardiac death in young individuals. One of the pathophysiological mechanisms implicated in HCM is ineffective force generation due to mutations in sarcomeric proteins. Although myosin light chains (MLCs) are known disease genes for HCM, the precise *in vivo* structural and functional role of particularly the *cardiac essential light chain-1 (cmlc-1)* is only poorly understood.

We recently isolated the zebrafish mutant *lazy susan (laz^{m647})* in a large-scale ENU-mutagenesis screen for recessive lethal mutations that perturb cardiac contractility. *Lazy susan* mutant embryos display absence of blood flow due to severely reduced contractility of both heart chambers. By positional cloning we were able to map the *lazy susan* locus to a genomic interval including an open reading frame encoding for the *cardiac essential myosin light chain-1 (cmlc-1)*. Sequencing revealed a nonsense mutation in the *cmlc-1* gene leading to the premature termination of protein translation and a carboxy-terminally truncated cMLC-1 in *laz* mutant hearts. Overexpression of wild-type cMLC-1 in mutant cardiomyocytes rescues the contractility defect. Injection of a morpholino-modified antisense oligonucleotide directed against the *cmlc-1* translational start site copies the severe *lazy susan* phenotype in 99% of injected wild-type embryos. Ultrastructural analysis showed that morpholino-mediated knock-down of cMLC-1 disrupts sarcomere assembly in cardiomyocytes. Interestingly, sarcomeres in *laz* mutant cardiomyocytes assemble normally. This demonstrates that, despite of the phenotypic similarities between cMLC-1 morphant and mutant embryos, ablation and truncation of cMLC-1 lead to severely impaired contractility by different mechanisms, suggesting a crucial role of the carboxy-terminus of cMLC-1 in the regulation of cardiac contractility.

In order to define the role of the cMLC-1 carboxy-terminus we mutated a putative phosphorylation site at amino acid position 195 (cMLC-1^{S195A}). Strikingly, overexpression of this phosphorylation-deficient cMLC-1 in *laz* mutant cardiomyocytes does not rescue the contractility defect. In contrast, introduction of a phosphomimetic amino acid (cMLC-1^{S195D}) fully restores cardiomyocyte contractility in *laz* mutants. Our results indicate for the first time an essential role of the carboxy-terminus of cardiac MLC-1 in force generation and demonstrate that phosphorylation of cMLC-1 controls cardiac contractility *in vivo*.

Bone Morphogenetic Protein antagonist Gremlin 2 promotes differentiation of embryonic stem cells to atrial

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Bone Morphogenetic Proteins (BMP) have pleiotropic effects on cardiac morphogenesis and cardiomyocytic differentiation. Besides forward BMP signaling, secreted BMP antagonists are also necessary for cardiac development indicating that fine-tuning the strength, or confining the zone, of BMP activity is required for proper cardiac development. The BMP antagonist *gremlin 2* is expressed in the pharyngeal arch mesenchyme next to the developing heart. We found that inactivation of Gremlin 2 causes severe defects in cardiac development. Although both atrial and ventricular cells are affected, atrial development is more sensitive to lack of Gremlin 2. Consistent with this idea, our results show that injection of *gremlin 2* mRNA in zebrafish embryos expands the atrial territory and restricts ventricular development. Our results also show that treatment of differentiating mouse embryonic stem cells with Gremlin 2 stimulates expression of atrial genes. Gremlin 2 induces atrial-specific transcription factors, whereas it blocks expression of suppressors of atrial differentiation. Our results suggest that Gremlin 2 controls the activity of signaling pathways that promote atrial fate leading to novel strategies for differentiation of pure populations of stem cell-derived atrial cardiomyocytes. This knowledge may also impact our treatment of degenerative atrial diseases.

The Netrin receptors Unc5b and DCC are required for parachordal vessel formation during vascular development

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Recent evidence has shown that axon guidance molecules play an important role in vascular development. In particular, we have shown (Wilson et al., 2006) that Netrin1a is required for the formation of the zebrafish parachordal vessel (PAV). Here we address the identity of the cells and receptors that receive this Netrin1a signal.

Zebrafish trunk vasculature development begins with the formation of the dorsal aorta (DA) and posterior cardinal vein (PCV). The PCV gives rise at ~32hpf to the secondary sprouts, which grow dorsally until they reach the horizontal myoseptum (HMS), then grow laterally into the superficial muscle, and then turn anteriorly and posteriorly to give rise to the PAV. Prior to and during PAV formation, Netrin1a is expressed by the muscle pioneers, which lie along the HMS. Knocking down *netrin1a* or blocking muscle pioneer formation both prevent formation of the PAV (Suli et al., submitted).

When we use morpholinos to knock down the known Netrin receptors Unc5b or DCC, the PAV also fails to form. In these morphants as well as Netrin1a morphants, secondary sprouts form from the PCV, but they fail to turn at the HMS to form the PAV. Thus, Netrin1a from the muscle pioneers acts to promote PAV formation, likely mediated through Unc5b and DCC. However, we are unable to detect *unc5b* or *dcc* mRNA in secondary sprouts by *in situ* hybridization, raising the question of whether these receptors act autonomously in endothelial cells, or perhaps elsewhere.

It has often been hypothesized that axons might guide endothelial cells, and netrin signaling through DCC and/or Unc5 homologs is known to be important in axon guidance for several neuronal types. In fact, the HMS is contacted by axons of the primary motor neurons. In particular, the Rostral primary motor neuron (RoP) develops shortly before the PAV, and courses along the HMS just as the PAV does. We are now testing whether Unc5b and DCC act in endothelial cells, or whether instead they may guide motor axons which are necessary for formation of the PAV.

Characterization of *tbx5b*, a novel paralog of *tbx5a*, during zebrafish cardiac development

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T-box proteins (tbx) are an evolutionarily conserved family of transcription factors comprised of 19 members in zebrafish *Danio rerio*. Gene paralogs (homologous genes that have evolved by duplication) have been reported in the zebrafish genome. Among the T-box family members, *no tail a/b*, *tbx2a/b*, and *tbx3a/b* represent examples of gene duplication. Here we present the cloning and characterization of *tbx5b*, a novel paralog to zebrafish *tbx5a*. The *tbx5a heartstrings (hst)* mutation results in severe cardiac abnormalities of the zebrafish two-chambered heart and absence of pectoral fins; and similarly, *Tbx5* in the mammalian four-chambered heart is implicated in congenital heart disease. Our studies show that like *tbx5a* and its mammalian orthologs *Tbx5* (mouse)/*TBX5* (human), *tbx5b* expression is critical for heart development. First, based on amino acid sequence, tbx5b is significantly diverged from tbx5a, while retaining the highest homology in the DNA-binding T-domain, indicating that tbx5b retained its function as a transcription factor. *tbx5b* expression is dynamic in the heart tube and dorsal eye, paralleling but not identical to the expression pattern of *tbx5a*. Surprisingly, however, *tbx5b* lacks expression in the fin bud, suggesting a divergence in function between the two genes. Embryonic heart tubes of *tbx5b* anti-sense morpholino-treated embryos do not undergo looping morphogenesis, a critical step in shaping the vertebrate heart. Moreover, *tbx5b* morphants demonstrate misregulation of cardiac valve markers, *bmp4* and *versican*. We tested the hypothesis that simultaneous loss of both zebrafish *tbx5* paralogs would result in compound cardiac defects. By knocking down *tbx5b* on an *hst*$^{-/-}$ background, we found that a linear heart tube does form, but with attenuated expression of the cardiac marker, *cmlc2* at 48hpf. In addition to a comparative assessment of their roles in development, we also examined the property of tbx5b protein to shuttle between the nuclear and cytoplasmic compartment, a feature previously demonstrated in our laboratory for tbx5a. Indeed, like tbx5a, tbx5b localizes to the cytoskeleton through direct contact with the actin-binding PDZ-LIM protein pdlim7. In summary, the zebrafish paralogs *tbx5a* and *tbx5b* have conserved sequence homology and functional properties. Our comparative analysis has identified similar and divergent roles for *tbx5a* and *tbx5b* in zebrafish development. These studies may lend further insight to the evolutionarily conserved role of T-box proteins in development across species, and to the functional consequence of TBX5 mutations in human genetic disease. Thus the identification of zebrafish *tbx5b* affords an opportunity to examine the evolution of gene function in relation to embryonic development.

VE-Cadherin is required for endothelial rearrangements during ISV Formation
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The intersegmental blood vessels (ISVs) of the zebrafish embryo serve as a paradigm to study sprouting angiogenesis in vivo. We have previously shown that during ISV formation endothelial cells (ECs) divide extensively during sprout outgrowth. Furthermore, by mosaic labelling experiments and examination of junctional proteins, we have shown that ISVs are multicellular tubes containing an extracellular lumen.
To examine the role of VE-Cadherin (VE-Cad) during ISV formation we have performed morpholino knockdown experiments. VE-Cad morphants display multiple cardiovascular defects. Initial sprouting of the ISV appears normal but they become disrupted during subsequent stages. Analysis of junctional proteins and in vivo time-lapse analyses indicate that in the absence of VE-Cad, ECs fail to perform cellular rearrangements, which are required for proper vessel formation. Taken together our findings suggest that VE-Cad is required for migratory behavior of ECs that occurs during vessel assembly. We propose a revised model of ISV formation, in which "scaffold formation" and "vessel assembly" participate as two distinct morphogenetic events and only the latter depends on VE-Cad function.

Hand2 Ensures a Proper Environment for Cardiac Fusion Through Control of Fibronectin Levels

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The vertebrate heart results from the fusion of two separate populations of cardiac precursors in the early embryo. How these populations come together, how they interact with their environment, and how their fusion is regulated is poorly understood. Here, we focus on the role of Hand2, a bHLH transcription factor and an essential regulator of heart development that appears to be particularly critical for this process. Mutation of the zebrafish *hand2* gene causes a striking cardiac fusion phenotype. Instead of exhibiting the proper migration of bilateral cardiomyocyte populations to form a heart tube at the midline, *hand2* mutants maintain two separated populations of cells that never fuse. The *hand2* mutant fusion defect may reflect a failure to establish a myocardial epithelium possessing apicobasal polarity. In *hand2* mutants, the cardiomyocytes do not form an epithelium, show misplacement of polarity markers, and display aberrant fibronectin deposition. Given the dramatic *hand2* cardiac phenotype, knowing the downstream components responsible for it is fundamental. Through gene expression profiling, we identify several transcripts that are differentially regulated in *hand2* mutants and may be essential for cardiac fusion. Notably, we observe upregulation of *fibronectin1 (fn1)* in *hand2* mutants. Moreover, overexpression of *hand2* results in a cardiac phenotype similar to that of *fn1* mutants. Based on this apparent reciprocal relationship between *hand2* and *fn1* function, we hypothesized that Hand2 controls the extracellular environment by downmodulating the exposure of cardiomyocytes to extracellular matrix components like Fn1. Indeed, heterozygosity for *fn1* in *hand2* mutants rescues aspects of the *hand2* mutant phenotype: notably, the bilateral populations of cardiomyocytes are able to fuse together at the midline. Consistent with our hypothesis, we determined through mosaic analysis that the *hand2* mutant environment prevents wild-type cardiomyocytes from fuse normally. In contrast, *hand2* mutant cardiomyocytes in a wild-type environment are able to integrate normally into the wild-type heart. Together, our data demonstrate that Hand2, through modulation of Fn1 levels, creates an appropriate environment for myocardial migration during cardiac fusion.

The role of telomerase in innate immunity and viral resistance of zebrafish

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Telomerase reverse transcribes telomere DNA onto the ends of linear chromosomes, protecting chromosome ends from recombination and fusion and retarding cellular aging. The loss of this enzyme can trigger cellular DNA damage responses in both the presence and absence of altered telomere integrity. In contrast to most normal somatic cells, which show little or no telomerase activity, T and B lymphocytes are able to transiently up-regulate telomerase in response to an immunological challenge. Telomerase activation in these immune cells is believed to mitigate the losses of replicative capacity and function caused by chronic antigenic stimulation, oxidative stress, and cellular aging. However, the role of telomerase in innate immune cells and the response of these cells to infection remain poorly understood. The zebrafish (*Danio rerio*) is an excellent vertebrate model for studying developmental immunity. The embryos of this fish are transparent and develop rapidly ex-utero, thus allowing for easy observation of fluorescence-labeled lymphoid organs and immune cells. After hatching, the zebrafish is exposed to the high microbial load of the aquatic environment at a time when adaptive immunity is still absent. Therefore, innate immunity plays an essential role in the survival of this specie at the early stages of life. In this study, we have used zebrafish embryos to determine the role played by telomerase in innate immunity and resistance to spring viremia of carp virus (SVCV). Knockdown of one or two components of telomerase with morpholinos resulted in impaired development and functions of immune cells, which led to increased viral susceptibility. These results indicate that an extracurricular role of telomerase is required for development of immunity.

Watching thymopoiesis in stable transgenic zebrafish embryos

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In zebrafish, the thymic anlage develops from the pharyngeal endoderm as a bilateral structure, first detectable at 48 hpf. It is colonized by lymphocyte progenitor cells, which differentiate into mature T cells. The thymus provides a special microenvironment for the interaction of lymphoid and stromal cells that is required to establish a self-tolerant repertoire of T cells.
Lymphocyte development, comprising the homing to the thymus and their intrathymic differentiation is directed by a complex network of different transcription factors. One of these key factors is *ikaros*. In zebrafish, *ikaros* expression is first detected at 24 hpf in the intermediate cell mass (ICM), the first hematopoietic structure detectable in the embryo. By 54 hpf and at later stages it is expressed in the thymus together with other marker genes characteristic of lymphoid differentiation, like *rag1*. Another important factor is *foxn1*, a regulator of thymic epithelial cell (TEC) differentiation. In zebrafish its expression is detectable from 48 hpf onwards.
In order to study the process of thymopoiesis in vivo, we generated *ikaros::eGFP* and *foxn1::mCherry* transgenic lines using modified BAC-DNA. These lines allow us to follow the development of the thymic epithelial anlage and the homing of prothymocytes to the thymus in long-term confocal time-lapse imaging. Using the *ikaros::eGFP* line, we describe the temporo-spatial characteristics of the migratory pattern of hematopoietic cells from the ICM, the AGM (aorta-gonad mesonephros) and the CHT (caudal hematopoietic tissue) to the thymic anlage. Using the *foxn1::mCherry* line, we describe the temporo-spatial development of the thymic anlage.
We also use these transgenic lines to characterize the factors influencing thymus development and T cell homing in vertebrates. The former is investigated by introducing various mutations affecting thymopoiesis, the latter by functional interference with gene-specific antisense morpholinos.
Of special interest to us is the process of thymus homing. We show that injection of morpholinos (MOs) directed against *foxn1* leads to aberrant migration of hematopoietic progenitor cells, indicating that the differentiation of TECs and the interaction of stromal and lymphoid cells is important for normal immigration of prothymocytes into the thymus and subsequent T cell development. We also show, that ccl25a/cxcl12a morphants show normal numbers of lymphoid progenitors but their thymus homing is impaired, indicating that these chemokines play an important role in thymus homing.

LPS enhanced neutrophil recruitment in a zebrafish embryo injury model is p38 MAP kinase dependent

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Recruitment of leukocytes is an important component of the complex inflammatory response to local injury and infection where the mechanisms of recruitment remain incompletely understood. Here we use a zebrafish embryo injury model to study and image this process. LPS was found to enhance neutrophil recruitment to a tail wound via the activation of p38 MAPK but was independent of zTLR4a and zTLR4b. zTLR4a and zTLR4b were differentially expressed in embryo and adult tissue. Zebrafish myelomonocytes did not display morphological activation when challenged with LPS but adult whole kidney marrow derived cells did respond to LPS stimulation in a dose-dependent manner with increased expression of TNFa and TNFb cytokine. Fish functional and transcriptional responses to LPS and other PAMP challenges are relatively unknown. The results presented in this study complement recent findings that suggest LPS recognition and TLR4 signalling in fish are different from the mammalian system. This model allows in vivo investigation of the mechanisms of LPS recognition in fish and evaluation of other modulators of leukocyte recruitment and the acute inflammatory process independent of TLR4 activation.

Transcriptome responses and toll.like receptor signalling during *salmonella* infection of zebrafish embryos

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Due to the clear separation of innate immunity from adaptive responses, the externally developing zebrafish embryo represents a useful *in vivo* model for identification of innate host determinants of the response to bacterial infection. We used microarrays and novel deep sequencing technology to perform a detailed time-course transcriptome profiling study and gene ontology analysis of the embryonic innate immune response to infection with two model *Salmonella* strains that elicit either a lethal infection or an attenuated response. The transcriptional response to infection with both the lethal strain and the avirulent LPS O-antigen mutant showed clear conservation with host responses detected in other vertebrate models and human cells, including induction of genes encoding cell surface receptors, signalling intermediates, transcription factors and inflammatory mediators. Furthermore, our study led to the identification of a large set of novel immune response genes and infection markers, the future functional characterization of which will support vertebrate genome annotation. Although microarrays and deep sequencing identified similar functional groups of genes, the unbiased nature of deep sequencing provided insights that microarray analysis could not have achieved, such as infection-dependent transcript isoform switching, expression of novel splice products not present in the current transcript and EST databases, and a high level of antisense transcription. Our transcriptome profiling study revealed specific regulation of Toll-like receptor 5 and downstream signalling components. Using knockdown analysis we showed that zebrafish TLR5 is required for the recognition of *Salmonella* flagellin, which is the first demonstration of a conserved TLR ligand specificity in this model. We also showed that zebrafish embryos use both MyD88-dependent and -independent signalling pathways and identified novel immune response targets of TLR5 and MyD88 signaling.

Using zebrafish to investigate the molecular control of hypoxic signalling during inflammation

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Diseases of neutrophilic inflammation are an increasing problem in the developed world, and remain largely untreatable. The genetic mechanisms by which neutrophil function is regulated remain poorly understood, and a greater knowledge of these mechanisms may lead to the identification of therapeutic targets. Hypoxia is a profound stimulus prolonging neutrophil functional lifespan. The hypoxia genetic pathway regulates the stability of the hypoxia inducible factor (HIF) protein in an oxygen dependent manner. In normoxia, HIF is phosphorylated by hydroxylases and targeted for degradation. In hypoxia, the hydroxylases are inactivated and HIF is stabilised allowing transcription and activation of downstream targets. However, the HIF pathway can be activated by inflammatory stimuli independently of hypoxia.

Neutrophils were visualised in the zebrafish using a transgenic line in which green fluorescent protein (GFP) is driven by the expression of the neutrophil specific gene *myeloperoxidase*. The hypoxia pathway was upregulated in these embryos by the addition of dimethyloxaloylglycine (DMOG) or by outcrossing to *vhl* or *fih* null TILLING mutants. Tail transection induces a self-limiting neutrophilic inflammatory response that can be observed using fluorescent microscopy techniques.

Activation of the hypoxic signalling pathway with DMOG was found to significantly delay the resolution of inflammation. This delay in resolution was not observed in $vhl^{-/-}$ or $fih^{-/-}$ mutant embryos. Neutrophil behaviour was investigated using a novel spinning disk confocal microscopy timelapse approach. The velocity and meandering index of neutrophils was not found to significantly differ after the upregulation of the hypoxia pathway by DMOG or in the TILLING mutants.

Using molecular biology techniques, dominant active and dominant negative forms of zebrafish *hif1*α have been cloned. Initial studies have indicated that these successfully upregulate or downregulate the HIF pathway respectively, by microinjection into a *phd3:GFP* transgenic line. PHD3 (prolyl hydroxylase 3) is a known downstream target of HIF1α and thus the *Tg(phd3:GFP)* line acts as a readout of the hypoxia pathway. These mutant forms of *hif1*α require further characterisation before specifically expressing them in neutrophils to observe their effects on neutrophil behaviour and the resolution of inflammation.

These studies highlight the potential of the zebrafish model to aid in our molecular dissection of the hypoxic signalling apparatus during *in vivo* physiological processes such as inflammation resolution.

Zebrafish Mast Cells Demonstrate Conserved Innate and Adaptive Immune Responses

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Mast cells (MCs) are multifunctional immune cells derived from hematopoietic stem cells that complete maturation where they take up residence, namely in tissues exposed to the external environment. These anatomic locations position them to play a critical primary regulatory role in eliciting both innate and adaptive immune responses. The zebrafish has emerged as a powerful new model system for studying infection and immunity owing to conserved cell biology, and ease of manipulation and phenotypic analysis due to ex-utero embryonic development. We were the first (Dobson et al., *Blood* 2008) to identify MCs in zebrafish gills and intestine and carboxypeptidase A5 (*cpa5*) as a developmental marker of both embryonic progenitors and mature MCs. Intraperitoneal injection of compound 48/80, a MC activator, resulted in MC degranulation and elevated plasma tryptase levels, which can be reduced following treatment with the MC stabilizing agent, ketotifen. Similarly, infection with heat-inactivated *A. salmonicida* or the fungal wall constituent, zymosan, resulted in zebrafish MC degranulation, increased plasma tryptase levels and interestingly, eosinophil recruitment. These results suggest MCs participate in innate immune responses to pathogens, which may be mediated through well-conserved Toll-like receptors. Mammalian MCs are better known for adaptive immune responses mediated through IgE/FcεRI signaling. We are characterizing an analogous pathway in the zebrafish. Structurally, we have demonstrated positive immunostaining in zebrafish MCs for a rabbit-derived polyclonal antibody that specifically targets the gamma subunit of the human high affinity IgE receptor. Using whole mount RNA in situ hybridization on 7 day embryos, we subsequently showed co-localization of zebrafish homologues of IgE receptor subunits with expression of *cpa5*, suggesting the presence of an IgE-like receptor on zebrafish MCs. Functionally, zebrafish MCs sensitized with mouse anti-DNP IgE followed by injection of DNP-BSA responded by degranulation and leukotriene production. Our studies reveal conserved MC function in zebrafish innate and adaptive immune reponses, effectively establishing the zebrafish as a novel organism for evaluating vertebrate MC activity. Ultimately, we will be able to exploit the zebrafish system as an *in vivo* platform for high-throughput screening of potential MC stabilizing/inhibiting agents, with a goal of identifying new effective therapies for allergic, inflammatory, and malignant MC diseases.

Notch signaling is required for mast cell development in the zebrafish

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The molecular pathways regulating mast cell (MC) development in vertebrates remain to be elucidated. The Notch signaling pathway is highly conserved in all metazoans and has been implicated in regulating hematopoietic stem cell induction and lineage cell fate decisions. Notch receptors and their ligands are expressed in a number of hematopoietic cells, including MCs. We were the first to identify zebrafish MC equivalents (Dobson et al., *Blood* 2008) and examine vertebrate MC transcriptional regulation *in vivo*. These studies demonstrated the significance of carboxypeptidase A 5 (*cpa5*) as a zebrafish MC-specific marker. We have now shown that the zebrafish Notch signaling mutant, *mind bomb*, displays profound loss of *cpa-5* expression. Similarly, wild type zebrafish embryos treated with Compound E, (Cpd E), a γ-secretase inhibitor that inhibits Notch signaling, show a similar phenotype. We previously identified *pu.1* and *gata2* as essential transcription factors for early MC development. Wild type zebrafish embryos treated at 50 μM Cpd E show decreased *gata2* expression, but wild type *pu.1* and *gata1* expression, suggesting a particular sensitivity of the MC lineage to Notch pathway inhibition mediated through *gata2*. At 75 μM Cpd E, we observed severe reductions in *gata2*, *pu.1*, *gata1*, and *mpo*, suggesting a broader dose dependent role for Notch pathway signaling in both myeloid and erythroid lineage development. Our data suggest that Notch signaling is a critical pathway necessary for MC development in the zebrafish. Reciprocal experiments overexpressing *notch* mRNA in wild type embryos and rescue experiments overexpressing *pu.1*, *gata-2* and other transcription factors in embryos in which the Notch pathway is absent (*mind bomb* and Cpd E treated) are underway to determine the specific signaling cascade downstream of Notch. A Notch green fluorescent protein (GFP) reporter zebrafish line has been generated in the laboratory, which will enable the tracking of Notch signaling in developing and migrating MCs. Moreover, a second transgenic line generated in the laboratory expressing the human *CKIT D816V* mutation found in the preleukemic condition, systemic mastocytosis, will be employed to test Notch pathway inhibitors in this condition. Parallel studies in a human mastocytosis cell line are also being undertaken. These studies promise key insight into the role of Notch signaling in MC development and the opportunity to use the zebrafish as an *in vivo* model for identifying novel therapeutic strategies in MC diseases.

ENU-mutagenesis in zebrafish identifies a specific requirement for Lsm8 in thymus development

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Proper development of T lymphocytes in the thymus is necessary for the establishment of acquired immune systems in vertebrates. Understanding the mechanisms for T lymphocyte and thymus development is crucial for controlling immunodeficiency syndromes and autoimmune diseases. However, the relevant molecular pathways are not fully understood. We have previously established a panel of ENU-mutagenized zebrafish lines with abnormal accumulation of rag1-expressing immature T-cells in the thymus. At the time of this writing, we have identified 10 mutations among 41 lines. Here we report on one of the mutants, WW18/10. Homozygotic WW18/10 mutants have no apparent developmental abnormality including craniofacial structures, but exhibit a reduction of immature T-cells in the embryonic thymus. Positional cloning identified a nonsense mutation in the Lsm8 gene. Lsm8 encodes a member of Sm-like proteins associated with U6 snRNA in the spliceosome. Possible functions of Lsm8 will be discussed.

Characterizing zebrafish defensins

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The β-defensins make up one of the largest groups of antimicrobial peptides and are important innate immune components in a variety of species throughout the animal and plant kingdoms. Although the β-defensins were first identified for their broad-spectrum anti-microbial activities, vertebrate β-defensins also have well-documented roles in the chemoattraction of adaptive immune cells such as immature dendritic cells, macrophages and T cells. This provides an important link between the innate and adaptive immune systems. The receptor through which beta-defensins chemoattract macrophages is as yet unknown.

This work builds on the initial reporting of three zebrafish β-defensin-like genes (Zou et al, 2007) and aims to assign functionality to these genes by analysing their expression, function and inducibility in both embryos and adult fish. This has been achieved using a combination of morpholino knockdowns, in-situ hybridisations and quantitative RT-PCR, in addition to *in vitro* anti-microbial assays.

In addition, the zebrafish system was exploited to further define the reported interactions between Human β-defensin 3 (HBD3) and the Mc1r receptor (Candille et al, 2007). Zebrafish are able to background adapt by dispersing the pigment (melanin) within their melanophores in order to appear darker in colour. This process can be observed under the microscope and is controlled by interactions between α-MSH and Mc1r. This system has been exploited using freshly prepared, cultured zebrafish melanophores to directly observe the effects that a panel of human β-defensins have on melanophore pigment dispersal both before and after the morpholino knockdown of Mc1r.

Overall, this work describes the novel use of the zebrafish model to investigate the function of both intrinsic and mammalian β-defensins. This also represents the development of a novel, valuable disease model for the study of β-defensins in this research group.

Candille SI et al (2007) A β-defensin mutation causes black coat color in domestic dogs. Science 318(5855) :1418-23

Zou J. et al (2007) Discovery of multiple beta-defensin like homologues in teleost fish. Mol Immunol 44(4) : 638-47

Specific combinations of CRFB chains constitute receptors for the virus induced IFNS in zebrafish

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The number of fish proteins identified as cytokines by sequence homology has grown tremendously, but the identity of their receptors is difficult to predict. The receptors of class II helical cytokines (interferons and IL-10-related cytokines) are heterodimers of CRFB transmembrane proteins, of which at least 13 members exist in the zebrafish genome. We have previously identified functionally the receptor of the first viro-induced interferon identified in the zebrafish (IFNϕ1, also known as IFN1 or IFNL1) as a dimer of CRFB1 and CRFB5. Since then, the number of fish viro-induced interferons has expanded, with two distinct classes defined according to the number of conserved cystein pairs. Zebrafish IFNϕ1 belongs to class I; the more recently described IFNϕ2 and IFNϕ3 belong to class II. While these three genes sit on a single chromosomic cluster, we have identified on a different chromosome a fourth sequence, encoding a class I member: IFNϕ4. By overexpression studies or injection of recombinant proteins, we found that these four IFNs induce the expression of antiviral genes such as viperin and increase the resistance of zebrafish larvae to a viral challenge, with IFNϕ1 appearing the most effective while IFNϕ4 has only marginal potency. Using CRFB gain- and loss of function experiments, we found that not all IFNs bind to the same receptor; the two class I IFNs signal through the previously described receptor, while the two class II IFNs rely on a different dimer. Both receptor complexes include a common short chain, associated to a specific long chain.

TIF1gamma regulates the erythroid and myeloid lineage output from haematopoietic progenitors

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During vertebrate development, primitive haematopoiesis is followed by a definitive wave that generates haematopoietic stem cells (HSCs), which maintain haematopoiesis throughout adulthood. HSCs arise in association with the ventral wall of the dorsal aorta. From 2dpf onwards in the zebrafish, they migrate to the caudal haematopoietic tissue (CHT, the foetal liver equivalent), where they co-exist with locally arising erythromyeloid progenitors, and finally to the thymus and to the kidney (the bone marrow equivalent). At present, little is known about how the differentiation potential of HSCs in the different niches is regulated. To address this, we made use of the zebrafish mutant *moonshine* (*mon*). *mon* harbours a mutation in the nuclear RING domain ubiquitin ligase, transcriptional intermediate factor 1γ (TIF1γ or Trim33), a factor essential for primitive red cell survival. Because *mon* mutants are devoid of primitive red cells, later waves of haematopoiesis can be studied without the interference of circulating primitive cells. At 4dpf, cells expressing erythroid and myeloid markers are found in the CHT. Although erythroid markers are also expressed in the CHT of *mon* mutants, no recovery of circulating red blood cells was observed, suggesting that these cells are unable to complete their maturation in the absence of TIF1γ. Thus, TIF1γ is required for the survival or differentiation of the erythroid cells arising *de novo* in the CHT. Surprisingly, TIF1γ-deficient embryos showed a dramatic increase in expression of myeloid markers in the CHT, suggesting TIF1γ is required to restrict myeloid cell fate in this haematopoietic niche. We have thus uncovered a novel role for TIF1γ in regulating the erythroid and myeloid differentiation output from haematopoietic progenitor cells residing in the CHT niche.

Live imaging demand-driven haematopoiesis during infection

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The rapid response of the innate immune system to a pathogenic challenge is critical for the ultimate wellbeing of the host. During normal 'steady state' haematopoiesis, haematopoietic stem cells (HSCs) undergo a step-wise reduction in lineage potential to generate the full complement of blood lineages, including myeloid leukocytes of the innate immune system that are crucial for host protection. These innate immune cells have a finite life span so a mechanism for rapid replenishment is essential. During the stressed state of infection it has long been known that the host has the potential to regenerate depleted immune cell stocks via 'emergency' or 'demand-driven' haematopoietic activity. However, the mechanism(s) through which it achieves this are incompletely understood. Recent studies have suggested a role for Toll-like receptor (TLR) signalling in skewing HSC differentiation towards the production of myeloid leukocytes during infection. TLRs are an ancient class of pattern recognition receptor that recognise highly conserved invariant components of pathogens and are well characterised for their ability to regulate immune responses of mature immune cell subsets. Using live imaging within transgenic reporter lines, we have previously demonstrated that zebrafish immune cells express the adaptor molecules necessary for transducing TLR-mediated signalling.

Using live imaging within transgenic *Tg(lyz:EGFP/DsRED2)* and *Tg(mpx:GFP)* reporter lines we reveal that zebrafish larvae demonstrate 'demand-driven' myelopoiesis in response to TLR ligand stimulation and a live bacterial challenge. Approximately 2 days following delivery of GFP-labelled *Salmonella typhimurium* into the hindbrain of 50 hpf embryos, and a robust innate immune response evidenced by the rapid infiltration of phagocytic *lyz:DsRED2*-expressing leukocytes, a massive expansion of myeloid leukocytes is observed within the haematopoietic niche bordered by the dorsal aorta and posterior cardinal vein. These leukocyte clusters are also highlighted by their expression of the *runx1P2:EGFP* transgene and their production is dependent on HSC emergence within the same region, as evidenced by their absence in Runx1-depleted larvae, suggesting an early definitive myeloid precursor identity. We are assessing the contribution of TLR signalling mediated through the different TLR adaptors towards this response as well as the capacity of different purified TLR ligands to replicate it. These studies will provide a novel means to directly observe demand-driven myelopoiesis within a whole animal context. They also have potential to further dissect the genetic pathways(s) that drive this process and to determine how these pathways differ to those controlling 'steady-state' haematopoiesis.

New insights into the evolution of lipopolyccharide (LPS) recognition and signaling

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It has long been established that lower vertebrates, most notably fish and amphibians, are resistant to the toxic effect of LPS. Furthermore, the lack of a TLR4 ortholog in some fish species and the lack of the essential co-stimulatory molecules for LPS activation via TLR4, i.e. MD-2 and CD14, in all the fish genomes and EST databases available, led us to hypothesize that the mechanism of LPS recognition in fish may be different from that of mammals. To throw light on the role of fish TLRs in LPS recognition, a dual-luciferase reporter assay to study NF- B activation in whole zebrafish embryos was developed and three different bony fish models were studied: (i) the gilthead seabream (*Sparus aurata*, Perciformes), an immunological-tractable teleost model in which the presence of a TLR4 ortholog is unknown; (ii) the spotted green pufferfish (*Tetraodon nigroviridis*, Tetraodontiformes) that lacks a TLR4 ortholog; and (iii) the zebrafish (*Danio rerio*, Cypriniformes) which possesses two TLR4 orthologs. Our results show that LPS signaled via a TLR4- and MyD88-independent manner in fish and, surprisingly, the zebrafish TLR4 orthologs negatively regulated the MyD88-dependent signaling pathway. We believe that the identification of TLR4 as a negative regulator of TLR signaling in the zebrafish, together with the absence of this receptor in most fish species, explains the resistance of fish to endotoxic shock and supports the idea that the TLR4 receptor complex for LPS recognition arose after the divergence of fish and tetrapods.

Negative regulation of Toll-like receptor (TLR) signaling: molecular and functional characterization of zebrafish MD1 and RP105

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Mammalian immune cells are able to sense bacterial lipopolysaccharide (LPS) by mean of TLR4/MD2/CD14 receptor complex. This response is negatively regulated by the RP105/MD1 receptor complex which acts as a decoy receptor for LPS. Interestingly, fish lack orthologs of MD2 and CD14, what may explain the inability of low concentration (ng/ml) LPS to stimulate fish immune cells. However, TLR4a, TLR4b RP105 and MD1 orthologs are all present in the zebrafish, although we have recently shown that zebrafish TLR4s are not involved in LPS signaling. In this study, we analyzed the expression profiles of RP105 and MD1 by real-time RT-PCR and their ability to physically interact. The results revealed that the transcript of *md1* was maternally transferred, while that of *rp105* was not detected until 9 hpf. These results suggest that zebrafish *md1* and *rp105* may not require each other to perform their functions at least in the first hours of development. On the other hand, both transcripts showed an ubiquitous expression in adult tissues, although their expression in the liver was very low. Injection of adult zebrafish with several pathogen-associated molecular patterns (PAMPs) resulted in a weak increase of the mRNA levels of *tlr4a*, *tlr4b* and *rp105* in the injection site, while that of *md1* was only induced by LPS. These results may indicate that zebrafish *tlr4* orthologs and *rp105* are simultaneously up-regulated at the transcriptional level in response to different PAMPs, suggesting a functional interaction between TLR4 and RP105 signaling pathways in the zebrafish. However, we found that zebrafish MD1 was unable to interact with RP105 and TLR4 orthologs. Studies are in progress using morpholino-mediated knock down to illuminate the functional roles played by TLR4, RP105 and MD1 in the zebrafish.

Molecular and functional characterization of zebrafish IFNg1-1

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Interferon gamma (IFNγ) is an important cytokine involved in the regulation of the innate and adaptive immune responses to viral and intracellular bacterial infections. Two IFNγ genes are expressed in immune cells of teleost fish and are potentially implicated in B- and T- lymphocyte responses. However, the in vivo relevance of IFNγ1-1 in bacterial and viral infections is poorly understood in fish. We report here the production of recombinant zebrafish IFNγ1-1 and its biological activity using bacterial (*Streptococcus iniae*) and viral (spring viremia of carp virus, SVCV) infection models. The results show that bacterial and viral infection failed to induce the expression of IFNγ1-1. In addition, stimulation of ZF4 cells with IFNγ1-1 was unable to induce the expression of Mx, contrasting the powerful effects exerted by IFNγ1-2 in these cells. More importantly, injection of fish with recombinant IFNγ1-1 did not affect their resistance to either viral or bacterial infection. Taking into account these results, together with the low sequence homology of zebrafish IFNγ1-1 with IFNγ of higher vertebrates and the lack of the conserved IFNγ superfamily domain (Pfam accession no. PF00714), we propose that zebrafish IFNγ1-1 is not a true IFNγ ortholog.

Zebrafish Numb and Numblike are involved in primitive erythrocytes differentiation

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Zebrafish hematopoiesis has been shown to be very similar to that in mammals with evolutionary conserved genetic programs which drive the formation of analogous blood cell types. In Vertebrates blood cells formation consists in two successive waves. The primitive hematopoiesis produces predominantly erythrocytes and only some primitive macrophages while definitive hematopoiesis provides long term Hematopoietic Stem Cells (HSCs) able to give rise to mature blood lineages.

The Notch signaling is an evolutionary conserved regulatory system implicated in regulating cell fate determination in various developmental processes included HSCs self renewal and blood lineages differentiation both *in vitro* and *in vivo*. One known inhibitor of Notch activity is the evolutionary conserved adaptor protein Numb. In mice, several lines of evidence suggest that Numb (Nb) and its homologue Numblike (Nbl) play redundant functions in specifying and maintaining neuronal differentiation. The expression of *Nb* and *Nbl* has been detected in most of the tissues of mouse embryos including the yolk sac and adult hematopoietic tissues in both mouse and human models. Hence, these findings rise the possibility that Nb and Nbl proteins could play a role also in the embryonic and adult hematopoietic system. It has been proposed that both Nb and Nbl are dispensable for hematopoiesis in adult mice but recent *in vitro* approaches provided evidences that Numb can modulate the specification of primitive erythrocytes through its interaction with Notch.

Numb and *Numblike* homologues have been identified and cloned also in zebrafish where they are expressed in the whole embryo from the first cleavage stage to organogenesis stages and by 24hpf their expression become restricted to the anterior half of the embryo. Using morpholino antisense oligonucleotide (MO) knock-down of Numb and Numblike we observed embryos in which circulating blood cells are absent or severely reduced starting from the earliest time point that circulation can be detected. In order to assess the presence of differentiated primitive erythrocytes in *nb/nbl* morphants we analyzed the erythroid hemoglobin content by o-dianisidine staining in 48hpf morphants and we observed that only few differentiated red blood cells were detectable. Furthermore, to gain insight into the erythropoietic defects we injected the *nb/nbl* morpholino in the transgenic line Tg(*gata1*: DsRed). Injected embryos at about 28-30hpf showed that, at this stage of development, the overall fluorescence of *nb/nbl* morphants start to appear strongly reduced and at 48hpf and 72hpf only a little amount of red cells is present within the sinus venosus. Taking together, our results provide the first *in vivo* evidence of an involvement of Numb and Numblike in erythrocytes determination and differentiation during primitive hematopoiesis.

The Hox cofactors Pbx and Meis1 act upstream of *gata1* to regulate primitive erythropoiesis

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Vertebrate embryos bearing deletions in individual *hox* genes display a partial loss of blood cells, demonstrating a role for these transcription factors in regulating certain aspects of erythroid lineage specification. However, the overlapping expression patterns and functional redundancy of posterior *hox* genes have prevented us from defining their role in regulating hematopoietic transcriptional gene networks. The specificity of Hox proteins is achieved through their interaction with other DNA-binding cofactors. These include the Three Amino acid Loop Extension (TALE)-class homeodomain transcription factors Pbx (Pre-B-Cell Leukemia Homeobox) and Meis (Myeloid Ecotropic Integration Site). Removal of Pbx generates an anteriorizing phenotype in the zebrafish hindbrain, in which rhombomeres 2-6 take on the identity of rhombomere 1. An identical phenotype results from the loss of *Hox1* gene products in *Xenopus*, indicating the significant role that TALE-class proteins play as Hox cofactors *in vivo*. In order to model global loss of Hox function in zebrafish primitive hematopoiesis, we have ablated the Hox cofactors Pbx and Meis1. Embryos lacking Pbx and Meis1 fail to produce visible circulating blood cells, and exhibit a severe reduction in the expression of *gata1*, the earliest marker of erythroid cell fate. Concomitant with a loss of *gata1*, Pbx and Meis1-depleted embryos initiate, but fail to maintain *scl* hemangioblast gene expression, and possess increased numbers of *pu.1*-positive myeloid cells. Furthermore, *gata1* overexpression is able to drive *scl* expression in Pbx and Meis1-depleted embryos. Combined, these results place Pbx and Meis1 upstream of *gata1* in the erythropoietic hierarchy. *hoxb7a*-overexpression rescues *gata1* expression in Pbx-depleted, but not Meis1-depleted embryos. We therefore propose a model whereby Hox acts in association with Pbx and Meis1, upstream of *gata1,* to specify the primitive erythropoietic cell lineage and inhibit myelopoiesis.

Using transgenic zebrafish to screen for small molecule inducers of inflammation resolution
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Rationale: Diseases of neutrophilic inflammation are common, affect many organ systems, and respond poorly to current therapies. There is a major unmet need to identify new ways to treat such diseases. Neutrophils are usually removed by macrophages having undergone apoptosis, but during inflammatory diseases survival signals delay this apoptosis leading to enhanced inflammation.
Methods: We have established a tractable model in transgenic zebrafish expressing GFP in the neutrophil lineage, in which inflammation resolution can be rapidly quantitated *in vivo*. Sterile physical injury to the tailfin of anaesthetised larvae leads to a reproducible and quantifiable neutrophilic inflammatory response which spontaneously resolves over time. This permits screening of compound libraries to identify compounds which accelerate inflammation resolution.
Results: Preliminary experiments have demonstrated the practicality of such screens, and have identified several lead compounds that serve as "proof of principle", demonstrating the utility of this approach in the identifcation of new immunotherapeutics. From the Spectrum collection (MSdiscovery), 960 compounds were tested, of which 12 were shown to have reproducible effects. These include several known anti-inflammatory agents. Of these, a number have been tested and shown to exhibit dose-dependent effects. Active compounds suppressed neutrophilic inflammation to levels below those seen with our positive control, a potent inducer of neutrophil apoptosis, pyocyanin. For example, in control fish the number of neutrophils present at the site of injury at 24 hours post-injury was 30.24+/-1.47. In pyocyanin treated fish it was 19.04+/-1.93, and for one compound it was 12.33+/-1.85 (mean+/- sem, $p<0.05$ for both treatments vs control, one-way ANOVA with Bonnferroni post-test correction, n=49, 24 and 9 respectively).
These compounds are under further investigation for their ability to modulate human neutrophil function and will be assessed for enhancement of resolution of inflammation in mammalian models of neutrophilic pulmonary inflammation.
Conclusions: These data show the ability of this model to identify novel therapeutics with dramatic immunomodulatory properties. Some of these compounds may be useful lead compounds for the identification of novel therapeutic entities.

Discovery and expression analysis of important markers of T-cell subsets in the zebrafish (*Danio rerio*)

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The zebrafish (*Danio rerio*) is now recognized as a useful vertebrate model to understand immunity and considerable progress has been made in understanding the molecular basis of normal white blood cell development. Despite this, however, very little is known about T-helper (Th) cell development in fish and whether cell subsets such as Th1, Th2, Th17 and Treg cells exist, as in mammals. Although CD4 and CD8 have recently been characterised in teleosts, the absence of other cell markers, which include T-cell specific transcription factors (Th-POK, STAT6, FoxP3 & T-bet) means we cannot firmly conclude that development and functional properties of T-cell subsets in fish parallel those of mammals. Using these transcription factors that play a critical role during development of T-cell subsets and looking at their distribution in a tissue specific and time specific manner would begin to reveal important details of the immune system of lower vertebrates.

Using the zebrafish genome and a synteny approach, we have identified CD4, CD8, Th-POK, STAT6, T-bet, and FoxP3 in zebrafish. Despite high levels of divergence with some of these genes at the sequence level, when compared with other vertebrates, these genes exhibit a striking conservation of gene order, suggesting that selection has maintained gene order among the vertebrates over hundreds of millions of years of evolution. Using quantitative PCR (Q-PCR), Whole *In Situ* Hybridisation (WISH) and a FoxP3 polyclonal antibody, the expression of T-cell subset markers is investigated in the developing and adult zebrafish.

Elucidating the roles of notch ligands, receptors and downstream targets in embryonic haematopoiesis and angiogenesis

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The Notch pathway has been shown to play an important role in the formation of definitive haematopoietic stem cells (HSCs) in both mouse and zebrafish embryos. In the zebrafish, it is thought that Hedgehog signalling from the notochord induces Vegf signalling in neighbouring somites, which in turn induces notch activity in the dorsal aorta. This then leads to induction of the HSC programme in cells in the ventral wall of the vessel, through up-regulation of genes such as *runx1* and *c-myb*. In support of this, previous work has shown that down regulation of notch signalling in the E3 ubiquitin ligase mutant *mindbomb*, leads to a loss of *runx1* expressing cells in the dorsal aorta. Conversely, overexpression of notch intracellular domain in zebrafish embryos leads to ectopic expression of *runx1* in the aortic roof and the vein.

Although a general need for Notch has been identified in the formation of zebrafish HSCs, the exact mechanisms by which this pathway regulates the process have yet to be elucidated. We present a detailed dissection of the role of Notch in initiating definitive haematopoiesis. Using morpholino knockdown and over-expression studies, we have identified the notch receptors and ligands responsible for induction of the HSC programme. We also present data suggesting that the putative notch target Gridlock is in fact an upstream regulator of the pathway. In addition, a search for other putative Notch targets in the dorsal aorta has revealed a role for two Notch target genes in regulating angiogenesis.

The role of RGS18 in hematopoiesis and megakaryopoiesis

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Hematopoiesis is a life-long developmental process that involves the differentiation of hematopoietic stem cells. Myelopoiesis and lymphopoiesis are controlled by hematopoietic growth factors, including cytokines, and chemokines that bind to seven-transmembrane G-protein-coupled receptors (GPCRs). Inactive heterotrimeric G-proteins are composed of α, β and γ subunits. Upon ligand binding, GPCRs stimulate Gα to release GDP and to bind GTP, resulting in the formation of active α-GTP and G-βγ, which regulate downstream effectors. Signaling is terminated when the Gα-subunit hydrolyses bound GTP to GDP, leading to reformation of inactive G-αβγ. GTPase-activating proteins can regulate heterotrimeric G-protein pathways by increasing the intrinsic GTPase activity of the Gα subunit. Regulators of G-protein signaling (RGSs) are a recently discovered protein family that can act as GTPase-activating proteins for Gα subunits. Several RGS proteins have been implicated in the function of mature myeloid or lymphoid cells.

Our lab mainly focuses on studying the role of RGS 18 in hematopoiesis and megakaryopoiesis, using zebrafish as a model. RGS18 is highly abundant in megakaryocytes, and is also detected specifically in hematopoietic progenitors and myeloerythroid lineage cells, but its role in megakaryopoïeses is not known. RGS18 can bind both Gαi and Gαq in vitro, enhance GTPase activity of Gαi, and attenuate signals from Gq-coupled receptors. As an initial step in understanding the role of RGS18 in megakaryopoiesis, we used the morpholino (MO) knock down technology. Two MOs, developed against two different sequences in the ATG region of RGS 18, were injected in one cell stage eggs of $CD41^{+}$-GFP zebrafish. Downregulation of RGS18 in KDs was confirmed by immunoblot. Both MOs caused a dose dependent decrease in total number of thrombocytes and secondary defects in blood vessel- and somite formation. The decrease in GFP-labeled thrombocytes was confirmed by flow cytometry analysis and Western blot. In situ results showed that the effects of the KD were not related to early hematopoietic defects but additional experiments are needed to find out in which phase of hematopoiesis/ megakaryopoiesis RGS18 is involved.

The second aim of the study is to unravel the pathway by which RGS18 is working.

In conclusion, this study showed for the first time a role for an RGS protein in megakaryopoiesis and thrombocyte formation.

A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish

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Intact barrier structures (e.g. epithelia around tissues, plasma membranes around cells) are required for internal homeostasis and protection from pathogens. Wound detection and healing represent a dormant morphogenetic program that can be rapidly executed to restore barrier integrity and tissue homeostasis. In animals, initial steps include recruitment of leukocytes to the site of injury across distances of hundreds of micrometers within minutes of wounding. The molecular and biophysical characteristics of the spatial signals that direct this immediate tissue response are unknown.

Due to their fast diffusion and versatile biological activities, reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) are interesting candidates for wound-to-leukocyte signaling. To investigate possible paracrine signalling by H_2O_2, we imaged its spatiotemporal dynamics, together with leukocyte motility, in an intact vertebrate tissue subjected to mechanical wounding. The zebrafish larval tail fin has become a popular vertebrate model system to study inflammatory and regenerative responses to wounds. Rapid leukocyte recruitment to the wound can be easily imaged, and the molecular dynamics of the tissue perturbed using morpholino knockdown, transgenic expression and pharmacology.

We measured H_2O_2 by expressing HyPer, a genetically encoded ratiometric sensor that is highly selective for H_2O_2 over other ROS. HyPer consists of the bacterial H_2O_2-sensitive transcription factor OxyR fused to a circularly permuted YFP. Cysteine oxidation of the OxyR part induces a conformational change that increases emission excited at 500 nm (YFP_{500}) and decreases emission excited at 420 nm (YFP_{420}). This change is rapidly reversible within the reducing cytoplasmic environment, allowing dynamic monitoring of intracellular H_2O_2 concentration. We introduced HyPer by mRNA injection into zebrafish embryos to induce global cytoplasmic expression.

This reporter revealed a sustained rise in H_2O_2 concentration at the wound margin, starting ~3 min after wounding and peaking at ~20 min that extended ~ 100-200 µm into the tail fin epithelium as a decreasing concentration gradient. Using pharmacological and genetic inhibition, we show that this gradient is created by dual oxidase (DUOX), and that it is required for rapid recruitment of leukocytes to the wound. This is the first observation of a tissue-scale H_2O_2 pattern, and the first evidence that H_2O_2 signals to leukocytes in tissues, in addition to its known antiseptic role.

Zebrafish miR-126 and miR-150 coordinately determine hematopoietic cell fate through c-Myb

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Precise regulatory mechanisms are required to appropriately modulate the cellular levels of transcription factors controlling cell fate decisions during blood cell development. The proto-oncogene *c-myb* functions at multiple stages during hematopoiesis, from hematopoietic stem- and progenitor cells to fully differentiated cell types, and can affect the definitive specification of lymphoid, myeloid, erythroid and megakaryocyte lineages. Given the spatio-temporal diversity of its developmental roles, the mechanisms controlling the timing and levels of c-Myb activity must be precise and tightly maintained.

Here, we demonstrate the direct regulation of c-Myb by miR-126 and the importance of the *miR-126/c-myb* interaction during definitive hematopoiesis in the zebrafish. We show that miR-126 specifically binds to a predicted target site within the *c-myb* 3'UTR to attenuate translation of its upstream coding region. Knockdown of miR-126 results in increased c-Myb protein levels and promotes erythropoiesis at the expense of thrombopoiesis *in vivo*, while concomitant knockdown of *c-myb* alleviates these effects.

We further provide evidence that specification of thrombocyte versus erythrocyte cell lineages is adjusted by the combined activity of the miRNAs miR-126 and miR-150. Both microRNAs are sequentially required but not sufficient individually to precisely regulate the cell fate decision between erythroid and megakaryocytic lineages during definitive hematopoiesis. Our observations support the notion that microRNAs not only act to provide precision to developmental programs but also are essential determinants in the control of variable potential functions of a single gene during hematopoiesis.

Gene/ Enhancer trap based screen identifies novel transgenic lines with reporter gene expression in haematopoietic and endothelial Cells

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Vertebrate haematopoiesis occurs in two waves. The first wave arises from the anterior and posterior lateral plate mesoderm which give rise to primitive myeloid and to primitive erythrocytes respectively. The definitive wave generates haematopoietic stem cells (HSCs), which maintain the blood system throughout adulthood. HSCs are able to self-renew and to give rise to progenitors that differentiate into mature cells of all blood lineages.
In zebrafish, the primitive erythrocytes and the definitive wave of haematopoiesis occur in the intermediate cell mass in the trunk of the embryo. As in other vertebrates, HSCs form in close association with the ventral wall of the dorsal aorta (DA). The cells subsequently enter the blood circulation via the vein, settle in the caudal haematopoietic tissue before seeding their final destination, the kidney, the site of adult haematopoiesis in the fish.
We have conducted a transposon based gene/enhancer trap screen with the aims a) to identify novel genes involved in vasculogenesis and haematopoiesis and b) to fluorescently label endothelial and haematopoietic cells at different stages of development. Besides identifying a number of transgenic lines with tissue-specific gene expression in various non- haematopoietic and non- endothelial tissues, we have also found two lines that show GFP expression patterns of interest. One transgenic line has expression in primitive erythrocytes while a second line shows reporter gene expression in primitive erythrocytes, in myeloid cells as well as in the ventral wall of the DA.
We are currently characterizing the lines further and will present a progress report at the meeting.

Characterization of medaka *C-MYB* mutant *BENI FUJI,* which displays defective hematopoietic progenitor differentiation; an insight into hematopoietic ontogenesis in medaka

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Vertebrate hematopoiesis is characterized by two evolutionary conserved phase of development; primitive hematopoiesis which is a transient phenomenon in the early embryos that generate primitive erythrocytes and macrophages, and definitive hematopoiesis that takes place in the later stages accompanied with the emergence of hematopoietic stem cell that generate all hematopoietic lineage.

Beni fuji (bef), was originally isolated as a medaka mutant that has apparently reduced number of erythrocytes in the peripheral blood. Positional-candidate cloning revealed that the *bef* mutants have a nonsense mutation in the *c-myb* gene. Previous studies have shown that *c-myb* is essential for definitive hematopoiesis and is widely used as a marker gene for the onset of definitive hematopoiesis. To analyze the phenotypes of *bef* mutants, we performed whole-mount *in situ* hybridization with gene markers of hematopoietic cells. At st32, the *bef* mutants showed decreased expression of erythrocyte marker α*-globin*, and myelomonocyte marker *l-plastin*, and the expression of neutrophil marker, *mpo1* was completely lost. These results suggest that the *bef* mutants have defects not only in erythrocytes but also in other myeloid which indicate that the definitive hematopoiesis is malignant. Surprisingly, in the early stages of st22, we observed a complete loss of *l-plastin* expression in the anterior region from where the primitive macrophage arises. This result suggests that *c-myb* may also function in the primitive hematopoiesis, potentially revealing a link between primitive and definitive hematopoiesis.

β-lapachone treatment causes apoptosis of red blood cells in zebrafish embryos

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β-lapachone, a novel antitumor agent, is currently in phase II clinical trials for the treatment of pancreatic adenocarcinoma, while its effect on embryonic development is unknown. Linear arrangement of ventricle and atrium, no circulation or circulation with few red blood cells, and pericardial edema were detected in 48-52 hours post fertilization (hpf) embryos treated with sub lethal doze (2μm) of β-lapachone. Severe yolk and pericardial edema was then detected in 96 hpf β-lapachone-treated embryos. There were abundant ROS production and DNA fragmentation in red blood cells of β-lapachone-treated embryos. Co-treatment β-lapachone with either NQO1 inhibitor, dicumarol, or calcium chelator, BAPTA-AM, could rescue red blood cell deficiency phenotype in β-lapachone-treated embryos. Cell number of neutrophil granulocytes and the development of T lymphocytes were also affected in β-lapachone-treated embryos. Visible *NQO1* mRNA is detected in the epidermis, olfactory bulbs, pharyngeal arches, otoliths, neuromasts of posterior lateral line, and intestinal bulb of embryos from different developmental stages. There were no toxicity effects on these tissues possessing detectable *NQO1* mRNA when treated with sub lethal doze of β-lapachone. These results indicate that red blood cell deficiency phenotype produced in β-lapachone-treated zebrafish embryos is also mediated through NQO1-dependent, ROS mediated apoptotic pathway.

Development of zebrafish models of UV induced melanoma

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Ultraviolet irradiation (UVR) is the most important environmental risk factor for the development of melanoma. We are studying the pathological effects of UVR-treatment on melanocytes and nevi in different genetic backgrounds, with the aim to generate a zebrafish UVR-induced melanoma model. Part of our knowledge and technology of UVR-induced melanoma are translated from that of a *Xiphophorus* hybrid (*X. maculates* Jp163B × *X.helleri*), a well established fish model for UVR-induced melanoma. Advantages of a zebrafish UV-melanoma model include the ability to elucidate the genetic changes that occur after UVR treatment and during melanoma development, as well as the ability to use small molecules to modulate the response to UVR.

Using *Xiphophorus* as a foundation for our work, we have built a UV irradiation chamber similar to the one used in the *Xiphophorus* melanoma models. Experiments were carried out to determine the UVR dosage and nuclear excision repair (NER) ability of zebrafish with different genetic backgrounds. Preliminary results showed the LD50 of UVB irradiation for 6-day-old wildtype and p53 mutant zebrafish is near 480J/M^2, but p53 mutant fish had a delayed response to UVB irradiation, indicating that genetic differences can affect the response to UVR in zebrafish. Using radioimmunoassay (RIA), we measured the two major photoproducts, cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone dimers ((6-4) PDs) induced in the DNA of adult zebrafish skin after UVB irradiation. At 24 hours post radiation (hpr), wildtype zebrafish (AB strain) had 63% of (6-4) PDs removed from their epidermal DNA by NER, whereas p53 mutant had only 31% of (6-4) PDs repaired. The p53 fish showed reduced NER of (6-4)PDs, comparable to similar experiments in mouse knockouts. This is further evidence that cancer genes in humans are also important for the response to UVR in zebrafish.

We are investigating the DNA damage repair ability and melanoma susceptibility after UVR in different mutant lines available in the lab. These lines are altered for pigmentation, tumor-suppressor genes or oncogenes. We have reasoned that some of these mutant lines are likely to develop melanoma after UVR exposure. With our zebrafish model system, we will be capable of addressing which molecular events (e.g. photoproducts and DNA repair) are responsible for initiating melanoma, how pigment and UVR spectrum interact, and which signaling pathways are crucial for nevi/melanoma transformation and melanoma progression.

Recapitulating Early Stages of Diabetic Retinopathy in Hyperglycaemic Zebrafish

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Diabetic retinopathy is the most common complication of type 2 diabetes and the leading cause of blindness in working-age people in developed countries. Some treatments can palliate progression of proliferative diabetic retinopathy (PDR) but a better comprehension of the initial, non-proliferative phase of the disease (NPDR) may reveal interventions that more effectively limit retinal damage. Early stages of diabetic retinopathy are mostly asymptomatic for patients thus, animal models of NPDR are essential.

Our objective was to develop a zebrafish model of diabetic retinopathy through the induction of morphological and/or functional changes recapitulating the neuronal damage and vascular microangiopathy observed in the retinas of diabetic patients. The inner retina in adult zebrafish is nourished by an intricate vascular network which shares many features with the human retinal vasculature.

Hyperglycaemia was induced in adult zebrafish by treating with 2% glucose on alternative days. 2% mannitol treatment was used as an osmotic control. After 30 days of treatment, retinal morphology and retinal vascular network were analyzed by immuno-histochemistry and transmitted electron microscopy. Ex-vivo electroretinograms (ERG) were conducted to assess electrophysiological function in the treated retinas.

Glucose-treated fish display neurodegenerative and vascular hallmarks of early stage diabetic retinopathy. In ~60% of glucose-treated fish, retinal histology is abnormal, displaying a pronounced degeneration of cone photoreceptors. In regards to the retinal capillaries of glucose treated animals, the interendothelial junctions are significantly more open, and the basement membranes are thicker, indicating a breakdown of the retinal blood-barrier function. ERGs confirm defects in cone photoreceptor function in the glucose treated fish.

Our data suggest that zebrafish are a useful animal model for research into diabetic retinopathy.

Using zebrafish embryos to investigate genes implicated in Alzheimer's disease pathology
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Alzheimer's disease (AD) is the most prevalent form of dementia. There is considerable evidence that AD is caused by accumulating amyloid beta peptides in the brain, as a result of amyloid precursor protein (APP) cleavage by secretase enzymes. The presenilin proteins are central to the gamma-secretase cleavage of the intramembrane domain of APP. Aberrant splicing and point mutations in the human presenilin genes, *PSEN1* and *PSEN2*, have been linked to familial forms of AD, through aberrant APP cleavage resulting in irregular amyloid beta formation. Zebrafish have orthologues of human *PSEN 1* and *2* and *APP*, therefore they are a useful tool for investigating some of the complex pathways that occur in Alzheimer's disease. We aim to understand the role these genes have in AD pathology by investigating their molecular biology in zebrafish embryos. We have demonstrated that low-level aberrant splicing of exon 8 in *psen1* transcripts in zebrafish embryos produces potent dominant negative effects that increased *psen1* transcription, cause a dramatic hydrocephalus phenotype, decreased pigmentation and other developmental defects. Similar effects are also observed after low-level interference with splicing of exon 8 in *psen2* transcripts. A microarray analysis was performed to analyse global gene expression changes to illuminate the molecular aetiology of these phenotypic effects. Of the 100 genes that showed greatest dysregulation after *psen1* or *psen2* manipulation, 12 genes were common to both treatments. Five of these have known function and showed increased expression. *Cyclin* G1 (*ccng1)* was of particular interest as the human CCNG1 protein shows increased immunoreactivity in the cytoplasm of neurons in human AD brains. Phylogenetic and conserved synteny analysis confirmed the orthology of zebrafish *ccng1* with human *CCNG1*. Expression of zebrafish *ccng1* in developing embryos at 24 hours post fertilization (hpf) was observed in the eye, tectum and somites. Decreased Ccng1 expression does not lead to any developmental defects and also cannot rescue the hydrocephalus or pigmentation phenotypes of embryos with aberrant splicing of *psen1* exon 8. Analysis of zebrafish *ccng1* function indicates that truncation of Ccng1 appears to cause developmental defects in the brain, notochord and somites, however, it does not decrease the level of normal *ccng1* transcript. The *CCNG1* paralogue, *Cyclin G2*, (*CCNG2),* is also expressed in zebrafiish (*ccng2*). Decreasing the expression of Ccng2 results in similar effects on embryo development as truncating Ccng1. Potentially, the truncated forms of Ccng1 interfere with Ccng2 function in a dominant negative manner. Splicing is observed to change in ageing cells, therefore, aberrant splicing of particular genes may play a role in sporadic forms of AD and other neurodegenerative diseases.

Zebrafish models for familial Alzheimer's disease

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Autosomal dominant mutations in presenilin 1 (*PSEN1*) lead to familial Alzheimer's disease (FAD), neuropathologically characterized by amyloid-β (Aβ) accumulation. Aβ is formed by PSEN1-mediated proteolytic cleavage of the amyloid precursor protein (APP). PSEN1 also cleaves Notch, a protein essential for correct neuronal differentiation during embryogenesis, maintenance of the neural stem cell niche and neurogenesis in adult. Some FAD-PSEN1 mutations lead to impaired Notch signaling whereas others are neutral. We aim to reveal the effects of expression of such different FAD-PSEN1 mutations in the embryonic and adult brain using zebrafish as a model system.

To this end, we utilize our recently characterized zebrafish mutant lacking functional Psen1. These Psen1-null mutants are viable and fertile, in contrast to *PSEN1*$^{-/-}$ mice. Psen1-null mutants show decreased cell proliferation and *de novo* neurogenesis, which is most pronounced in the cerebellum. In addition, Delta/Notch signaling is impaired. The zebrafish Psen1-null mutant is the first vertebrate model with targeted gene knockout of PSEN1 compatible with life. As a result, we have a unique opportunity to study effects of FAD-PSEN1 with respect to Psen1 loss-of-function in the adult brain. By misexpression of human FAD-PSEN1 mRNA in Psen1-null embryos, we aim to dissect the effects of the PSEN1M146V, decreasing Notch signaling *in vitro*, and PSEN1L286V, neutral towards Notch *in vitro*, mutations during embryogenesis. In addition, we have generated novel transgenic FAD-PSEN1 lines expressing mCherry-fluorescently tagged human FAD-PSEN1 under the zebrafish *psen1* promoter region. In these lines we are investigating effects of FAD-PSEN1 on the neural stem cell compartment during embryogenesis and in adult brain in relation to Notch and APP signaling pathways.

Deletion of the WD40 domain of LRRK2 in zebrafish provides a model for Parkinson's disease

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LRRK2 plays an important role in Parkinson's disease (PD), but its biological functions are largely unknown. Here, we cloned the homolog of human LRRK2 (zLRRK2), characterized its expression and investigated its biological functions in zebrafish. The blockage of zLRRK2 protein expression by morpholinos caused embryonic lethality and severe developmental defects such as growth retardation and loss of neurons. In contrast, deletion of the WD40 domain of zLRRK2 by morpholinos targeting splicing did not induce severe embryonic developmental defects; rather it caused Parkinsonism-like phenotypes, including loss of dopaminergic neurons in midbrain and locomotion defects. These neurodegenerative and locomotion defects could be rescued by over-expressing zLRRK2 or hLRRK2. Interestingly, administration of L-dopa could also rescue the locomotion defects, but not neurodegeneration. Taken together, our results clearly indicate that zLRRK2 is a true ortholog of hLRRK2 and that the deletion of WD40 domain of zLRRK2 provides a good disease model for PD.

tRNA SPLICING ENDONUCLEASE MUTATIONS CAUSE PONTOCEREBELLAR HYPOPLASIA

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Pontocerebellar hypoplasia (PCH) represents a group of neurodegenerative autosomal recessive disorders with prenatal onset (PCH1-6). Children suffer from severe mental and motor impairments due to atrophy or hypoplasia of the cerebellum, hypoplasia of the ventral pons, microcephaly and variable neocortical atrophy. The disease is progressive and usually patients die before they reach adulthood.

We identified a common mutation in TSEN54 (A307S) in the majority of European PCH2 patients. TSEN54 is one of the four subunits of the tRNA splicing endonuclease (TSEN34, TSEN2 and TSEN15). The TSEN complex is responsible for the splicing of intron containing tRNAs and also plays a role in pre-mRNA 3'end formation. In PCH patients without the A307S mutation, we identified missense and nonsense mutations in TSEN54, TSEN34 and TSEN2 subunits.

In situ hybridization for TSEN54 using LNA/2OME probes revealed that TSEN54 is highly expressed in neurons of the pons, cerebellar dentate and olivary nuclei. Northern blot analysis of tRNA-Tyrosine from fibroblasts of 3 patients did not show unspliced products.

The molecular pathways behind pontocerebellar hypoplasia remain unclear. In order to study the mechanisms underlying PCH we are currently establishing zebrafish models for pontocerebellar hypoplasia. As a first step we performed knock down of TSEN54 or TSEN2, employing both ATG and splice site morpholino oligonucleotides and observed neurodevelopmental phenotypes, most prominently affecting brain development. These data suggest that morphants may phenocopy some of the aspects of pontocerebellar hypoplasia.

Dithiocarbamates are teratogenic to zebrafish through inhibition of lysyl oxidases

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Dithiocarbamates (DTC) are a class of compounds which are extensively used in agriculture as pesticides. As such humans and wildlife are undoubtedly exposed to these chemicals. Although DTC are thought to be relatively safe due to their short half lives it is well established that they teratogenic to developing vertebrates. In zebrafish these teratogenic effects are characterized by distorted notochord development and shortened anterior to posterior axis. DTC are known copper chelators but this does not fully explain the observed teratogenic effects. We show here that DTC cause malformations in zebrafish that highly resemble teratogenic effects observed by direct inhibition of a group of copper containing enzymes termed lysyl oxidases (LOX). Additionally, we show that DTC directly inhibit LOX in vitro and demonstrate that partial knockdown of three lysyl oxidases, lox, loxl1 and loxl5b sensitizes the developing embryo to DTC exposure. Taken together, these results indicate that teratogenic effects caused by DTC are, at least in part, caused by direct inhibition of LOX activity. These findings have implications for risk assessment of DTC.

Development of semicircular canals in the zebrafish inner ear

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We are using zebrafish as a model system for human deafness and vestibular disorders. *big ears (bge)* is an adult viable zebrafish mutation isolated in the Tübingen 1996 mutagenesis screen. The *lauscher (lau)* mutant is allelic to *bge* by complementation assay. The inner ears of these mutants become swollen at the end of the third day of embryogenesis. Epithelial projections forming the semicircular canal system show morphological and gene expression abnormalities and fail to fuse correctly. Preliminary data suggest that the swelling is a secondary result of the failure of the projections to fuse in the mutant. We have identified the *bge* gene by genetic mapping and a candidate gene approach. Orthologous genes have been cloned in the human and mouse, but their function is still unknown. Expression of *bge* appears to be ear-specific, and is restricted to the epithelial projections of the developing semicircular canal system. Point mutations were found in cDNA and genomic DNA for *bge* and *lau,* and have been confirmed by PCR-based genotyping assays. Both are missense mutations that predict the substitution of conserved amino acid residues in the protein. A splice site morpholino designed to knock down function of the *bge* gene phenocopies the semicircular canal projection defects seen in the mutant.

Zebrafish as a model for neurodegenerative diseases: functional analysis of *GIGYF2*, a candidate gene for Parkinson's disease

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Parkinson's disease is the second most common neurodegenerative disorder, after Alzheimer's disease. It is characterized by depigmentation of the substantia nigra (SN), caused by the selective and progressive loss of dopaminergic (DA) neurons, leading to resting tremor, bradykinesia, postural instability, and muscular rigidity. Family-based whole-genome linkage scans have already mapped 12 chromosomal loci (*PARK1–PARK13*) responsible for rare forms of autosomal dominant or recessive PD. However, for some of these loci, the specific gene remains elusive. *GIGYF2* (Grb10-Interacting GYF Protein-2) has recently been proposed to be the causative gene at the PARK11 locus, however, further studies did not confirm its association with the disease. Therefore, to elucidate the role of *GIGYF2 in vivo*, we cloned the zebrafish homolog *gigyf2* and determined its spatio-temporal expression during development by whole mount *in situ* hybridization technique. We found that *gigyf2* is expressed in the embryo central nervous system, as well as in other non-neuroectodermal territories. Moreover, *gigyf2* loss of function experiments, by means of morpholino injection, did not cause drastic morphological brain alterations or loss of DA neurons during development. Therefore, our *in vivo* results suggest that *gigyf2* is not necessary for the proper differentiation of the DA system in zebrafish, supporting recent genetic data that do not confirm *GIGYF2* as a PD gene.

Splice factor deficiency leads to photoreceptor degeneration in a zebrafish model for retinitis pigmentosa

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Approximately 1 in 3500 individuals suffers from Retinitis pigmentosa (RP), a genetic eye disease characterized by the progressive degeneration of photoreceptor cells in the neural retina. Mutations in more than 100 genes have been identified as cause for RP, but a large number of disease causing genes still remains unknown. The majority of known RP genes encodes for proteins involved in photoreception. Surprisingly, however, RP mutations have also been identified in housekeeping genes required for general cellular processes. These account for a considerable percentage of RP cases and include mutations in PRP3, PRP8 and PRP31, which are core components of the tri-snRNP complex implicated in pre-mRNA processing.

Aim of our project is to elucidate, how defects in general splicing factors lead to cell-type specific RP phenotypes. Our working hypothesis proposes a general weakening of the splicing machinery that primarily affects processing of a distinct subpopulation of retina expressed pre-mRNAs, thereby mediating a tissue specific effect. Gene knock-down of PRP8 and PRP31 by morpholino injection into zebrafish embryos leads to retina defects and irregular organization of the photoreceptor layer. Behavioural tests to measure the opto-kinetic response (OKR) furthermore suggest that PRP8 and PRP31 morphants exhibit severe visual deficits. In order to identify inefficiently spliced and ultimately degraded target pre-mRNAs in these morphants, we performed microarray assays using cDNAs derived from PRP8 and PRP31 deficient retinae. Our results suggest that a significant number of retina expressed genes is down-regulated in PRP8 and PRP31 morphants. Several of the identified transcripts represent already known RP disease genes. In addition, we identified potential new mediator genes, which so far have not been associated with an RP pathomechanism. Preliminary characterization of selected novel candidates suggests that their knock-down can induce a RP-related phenotype in zebrafish. Using semi-quantitative RT-PCR, we furthermore detected inefficiently spliced introns in several of the analyzed transcripts.

Taken together, our study shows that zebrafish is an excellent model to recapitulate aspects of human RP and to elucidate the underlying pathomechanism. This model can be used to identify new candidates, so far not associated with RP, to broaden the spectrum of disease causing genes as a valuable contribution to clinical diagnostics for RP predisposition.

Expression of H-RASV12 in a zebrafish model of Costello syndrome causes cellular senescence in adult proliferating cells

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Constitutively active, 'oncogenic' H-RAS can drive proliferation and transformation in human cancer, or be a potent inducer of cellular senescence. Moreover, aberrant activation of the Ras pathway owing to germline mutations can cause severe developmental disorders. In this study we have generated transgenic zebrafish that constitutively express low levels, or can be induced to express high levels, of oncogenic *H-RAS*. We observed that fish carrying the integrated transgene in their germline display several hallmarks of Costello syndrome, a rare genetic disease caused by activating mutations in the gene *H-RAS,* and can be used as a model for the disease. In Costello-like fish, low levels of oncogenic *H-RAS* expression are associated with both reduced proliferation and an increase in senescence markers in adult progenitor cell compartments in the brain and heart, together with activated DNA damage responses. Overexpression of *H-RAS* through a heat-shock-inducible promoter in larvae led to hyperproliferation, activation of the DNA damage response and p53-dependent cell cycle arrest. Thus, oncogene-induced senescence of adult proliferating cells contributes to the development of Costello syndrome and provides an alternative pathway to transformation in the presence of widespread constitutively active H-Ras expression.

A zebrafish model of Alzheimer's Disease

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Our ageing society is confronted with a dramatic increase of patients suffering from Tauopathies, which include Alzheimer's disease and certain Frontotemporal Dementias. These disorders are characterized by typical neuropathological lesions including hyperphosphorylation and subsequent aggregation of Tau protein and neuronal cell death. Currently, no mechanism-based cures are available. Genetically modified animals are invaluable models to understand the molecular mechanisms of pathology and screen for disease-modifying compounds. We have generated the first Tau-transgenic zebrafish, which rapidly recapitulate key pathological features of Tauopathies including phosphorylation and conformational changes of human Tau protein, tangle formation, as well as neuronal and behavioral disturbances and cell death. In contrast to existing vertebrate models zebrafish larvae are ideally suited for both *in vivo* imaging and drug development due to their optical transparency and small size. Therefore, the transgenic fish are important tools better understand the pathology of Tauopathies and develop treatment approaches. We could already demonstrate for the first time that neuronal cell death can be imaged by time-lapse microscopy *in vivo*. Furthermore, we used our fish-model to identify new compounds targeting the Tau-Kinase GSK3β, since phosphorylation of Tau is believed to be a trigger for disease progression. We identified a novel highly active GSK3β inhibitor, which we developed by rational drug design and validated for *in vivo* activity in the transgenic fish model.

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DISC-1 is an essential modulator of the wnt pathway

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We have begun to use the zebrafish as a tool to study the function of DISC-1, a gene implicated in the etiology of schizophrenia by interacting with GSK3β. We define a "tool" as an animal system that offers useful insight into function of a disorder risk gene, but that may not recapitulate the human phenotype. Despite low overall amino acid similarity, highly conserved domains are present in the DISC-1 gene in zebrafish and mammals. Zebrafish DISC-1 shows maternal and zygotic expression and is strongly expressed in the developing central nervous system. In order to test the embryonic function of DISC-1, we decreased zygotic gene function by injecting morpholino-modified antisense oligos (MOs) targeting splice sites. The D1MO was directed against the splice donor site between exon 1 and intron 1/2, and ablates all normal DISC-1 RNA. D1MO injected embryos ("morphants") show a very strong phenotype, apparent by early somitogenesis, characterized by a massive deficit in neuronal outgrowth and poor fasciculation. We noticed that the gross phenotypes resulting from DISC-1 and Wnt8b loss of function are similar, and showed that partial loss of function of each resulted in a strong phenotype, demonstrating synergy between these genes. Several additional lines of evidence indicate that DISC-1 functions in the Wnt pathway. First, similar changes in forebrain gene expression are observed after loss of DISC-1 or loss of Wnt8b function. Second, loss of DISC-1 function decreased reporter gene expression in a Wnt-responsive reporter zebrafish line, TOPdGFP. Third, a GSK3β inhibitor is able to rescue reporter and endogenous gene expression after DISC-1 loss of function. Fourth, increasing β-catenin levels, through an inducible fusion protein, rescues the DISC-1 loss of function phenotype. Finally we were able to rescue the morphant phenotype with human DISC-1 demonstrating the fundamental role of DISC-1 in modulating GSKβ during zebrafish development.
In conclusion, our data identify DISC-1 as an essential positive modulator of the canonical Wnt pathway, and confirm the efficacy of using zebrafish as a tool to explore the function of genes implicated in human mental health disorders.

Functional characterization of two human disease mutations, PRP31-SP117 and PRP31-AD5, in a zebrafish model for Retinitis pigmentosa

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Retinitis pigmentosa (RP) is an inherited eye disease with a prevalence of approximately 1/3500, characterized by a progressive degeneration of photoreceptor cells in the retina, which firstly affects rods and secondarily results in the loss of cones. Different mutations in the general splicing factor PRP31 have been identified as a major cause for the autosomal-dominant form of RP in humans. So far, mostly cell culture models have been used to study the mechanisms underlying RP caused by PRP31 mutations and conflicting results have been obtained from such *in vitro* approaches. We have used transgenic zebrafish as a powerful animal model for human diseases, to investigate by which mechanisms two human mutations (SP117 and AD5), which cause a frameshift at amino acids 256 and 371 respectively and might result in truncated PRP31 proteins (277 and 469 aa; wildtype 499 aa), lead to photoreceptor cell degeneration. After injection of RNAs encoding both mutant PRP31 variants into zebrafish embryos, we found that the SP117 protein degraded quickly and was no longer detectable at 11hpf, whereas the AD5 variant had a similar stability as injected wildtype PRP31 and showed continuous high levels of expression at 14hpf. For an *in vivo* assay of protein localization and function, we next generated a rhodopsin-EGFP (rho-GFP) transgenic line, which expresses GFP in the cytoplasm of rods. rho-GFP transgenic embryos were then injected with plasmids encoding wildtype or mutant PRP31 fused in frame to mCherry under the control of the rhodopsin promoter. Embryos injected with wildtype PRP31:mCherry showed transient expression in the nuclei of rods, similar to the situation in AD5:mCherry injected embryos. In contrast, in SP117:mCherry injected embryos, the majority of mCherry signal was detected in the cytoplasm of rods indicating aberrant subcellular localization of the mutant protein. In addition, we found that transient expression of the AD5 mutant resulted in a significant reduction of rhodopsin expressing rod photoreceptors, while expression of SP117 or wildtype PRP31 did not change the number of rhodopsin expressing cells. Hence, our *in vivo* data suggest that the two analyzed PRP31 mutations act by different mechanisms: Expression of AD5 mutant protein results in rod degeneration possibly in a dominant-negative fashion, whereas mutant SP117 protein is mislocalized to the cytoplasm and unstable and causes rod degeneration by haploinsufficiency due to nonsense-mediated mRNA decay (NMD). Our zebrafish studies thus provide novel insight into the pathomechanism of both mutations and have implications for future discriminatory therapy of this human disease caused by different mutations.

Fishing for neurorpotectants from Chinese medicine

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Neurodegenerative disorders such as parkinson's, alzheimer's and multiple sclerosis represent increasing severe public health issues, which is lack of effective treatment so far. Chinese medicine (CM) has a long history of application in Asia societies for treatment of neurodegenerative disorders. In an effort to find effective neuroprotective ingredients from Chinese medicine, herein, 14 known pure compounds from CM and 2 herbal extracts were investigated for neuroprotective activities by a combined model of PC12 cell *in vitro* and zebrafish *in vivo*.

In present study, selected neurotoxin (6-OHDA and MPTP) against dopaminergic neuron and non-specific oxidative agents (*L-2* hydroxyglutaric acid, LGA) were used to induce neuronal cell damage in both cell culture and zebrafish models. Among these compounds and extracts tested, quercetin, a well-known flavonoid with anti-oxidative activity, and ethanol extract of *Allpinae Oxyphyllae* (AOE), exhibited significant neuroprotective activity against 6-OHDA induced PC12 cell death and 6-OHDA and MPTP induced dopaminergic neuron loss in zebrafish. Moreover, both quercetin and AOE could notably prevent LGA-induced neuronal apoptosis in zebrafish by dose-dependent manner. However, two pure compounds, namely chrysin (a flavonoid with structure-similar to quercetin) and protocatechuic acid, isolated from AOE, showed mild neuroprotective in zebrafish model, but not in PC12 cell model. It may be due to these chemicals with functional conservation and favorable pharmacokinetic properties for activity in zebrafish. In future study, the active metabolites and synergistic interaction of different compounds in the AOE needed to be elucidated.

To the best of our knowledge, this is the first study of application of zebrafish model for neuroprotectants screening from CM. The results of this research are expected to provide a scientific basis for clinical use of the selected CM and the identification of new potential compounds leading to safe and effective drug development for the treatment of neurodegenerative disorders.

Biomedical Properties of a Series of Ruthenium-NHC Complexes Based on In Vitro Oxidant Activities and In Vivo Evaluation of Biosafety in Zebrafish Embryos

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Over the last years, ruthenium complexes have demonstrated a great potential in the design of new metal-based drugs. Besides the metal centre, the ancillary ligands play an important role, defining the final features of the complex. In this sense, N-heterocyclic carbene (NHC) ligands have demonstrated their capability to support a wide variety of metals. Recently, the biomedical applications of NHC-based metal complexes are beginning to attract great interest, gathering biomedical, biomolecular and organometallic chemists in the search of new and improved metallodrugs. However, only little data with regard to their toxicity have been available until now, and no data are available in the literature on animal models. In this work, we have studied the *in vitro* antioxidant/pro-oxidant activity of a series of Ru(II)-NHC complexes and their toxicity using the zebrafish model. In the *in vitro* assay, two of the Ru(II)-NHC compounds showed significant pro-oxidant dose-dependent activity at the highest concentration, whereas a ruthenium precursor ($RuCl_2(dmso)_4$) did not show any pro-oxidant behavior at any studied dose. In the zebrafish embryotoxicity test, the two ruthenium compounds and the ruthenium precursor were analyzed in comparison with three reference compounds: SDS, $CuSO_4$ and tert-Butyl hydroperoxide (TBH). The two ruthenium compounds were less toxic than SDS and $CuSO_4$, while the ruthenium precursor did not present loss of viability at the maximum dose used, indicating that the precursor is the safest compound of the series. Additionally, morphological changes in embryos due to the treatment were studied. At the lowest studied dose, the embryos incubated with ruthenium compounds, ruthenium precursor or with TBH did not present differences with the controls, showing clearly a well developed body and being out of their chorions at 72 hpf. However, at the same concentration the embryos treated with $CuSO_4$ remained inside of the chorion, while the animals treated with SDS result dead presenting opaque coloration by coagulation of their organs. With a higher dose, the embryos treated with the ruthenium precursor and TBH did not present differences in comparison to controls, while the animals incubated with SDS or copper sulfate were dead. However, the embryos treated with the ruthenium compounds remained inside of the chorion, suggesting a delay in the egg development. At the highest concentration, the totality of incubated embryos with the compounds died, except the controls and the animals treated with the ruthenium precursor that remained inside of the chorion. The cardiotoxicity was studied as well, and the influence of the ruthenium compound treatments in the heart rate was analyzed in comparison to the reference compounds. The results indicate that the most toxic compound was the SDS, followed by the copper sulfate, confirming the viability results. The safest evaluated substance was again the ruthenium precursor showing no alteration except for the highest concentration, where a minor decrease in the heart rate was detected.

In conclusion, this is the first study testing Ru(II)-NHC complexes in an animal model, the zebrafish embryo. The results showed that the behavior of the ruthenium complexes and the ruthenium precursor evaluated here present a reliable correlation between the *in vitro* oxidant activity and the toxicological parameters studied in zebrafish embryos that are related to the same time to their structures.

Validation of mutations related to developmental human brain diseases using zebrafish

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In collaboration with human genetics research groups at the Université de Montréal, we performed large-scale genomics screening for mutations of synaptic genes in patients with developmental brain disorders, including autism and schizophrenia. However, the initial identification of mutations related to human disease often lacks biological validation of their pathogenic nature. We are therefore using zebrafish to screen potential gene variants by knocking down the expression of the homologous zebrafish gene to yield a phenotype which we then attempt to rescue with the native human gene and in comparison to possible disease-related variants. For each human gene, we start by identifying the zebrafish homolog from the genomic database and designing a morpholino antisense oligonucleotide to selectively target translation. We next attempt to rescue the knockdown phenotype by expressing the native human mRNA upon co-injection with the morpholino. If rescue is observed, we compare the effectiveness of the disease-related sequence variants.
Using this experimental approach we successfully studied several mutations in genes related to human spinal cord diseases. The genes tested include *ALS2* related to amyotrophic lateral sclerosis, *AP1S1* linked to skin and spinal cord disorders and *SPG8* associated with hereditary spastic paraplegia. We have found that knockdown of the zebrafish homologues genes yielded specific phenotypes which included abnormal motility, led to changes in neural population or defects in spinal axon projections. In the cases tested, the phenotype could be rescued by overexpression of the native human mRNA but not by some of the disease-related alleles, including novel mutations, thus establishing the usefulness of this approach. For our genomics project we have identified several inherited as well as *de novo* mutations of synaptic genes that we are validating in zebrafish. Zebrafish thus provide a useful model for validating the pathogenic nature of human brain disease-related alleles.

G93A-SOD1 transgenic zebrafish as a model of amyotrophic lateral sclerosis
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Axonal degeneration precedes symptom onset and motor neuron (MN) cell death associated with amyotrophic lateral sclerosis (ALS; MN disease), making it an appealing target for therapeutic intervention prior to the irreversible loss of MNs. Detailed observations of MN axons and neuromuscular junctions (NMJs) are possible in zebrafish; therefore, they provide an excellent *in vivo* system to study early events in ALS onset and progression, as well as mechanisms that underlie neuroprotection. Transient genetic manipulation of zebrafish to express G93A-SOD1, a mutation associated with familial ALS, results in defects in MN outgrowth and axonal branching. To study the mechanisms of neurodegeneration and identify novel treatments for ALS, stable G93A-SOD1 transgenic zebrafish have been generated. These G93A-SOD1 transgenic zebrafish demonstrate a progressive loss of swimming ability. We are currently characterizing this model of ALS by analyzing NMJ integrity, axonal morphology and survival in order to understand the mechanisms of axonal degeneration associated with ALS. Knowledge obtained from the characterization of stable transgenic zebrafish as a model of ALS will greatly advance our ability to treat ALS by identifying points for therapeutic intervention throughout the course of MN degeneration.
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Using zebrafish to investigate presenilin, γ-secretase, and APP for Alzheimer's disease research

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Introduction: An integrated understanding of the exact molecular and cellular basis of Alzheimer's disease (AD) remains elusive. Histological analyses of AD patients have observed lesions containing extracellular deposits of amyloid-beta peptides (Aβ) and intracellular neurofibrillary tangles (NTF). Aβ peptide is a cleavage fragment of the Aβ precursor protein (APP). Aberrant proteolytic processing of APP by γ-secretase results in an imbalance between Aβ production and clearance that appears to promote neuronal dysfunction and death. Presenilin proteins form the catalytic cores of γ-secretase complexes. Genetic studies have discovered mutations in *APP* and *PRESENILIN 1* and *2* that contribute to familial autosomal dominant AD. *Danio rerio* provides an effective vertebrate model for investigating the molecular bases of AD pathology as it possesses orthologues of human *PSEN1* and *2*, and *APP*.
Results: 1) We have demonstrated that disruption of *PSEN1* transcript splicing can have potent dominant negative effects on the function of *PSEN1* and the related gene *PSEN2*. We hypothesise that aberrant splicing of *PSEN1* in ageing neural cells may contribute to sporadic AD. 2) We have synthesized mRNA encoding *PSEN1* truncations after exon junctions. When injected into embryos, particular truncations have a dominant negative effect. We hypothesise that the aberrant splicing of *PSEN* (above) produces truncated proteins that act in a dominant negative manner by invading γ-secretase complexes to prevent the formation of the catalytic site. 3) Currently there is no *in vivo* assay appropriate for investigating γ-secretase activity. We have begun development of transgenic zebrafish that produce a modified APP substrate that can be used to observe changes in γ-secretase activity. 4) We attempted to develop a transgenic model of amyloid toxicity in zebrafish by expressing the 42 amino acid residue form of human Aβ specifically in melanocytes using the *mitfa* promoter. From 16 months of age some of these adult fish showed progressive loss of melanocytes. We are currently modifying this transgenic model to display an earlier phenotype. This could prove useful for analysis of drugs intended to ameliorating amyloid toxicity.

Acetaminophen-Induced Nephrotoxicity in Zebrafish

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Acetaminophen (APAP) is widely used as an analgesic and antipyretic drug for decades. In mammals, over-dosed of APAP leads to liver and kidney damages but the toxic effects of APAP is little known during embryogenesis. Here, we used a green fluorescenct kidney line, *Tg(wt1b:eGFP)*, as a model to observe the APAP-induced nephrotoxicity dynamically. We carried out a series of exposure experiments with different concentrations (340, 3400, 6800ppm), durations (12, 24, 36, 48, 60h) and onsets (12, 24, 36, 48, 60hpf). Results showed that zebrafish embryos exhibited no evident differences in survival rates and morphological changes in the vehicle-control (0ppm) and 340ppm APAP-exposure (12-72hpf) groups. In constrast, after higher dose (3400, 6800ppm) treatment, embryos displayed malformed kidney phenotypes which were classified as three groups: (1) mild defects: curved and cystic pronephric tubule (pt) and the pronephric duct (pd) but with a normal glomerulus (gl); (2) morderate defects: mild defects but with a cystic and atrophic gl at normal position; (3) severe defects: similar to morderate defects but gl are separated by midline. The percentages of embryos with malformed kidney phenotypes increased as the exposure dosages of APAP increased. Specially, under the same exposure duration (ex: 12h) and dose (3400ppm), embryos displayed higher percentages of severe defects at earlier onset of exposure (ex: 12-24hpf), whereas embryos displayed higher percentages of mild defects at later exposure onset (ex: 60-72hpf). When the exposure duration is longer then 24h 6800ppm APAP, no surviving embryos were observed by 72hpf. These results indicated that APAP-induced nephrotoxicity depended on the exposure dose, durations and onsets. Furthermore, whole-mount in situ hybridization revealed embryos after APAP treatment had reductive wt1b expression domains. Taken together, we suggest that treatment with APAP leads to down regulation of wt1b expression, and consequently causes malformed kidney phenotypes.

Neuromuscular junction formation in Dok-7 deficient zebrafish embryos

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Congenital myasthenic syndromes (CMS) arise from genetic defects that affect transmission at the neuromuscular junction (NMJ). Causal mutations in presynaptic nerve terminal, synaptic cleft, and postsynaptic apparatus proteins have been identified. Recently, mutations in a novel CMS gene, *DOK*("downstream of kinase") 7, were found to cause an autosomal recessive form of myasthenia. *DOK7* mutations have emerged as one of the major genetic defects in CMS, accounting for about 10% of genetically diagnosed CMS cases. In contrast to other CMS subtypes, patients with *DOK7* mutations do not benefit from long-term therapy with esterase inhibitors.
The precise function of the Dok-7 protein at the NMJ has not been elucidated, yet. Okada and co-workers generated mice lacking Dok-7 to explore its role in vivo. However, Dok-7 deficient mice were immobile at birth and died shortly thereafter. The zebrafish is an established model of vertebrate development and is also receiving increasing attention as a model of human disease; mutations that are lethal in mammals at early stages of development can be studied in the zebrafish. We therefore investigated the role of Dok-7 in endplate development and endplate maintenance in a zebrafish model.
Downregulation of Dok-7 expression by injection of an antisense morpholino oligonucleotide into fertilised zebrafish eggs revealed first abnormalities of NMJ patterning in zebrafish embryos at 48h post-fertilization (hpf). By this time, motor axons in wild-type embryos have extended branches into and formed synapses with laterally located muscle fibres. Very early stages of NMJ formation do not seem to be affected in Dok-7 deficient embryos. These results might imply a role of Dok-7 in stabilising and branching of NMJs.
Our results may help to determine the molecular mechanisms through which *DOK7* mutations compromise neuromuscular transmission in CMS patients. Furthermore, zebrafish may be a suitable model organism for testing novel treatments for patients with *DOK7* mutations.

New screening for photoprotective compounds using zebrafish as a vertebrate *in vitro/in vivo* model

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Skin overexposure to solar ultraviolet radiation (UVR) leads to deleterious effects such as photooxidative stress, sunburn, immune suppression, photoaging and skin cancer. Cells culture models have been mostly used to perform quantitative studies of radiation cytotoxicity; however, the response of cells under culture conditions does not reflect the *in vivo* response of a whole organism.

In recent years, zebrafish (*Danio rerio*) has increased its importance as a vertebrate model both for toxicology assays and screening of therapeutic agents, due to its transparency, fast development and easiness to handle in multiwell plates.

In the present study, using the zebrafish embryo model, a new fast and easily to perform method to detect antioxidant and photoprotective properties of natural extracts and other compounds has been developed. The photoprotective effect of a hydrophilic extract of the fern *Polypodium leucotomos* (PL) has been investigated besides another compounds, known for their antioxidant and photoprotective properties, like (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and L-Ascorbic acid (Vitamin C). Zebrafish embryos (24 hpf) were exposed to UVB radiation, with or without previous incubation with different concentrations of the compounds. Irradiated embryos treated with the different compounds were compared with those untreated by evaluating the morphological abnormalities and viability up to 48 hpf. Evaluation of DNA photodamage was assessed by quantification of apoptotic cells using Acridine Orange vital fluorescent dye. A significant increase in the viability and a decrease in the apoptotic cells percentage of the PL and Trolox treated irradiated embryos were observed. Much less photoprotective effect was observed in irradiated embryos treated with Vitamin C. Affected biochemical pathways are being studying by using cDNA microarrays (Affymetrix: 'Genechip Zebrafish Genome Arrays') and the mechanisms underlying the exposure conditions will be presented.

Our results confirm the use of zebrafish model to determine the effects of UVB radiation in terms of photoxidative stress and photodamage.

The role of the tumour suppressor LKB1 in development and cancer

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Germline mutations in the serine-threonine kinase LKB1 causes Peutz-Jeghers syndrome, a gastrointestinal hamartomatous polyposis disorder with increased predisposition to malignancies of epithelial origin, in particular of the gastro-intestinal tract. LKB1 activates AMPK under low energy conditions and that leads to growth suppression through several pathways including inhibition of the mTOR pathway. Additionally, LKB1 is associated with cell polarity. To gain insight into how LKB1 mediates its effects during development and cancer, we have identified two mutations in the single zebrafish LKB1 ortholog by TILLING. Both mutations result in a premature stop codon within the kinase domain.

Homozygous *lkb1* mutants are morphologically indistinguishable from wt siblings the first 5 days of development. From day 5 onwards, when yolk absorption is completed, we observe emaciation of the *lkb1* mutants concurrent with an abrupt collapse of the intestinal villi and flattening of the epithelium. Perhaps surprisingly, cell polarization of intestinal epithelial cells is not affected but rather resembles those of starved wild-type larvae at around 11 days post fertilization (dpf). Consistent with this finding, we have shown by histochemistry that *lkb1* mutants display defects in energy metabolism control resulting in premature starvation and death of the larvae at 8 dpf.

Currently, we are investigating which pathways are aberrant regulated in *lkb1* mutants at the molecular and transcriptional level. In addition, we recently started the analysis of adult aged *lkb1*/+ fish to establish whether LKB1 acts as a tumor suppressor also in fish.

Zebrafish as a new model to study mitochondrial disease

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Mitochondrial energy production by oxidative phosphorylation (OXPHOS) is fundamental to life. Deficiencies lead to many rare early onset neurological syndromes, but are alos involved in common disorders, like cardiac diseases and type 2 diabetes. Defects in mitochondrial biogenesis and particularly mtDNA replication are key factors in these conditions. The mtDNA replicating polymerase gamma (POLG) is crucial and POLG mutations cause a broad range of phenotypes in humans, ranging from failure to thrive, progressive external ophthalmoplegia to epilepsy and liver failure. POLG mutations possibly account for about 25% of all OXPHOS patients. Our aim is to develop a zebrafish model to investigate how mtDNA replication defects lead to OXPHOS deficiencies. An embryonic model of POLG deficiency will be established by morpholinos allowing a gene dosage dependent approach for the whole organism. To create a transgenic POLG model, the dominant-negative Y955C mutation will be expressed by transposon-mediated transgenesis. This mutation drastically lowers polymerase catalysis, induces replication errors and leads to oxidative damage in yeast and mice. Phenotypes and mitochondrial parameters will be determined based on disease manifestation in patients. In particular, heart, brain, muscle and liver will be investigated, because these organs mainly rely on OXPHOS for their energy production and are most often affected in patients with mtDNA replication defects. When established, these models are of great value to unravel the largely unknown pathological processes in mtDNA replication defects and the associated phenotypes. As no effective treatment for OXPHOS disorders exists, identification of compounds that can reduce or delay pathology is of major importance. The established zebrafish models are perfectly suited for this. Moreover, this does not only apply to the severe neurological syndromes, but also to common and age-related diseases in which mtDNA copy number and mitochondrial function is critical.

Analysis of disease associated splice factors in a zebrafish model for retinitis pigmentosa

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Retinitis Pigmentosa (RP), an inherited disease leading to blindness, is characterized by progressive rod photoreceptor degeneration with secondary loss of cone photoreceptor cells. The majority of RP-causing mutations affect proteins involved in photoreception. However, mutations in at least three constitutive mRNA splice factors, *hPrp3*, *hPrp8* and *hPrp31* have been described that lead to RP. These factors are components of the so-called U4/U6.U5 tri-snRNP particle, an essential part of the pre-mRNA splicing machinery. By screening for RP patients carrying new mutations in splice factors, we identified *hPrpf4*, another well known tri-snRNP protein as a new RP candidate gene. Biochemical analysis of mutant hPrp4 revealed defective tri-snRNP association. Furthermore we have established a zebrafish knockdown model to gain insight into the mechanism, by which defects in ubiquitously expressed pre-mRNA processing factors cause photoreceptor specific degeneration. In the zebrafish system, mild gene knockdown of *zfPrp31* or *zfPrp4* leads to severely reduced expression levels of rhodopsin, even though the remaining embryo apparently develops normally. Behavioural tests indicate that *zfPrp31* and *zfPrp4* morphants exhibit deficits in visual cognition. Thus, we could mimic the main features of RP in zebrafish larvae. In microarray analysis of *zfPrp31*-morphant retinae, a considerable number of retina-specific genes turned out to be significantly down-regulated, compared to housekeeping genes. Together our data are consistent with the idea that mutations affecting the spliceosomal activity cause RP by influencing splicing of retina specific transcripts.

Zebrafish, a new model to study rett syndrome

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Rett syndrome (RTT) is a X-linked dominant neurological disorder that affects almost exclusively females, occurring with a frequency of up to 1/10,000 live female births and causing foetal demise in males. After an early period of apparently normal or almost normal development (until 6-18 months of age), this disorder produces a profound mental disability, reduction in speech and purposeful hand movements and reduced brain growth. It is now known that approximately 80% of classic RTT patients have a mutation in the gene encoding methyl-CpG binding protein 2 (MeCP2), a transcriptional repressor involved in chromatin remodeling and the modulation of RNA splicing.

Although several murine RTT models were obtained, we propose zebrafish as a powerful vertebrate system to investigate the role of MeCP2 during embryonic development. z*MECP2* was previously mapped to linkage group 8 and it is highly similar to mammalian MECP2. Relatively high levels of its expression were found in embryos at 1 to 4 h postfertilization (hpf), after 24 hpf, and in adult brain and eyes.

Using a specific antisense morpholino oligonucleotide (*MECP2*MO) to knockdown gene activity, we observed morphological and behavioral defects resembling phenotypes in RTT. In fact, z*MECP2*MO injected embryos display spontaneous and touch-provoked motility defects. Moreover, morphants showed a strong reduction of both cephalic nervous system and otic vesicles. Midbrain-hindbrain boundary (MHB) is thin, and both midbrain and hindbrain ventricles appear inflated. A preliminary analysis of morphants with neural marker (*otx3*, *wint1*, *pax2a*) suggests that z*MECP2* is not very essential to early brain development, but it could be necessary in neuronal maturation and maintenance as described in mammals. After the loss of function of z*MECP2,* we detected that neuromuscular axons of morphants can display slight problem in the pathfinding, suggesting a function for z*MECP2* in terminal neuronal differentiation.

In order to characterize in more detail the effects of MECP2 knock-down during zebrafish embryonic development we are currently analyzing the cytological and histological structure of the nervous system and the neuromuscular synapse formation.

Modeling Frontotemporal Lobar Degeneration in zebrafish

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Frontotemporal Lobar Degeneration is the second most common form of dementia in patients under the age of 65. The Tar-DNA binding protein of 43 kDa (TDP-43) was recently identified to be the major protein species to be aggregated in familial and sporadic cases of Frontotemporal Lobar Degeneration with ubiquitin positive inclusions (FTLD-U). So far it is not known how TDP-43 aggregation contributes to the pathogenesis of FTLD-U. Either the aggregates or oligomeric intermediates confer toxicity to the neurons, or alternatively the loss of TDP-43 due to sequestration to the aggregates results in toxicity. To distinguish between these possibilities we chose the zebrafish to generate an animal model recapitulating aspects of 'TDP-43 Proteinopathies'.
In zebrafish there are two close homologues to the human TDP-43 protein, Tar-DNA Binding Protein (TARDBP) and Tar-DNA Binding Protein Like (TARDBPL). Both proteins are widely expressed during early development. To test the hypothesis if TDP-43 loss-of-function is toxic and to get further insight into its physiological function, we knocked down TARDBP and TARDBPL by antisense gripNA injections. To investigate if gain-of-function of TDP-43 is sufficient to induce aggregation and neuronal cell death we generated several transgenic zebrafish lines expressing either human wildtype TDP-43 or different mutants of TDP-43. Characterization of the TDP-43 gain- and loss-of-function zebrafish will be presented. These zebrafish models are valuable tools in understanding the pathomechanism leading to FTLD-U in humans, especially since there are currently no other animal models available.

Characterization and expression of *slc2a10*, the zebrafish ortholog of the human gene involved in arterial tortuosity syndrome

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Arterial tortuosity syndrome (ATS) (OMIM#208050) is a rare autosomal recessive connective tissue disease, characterized by widespread arterial involvement with elongation, tortuosity, and aneurysms of the large and middle-sized arteries, and pulmonary arteries stenosis. Other typical signs include characteristic dysmorphyc features and connective tissue manifestations, *i.e.*, soft, hyperextensible wrinkled skin and skeletal abnormalities. Although ATS has been recently reported to be due to loss-of function mutations in the *SLC2A10* gene, encoding for the facilitative glucose transporter type 10 (GLUT10), the role of this transporter in the pathogenesis of ATS is still unknown. We have searched for the *SLC2A10* ortholog gene (*slc2a10*) in *Danio rerio*. Computational analyses and sequencing of cDNA from adult fishes and embryos at different developmental stages, allowed us to define the genomic organization of *slc2a10* and his entire coding sequence. The gene, located on chromosome 11, has an exons/introns structure comparable to that of the human counterpart, consisting of five exons separated by four introns. The consensus open reading frame (1.542 bp) encodes a polypeptide of 513 amino acids. The zebrafish *glut10* protein shares a 45% sequence identity with the human protein, maintains the typical 12 transmembrane hydrophobic domains structure and shares the signature sequence motifs involved in glucose transport activity. RT-PCR analysis in adult zebrafish tissues revealed that *slc2a10* gene is expressed in liver, heart, gills, eyes, brain, spleen, swim bladder, ovary and caudal fin. Quantitative real-time PCR analysis showed that *slc2a10* is a maternal gene, since the transcript is present already in the zygote and in the early embryonic stages and decreases from the segmentation onset to the early larval stages. By *in situ* hybridization we observed that the *slc2a10* expression is ubiquitous until mid-somitogenesis. However, at 13.5-14 hpf (8-10 somites) the signal appeared to be restrict to the otic placode and to the notochord, whereas at 19 hpf (20-25 somites) the expression was observed in the caudal notochord and remained up to 1-day-old embryos. In 48 hpf embryos, a distinct hybridization signal was detected in the otic vesicle; in 4-day-old *larvae* the *slc2a10* transcript was present in the cardiac region. These data suggest that *glut10* should play a role in vascular development in zebrafish.

Analysis of Alzheimer's disease induced neurotoxicity using zebrafish

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Alzheimer's Disease (AD) is the most common form of dementia and is characterized by Aβ protein aggregates, intracellular neurofibrillary tangles and neuropil threads, which consist of the microtubule-associated tau protein, and neuronal degeneration. Familial Alzheimer's Disease (FAD) mutations, which can be found in the β-Amyloid Precursor Protein (hAPP) or in one of its proteases, Presenilin1 or 2 (hPS1/hPS2) lead to an increase of $A\beta_{42}$, which is more prone to aggregation. It is still unclear how the amyloid burden induces neuronal cell death and if Aβ oligomers or aggregates are the neurotoxic species. Moreover the role of the Tau protein and its aggregates is not fully understood.
We chose the zebrafish as an animal model to overexpress high amounts of hAPP or hTau respectively to study aspects of AD *in vivo*. We generated zebrafish stably overexpressing $hAPP^{swe}$ or $hTau^{P301L}$ in combination with DsRed as a fluorescent marker protein under the control of the neuronal promotor HuC. To further increase the Aβ burden and to induce tau pathology at the same time, we generated a quadrouple transgenic fish overexpressing $hAPP^{swe}$, $hPS1^{L166P}$, $hTau^{P301L}$, and DsRed. To distinguish between toxicity induced by Aβ and Tau we will challenge the transgenic fish to promote either aggregation of Aβ or to enhance aggregation of Tau protein. Progress in the identification of Aβ or Tau induced neurotoxicity will be presented.
In summary, these transgenic zebrafish lines will serve as a valuable tool to monitor the effect of high amounts of APP and Tau protein *in vivo*.

Dynamin-2 Function in Embryogenesis and Skeletal Muscle Formation
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Dynamins are mechanochemical GTPases involved in a wide variety of cell and organelle fission events. Classical dynamins are critical components of clathrin-mediated endocytosis, where they contribute to the release of newly-formed endosomes. Recently, mutations in dynamin-2 were found to cause two human neuromuscular disorders, a dominant form of centronuclear myopathy and a dominant form of hereditary motor and sensory neuropathy. While dynamin-2 has been extensively studied in intracellular processes, its specific function in nerve and muscle development is unclear. In order to examine this question, we looked at the zebrafish homologue of human dynamin-2. In this study, we used morpholino-mediated knockdown of dynamin-2 to determine the effect of dynamin-2 deficiency on developing zebrafish embryos. Overall, dynamin-2 knockdown caused global abnormalities, including a shortened body structure, small muscle compartments and underdeveloped head and eyes. It also resulted in severe edema within the first 48 hours of life, despite the presence of cardiac contractions.
Our subsequent analysis focused on skeletal muscle development during embryonic and early larval stages. Behavioral analysis revealed severe motor defects in dynamin-2 morphant embryos and larvae. At 24 hpf, spontaneous coiling is severely reduced or absent in dynamin-2 morphant embryos. At 48 hpf, morphant embryos show reduced response to mechanosensory stimulation. Immunohistochemistry revealed general disorganization and hypotrophy of individual larval muscle fibers. Notably, ultrastructural analysis showed significant disorganization of both the sarcoplasmic reticulum and transverse (T-) tubules. We hypothesize that dynamin-2 plays a critical role in the formation and maintenance of the tubuloreticular network in muscle cells, and that disruption of this function leads to the phenotype seen in patients with centronuclear myopathy. We are currently generating transgenic zebrafish that express dynamin-2 mutations associated with centronuclear myopathy. The analysis of dynamin-2 and associated proteins in zebrafish muscle offers a highly promising model for the study of centronuclear myopathy, and will lead to a greater understanding of the cellular dysfunction that underlies this disease.

A novel functional role of iduronate-2-sulfatase in zebrafish early development

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Sulfated glycosaminoglycan chains of extracellular matrix and cell membrane-tethered proteoglycans exert specific cellular functions by interacting with a broad spectrum of morphogens and growth factors.
In humans, a congenital impaired catabolism of sulfated glycosaminoglycans is associated with severe metabolic disorders. Here, we report on the identification and characterization of a zebrafish iduronate sulfatase orthologue. By knocking down its function with antisense morpholino oligos, we demonstrate that iduronate sulfatase plays a critical role during early vertebrate development and its downregulation may be responsible for severe developmental defects, including a misshapen trunk and abnormal craniofacial cartilages. We show that the altered cartilage patterning is mediated by depauperation of *sox10*-expressing neural crest cell precursors. Through the application of a transactivation reporter assay, we also provide a molecular proof that increased TGFβ signalling is tightly associated with downregulation of iduronate sulfatase function. Our results provide important insights into the mechanisms underlying lysosomal storage disorder pathogenesis.

Role of Collagen XVIII in eye development

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Collagen XVIII is a non-fibrillar collagen with heparan sulfate side chains that occurs in wide variety of tissues in association with basement membrane. Three isoforms (short, intermediate and long) differing by their N-terminal domains are obtained from two distinct promotors. Mutations in short variant of human collagen XVIII cause Knobloch syndrome, a recessive disorder with high myopia and vitreoretinal degeneration. The lack of type XVIII in col18a1-null mice results also in ocular abnormalities.

We previously reported on the expression pattern of collagen XVIII in zebrafish from segmentation to hatching period using a probe complementary to the endostatin domain which is common to the three variants. Several tissues such as central nervous system, notochord, pronephric ducts were found to express collagen XVIII mRNA (1). We further showed transcript expression in the developing eye at 24 hpf and 72 hpf. In preliminary knockdown experiments, injection of MOs against the three variants resulted in decreased eye size of the embryos suggesting a role for collagen XVIII in zebrafish eye development.

The Fgf signalling which requires HSPG (heparan sulfate proteoglycan) binding for activity was also shown to play a central role in the differentiation of the retina. At 24 hpf, Fgf8 colocalizes with collagen XVIII in some structures important for the eye development such as the MHB and the optic stalk. The aim of our study was thus to investigate the functional consequences of collagen XVIII morpholino-knockdown on the eye development and the potential link with Fgf signalling.

The lack of all 3 isoforms did not affect the expression of Fgf8. However, the transcription factor pea3, a target of Fgf8 signalling, was specifically reduced in an area around the lens in 24 hpf and 48 hpf morphants. The differentiation of retinal ganglion cells was also disturbed in collagen XVIII MOs injected-larvae. This phenotype was similar to the retina phenotype observed in zebrafish larvae exposed to the FGFR inhibitor SU5402. A histological study confirmed a disorganization of retinal cells in 72 hpf injected-larvae. Finally, the vascularization of the eye was also significantly reduced.

Our study suggests that collagen XVIII may control Fgf signalling pathway possibly through the interaction between its heparan sulfate chain and thus confirms the importance of a link between HSPG and Fgf in the development of organs such as the eye.

(1) Haftek Z *et al.*, Gene Expr Patterns. 2003 Jun;3(3):351-4.

polb^{ne2385} – a novel zebrafish model for neurodegeneration?

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Genomic DNA in all cells of the body is constantly exposed to endogenous and exogenous events leading to DNA damages, which, if not repaired, can cause genetic mutations, instability of the genome and cell death. The major endogenous cause of damages to the DNA is oxidative stress due to production of reactive oxygen species during normal metabolic activity of the cell. To repair damages of sugar and base groups the cells are provided with a robust repair machinery, of which base excision repair (BER) and nucleotide excision repair(NER) are two major pathways. Key enzymes in these pathways include DNA glycosylases, AP endonucleases, DNA polymerases and DNA ligases.

Some forms of cancer, premature ageing and neurodegenerative diseases have been linked to defects in important components of the DNA repair machinery. Certain cell types, in particular some types of neurons, are more vulnerable and undergo degeneration to a larger extent than others. Today it is not known what is causing this increased sensitivity, but intrinsic properties, such as transmitter content, higher levels of metabolic activity leading to production of more reactive oxygen species and/or different capacity to buffer for oxidative stress, of these neurons could provide one explanation. These intrinsic properties may, in turn, induce higher levels of DNA damage, and can therefore explain why some neurons are affected more and/or earlier during the degeneration process if the DNA repair machinery is not functional.

Here we describe a novel DNA polymerase beta mutant (*polb*^{ne2385}) identified in an *in situ* hybridization-based ENU screen. This mutant carries a point mutation leading to the splicing-out of exon 2 and a frame-shift. We show that this mutation triggers *polb* mRNA degradation, thus generating a null phenotype. Homozygous larvae show abnormal morphological and behavioural phenotypes including weak escape response and they die after about one week. Expression of dopaminergic and serotonergic markers is reduced, while markers for other neuronal populations, as well as early brain patterning markers, are unchanged. Thus, this mutant shows a specific loss of some neuronal identities. We are currently exploring whether it can be used as a novel model for selective neurodegeneration to elucidate some of the possible mechanism behind this group of diseases.

Analysis of cohesin and condensin genes during zebrafish development

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During cell division, the multi-subunit complexes cohesin and condensin have important roles in sister chromatid cohesion and chromosome condensation, respectively. Mutations in the cohesin subunits *SMC1A* and *SMC3*, as well as mutations in the cohesin loading factor *NIPBL*, have been shown to cause the genetic disorder Cornelia de Lange Syndrome (CdLS) in humans. CdLS patients have severe developmental abnormalities, including facial dysmorphism, growth retardation, upper limb anomalies, and mental retardation. It has been shown recently that cohesin does not only function in cell division, but also has key roles in tissue-specific development, such as axon guidance and pruning, skeletal patterning, and hematopoiesis. The mechanism underlying is so far poorly understood, but it has been suggested that cohesin proteins may be involved in transcriptional regulation of developmental genes.
To further understand non-canonical roles of cohesin, we analyzed the expression of cohesin subunits in the developing zebrafish embryo and compared it with expression of condensin subunits and the distribution of proliferating cells. Cohesin and condensin subunits are dynamically expressed in a similar, but not identical pattern, indicating a diverse function of the two complexes. The expression pattern of cohesin subunits does not completely overlap with zones of proliferation, supporting the existence of non-proliferative roles. These results suggest that while both complexes are involved in cell division, alternative developmental functions that may underlie CdLS are possible.

Depletion of zebrafish fukutin family protein activities extends the phenotypic spectrum from dystroglycanopathy to lamininopathy

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Muscular dystrophies associated with hypoglycosylation of alpha-Dystroglycan are commonly referred to as dystroglycanopathies, in which mutations affect at least six known or putative glycosyltransferase genes, including *POMT1*, *POMT2*, *POMGnT1*, *fukutin*, *fukutin-related protein (fkrp*) and *LARGE*. Allelic mutations in each of these genes can lead to a wide spectrum of clinical severity, ranging from a congenital onset of severe muscular dystrophy with brain malformation to a late onset of milder limb-girdle muscular dystrophy without mental retardation. To date, POMT1, POMT2 and POMGnT1 have been shown to catalyze the *O*-glycosylation of alpha-Dystroglycan. Over-expressing LARGE circumvents glycosylation defects in cells from some dystroglycanopathy patients, suggesting that LARGE may stimulate an independent sugar modification. The function of Fukutin and FKRP, however, as well as the pathological mechanisms leading to the wide range of clinical severity still remains unclear.

To model dystroglycanopathy in zebrafish, we have recovered a novel *dystroglycan* nonsense allele and inhibited Fukutin family protein function by low-to-high doses of antisense morpholino oligonucleotides (MOs). We show that removal of zebrafish Fukutin or FKRP not only causes reduced glycosylation of alpha-Dystroglycan but also elicits notochord defects associated with loss of Laminin function. Importantly, a complete absence of Dystroglycan leads to loss of Dystrophin localisation, but not loss of Laminin function. The absence of Laminin localisation in *fukutin* or *fkrp* MO-injected embryos is mainly a consequence of defective post-translational processing. Taken together, our results strongly suggest that Fukutin family proteins play a pivotal role on the secretion of Laminin, possibly through a novel glycosylation mechanism, and that loss of Laminin function together with hypoglycosylation of alpha-Dystroglycan underlies the wide clinical spectrum of some forms of muscular dystrophies.

The zebrafish histaminergic system: telencephalic projections, co-transmitters and afferent innervation

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Histamine is a modulatory neurotransmitter involved in e.g. psychiatric disorders and neurodegenerative diseases. Learning, memory, anxiety and sleep are functions regulated by histamine and other neurotransmitters. In zebrafish, lateral telencephalic pallium is proposed to be homologous to the mammalian hippocampus, selectively involved in spatial learning and memory. The medial telecephalic pallium is considered homologous to the mammalian amygdala processing emotions, e.g. anxiety-like behavior. 3D confocal microscopy combined with immunohistochemistry of larval zebrafish brains showed that histaminergic fibers projected from the caudal zone of periventricular hypothalamus to the telencephalon, where some fibers crossed at the commissura anterior to contralateral side to innervate the central part of dorsal telecephalic together with the ipsilateral fibers. Histamine-producing neurons are found exclusively in the hypothalamus which is known to regulate body temperature, hunger, thirst and circadian rhythm. In order to understand the underlying mechanisms of and the role of histamine in these different physiological functions, we identified co-transmitters of the histaminergic neurons in the posterior hypothalamus of zebrafish by immunohistochemistry. In adult zebrafish GABA, galanin and thyrotropin releasing hormone (TRH) were co-localized with histamine in some neurons. TRH-immunoreactive fibers made close contacts with histaminergic neurons. Similar innervation pattern was observed between the histaminergic neurons and the fibers containing the endogenous opioid peptide, met-enkephalin. No met-enkephalin was observed in histaminergic neurons. Taken together, we show that histamine is co-localized with GABA, galanin and TRH in zebrafish which is in accordance with previously published data from rodents. Galanin, GABA, TRH and met-enkephalin are also known to be involved in mediating the physiological functions of hypothalamus, suggesting that together with histamine these neurotransmitter systems can modulate the response of hypothalamus to different stimuli. Previous studies have shown that manipulating the histaminergic system affects learning and memory. Here we present results that are in accordance with those as we show that histamine fibers innervate dorsal telencephalon, an area involved in cognition.

A zebrafish model of charcot-marie-tooth 2D

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Cardiac valves derive from endocardial cells and function throughout the life of vertebrates to prevent retrograde blood flow. In a large scale ENU mutagenesis screen, we identified a number of mutants in discrete stages of valve development. In one of this lines, we named *metronome,* homozygous mutants show retrograde blood flow from 72 hours post fertilization (hpf) onwards, as a result of an impaired cardiac valve development. In parallel, these mutants become progressively immotile by 96 hpf. We found that although filamentous actin is present, the myofibrils of *metronome* mutants lack the organised fibrillar arrangement seen in wild type siblings. We mapped the mutation with positional cloning and we located it in clone DKEY-276I5 in linkage group 24. We have found that *metronome* carries a recessive point mutation in the glycyl t-RNA synthetase (*gars*). Mutations in the human orthologue of *gars* are responsible for an adult onset distal neuropathy, Charcot Marie Tooth disease (CMT) type 2D and spinal muscular atrophy type V. The mutation in our animal model (T130K) resides next to a previously described human mutation (L129P). Aminoacyl-tRNA synthetases have a broad repertoire of functions beyond translation (they catalyze the first committed step of protein synthesis by linking an aminoacyl to its respective tRNA), including transcriptional and translational regulation as well as cell signalling.

We have phenocopied the *metronome* phenotype by morpholino injections in wild type embryos. *gars* T130K does not rescue GRS1 mutant yeast and is not associated with cytoplasmic granules in mouse motor neurons cells in vitro, similar to the most severe human mutations (in collaboration with Antony Antonellis and Eric Green, NHGRI, NIH). Using an anti-human (GARS) antibody we have shown that there is a maternally provided protein in the homozygous mutant embryos, which explains why there is no obvious phenotype up to 60 hpf. In parallel, we have indications that T130K interferes with the proper dimerization of GARS, providing an insight in the pathophysiology of CMT. Moreover, we have shown that *gars* is highly expressed during the developing brain outlining the ventricles, besides a ubiquitous basal expression.

We will present our latest data on the mechanism of GARS function in this in vivo model of CMT2D.

Cardio-facio-cutaneous syndrome alleles are active during zebrafish development and are sensitive to small molecule inhibitors

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The Ras/MAPK pathway is critical for human development, and plays a central role in the formation and progression of most cancers. Children born with germ-line mutations in BRAF, MEK1 or MEK2 develop cardio-facio-cutaneous (CFC) syndrome, an autosomal dominant syndrome characterized by a distinctive facial appearance, heart defects, skin and hair abnormalities, and mental retardation. CFC syndrome mutations in BRAF promote both kinase-activating and kinase-impaired variants. CFC syndrome has a progressive phenotype, and the availability of clinically active inhibitors of the MAPK pathway prompts the important question as to whether such inhibitors might be therapeutically effective in treatment of CFC syndrome. To study the developmental effects of CFC mutant alleles *in vivo* we have expressed a panel of 28 BRAF and MEK alleles in zebrafish embryos to assess the function of human disease alleles and available chemical inhibitors of this pathway. We find that both kinase-activating and kinase-impaired CFC mutant alleles promote the equivalent developmental outcome when expressed during early development. BRAF CFC mutations promote an additive effect during development, consistent with both the kinase-active and kinase-impaired BRAF CFC mutations acting as gain-of-function mutations during development.

Treatment of CFC-zebrafish embryos with inhibitors of the FGF-MAPK pathway can restore normal early development. Importantly, we find a developmental window in which treatment with a MEK inhibitor can restore the normal early development of the embryo, without the additional, unwanted developmental effects of the drug. CFC syndrome has a progressive phenotype, and as many of the phenotypic effects develop postnatally, patients may be helped by systemic therapies after birth. Our work shows the zebrafish system as a tractable tool for medical and research geneticists to explore allele activity and therapeutic potential, and establishes a foundation to propel forward the clinical discussion and scientific strategy for assessing the suitability of using currently available cancer drugs to treat the progressive phenotypes of CFC in children.

Down regulation of Hccs in medaka recapitulates the phenotype observed in Microphthalmia with linear skin lesions (MLS) syndrome

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The Microphthalmia with linear skin defects (MLS) syndrome is an X-linked dominant male-lethal neuro-developmental disorder associated to mutations in the holocytochrome c-type synthetase (*HCCS*) transcript. Female patients display unilateral or bilateral microphthalmia and linear skin defects, additional features include CNS malformation and mental retardation. *HCCS* codifies a mitochondrial protein that catalyzes the attachment of heme to both apocytochrome c and c1 necessary for proper functioning of the mitochondrial respiratory chain. The molecular mechanisms underlying the eye and brain developmental anomalies in this disease are still unknown. Previous studies demonstrated the early lethality of mouse embryonic Hccs knock-out stem cells. We thus decided to generate a model for this disease in medaka fish (*Oryzia latipes*). This model will allow us to overcome the possible embryonic lethality using graded concentrations of either the mutated mRNA or the morpholinos. Specific morpholinos directed against the *HCCS* transcript have been designed and injected. Our experiments have determined that the morpholinos effectively downregulate the expression of the *olhccs* gene. Gain of function studies did not result in an aberrant phenotype. Instead injection of both the mutated protein (dominant-negative) and two different morpholinos resulted in a pathological phenotype, which resembles the human condition. As expected, morphants displayed microphthalmia, coloboma, and microcephaly. In addition, unexpectedly, absence of blood pigmentation was also observed. Analysis with specific markers (e.g, Pax6, Six3.2, Otx2, Rhodopsin, Crx, Chx10, Ath5, Syntaxin) showed an abnormal formation of the retinal-pigmented epithelium (RPE) and defects in differentiation of the ventral neural retina. RNA *in situ* hybridization studies revealed an abnormal domain of expression for connexin43, recently shown to be important for cell proliferation and apoptosis. Tunel and pHH3 assays on morphants, revealed abnormalities in cell proliferation and programmed cell death. Altogether this data suggest a possible role of these mechanisms in the pathogenesis of MLS syndrome and further studies are ongoing to explain the pathogenetic link between Hccs inactivation and the eye abnormalities observed in morphants and affected patients.

Functional characterization of the *SLC7A6OS* gene in *danio rerio*

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SLC7A6OS is a novel protein coding gene representing a natural antisense transcript (NAT) of *SLC7A6* (SoLute Carrier family 7 member A6) in the human genome.
RT-PCR studies demonstrated that *SLC7A6OS* is expressed in all tissues and cell lines we tested. We failed to obtain experimental evidence for a co-regulation of the *SLC7A6/SLC7A6OS* cis-NAT gene pair in cell models. Transient expression in HeLa cells demonstrated that SLC7A6OS is a soluble protein mainly localized in the cytoplasm. While the *SLC7A6OS* gene is conserved during evolution, the encoded protein shows no significant sequence identities with other polypeptides of known functions in vertebrates. Although we failed to identify relevant protein domains or functional sites that might provide clues about the biological role of this polypeptide, a low but probably significant level of sequence identity has been detected with *Saccharomyces cerevisiae Iwr1*. *Iwr1* is a factor that associates to RNA polymerase II through the binding to RBP3 (RNA polymerase II subunit 3). This finding suggests that SLC7A6OS may play a role in the regulation of gene expression.
More recently, we decided to study the biological role of *SLC7A6OS* in *Danio rerio*. We have identified the putative *slc7a6os* zebrafish ortholog on chromosome 7 adjacent to *slc7a6*, although there are no evidences that these two genes are NATs in the genome of the teleost. *Slc7a6os* zebrafish protein is 46% identical to the human counterpart and it also shows a cytoplasmic localization when transiently expressed in HeLa cells. Real time RT-PCR studies demonstrated that *slc7a6os* is a maternal gene, expressed from the zygote stage to the adult fish. Whole mount *in situ* hybridization experiments showed that *slc7a6os* is expressed predominantly in the central nervous system during embryogenesis, and also in the thymus and neuromast of lateral line, during the hatching period. We have investigated the function of the *slc7a6os* gene by morpholino-induced gene knockdown. The phenotypes observed in injected embryos suggest that *slc7a6os* is required for early neural development.

Understanding PINK1 mechanism by gene expression arrays

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One of the challenges in Parkinson's disease (PD) is identifying the biochemical pathways affected by genetic alterations in some key genes causing hereditary PD. Mutations in the *PINK1* gene that cause autosomal recessive PD have been described. The Pink1 protein product localises to the mitochondria and cytoplasm. The function of *PINK1* is not clearly known but it is a protein kinase, mutations of which enhances sensitivity to ubiquitin proteasome system inhibitors and lowers the threshold to apoptotic cell death. PINK1 mutations may lead to mitochondrial dysfunction and increased sensitivity to cellular stress through a defect in the apoptosis pathway.

This study aims at finding novel targets for Pink1. Microarrays on small amounts of RNA can give useful unbiased information of pathways and genes involved. In order to understand the mechanism and different pathways that are altered after PINK1 knockdown with the morpholino oligonucleotides and performed a microarray experiment. A set of four replicates from four different sets of fish were made and hybridised on two-colour gene expression arrays from Agilent. Data was analysed using the R and IPA program programs.

We found 237 genes being significantly altered in PINK1 morphants compared to the controls (level of confidence: $p>0.01$ and log fold change values from -1.6 to +0.9). When the 237 genes were organized into functional categories in biological process pathway by their GO terms, the pathways that were identified as significantly affected (cut off: more than 50% of the genes altered) are: specification of organ axis polarity, cerebellum formation, hindbrain formation, cerebellum development, cerebellum morphogenesis, glutathione biosynthetic process, specification of axis polarity and amoeboidal cell migration. Most of these pathways have links to different forms of PD. The pathways that were significantly altered ($p>0.04$) based on the commercially available IPA program were cholesterol biosynthesis, oxidative stress, TGF-beta signalling, hypoxia inducible factor signalling pathway, apoptosis and p53 signalling and mitochondrial dysfunction. PINK1 knockdown in zebrafish thus alters several important pathways, which will enable detailed studies on individual genes and novel mechanisms.

Identification and characterization of the putative co-orthologs of *MCOLN1*, the gene mutated in mucolipidosis type IV

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Mucolipidosis type IV (MLIV, MIM 252650) is an autosomal recessive lysosomal storage disorder that causes mental and motor retardation as well visual impairment. MLIV is caused by mutations in the *MCOLN1* gene, which codes for mucolipin-1 (TRPML1), a member of the large family of transient receptor potential cation channels (TRP). In mammals, the mucolipin family includes other two members: mucolipin-2 (TRPML2) and mucolipin-3 (TRPML3) encoded by *MCOLN2* and *MCOLN3* genes, respectively. We have identified in the past the gene responsible for MLIV and subsequently we started to study the pathogenetic mechanism of the disease. More recently, we decided to study the biological role of the *MCOLN1* gene in zebrafish. A zebrafish transcriptome- and genome-wide search using the sequences of the MCOLN polypeptides revealed the presence of five different fish genes related to human mucolipins. Being our interest focused on MLIV, we concentrated our efforts on the functional characterization of *mcoln1.1* and *mcoln1.2*, the putative co-orthologs of human *MCOLN1*. Transient-expression experiments in human HeLa cells demonstrated that fish *mcoln1.1* and *mcoln1.2* proteins, similarly to human mucolipin-1, localize to late endosomal/lysosomal compartments. Whole mount *in situ* hybridization experiments show interesting peculiarities in terms of expression pattern of the two zebrafish genes during development. In particular, the expression of *mcoln1.1* in the intermediate cell mass (ICM), suggests that this gene, like the human TRPML1 counterpart, may play a role in iron metabolism. Morpholino-mediated knockdown of zebrafish *mcoln1.1* causes alterations in vascular development. A more detailed phenotypic characterization of *mcoln1.1* morphants is currently underway. Morphological effects due to the ablation of *mcoln1.2* function are also being investigated.

A Fish Model for Duchenne Muscular Dystrophy

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In humans, mutations within the structural muscle gene *Dystrophin* (*DMD*) lead to Duchenne Muscular Dystrophy, one of the most common, lethal disorders. Currently, the mouse null mutant for *dmd*, the *mdx* mouse (Hoffman et al., Cell 1987), is the mainly used model for Duchenne Muscular Dystrophy, even though the phenotype of the *mdx* mouse is extremely mild and does not reflect the human condition.

Our laboratory identified the *sapje* (dmd^{ta222a}) zebrafish mutant as a carrier of a null mutation in the orthologous *dmd* gene (Bassett et al., Development 2003). Further survey of the phenotype revealed that the progression of the muscle atrophy is comparable to that observed in humans. We documented extensive skeletal muscle wasting throughout the larvae lifespan of dmd^{ta222a} homozygotes. Necrotic myofibres are replaced by mono-nucleated infiltrates, interstitial connective tissue, as well as neutrophils, indicative for inflammation. In addition, dmd^{ta222a} homozygous mutants are characterised by progressively exhausting muscle precursors and broader variation of muscle fibre cross sectional areas. Due to its profound phenotype, the dmd^{ta222a} fish closely resembles symptoms of the human disease.

We performed a non-complementation screen that led to the identification of novel *dmd* null alleles. The mutations of the three novel *dmd* alleles (dmd^{pc1}, dmd^{pc2}, and dmd^{pc3}) encode premature stop codons causing loss of the gene product. Two of these mutants (dmd^{pc2} and dmd^{pc3}) carry a null mutation in exons (32 and 34, respectively) that are located in the highly repetitive rod domain, which is partially disposable for the function of Dmd. In addition, exclusion of either of these exons from the mature mRNA does not disrupt the open reading frame, giving us the unique opportunity to perform exon skipping experiments in live *dmd* zebrafish mutants. Two exon skipping protocols are presented: one that induces a mild skipping of exon 32 that does not lead to a phenotype rescue, and another one that induces strong skipping of exon 32, leading to a rescue of the dmd^{pc2} homozygous phenotype. The data suggest that, due to the severity of the *dmd* phenotype, we are in the unique position of comparing different levels of rescue and thereby using the *dmd* zebrafish as a model to systematically test therapeutic strategies for treatment of Duchenne Muscular Dystrophy.

Glia-Neuronal Interactions and Myelination in Zebrafish
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Myelination is a tightly regulated process that requires communication between neurons, myelinating and non-myelinating glia. To illuminate these communication processes, we work with the zebrafish as model organism. Due to its transparent, fast and extra-maternally developing larva, this species is particularly suitable to visualize complex cellular interactions in live imaging experiments. We follow two strategies to investigate the contribution of astrocytes to the formation and maintenance of myelin.
In the first approach, we aim to genetically ablate astrocytes at specific time points. We created transgenic zebrafish expressing diphtheria toxin subunit A (DTA) under the control of an astrocyte specific promoter. Expression of the toxic protein is induced via the cre/loxP system which in turn is heat inducible. Ablation of astrocytes resulted in deformation of the body axis and characteristic "curly tail" phenotypes, as well as an increased level of cell death in deformed zebrafish. Currently, we are investigating the ablated larvae via immunohistochemistry to investigate effects on neurons and glia after DTA induction in astrocytes.
In a second approach, we use the zebrafish as model organism for the leukodystrophy Alexander disease (AXD), a very rare but severe human genetic disease. AXD is characterized by rapid and widespread white matter degeneration. On a molecular level, mutations in the gene for glial fibrillary acidic protein (GFAP), an astrocyte specific protein, are causal for the disease. It is unknown how a primary defect in astrocytes causes extensive loss of white matter. In AXD patients, mutated GFAP is the main component of intracellular aggregates called Rosenthal fibers. To monitor aggregation dynamics and possible clearance mechanisms, we established transgenic zebrafish lines expressing wildtype and mutant GFAP fused to eGFP as a reporter. GFP positive aggregates were detectable with onset of GFAP expression. We found that formation of the aggregates is a dynamic process: aggregates are mobile in astrocytes, dissolve and re-aggregate during cytokinesis. Currently, we are investigating effects of various drugs on clearance of the aggregates. Additionally, we are performing biochemical and EM analysis to show different aggregation states and potential degradation products.

Functional characterization of mutant TDP-43 in zebrafish

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Amyotrophic lateral sclerosis (ALS) is an adult-onset rapidly progressing neurological disorder characterized by loss of motor neurons, cells which control muscle movement. Approximately 10% of ALS cases are familial (FALS). Recently, we made the breakthrough discovery of a considerable number of *TARDBP* mutations in FALS and sporadic ALS (SALS) patients, the gene encoding TDP-43, the protein that aggregates in inclusion bodies from the spinal cord of ALS patients. For this project, we have developed a series of human TDP-43 cDNA constructs tagged at both the C-terminus and N-terminus which I will use to determine mutant and WT TDP-43 expression in cell lines, primary motor neurons and zebrafish embryos. Here we demonstrate that mutant TDP-43 causes a specific motor neuron disorder through a toxic gain of function since expression of mutant TDP-43, but not WT, causes selective motor neuron toxicity in primary spinal cord cultures. Further, expression of mutant causes a specific motor phenotype and motor neuron axonal defects in zebrafish embryos. On the other hand, decreased expression using anti-sense morpholino oligonucleotide (AMO) of TDP-43 yielded a similar phenotype in zebrafish; thus a specific loss of function may also be involved in the selective vulnerability of motor neurons to mutant TDP-43. A further characterization of these models will allow us to unravel molecular partners of mutant TDP-43 as well as to allow a better understanding of specific mechanisms of disease involved in mutant TDP-43 caused motor neuron degeneration. We are also generating *Tardbp* knock-outs in zebrafish as well as mutant TDP-43 transgenic fish lines. These lines will be functionally characterized to determine whether they properly model motor neuron disorders. These lines can then be tested for the screening of a large number of pharmaceutical compounds in transgenic zebrafish expressing mutant TDP-43, thus directly opening avenues for the development of therapies that could delay or prevent disease onset and progression in all ALS patients.

Zebrafish mitofusin-2 knockdown: a new model for CMT2A neuropathy?

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Mitofusin 2 (MFN2) is a large dynamin-like GTPase protein, located in the outer membrane of mitochondria and at the surface of the endoplasmic reticulum. Mfn2 is essential to maintain the organization of mitochondria in cells and it may play a role in the modulation of calcium exchanges between these two cellular compartments. MFN2 gene mutations are associated with Charcot-Marie-Tooth disease type 2A (CMT2A), an hereditary axonal neuropathy characterized by distal muscle weakness and atrophy.
To investigate the function of this gene and its role in the pathogenesis of CMT2A, we have identified the zebrafish MFN2 ortholog and used a morpholino antisense oligonucleotide to knockdown mitofusin function. Morphant embryos showed motor impairment. Morphologically they displayed curved tails, disorganized somites, small and underdeveloped eyes and an extensive dilatation of the brain ventricles. Interestingly, this phenotype resembles the central nervous system abnormalities and optic atrophy observed in some CMT2A patients. Shorter and abnormal motor neuron axons were observed on 48 hpf morphant larvae, suggesting that these alterations are likely related to the movement deficits observed in morphants at this stage.
Our preliminary results suggest that the abnormalities observed in MFN2 morphants are consistent with the human pathology. Given that a reliable animal model for CMT2A is still lacking, we propose zebrafish as a new and useful tool to dissect the pathogenetic mechanisms underlying CMT2A.

Nephrocyatin-4 is a ciliary protein required for control ao wnt/b-catenin versus wnt/pcp balance and ciliogenesis in zebrafish embryos

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Nephronophthisis, a heterogeneous group of autosomal recessive cystic kidney disease, is caused by mutations in *NPHP* genes (*NPHP1-11*), encoding nephrocystins. The nephrocystins share a common subcellular localisation at the primary cilium. Primary cilia play a pivotal role in regulating canonical Wnt/β-catenin and non canonical Wnt/PCP (planar cell polarity) pathways. Wnt/PCP is required for polarized convergent extension (CE) movement during gastrulation. The cytoplasmic protein Dishevelled (Dvl) is the central activator of both Wnt pathways. Proper cilia structure and motility are required in the Kuppfer's vesicle to establish left-right asymmetry, and in the pronephros for effective urinary excretion.

Here we investigated nephrocystin-4 function *in vivo,* by loss of function experiments using two independent morpholinos® against *nphp4* in zebrafish embryos. Both morpholinos® led to abnormal CE during gastrulation, left-right asymmetry defects, and pronephric cysts.

The CE phenotype suggests that *nphp4* interacts with the Wnt/PCP pathway. We showed genetic interaction of *nphp4* with a core Wnt/PCP gene, *vangl2*. A specific activator of the Wnt/PCP, myristilated-palmitoylated Dvl2, rescued the CE phenotype. Conversely, we checked whether nephrocysin-4 regulates the canonical Wnt pathway. Using the β-catenin reporter TOPFLASH in HEK293 cells, we showed that nephrocystin-4 overexpression led to a significant decrease in β-catenin activation. We also demonstrated that nephrocystin-4, inversin (*NPHP2*) and Dvl2 are part of the same protein complex.

To investigate if the laterality defects and pronephric cyst formation were linked to ciliogenesis defects, we measured cilia length and motility. Cilia length was significantly reduced in monociliated cells of the Kuppfer's vesicle and pronephric duct of *nphp4*-morphants, supporting a role of nephrocystin-4 in ciliogenesis. However, cilia motility of multiciliated cells was preserved in pronephric cysts, arguing against an altered cilia-driven fluid flow. In most of *nphp4*-morphants, pronephric cysts are related to obstruction of the cloaca.

Altogether our data support a role for nephocystin-4 as both an activator of the Wnt/PCP pathway and a repressor of the Wnt/β-catenin pathway, possibly via its interaction with Dvl. Moreover, nephrocystin-4 is required for ciliogenesis in the Kuppfer's vesicle and the pronephric duct.

Modelling muscular dystrophy and exploring therapeutic strategies in zebrafish

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Mutations in the human Laminin-alpha2 (*LAMA2)* gene result in the most common form of congenital muscular dystrophy, MDC1A. We have recently identified a zebrafish model of MDC1A called *candyfloss* (*caf*), which carries a loss-of-function mutation in the zebrafish *lama2* gene. Homozygous *caf* mutants display a progressive muscle detachment phenotype, which on a cellular level closely resembles that of human MDC1A patients.

We have compared the pathomechanisms leading to muscle fibre detachment in the *caf* model with the previously characterized dystrophin-deficient sapje (*sap*), a zebrafish model for Duchenne`s muscular dystrophy. Fibre loss in *sap* is due to disruption of the Dystrophin Glycoprotein Complex (DGC), which leads to sarcolemmal rupture and subsequent necrosis. In contrast, fibre retraction in *caf* is caused by attachment failure of the extracellular matrix to the sarcolemma, which itself maintains its integrity. Using single fibre labelling techniques we show that in *caf* mutants, the majority of retracted muscle cells remain viable but non-functional for several days after detachment before undergoing apoptosis. Despite this delayed muscle fibre death, the overall number of affected fibres in *caf* is greater than in *sap*, leading to a more severe phenotype with earlier mortality. Lastly, since fibre loss in *caf* is ultimately due to apoptosis, we use a combination of pharmacological and genetic approaches to investigate the potential of manipulating apoptosis for the treatment of the *caf* dystrophic phenotype.

The zebrafish model for the study of neurotoxins mechanisms

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This project aims at studying the molecular mechanisms of action of three groups of presynaptic protein neurotoxins on the neuromuscular junction of the zebrafish to gather novel information on and their mode of binding, membrane interaction and target modification.
The first group of neurotoxins includes the clostridial neurotoxins: seven botulinum neurotoxins (BoNT/A to /G) and one tetanus neurotoxin (TeNT); they are produced by anaerobic bacteria of the genus *Clostridium*, and cause botulism and tetanus, respectively. These neurotoxins paralyze the neuromuscular junction for long time periods following their zinc-endopeptidase activity specific for the key components of the apparatus of neuroexocytosis, the three SNARE proteins: VAMP, SNAP-25 and syntaxin (1).
The second group consists of neurotoxins isolated from the venom of Australian and Asiatic snakes belonging to the Elapid family. These presynaptic toxins are endowed with PLA2 activity and their physiological target *in vivo* is the neuromuscular junction, where they block neuroexocytosis by causing degeneration of the nerve terminal and paralysis.
The third group includes spider neurotoxins, mainly latrotoxins, which cause neurodegeneration and paralysis by allowing a massive entry of calcium into the nerve terminal.
In any case, these paralysis are followed by a complete recovery of function and our experiments are aimed to the understanding of the cellular and molecular events at the base of toxin mechanism of action and of the process of neuromuscular junction regeneration. The zebrafish model offers a range of genetic tools which allows one to test the role of selected components of the neuromuscular junction in the mode of toxin action and/or in the ensuing process of regeneration. In addition this animal offers the unique possibility of imaging the neuromuscular junction and this should allow us to incorporate fluorescent tags on selected protein components of the neuromuscular junction and to follow their behaviour in a time and spatially resolved mode. In addition fluorescent tagged toxins can be used and their redistribution and localization at the neuromuscular junction can be followed.
The ensemble of these approached will throw new light on the molecular mechanism of intoxication by these diverse presynaptic neurotoxins and at the same time will provide novel information on the process of regeneration of the neuromuscular junction after degeneration.

Positional cloning and functional analysis of the zebrafish *albino* mutant, a novel model for human pigment variation

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More than a hundred genes have been associated with various pigment phenotypes across the animal kingdom. Many of these genes play a substantial role in the evolution of species as well as being implicated in disease. While mammalian melanocytes export their melanosomes to other tissues, for example epidermis and hair, zebrafish melanophores retain melanosomes within their cell bodies and traffic them dynamically throughout the cell. Also, as zebrafish melanosomes are easily visualized and remain in the cells, they offer a powerful model system to study the biological function and trafficking of melanosomes *in vivo*.

We have mapped and isolated the *albino* mutant to a region located on chromosome 21 containing an early stop in the solute carrier family 45, member 2 (SLC45A2). The ortholog of SLC45A2 in mouse, AIM1 has been implicated in the underwhite coat color phenotype while the human ortholog, MATP has been shown to cause oculocutaneous albinism type 4, and is tightly associated with the natural variation of human skin color. It is a member of the same transmembrane family as SLC24A5, which is affected in the zebrafish mutant *golden*, and which has also recently been described as playing a role in human skin color variation. Beyond this, little functional data exists. Through the analysis of multiple alleles we hope to achieve a better understanding of SNPs leading to color variation in zebrafish.

We have used electron micrograph analysis to analyze the melanosome morphology of wildtype, *albino* and *golden* fish as well as immunofluorescence to show the localization of the products of these genes. As an additional tool we have created a transgenic line expressing a UAS inducible fusion protein of melanoregulin, Melanoregulin was identified in a global gene expression screen for melanophore-related genes and has been shown to specifically localize to melanosomes. Taken together we present a model system capable of greatly expanding our knowledge of pigment variation as well as the function of melanosomes themselves.

Identification of small molecules that modulate infection of *pseudomonas aeruginosa* in zebrafish embryos

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The increasing prevalence of antibiotic resistance among clinically important human pathogens is a growing threat to human health. There is a clear need to identify and develop novel classes of antibiotics to combat the growing emergence of antibiotic resistant strains. Historically, antibiotics have been identified by their ability to kill (bacteriocidal) or inhibit the growth (bacteriostatic) of bacteria *in vitro*. This methodology selects for small molecules that target bacterial processes essential for growth, such as cell wall synthesis, DNA replication, RNA transcription, and protein synthesis. While historically this approach has been highly effective, no novel classes of clinically relevant antibiotics have been discovered in over 40 years, with the exception of the recent development of the narrow spectrum drugs daptomycin and linezolid. Importantly, screening bacteria for small molecules that kill or inhibit growth *in vitro* ignores a wide range of potentially druggable targets such as bacterial virulence factors, bacterial genes essential for viability in the host but non-essential *in vitro*, and host proteins themselves. Given the current gap in our ability to develop novel antibiotics by traditional *in vitro* based methods and the growing demand for such drugs, there is a need to reconsider both novel methodologies to identify antibiotic-like molecules as well as alternative ways antibiotic-like molecules could act (novel targets). We are exploring an alternative way of identifying small molecules that have antibiotic-like properties by conducting a chemical screen in a zebrafish embryo infection model. This will allow us to identify compounds that rescue embryos from lethal challenge with the bacterium *Pseudomonas aeruginosa*, one the one of the most common causes of antibiotic resistant, nosocomial infections in developed countries. We have found that *P. aeruginosa* infection of zebrafish embryos resembles acute *P. aeruginosa* infection in rodent hosts. For example, virulence factors required for infection are conserved from rodents to zebrafish (genes involved in type three secretion (*pscD*) and quorum sensing (*lasR* and *mvfR*)) and zebrafish myeloid cells are required for combating infection, similar to rodent models. We have also found that immersion of infected embryos in the anti-*Pseudomonal* antibiotics ciprofloxacin or imipenem rescues infected embryos from lethal challenge, demonstrating the feasibility of conducting a chemical screen for small molecules that attenuate infection. We are currently screening a chemical library composed of 1120 compounds with known biological activity for compounds that rescue embryos from lethal *P. aeruginosa* challenge. We plan to use compounds identified in this screen to explore the hypothesis that bacterial virulence factors, bacterial *in vivo* essential genes, and host gene products are viable targets for antibiotic development.

Chemical-genetic screening for small molecules that alter pigment cell development

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Genetic screening in zebrafish has revealed a wealth of mutations in pigment cell development, patterning, and differentiation. In zebrafish, the three pigment cells types are derived from the neural crest: the black/brown melanocytes, the yellow xanthophore, and the silver iridophores. We aim to understand normal pigment cell development and regeneration pathways, with a view to how these pathways may behave during human development and pigmentation, as well as in melanocyte disorders, such as vitiligo, premature hair graying, and melanoma. To complement the available genetic mutants lines, and to identify important molecular tools, we are screening small molecule libraries for compounds that specifically affect the ontogeny and biology of the pigment cells - their development from precursor/stem cells, their proliferation, migration, differentiation, interaction with their environment, and death. In addition to the effects on wild type pigment cells, we are also screening for chemicals that suppress or enhance genetic mutant lines that are deficient for melanocyte and irridophore development and movement, as well as suppressors of small molecules that specifically target melanocytes for cell death. Combined screening results from this screen will be presented, and insight into chemical control of pigment cell pathways will be discussed.

Zebrafish as a model organism for lowe syndrome

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Lowe syndrome is a rare X-linked disease which affects 1 in 100,000 male births, causing disruption to specific tissues, namely those of the brain, eyes and kidney. The genetic cause of the disease has been mapped to mutations within a ubiquitously expressed inositol polyphosphate 5-phosphatase enzyme, termed OCRL1 (oculocerebrorenal syndrome of Lowe). However it is currently unknown how these mutations, which lead to loss of protein expression and activity, cause the disease phenotype.
Here we aim to elucidate how the loss of OCRL leads to Lowe syndrome utilising zebrafish as a model organism, studying the effects of OCRL knockdown by morpholino and subsequent rescue on the development of zebrafish embryos.

Role of the *parl* genes in Parkinson's Disease etiology using *Danio rerio* as a model

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Familial Parkinson's disease (PD) is associated with mutations in a number of genes, including *PINK1* and *Parkin*. Another candidate gene that may play a role in dopaminergic (DA) neuron death codes for the evolutionarily conserved presenilin-associated rhomboid-like (PARL) protease, located in the inner mitochondrial membrane where it plays a role in regulated intramembrane proteolysis (RIP). Surprisingly, this typically hydrophilic enzymatic reaction takes place in the hydrophobic environment of the lipid bilayer. Thus, it is executed by a unique class of proteases, called intramembrane-cleaving proteases (I-Clips) which include the rhomboid family of proteases. *rhomboid-7*, the *Drosophila* orthologue, is genetically upstream and in the same pathway as *PINK1* and *Parkin*. Zebrafish have two *parl* paralogues, which we arbitrarily designated *parl1* and *parl2*. We found that *parl1* is not expressed maternally, but is present from 1 day post-fertilization (dpf) to 7 dpf and is expressed in the adult brain, muscle, liver and heart tissues. *parl2* maternal expression is low, but is highly expressed from 1 - 7 dpf, as well as in the adult brain and liver. No *parl2* transcript was detected in the heart and muscle tissues. When combined, these expression patterns are comparable to the ubiquitous expression of *parl* seen in human adult and fetal tissues. The *parl1* start codon is not annotated by the Ensembl database. We identified a putative ORF upstream of the mis-annotated exon 2 in the database which was confirmed by PCR and sequencing. With the start codon located, we designed translation blocking antisense morpholino oligonucleotides (MOs). Knockdown of *parl1* or *parl2* results in mild neurodegeneration and perturbed patterning of DA neurons in the ventral diencephalon. Remarkably, double knockdown of both zebrafish *parl* genes results in 52% - 87% lethality, while surviving embryos show mildly mis-patterned DA neurons in lower densities at 3 dpf. It is hoped that studying the *parl* genes in zebrafish will help elucidate their contribution to the etiology of PD in humans. Funded by CIHR and the Ottawa chapter of the Parkinson Society of Canada.

Developing an early onset melanoma model in zebrafish suitable for forward chemical genetic screening

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It is increasingly apparent that target-based drug discovery is not delivering adequate numbers of clinical candidates for treatment of malignancies. The main problems encountered are target identification, validation and late stage attrition. New complementary techniques are required to increase the success rate of cancer drug discovery. Phenotype-guided discovery, using chemical genetic screens, provides an alternative strategy that circumvents many of the issues associated with target-based discovery. Zebrafish is a vertebrate model uniquely suited to such high throughput screens, intriguingly such a relevant neoplasia models has recently been established in our group. Expressing oncogenic V12HRas in zebrafish melanophores using the zebrafish mitfa promoter, results in tumour formation in transgenic fish within 4 weeks, enabling screening for novel anti-cancer therapeutics. The tumour model exhibits marked melanophore hyperplasia and dysplasia from very early larval stages. Melanophores also exhibited increased activation of MAPK and PI3-kinase signalling, both pathways are often over activated in human melanomas. The early onset and easy visualization of the mitfa-V12HRas phenotype makes this a promising model to screen small molecule libraries for entities that could antagonize neoplastic transformation. A pilot screen using a panel of known MAPK and PI3-kinase inhibitors was carried out to further validate this model as a potential drug screening vehicle.

The Role of PTEN in Development and Cancer

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PTEN is one of the most frequently mutated tumor suppressor genes in progression of cancer. In general it is known that PTEN counteracts the PI3kinase/Akt/PKB pathway, which is involved in cell growth, proliferation and survival. Functional analysis in mouse, *Drosophila* and *C.elegans* demonstrated that PTEN plays an important role during development, since in these model organisms, PTEN deletion causes embryonic lethality. The zebrafish genome encodes two *pten* genes, *ptena* and *ptenb*, which seem to have a partially redundant role during early development. Single loss of function does not appear to induce phenotypic changes and mutant embryos are viable and fertile. Concomitant loss leads to a dramatic phenotype. Double knock out mutants pass through early embryogenesis, however from 3 dpf onwards the embryos display developmental defects. Up to day five the embryos show severe malfunction marked as hyperplastic-dysplastic defects. Double mutants exhibit enhanced cell proliferation and enhanced cell survival after γ-irradiation compared to siblings. Now, we focus to use the powerful tool of zebrafish lacking PTEN for studying cancer progression. Using state of the art techniques like zf cell culture and transplantation of tumor cells we investigate the role of PTEN during tumorigenesis *in vitro* as well as *in vivo*.

Cxcr7 and tumor angiogenesis in zebrafish

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Angiogenesis, the growth of new blood vessels from pre-existing ones, plays an important role in tumor growth and metastatic dissemination. CXCR7 (RDC1, CCX-CKR2) has been identified as a chemokine receptor that binds CXCL11/ ITAC and CXCL12/SDF-1 chemokines. Recent studies have shown that CXCR7 is expressed by tumor-associated vessels but not by the normal vasculature, thus suggesting a role for this receptor in tumor angiogenesis. Here, the zebrafish *cxcr7* ortholog was cloned and its expression during development was investigated by RT-PCR analysis [with significant levels of *cxcr7* transcript being detectable from 3 hours post-fertilization (hpf) onwards] and by whole mount in situ hybridisation. During late somitogenesis *cxcr7* is strongly expressed within somites and in central nervous system precursors. At 24 hpf the expression of *cxcr7* in the brain is maintained, associated with a distinct expression in the spinal cord. At 48 hpf *cxcr7* is expressed in branchial arches and in posterior lateral line whereas the expression in the brain is limited to diencephalon and midbrain.
Knock down of *cxcr7* expression by injection of an ATG-targeting morpholino leads to a severe impairment of the systemic circulation in zebrafish embryos. This is paralleled by an interruption at the level of the anterior aortic bifurcation, as shown by loss of expression in this region of the endothelial markers *fli1a* and *VE-cadherin*. Also, transgenic tg(*kdr*:EGFP) embryos injected with *cxcr7* morpholino showed a delay in the development of intersomitic vessels. Furthermore, using a zebrafish/tumor xenograft assay developed in our laboratory, we found that *cxcr7* downregulation strongly inhibits tumor angiogenesis when zebrafish embryos are grafted with pro-angiogenic mammalian tumor cells.
In conclusion, *cxcr7* appears to be involved in the development of the vascular system in zebrafish embryo. Also, our data support a role for this chemokine receptor in tumor angiogenesis.

Oncogenic *NUP98-HOXA9* suppresses cellular apoptosis and reprograms myeloid hematopoiesis in transgenic zebrafish

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The *NUP98-HOXA9* fusion oncogene results from a t(7;11)(p15;p15) chromosomal translocation and is associated with inferior prognosis in *de novo* and treatment-related acute myeloid leukemia (AML), as well as blast crisis in chronic myeloid leukemia (CML). *HOXA9,* belonging to the highly-conserved *HOX* gene family of developmental transcription factors is critical for vertebrate hematopoiesis. Elucidating the activity of oncogenic *NUP98-HOXA9* may reveal universal mechanisms of leukemogenesis and lead to the design of targeted therapies. The zebrafish is a robust model for studying vertebrate hematopoiesis and leukemogenesis, by virtue of its *ex utero* development, and conserved genetics and cell biology. We have engineered a transgenic zebrafish harbouring the human *NUP98-HOXA9* translocation, and report two novel *in vivo* phenomena: (1) *NUP98-HOXA9* leads to suppression of cellular apoptosis and abnormal cell cycling following exposure to ionizing radiation. Irradiated transgenic embryos displayed reduced levels of the conserved apoptotic marker, activated caspase-3, and persistent levels of the cell cycle marker, phosphorylated-Histone-H3. Additionally, *NUP98-HOXA9* resulted in differential expression of prominent genes in the apoptotic response. Notably, we observed downregulation of the *puma* pro-apoptotic gene, and upregulation of the *bcl2* and *bcl-x$_L$* anti-apoptotic genes. These data postulate suppression of caspase-3-dependent apoptosis as a mechanism for *HOXA9*-mediated oncogenesis *in vivo*. 'Rescue' experiments using a morpholino to *bcl-x$_L$* have suggested insufficiency of single-target therapy, and that synergistic, multi-target interruptions may be required to restore radiosensitivity. (2) *NUP98-HOXA9* perturbs zebrafish embryonic hematopoiesis, leading to upregulated expression of myeloid-specific genes, *pu.1*, *lysC*, and *l-plastin*, and downregulation of the erythroid-specific gene, *gata1*. These changes in gene transcription appeared to affect both 'primitive' and 'definitive' phases of zebrafish hematopoiesis, including the recently-described erythro-myeloid progenitors (EMPs), suggesting *NUP98-HOXA9* reprograms blood cell precursors to a predominantly myeloid fate. Preferential upregulation of *pu.1* during 'primitive' hematopoiesis further implies an impairment of terminal myeloid differentiation. Taken together with the anti-apoptotic phenomenon, these data suggest a mechanism underlying *NUP98-HOXA9*-mediated leukemogenesis and provide an unprecedented opportunity for chemical modifier screens to identify promising therapeutic agents that restore a normal phenotype.

Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model

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Aberrant regulation of cell migration drives progression of many diseases, including cancer, cell invasion and metastasis formation. Analysis of tumour invasion and metastasis in living organisms to date is difficult and involves complex and time consuming investigative techniques. For primary human tumours we establish here a simple, fast, sensitive and cost-effective in vivo model to analyse tumour invasion and metastatic behaviour.

We labelled small explants from gastrointestinal human tumours with fluorescent live dye (CM-Dil) and investigated their metastatic behaviour after transplantation into 48hpf zebrafish embryos. Due to its transparency, zebrafish embryos are an excellent *in vivo* model to follow real-time invasion, migration and micrometastasis formation.

Xenografts of primary human tumours implanted into the yolk of 48hpf zebrafish embryos showed invasiveness and micrometastasis formation within 24 hours after transplantation, which was absent when non-tumour tissue was implanted. Furthermore, primary human tumour cells, when organotopically implanted in the liver of 72hpf zebrafish embryos, demonstrated invasiveness and metastatic behaviour, whereas primary control cells remained in the liver. Pancreatic tumour cells showed no metastatic behaviour when injected into cloche $^{-/-}$mutant embryos, which lack a functional vasculature.

In conclusion, our results show that the zebrafish in its embryonic stage is a useful *in vivo* animal model for rapid analysis of invasion and metastatic behaviour of primary human tumour specimen.

A zebrafish engraft model reveals a role for the PDZ/LIM genes LMO7, Mystique and RIL in metastatic behaviour of human pancreatic cancer cells

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PDZ as well as LIM domains are interaction modules, present in a large variety of proteins with diverse biological functions and assorted additional domains. In zebrafish, we identified eleven genes that encode for both a PDZ domain, and one or several LIM domains: five genes of the ALP subfamily (ALP, ALP-like, Elfin, Mystique, and RIL) three of the Enigma subfamily (Enigma, Enigma Homolog, and ZASP), the two LIM kinases (LIMK1 and LIMK2), and the LIM only protein 7 (LMO7). Functionally, all proteins of the PDZ/LIM family share an important trait, they can associate with the actin cytoskeleton.

In this study, we analyzed the effects of knock down of PDZ/LIM genes on migration and metastatic behavior of human pancreatic cancer cells. Using a zebrafish xenotransplantation model, we were able to follow metastasis formation and progression *in vivo* and real time. We implanted human pancreatic cancer cells (PaTu8988-T) into the yolk sac of 2 day post fertilization (dpf) embryos and monitored invasion and metastatic behaviour by confocal laser scanning microscopy. Prior to injections into zebrafish embryos, PaTu8988-T cells were treated with gene-specific small interfering RNAs (siRNAs) for Mystique (PDLIM2, SLIM), RIL (PDLIM4) or LMO7 (PCD1; FBX20).

In comparison to control embryos, treatment of pancreatic cancer cells with either one of these siRNAs led to reduced migration and inhibition of the formation of micrometastasis. These results suggest that the three PDZ/LIM genes Mystique, LMO7 and RIL are important for the migratory capacity and metastatic behaviour of this highly-aggressive pancreatic cancer cell line.

Validating novel cancer genes in a zebrafish melanoma model

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We have established a model of melanoma progression in zebrafish that is based on the Gal4-UAS system, where the crossing between a melanocyte-specific Gal4 line with a UAS:H-RAS^{V12} line results in melanoma development with a high frequency.
This binary system allows for target expression/knock down of modulators of oncogene activity in the same cells that undergo transformation, thus allowing precise tumor-specific manipulation of gene expression.
We will use this approach to restore the expression of tumor suppressor genes specifically in melanoma cells using the UAS system. Besides known candidates we will test 101 novel candidate cancer genes identified with a bioinformatics approach (Rambaldi et al., 2008). These genes were selected for the low frequency of duplication and the high connectivity in protein networks. As a first step towards the choice of the candidate tumor suppressor to re-express in our melanoma model we carried out quantitative analysis of expression of the 101 genes in several melanoma samples. Data obtained by Real time-PCR analysis from zebrafish melanoma tissue (versus normal skin) demonstrate that the expression of many novel candidate cancer genes is down-regulated (potential tumor-suppressor genes) in zebrafish melanomas whereas only a few are up-regulated (potential oncogenes). We will perform a similar analysis in human melanoma tissue and in melanoma cell lines. The next step will be to restore the expression of the most significantly repressed candidates in our melanoma model. All this work is ongoing and the data obtained will be presented and discussed as well as the strategy used to identify and validate the novel tumor suppressor genes.

Rambaldi, D *et al*, (2008) Low duplicability and network fragility of cancer genes. *Trends Genet* 24(9):427-30.

Chemical-genetic Approaches in Zebrafish and Yeast Identify Novel Compounds that Sensitize Melanocytes for Cell Death

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Metastatic melanoma is one of the most aggressive cancers, and resistant to all chemotherapy, with afflicted individuals having a median life expectancy of less than one year. Clues to why melanoma is so difficult to treat may come from the nature of the melanocyte itself. Derived from highly motile neural crest cells, melanoma has high metastatic potential, and armed with enhanced survival and anti-apoptotic capabilities, melanocytes are naturally resistant to cytotoxic agents. Understanding the ontogeny and biology of the melanocyte - their development from precursor/stem cells, their proliferation, migration, differentiation, interaction with their environment, and death - may be highly informative for new therapeutic approaches to melanoma. We study these processes using the zebrafish model system, which allows the visualization of melanocytes in live tissue as well as their progression to melanoma.
Well suited to high-throughput genetic and chemical screening, zebrafish are becoming widely used in the drug-discovery process for target validation, disease modeling, toxicology and target and lead compound discovery. Screening 1600 bioactive compounds has allowed us to identify a small molecule panel that alters distinct aspects of melanocyte biology in zebrafish. One novel compound, BIO1E7 appears to specifically sensitize zebrafish melanocytes for cell death by tyrosinase-independent pathway. Systematic genetic profiling of 6000 yeast gene deletion mutants for compound sensitivity has enabled us to identify BIO1E7 to interact genetically with the DNA damage repair pathways. In human melanoma cancer cell lines, we find BIO1E7 induces reactive oxygen species (ROS), a G2/M-phase cell cycle arrest, and apoptosis. Like in yeast, we find that mouse melanocytes deficient for the nucleotide excision repair are especially sensitive to BIO1E7, indicating that the BIO1E7 mode-of-action is conserved. We are currently exploring the direct mechanism of BIO1E7 action, and the effects of BIO1E7 on mouse melanocytes in situ. Given that melanocytes are have inherent mechanisms to coop with DNA damage induced apoptosis, we suggest that the BIO1E7 mechanism may reveal a new point of vulnerability for the melanocyte.

Functional analysis of the tumourigenic transactivator Cited1 in zebrafish

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The Non-DNA binding transactivator Cited1 is the founding member of the CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich carboxyl-terminal domain (Cited) family. All family members have in common the highly conserved carboxy terminal transcription activator domain that bind the transcriptional integrators CBP and p300 and regulate their dependent transcriptional responses. Human hCITED1 has previously been implicated in the progression of melanoma and other malignancies through its miss-expression. hCITED1 have also been shown to be reliable markers of papillary thyroid carcinoma (PTC). Yet, there are only a few functional data on Cited1, which suggest that Cited1 might keep cells in an undifferentiated state. Developmental processes like proliferation and differentiation of progenitor cells mirrors in great parts cancer formation. To better understand Cited1 functions we have cloned the zebrafish ortholog of the mammalian *Cited1* and analyze its role in development. Zebrafish z*fcited1* expression is first detected by in situ hybridization at 11hpf in the otic vesicle and at 18hpf in a spatial restricted pattern in the neuroectoderm. After somitogenesis z*fcited1* expression becomes restricted to the subventricular zone of the hindbrain. We found, that z*fcited1* is co-expressed in a subset of *pcna* expressing proliferating cells, but never in neuron precursors expressing *deltaA* or freshly differentiated neurons expressing *elavC*. Further, expression of z*fcited1* is lost in the notch-pathway mutant *mind bomb*, where too many neuronal progenitors undergo neuronal differentiation. To elucidate the function of z*fcited1* we performed loss of function and gain of function experiments. A morpholino knock down of z*fcited1* resulted in p53 dependent apoptosis presumably as an off target effect. The over expression of z*fcited1* mRNA also induced p53 dependent apoptosis. Embryos co-injected with a p53 morpholino and z*fcited1* mRNA survive and show a dramatic increase in size of the hindbrain and disturbed neurogenesis. The same phenotype is observed using hCITED1 mRNA. In both cases the phenotype is not fully penetrant. This phenomenon could be explained by the reported proteolytic degradation of hCITED1 through an O-box. The putative protein sequence of zfCited1 contains the same O-box and additionally a D-box. Indeed using a zfCited1-GFP construct we can show a fast degradation of the zfCited1-GFP protein after mRNA injection. hCITED1 negatively regulates the Wnt pathway by binding b-catenin. Contrary to the hCITED1 we show that zfCited1 is not able to bind b-catenin in a LEF/TC reporter luciferase assay. In summary we conclude that z*fcited1* is involved in regulation proliferation and to hold the progenitor status of cells. Despite the different ability of hCITED1 and zfCited1 to bind b-catenin we reason that further functional analysis of the zfCited1 can help to understand hCITED1 function in tumor formation and progression.

Molecular and Genetic Approaches to Melanoma Development

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The incidence of melanoma is rapidly rising, and treatment of advanced stages has a low success rate. One way in which melanoma can occur is from benign nevi. The most frequent initiating mutation of nevi is the constitutively activating mutation in *BRAF* (V600E). Activation of the MAPK pathway by $BRAF^{V600E}$ causes proliferation of melanocytes before senescence pathways are activated. We utilise zebrafish to study melanoma because of their tractable genetics and transgenics and ease of study of melanocytes and tumour development. Crossing zebrafish carrying the $BRAF^{V600E}$ mutation with lines containing loss of function mutations in *p53*, *ptena* and *ptenb*, we are able to generate melanomas arising in differing genetic backgrounds. Utilising these models will allow us to examine histological, cellular and behavioural characteristics between melanomas arising from differing genetic backgrounds.

Antibodies are important and effective tools to study the cellular and molecular changes during melanoma progression. The ease of generating large numbers of individuals with nevi and melanoma allows the zebrafish to be an ideal model for this. However, the lack of reliable zebrafish antibodies in the field may hamper this effort. In response to this we are optimising a panel of antibodies raised against highly conserved regions of human peptides. We are taking a highly systematic approach to optimisation using both human, zebrafish and medaka tumour cores in a tissue microarray. Successfully validated antibodies will be tested on both developing tissues of young zebrafish as well as a range of tumour cores.

Role of Sdf1 in melanoma progression and pigment cell movement in medaka

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Chemokines are an abundant family of small secreted signal molecules, first identified in the lymphatic system due to their role in the migration and homeostatsis of immune cells. However, tumor cells exploit chemokine signals for progression and metastatic guidance. The chemokine-recpetor pair Sdf1/Cxcl12-Cxcr4 has become of great interest, as it is suspected to drive tumor progression and formation of secondary tumor nodules in distinct organs. High levels of the Sdf1-receptor Cxcr4 have been identified in human malignant melanoma, a highly aggressive, metastasizing tumor with very low survival rates.
To investigate the role of Sdf1/Cxcl12 and its corresponding receptors Cxcr4 and Cxcr7 during melanoma progression in detail, we started to characterize the orthologous genes in a transgenic medaka melanoma model. In this model the oncogene Xmrk, a constitutively active mutant version of the epidermal growth factor receptor, is exclusively expressed in pigment cells. High levels of *xmrk* expression result in malignant transformation of melanophores and xanthoerythrophores into diverse forms of pigmentary lesions, including melanoma. Quantitative RT-PCR analysis revealed different expressional levels of *sdf1*, *cxcr4* and *cxcr7* in different tumor types investigated. Exceptional high expression of *sdf1b* and *cxcr4b* was detected in tissues colonized by invasive melanoma. Trans-well migration assays indicate that Xmrk expressing mouse melan-a cells, which express Sdf1 receptors but not the chemokine, are attracted to a source of Sdf1 in vitro. To additionally investigate guidance of tumor progression in vivo, we established transgenic medaka lines which specifically express *sdf1* genes in pigment cells. Initial transient experiments show that individual cells expressing increased levels of *sdf1* show altered pigment cell movement in vivo. Further investigation of double transgenic lines overexpressing Xmrk and Sdf1 in the same cell will contribute to a better understanding of the influence of chemokine signals on melanoma progression and tumor spreading.

The zebrafish, a new model to study the role of telomerase and telomere in stem cell behaviour

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Telomerase plays a primary role in the maintenance of telomeres and is expressed in the stem cell compartment of several adult tissues and tumor cells of human and mouse. However, most somatic cells and tissues lack telomerase activity. Therefore, telomere length and telomerase activity have recently been proposed as stem cell markers.
Little is known about the expression and functions of telomerase in non-mammalian vertebrates. The zebrafish shows a high degree of genetic and physiological similarity with mammals and it offers several advantages, including small size, high fecundity and manageable for highthroughput screening. We have used this model to investigate the roles played by telomerase and telomere length in the behaviour of stem cells. Thus, we perform a complete study about telomerase activity and telomere length among a broad range of tissues and ages from zebrafish and found that telomerase expression and telomere length increased during the zefrafish life. However, both telomerase activity and telomere length drastically declined in senescent fish. We also observed that telomerase expression was upregulated in regenerating fins. Finally, we have characterized the minimum regulatory sequence of the zebrafish telomerase gene in order to generate a transgenic zebrafish that will allow us to identify *in vivo* cells with high telomerase activity, i.e. progenitor/stem cells.

Zebrafish PICH plays a critical role in sister chromatid separation during mitosis

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In mitosis, spindle assembly checkpoint ensures even separation of sister-chromatids to each daughter cell. Failure in this process results in the loss of genetic information and may cause the disease of genetic instability, cancer. Mitotic kinases orchestrate the spindle assembly checkpoint and became attractive targets for anti-cancer drug development. Among the mitotic kinases we became interested in Plk1 and its interaction partner PICH (Plk1-interaction checkpoint helicase). PICH is a putative component of spindle assembly checkpoint as a tension sensor and also involved in DNA decatanation during sister chromatid separation. Despite the considerable interest in PICH, studies exploring the function and importance of PICH during normal cell division are lacking. Therefore we adopted zebrafish embryogenesis model for the easiness of genetic manipulation and abundance of mitotic cells. First, we identified zebrafish ortholog for PICH (zPICH) though bioinformatics. zPICH was readily associated with Plk1 and localized to kinetochores from prometaphase to metaphase, similar to human PICH. zPICH expression was highest at the cellular regions undergoing vigorous proliferation in zebrafish embryos. When the level of zPICH was knocked-down, anaphase bridges were frequently observed, suggesting crucial role of zPICH in proper chromosome segregation. In order to analyze the cell division in detail, we performed time lapse microscopy and found incomplete chromosome separation and cytokinesis for the morphants. These data demonstrate that the zPICH is indeed a human PICH ortholog, which is critical for the sister-chromatid separation. Our results also suggest that the zebrafish model could provide a valuable tool to study the role of mitotic regulators during normal cell cycle and may become a useful vessel to screen drugs targeting them.

Molecular and functional characterization of trim8 genes in zebrafish

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TRIM8 is a member of the TRIM/RBCC gene family. TRIM encode proteins characterized by the presence of the tripartite motif, which consists of a RING finger, one or two zinc-binding motifs named B-box and an associated Coiled-coil region (RBCC). In spite of highly conserved structure, which suggests a common basic function, TRIM proteins are involved in a broad range of biological processes and are implicated in several pathological conditions such as Mendelian genetic diseases, cancer development and viral infection.

Bionformatic analysis, ChIP and luciferase assays revealed that *TRIM8* regulation is modulated by four p53 responsive elements located in the first intron of *TRIM8* gene. Importantly, we showed that *TRIM8* interacts with and increases the p53 protein stability and it affects p53 transcriptional activation of p21 in mammalian cell lines.

TRIM8 is highly expressed in brain and is located within the 10q24.3, a region mostly involved in deletions and rearrangements in brain cancer. Recent investigations revealed that the human *TRIM8* gene is down-regulated in a number of glioblastomas and astrocytomas, and studies in mammalian cells suggest it could be involved in a tumor suppression mechanism through the interaction with p53. Our goal is to analyze expression of *Danio rerio TRIM8* ortholog(s) and loss-of function phenotype, in order to clarify its developmental and biological role in this animal model.

A zebrafish genome-wide search allowed us to detect two orthologs, named *trim8.1* and *trim8.2*, located on chromosome 13 and 12 respectively. Bioinformatic analysis indicates that both *trim8.1* and *trim8.2* possess the p53 responsive elements and encode a protein with the typical tripartite motives. Zebrafish *trim8.1* and *trim8.2* expression is detected by RT-PCR starting from early stages of development. *In situ* hybridizations show a similar expression pattern in the developing central nervous system (forebrain and hindbrain) and eye, in particular in the cerebellum, tectum and retina. We are now generating *trim8.1* and *trim8.2* knockdown by microinjection of specific morpholino, in order to evaluate the consequences of the silencing of each and both zebrafish *trim8* genes.

In order to confirm the data obtained in mammalian cells and to shed light on the involvement of TRIM8 in brain tumors onset through p53 interaction, we plan to test the reciprocal effects of *trim8.1* and *trim8.2* knockdown/over-expression on *p53* expression and, vice versa, the effect of *p53* silencing/up-regulation on the expression of *trim8* genes.

Generation and analysis of zebrafish liver cancer by overexpressing k-rasv12 using mifepristone-inducible cre/lox system

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With histology and gene expression studies of zebrafish tumors closely resembling that of the human, the zebrafish (*Danio rerio*) has recently emerged as a promising model system to study human cancers. Ras signaling has been at the leading edge of signal transduction and molecular oncology, yet a complete understanding of Ras in human cancer remains elusive. Although *KRAS* mutations occur in vast majority of human cancers, very little is known about the molecular mechanisms that K-ras drive in tumorigenesis. To study the molecular mechanisms of K-ras oncogenesis, we developed a zebrafish liver cancer model through generation of stable transgenic lines expressing activated K-ras^{V12} in the liver. Using *Activator/Dissociation* transposable elements from the maize, we first introduced zebrafish *kras^{V12}* oncogene fused with *EGFP* reporter gene under the liver-specific *lfabp* promoter and obtained germline transmitted transgenic zebrafish. We observed tumor growth in EGFP-positive livers but also a high mortality in EGFP-positive F_1 zebrafish, which hindered the maintenance of these transgenic zebrafish for long-term research. We next used a combination of the mifepristone-inducible LexPR gene expression system and Cre/loxP recombination system to control the expression of oncogenic K-ras^{V12}. Activation of this system successfully induced liver tumor phenotypes, mostly hepatocellular carcinoma, as depicted in our histological examination. By using the inducible transgenic system, we found that the liver tumor could be induced from both fry and adult and we also noticed some GFP-positive tumors in some aged fish, raising the possibility of tumor metastasis. A cDNA microarray approach was then employed to analyze the gene expression profiles of K-ras^{V12} induced liver tumor formation. Several Ras oncogene family members and genes involved in the MAPK signaling pathway are significantly up-regulated. Other pathways involved in tumorigenesis are also deregulated in our K-ras^{V12} transgenic model. Consistent with this, our miRNA array data indicated the down-regulation of several miRNAs known to be involved in regulation of expression of kras oncogene. Further analyses of microarray data may help to investigate molecular mechanisms and search for signature pathways leading to K-ras^{V12} induced liver oncogenesis. We recently also applied the mifepristone-inducible system to transgenic zebrafish expressing K-ras^{V12} in the pancreas and have also successfully induced tumor formation in this organ and thus demonstrated the general application of the inducible Cre-loxP system in development of transgenic tumor models in zebrafish.

Zebrafish brain represents a reliable niche for xenotransplanted human glioblastoma derived cells

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Glioblastoma (GBM) is one of the most common primitive malignant tumours, occurring in the central nervous system, which has been shown to contain functionally important subsets of cells with stem-like properties, known as cancer stem cells (CSCs). These subsets of stem cells express primitive markers (i.e. CD133, SOX2, MSI1, BMI1 and Nestin) and are most probably related to the high incidence of relapses in GBM. The presence of CSCs in GMB has been associated with the hypoxic microenvironment typical of brain tumours niches. Thus, a deeper understanding of the parameters characterizing the hypoxic microenvironment, affecting and preserving cancer cells, can help in designing strategies and tools aimed to cure cancer. In recent studies, the zebrafish embryo has been exploited as a novel *in vivo* model to recreate a transparent and reliable tumour cell niche. We optimized parameters for xenotransplanting primary GBM-derived cells into brain ventricles of zebrafish albino embryos, at 48 hours post-fertilization. Transplanted cells were followed for at least 10 days post injection. Cells integrated successfully into the brain and could be tracked, *in vivo*, by GFP expression or vital staining with a fluorescent dye. By immunocytochemical analyses and *in situ* hybridisation, tumour grafted cells were checked for the expression of CD133, Nestin and the hypoxic marker Hif-1α. As controls, we used *ACTB* antisense riboprobes and anti-human nuclei antibodies, able to detect all transplanted human cells. Our preliminary observations indicate that at 48 hpf stage, zebrafish CNS microenvironment preserves the GBM immature phenotype. Thus, zebrafish embryos, by mimicking an *in vivo* hypoxic microenvironment, appear as valuable biosensors for human brain tumours and CSCs behavior studies.

Heritable Zebrafish T Cell Cancer Models from a Mutagenesis Screen

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T cell lymphoblastic lymphoma (T-LBL) and leukemia (T-ALL) are common pediatric malignancies. These diseases have poor prognoses, and their treatments often cause significant morbidities. Unlike many childhood cancers, most T cell neoplasias carry no pathognomonic cytogenetic finding. Consequently, insight into their molecular pathogenesis is incomplete. One key transformation mechanism in T cell malignancy is established, as NOTCH1 activation occurs in about 50% of T-ALL cases. However, many other genetic lesions in these diseases are unknown. To address this deficiency, we pioneered a zebrafish phenotypic screen designed to create new genetic models of T cell neoplasia. We used ENU to randomly mutagenize the germline of adult transgenic zebrafish with T cell-specific GFP expression (*lck::EGFP*). Then, via fluorescence microscopy, we screened their progeny at juvenile stages for abnormal T cell phenotypes, including the development of GFP^+ tumors.

Here, we describe the identification and characterization of three different mutants with heritable T cell cancer predisposition. Two mutants exhibit dominant inheritance, while one acts recessively. In all three, disease penetrance is incomplete, demonstrating that other somatically-acquired genetic 'hits' are required for malignancies to develop. In each mutant, disease incidence, histologic infiltration, and cellular morphologies resemble human T-LBL and T-ALL. Expression profiles confirm neoplasms are T lineage, and T cell receptor analyses of tumor cells verify their clonality. Malignant cells are transplantable, radiation-sensitive, relapse-prone, and contain leukemia-initiating cells, like their human correlates. Overall, all three mutants recapitulate human T cell malignancy, showing heritable disease predisposition.

Current efforts are focused on cloning the mutations conferring inherited cancer risk in each line and the discovery of additional non-inherited genetic lesions contributing to neoplastic transformation. Specifically, comparative genomic analyses have revealed regions with copy number gains or losses in malignant tissues, but not matched control samples, with several recurrent copy number aberrations seen thus far. In sum, we have identified new models of T-LBL and T-ALL. These mutants should provide exciting experimental platforms for study of this important class of human cancers.

Evaluation of Small-molecule PLK1 Inhibitors Using Zebrafish

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PLK1 (Polo like kinase 1) is one of the key regulators which control mitotic entry, spindle assembly, chromosome segregation, and cytokinesis in cell cycle. Since PLK1 expression is abnormally up regulated in several tumors, it has been regarded as a good target for cancer therapy. A number of PLK1 small molecule inhibitors have been developed as chemical biology tools and potential anti-cancer drug candidates. However, the specificity of these inhibitors is not thoroughly examined. Here, we use whole zebrafish embryo assay coupled with genetic analysis to address this issue. Bioinformatics analysis revealed zebrafish genome has all of the PLK family members which share very high homology with their human counterparts. In particular, PLK1 has a nearly identical 3-D structure between zebrafish and human. To determine if zebrafish can be used to evaluate specificity, we selected two published PLK1 inhibitors, LFM-A13 and ON01910, and one homemade PLK1 inhibitor, PLK1-Yang. When added at 2-cell stage, all of these inhibitors prevented embryos from dividing and caused cells to fuse into one large cell. When added at shield stage, a later stage when zygotic mRNA transcription program is initiated, embryos survived for 3 days but showed different phenotypes for each compound. Embryos treated with LFM-A13 were relatively normal. Embryos treated with ON01910 didn't properly develop trunk and tail while the head structure was relatively normal. Embryos treated with PLK1-Yang had shorter body axis and deformed head structure, which were similar to the phenotypes of zebrafish that are genetically deficient of plk1. These data suggest that the three PLK1 inhibitors had different off-target activities and PLK1-Yang was most specific to PLK1 among them. Our studies also suggest that zebrafish can be used as an efficient in vivo model to rapidly evaluate specificity of small molecule chemical inhibitors designed to other target proteins.

Formation of liver tumor by inducible expression of c-Myc in a tet-on transgenic system in zebrafish

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C-myc (*MYC*) is frequently observed to be overexpressed in human cancers. Its role in tumorigenesis has also been shown in transgenic models of rodents. Although *c-myc* overexpression has been found in intestinal adenomas and hepatic adenomas in an APC-deficient zebrafish, its function in transgenic zebrafish studies has only been proved in T-cell leukemia. Despite that these studies have indicated that *c-myc* is involved in tumorigenesis, the exact mechanism of *c-myc* involving in the tumor initiation and progression and its target genes remains undetermined. In order to investigate the function of c-myc as well as its underlying biological processes involved in liver cancer formation, we have established a transgenic zebrafish model by using the Tet-on system to conditionally express mouse *c-Myc* in the liver under the control of the zebrafish *lfabp* (*liver fatty acid binding protein*) promoter. After 14 days of treatment with the inducer Doxycyclin starting from 21 dpf, 100% (n= 74) of transgenic fish showed apparently enlarged abdomen, whereas none (n=23) of the non-transgenic siblings showed such phenotype. Histological analysis showed that overexpression of mouse *c-Myc* in zebrafish liver caused hyperplasia of liver at early stage and followed by the development of hepatocellular adenoma when *c-Myc* was overexpressed for a longer time. Brief withdrawal of Doxycycline greatly reduced the *c-Myc* expression and alleviated the enlarged liver phenotype. Compared to the activation of *c-Myc* from 21 dpf, less time is needed to obtain liver overgrowth when *c-Myc* was activated from early developmental stages. In addition, we also observed in some of the treated embryos that liver cells were detached and migrated to other tissues including blood. Our observations indicated the potential of our *c-Myc* transgenic zebrafish as a model in the study of liver cancer progression and reversal. In the ongoing experiments, we are going to activate transgenic *c-Myc* at different ages to investigate its role in liver cancer formation during different stages of development and growth. Microarray studies will be carried out for understanding of the c-myc pathway from transcriptomic analyses and we expect that these experiments will provide new insights into mechanism of c-myc involving in the liver cancinogenesis.

Zebrafish is a powerful system in studying Aurora-A function in mitosis and development
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Aurora-A is a serine/threonin kianse, which regulates many intricate processes during mitosis. It is localized at centrosomes and microtubules during mitosis, implicating roles in the control of centrosome and spindle function. Since the discovery of Aurora-A overexpression in cancer cells and induction of cell death with its inhibition, Aurora-A specific inhibitors drew much attention as promising anti-cancer drugs.
Despite the emerging interest for clinical applications, our understanding how Aurora-A functions in normal cells and organisms is poor. Here, we performed loss-of-function experiments in zebrafish embryos, as the model stages normal cell cycle and development. In order to block the function of zebrafish Aurora-A (zAurA) we utilized a translation blocking morpholino or a small molecule inhibitor, MLN8054. After the morpholino injection or the inhibitor treatment, zebrafish developed short trunk with severe growth retardation and cell death. The developmental defects might be attributable to the abnormal mitotic progression as the cells in the morphants manifested monopolar spindles and increased expression of mitotic markers suggestive of mitotic arrest. The embryos treated with MLN8054 also exhibited abnormal spindle formation including monopolar and multipolar spindles, similar to the morphants. In order to analyze the fate of individual cells after blocking zAurA, we generated Histone H2B-GFP transgenic zebrafish and carried out time-lapse microscopy. In the live-imaging, the morphants displayed mitotic delay and/or mitotic slippage in most cells. Intermittently, we also observed chromosome bridges and cell death. Taken together, our data demonstrate that zAurA is essential for the bipolar spindle formation and that its loss primarily results in mitotic arrest. Our study reveals that zebrafish is a powerful model in studying mitosis. Furthermore, zebrafish embryogenesis is an efficient *in vivo* system to test the efficacy of mitotic inhibitors, let alone assess the functions of mitotic kinases.

Kidney Damage and Regeneration in Zebrafish Larvae
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The vertebrate kidney has an innate ability for recovery and regeneration following acute injury. During the recovery phase renal epithelial tubular cells are known to proliferate actively and differentiate to reconstitute tubular epithelium. The regeneration process is thought to have many parallels with the growth and maturation that occur during kidney organogenesis. However, the source of proliferating cells that repopulate the injured nephron, and the relative contribution of intra-renal versus extra-renal cells is poorly understood. A better understanding of the mechanisms of nephron repair may provide important clues for the development of new therapies for the treatment of acute kidney injury. Fish possess a remarkable regenerative ability and are able to respond to renal injury by nephron neogenesis. The zebrafish pronephric kidney thus provides a simplified and suitable model for the study of kidney regeneration. To explore the link between kidney repair and embryonic development we induced gentamicin mediated proximal tubular damage in the larval zebrafish kidney. To model damage in real-time, we utilized transgenic lines for *lhx1a* and *pax2a*, two genes known to play key regulatory roles during kidney embryonic development. We assayed for damage in terminally differentiated pronephric kidneys that do not express either reporter. The goal was to determine if we could visualize reactivation of either reporter and if so whether cells expressing GFP were found in the existing tubules or in a distant population of cells. In both *Tg(lhx1a:eGFP)* and *Tg(pax2a:eGFP)* lines we saw reactivation. Our current data suggests that the GFP positive cells are localized outside the kidney and could possibly be ascribed to a local population of progenitor cells. In the future, by determining the origin of the reactivated cells and being able to manipulate their numbers, we hope to develop methodologies that could eventually be used as an alternative therapy for acute kidney injury.

Canonical Wnt Signalling in the Development of the Zebrafish Optic Tectum

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The optic tectum (OT) is one of the primary processing centers of visual information in vertebrates. During the embryonic development of teleost fish, it develops from the simple neuroepithelium of the mesencephalic alar plate into a complex, multilayered structure, containing at least eleven different cell types. The continuous post-embryonic growth of the OT shows that its germinative zone retains proliferating cells during the whole life of the fish, providing a good model to study neural stem cell maintenance and post-embryonic neurogenesis.
We provide evidence for the existence of a Wnt-responsive, proliferative cell population in the dorsal OT. These cells are epithelial in their character and abut the mesencephalic roof-plate, which is the likely source of Wnt signals. With a combination of loss-of-function and gain-of function experiments, we show that Wnt signalling is essential for cell-cycle progression in these cells. We hypothesize that this population of cells contains the neural stem cells of the OT and the proximity of the roof-plate defines the niche where these stem cells can persist.

A small molecule screen for motor neuron regeneration in zebrafish

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Recently, we have demonstrated that adult zebrafish are capable of motor neuron regeneration (Reimer et al., 2008, J Neurosci 28:8510-8516). However, humans cannot do this, for example in motor neurone diseases, and it remains a mystery as to why not. Small molecules could be useful in stem cell therapy of motor neuron diseases and there is a need for novel small molecules that drive motor neuron differentiation from stem cells. In order to find such compounds, we use transgenic embryonic zebrafish containing motor neurones that express green fluorescent protein (GFP) in drug screens. We have identified small molecule 'hits' that alter axonal outgrowth in HB9:GFP transgenic embryos. We are testing these compounds in islet-1:GFP transgenic fish to determine whether compounds retard or accelerate differentiation of late born motor neurons. We have identified a number of small molecules that influence motor neuron differentiation, and have started to characterise the signalling pathways on which these compounds act during motor neuron differentiation in development and adult regeneration. This may ultimately contribute to the development of therapeutic approaches to motor neuron diseases.

We thank Drs. D. Meyer and H. Okamoto for transgenic fish lines and Dr Mike Tyers for providing compounds. This work is supported by a College of Medicine and Veterinary Medicine BioQuarter grant.

Forward Genetic ENU Chemical Mutagenesis Screen for Replacement Tooth, Craniofacial and Skeletal Mutants in Zebrafish

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Zebrafish exhibit continuous replacement tooth formation throughout their lives, providing a unique opportunity and model to identify genes participating in this process. We are currently performing a forward genetic, chemical mutagenesis screen to identify novel zebrafish mutants exhibiting mineralized tissue phenotypes. Our screen consists of an optimized Alizarin red staining protocol for juvenile zebrafish ~15mm-25mm in length, based on the fact that wild type specimens of this size have formed all of the skeletal elements of the axial and craniofacial skeleton, and exhibit both primary and replacement teeth. We performed weekly ENU mutagenesis of ten males per week followed by out-crossing each mutagenized male, to generate 1,084 individual F1s, each of which was crossed to wild type AB fish to generate 131 individual F2 families to date. F3 families, including homozygous recessive, heterozygous, and wt sibling genotypes, are currently being screened for mineralized tissue defects. To date, we have screened 27 F3 families, six of which exhibit early lethal homozygous recessive phenotypes. We are also continuing characterizations of novel mutants identified in an earlier pilot screen performed by this laboratory. Our screen is novel, and geared to identify pathologies relevant to human disease, in that it focuses on relatively late developmental defects, in mineralized tissues, in both homozygous recessive and heterozygous mutants, exhibiting viable defects. We expect that the proposed studies in zebrafish will result in the elucidation of molecular signaling networks mediating mineralized skeletal tissues, and replacement tooth formation, which may eventually facilitate the development of educated, clinically relevant, molecular-based, mineralized tissue replacement therapies in humans. This research is being supported by NIH grant DE018043 (PCY).

Analysis of gene expression following acute hypoxic stress in zebrafish adult hearts

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Mammalian adult heart has little capacity to repair after severe injury, although cardiac niches containing stem cells and early lineage-commited cells are present. In case of myocardial infarction, necrotic myocardium is replaced by non-contractile scar tissue, while the remaining muscle undergoes pathologic hypertrophy. Zebrafish has recently become a popular regeneration model system thanks to its ability to replace lost or damaged tissue, including fin, spinal cord and heart. Therefore, to elucidate mechanisms of cardiac regeneration/repair would have interesting therapeutic implications.

To simulate the ischemic injury of myocardial infarction caused by coronary artery occlusion, we set up a treatment to cause acute hypoxic stress in adult zebrafish. In order to elucidate possible mechanisms of injury repair, we focused our attention on the search for cardiac progenitors.

Adult zebrafish heart expresses genes involved in cardiac development and, at low levels, a gene homologous to a mammalian stemness marker (*kita*). We observed the up-regulation of *kita* 18 hours after zebrafish exposure to hypoxia and we are currently analyzing possible differential expression of other key development genes that might be involved in the repair of localized ischemic damages and might suggest a mechanism of differentiation of cardiac progenitors present in the adult heart.

Expression profiling of neural precursor cell markers in the adult zebrafish optic tectum

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In adult teleost brain, proliferating cells are observed in broad area while these cells are located in the restricted regions in mammalian brain. In the optic tectum of adult zebrafish brain, most of proliferating cells are distributed in the caudal margin of periventricular gray zone (PGZ). However, whether these cells have properties of neural stem or precursor cell is still unknown. To confirm this point, we examined expression patterns of known neural stem/precursor cell markers along with bromodeoxiuridine (BrdU) labeling in the optic tectum PGZ of adult zebrafish. We found that the PGZ of optic tectum is largely divided into three distinct regions, one mitotic region and two non-mitotic regions which we designate as superficial and deep layer of PGZ. These regions are distinguished by the differential expression patterns of neural stem/precursor or neuronal cell marker genes, such as *pcna*, *gfap*, *sox2*, *fabp7a*, *cntfr*, *msi1, elavl3* and *neurod*. We also analyzed cell linage of proliferating cells from 24 hours to 2 months after BrdU incorporation. We found that few BrdU-positive cells were still observed in the mitotic region in 2 months after BrdU incorporation. We also found that most of the cells exited cell cycle and showed strong *elavl3* expression between 2 weeks and 1 month after BrdU incorporation. Then, these cells differentiated into the cells in the superficial layer of PGZ. Interestingly, some populations of BrdU-positive cells which ceased cell proliferation were *elavl3*-negative and maintained expression of *sox2*, *fabp7a*, *cntfr* and *msi1*. In contrast to *elavl3*-positive cells, these cells incorporated into deep layer. These findings suggest that most of the progenitor cells differentiated into cells consisting mature PGZ. However, some populations may not differentiate and maintain neural stem/progenitor-like properties in the adult optic tectum.

Expression of Telomerase and Telomere Length are Unaffected by Either Age or Limb Regeneration in danio rerio

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The zebrafish is a rapidly growing model for studying many aspects of biology. Recently, *ztert*, the teleost homolog of the mammalian telomerase gene has been cloned and sequenced. In contrast to humans, it has been shown that the zebrafish maintain telomerase activity for much of its adult life. We have looked systematically at several individual organs of the zebrafish with regard to both telomere length and telomerase activity through stages of its adult life. Heart, gills, kidney, spleen, liver, and intestine were evaluated at 3 months, 6 months, 9 months, and 2 years old by Southern blot analysis and TRAP assay. We find that telomeres do not appreciably shorten though the lifespan of the zebrafish in any organ. In addition, there was little difference in telomere lengths between organs. All tissues examined also expressed similar and abundant amounts of telomerase activity at all time points. Even following limb regeneration performed by fin clipping, no shortening of telomeres was observed. We speculate that the retention of lifelong telomerase activity is necessary in the zebrafish as it as an organism with a tremendous regenerative capacity. The study of the zebrafish's ability to maintain lifelong telomerase activity may be helpful in unraveling the complexity involved in the telomere maintenance (or lack thereof) in other species such the mouse or human.

Transcriptomics approach to investigate zebrafish heart regeneration

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Ischemic cardiopathy is the leading cause of death in the world, for which efficient regenerative therapy is not currently available. In mammals, after a myocardial infarction episode, the damaged myocardium is replaced by scar tissue featuring collagen deposition and tissue remodelling with negligible cardiomyocyte proliferation. Zebrafish, in contrast, display an extensive regenerative capacity as they are able to restore completely lost cardiac tissue after partial ventricular amputation. Due to the lack of genetic lineage tracing evidence, it is not yet clear if new cardiomyocytes arise from existing contractile cells or from an uncharacterised set of progenitor cells. Nonetheless, several genes and molecules have been shown to participate in this process, some of them being cardiomyocyte mitogens in vitro. Though questions as what are the early signals that drive the regenerative response and what is the relative role of each cardiac cell in this process still need to be answered, the zebrafish is emerging as a very valuable tool to understand heart regeneration and devise strategies that may be of potential value to treat human cardiac disease. Here, we performed a genome-wide transcriptome profile analysis focusing on the early time points of zebrafish heart regeneration and compared our results with those of previously published data. Our analyses confirmed the differential expression of several transcripts, and identified additional genes with differentially regulated expression during zebrafish heart regeneration. We validated the microarray data by conventional and/or quantitative RT-PCR. For a subset of these genes, their expression pattern was analyzed by in situ hybridization and shown to be upregulated in the regenerating area of the heart. The specific role of these new transcripts during zebrafish heart regeneration was further investigated ex vivo using primary cultures of zebrafish cardiomyocytes and/or epicardial cells. Our results offer new insights into the biology of heart regeneration in the zebrafish and, together with future experiments in mammals, may be of potential interest for clinical applications.

Hypoxia and reoxygenation injury response in the zebrafish adult heart

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Background: The adult zebrafish heart is known to regenerate following amputation of the ventricular apex. The mechanisms for this response are poorly characterized.

Objective: It was examined whether systemic hypoxia followed by reoxygenation damages the adult zebrafish heart and, eventually, whether a myocardial regenerative response occurs after injury.

Methods and Results: Wild type (WT) adult zebrafish were kept either in 5% (hypoxic; H) or in 80% (normoxic control; C) O_2 fish water for 15'. Thereafter H fishes were returned to normoxic water and the heart was removed for analysis at different time points between 3h and 24h after H treatment. Apoptotic DNA fragmentation was measured by TUNEL staining of heart serial sections; 12h after H there was a 5-fold increase in the number of apoptotic nuclei vs C (n=2 in each group). Determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cardiac tissue lysates was performed to assay internucleosomal degradation of genomic DNA occurring during apoptosis; 14h after H histone-associated DNA fragments were 6-fold higher than in C (n=5 in each group; $p<0.05$). Caspase-3 activation was evaluated by immunoblot analysis of the whole heart; activated caspase-3 was absent in C hearts, it was detected at very low expression level 6h after H and was strongly upregulated 14 and 18h after H (n=2 at each time point). The expression of different genes involved in programmed cell death was determined by quantitative RT-PCR on RNA isolated from the whole heart 3-6h after H. It was found a 2-6–fold upregulation of genes involved in apoptosis (*bax*, *bcl-2*, *bcl2l10*, *bik* and *api-5*), of the stress response gene heme oxygenase1 (*hmox1*) and of the erythropoietin (*epo*) gene (n=3 in each group). Finally, cardiac function was assessed by echocardiography (High-frequence Ultrasounds System, 70 MHz,Vevo770 VisualSonics) 16h after H. Zebrafish exposed to H (n=5) exhibited a significant decrease in function as determined by valvular annular plane systolic excursion *vs* C (from 0.71±0.1mm to 0.55±0.1mm , n=5, $p<0.05$). In order to investigate whether hypoxia and reoxygenation injury triggered a regenerative response cardiac DNA synthesis was determined by BrdU immunofluorescence as an indicator of cell proliferation; 24h after H $BrdU^+$ nuclei were 9.20% of all heart nuclei vs 0.82% in C.

Conclusions: Under the conditions of the present study systemic hypoxia followed by reoxygenation induces significant cardiac damage associated with a marked decrease in ventricular contractility. Further, it was observed an increase in DNA synthesis which may lead to cardiac regeneration and repair.

***Prokineticin 2* expression is associated with neural repair of injured adult zebrafish telencephalon**

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It is well known that teleost fish, including zebrafish, display an intense proliferative activity in several brain regions throughout their life. We used this feature to analyse the distribution of *prok2* transcripts in adult zebrafish brain and during injury-induced telencephalon regeneration. Prokineticin 2 (Prok2) is a secreted protein that regulates diverse biological processes including olfactory bulb neurogenesis in adult mammals. However, its precise function in adult neurogenesis is not yet fully understood. First, we characterized the zebrafish *prok2* gene and showed that its transcription was mainly restricted to neurons in almost all proliferating areas in adult zebrafish brain. Using a novel paradigm of telencephalon injury in adult zebrafish, we observed that brain lesions induced a dramatic increase in cell proliferation within the injured hemisphere in regions located both adjacent and distal to injury sites. Our data also strongly suggest that this proliferation was followed by the migration of newly generated neurons towards injury sites. During the very first days following injury, we observed the transient appearance of *prok2*-expressing cells in regions around the lesion, and a few days later in broad cell rows extending from cortical regions of telencephalon ventricles to injury sites. Ectopic transcription of *prok2* was no longer detected when the wound healing process was close to completion, showing that ectopic prok2 transcription parallels neuronal regeneration. Taken together, our results suggest that Prok2 may be involved in both adult neurogenesis and post-lesion neuronal regeneration.

The role of Notch/Delta signaling in adult fin regeneration

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Some nonmammalian vertebrates like urodele amphibians and teleost fish are capable of completely replacing damaged or lost body parts. This ability of regeneration in adult animals is a striking example of postembryonic morphogenesis and is thought to involve stem cell proliferation and/or dedifferentiation of somatic cells, cell differentiation and migration and tissue patterning. Although several factors and pathways involved in regeneration have been identified recently still major questions concerning genetic and cellbiological interactions within the regenerating tissue remain unsolved.
We and others have recently shown that the canonical Wnt/ß-catenin signaling pathway is required for zebrafish fin regeneration. Using microarray based transcriptional profiling of regenerating fins in which we specifically blocked or enhanced Wnt signaling we have identified several Notch/Delta pathway components as being regulated by Wnt/ß-catenin signaling during fin regeneration. The Notch/Delta pathway is highly conserved across metazoa and has a pivotal importance during embryonic development. Previously, Notch signaling has been found to promote tail regeneration in Xenopus tadpoles and to be required for the regeneration of rat tracheal epithelium, mouse satellite cells and zebrafish lateral line hair cells. However, to our knowledge nothing is known about Notch signaling in zebrafish fin regeneration. Using a heatshock-inducible line to overexpress the Notch1a intracellular domain (N1aCD) we found that Notch gain-of-function inhibits fin regeneration. N1aCD overexpression after wound healing and formation of the progenitor cells of the blastema has taken place is still sufficient to interfere with regenerative outgrowth, indicating that Notch signaling has a function in controlling proliferation and differentiation of blastema cells. Supportingly the expression pattern of the blastemal marker *msxb* is massively broadened at six days post amputation as compared to the control. These preliminary results suggest a prolonged maintenance or even ectopic induction of blastemal cells at the cost of differentiated tissue in the Notch gain-of-function situation. Thus, we hypothesise that Notch/Delta signaling enhances proliferation and delays differentiation of blastemal cells, a function that this pathway is also known to exert on other progenitor cells in various developmental processes. We are currently analyzing the morphological and molecular details of this phenotype by histology as well as in-situ and antibody staining. Additionally we are establishing transgenic lines to study the Notch signaling loss-of-function effects.

Asb11 is a muscle satellite cell marker important for regeneration

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Muscle satellite cells are progenitor cells that are involved in formation and growth of adult muscle, as well as regeneration following injury or disease. To fulfill their role in muscle maintenance, hypertrophy and repair, satellite cells are activated from their quiescent state to produce myoblast precursors. Various molecular markers have been identified in quiescent satellite cells, such as Pax7, saliomucin CD34 and the adhesion molecule M-cadherin. Here, we identified a novel muscle satellite cell marker, ankyrin and SOCS box containing 11 (Asb11). We show that Asb11 co-localizes with BrdU long term label retaining cells. We induced muscle injury in the dorsal flank of the zebrafish muscle, and observed the proliferative and regenerative response. By comparing responses in wild-types, Asb11-/- homozygous mutants as well as mutants with gain-of-function of canonical Wnt signalling at various time-points post-injury, we show that Asb11-/- is less effective in regeneration in response to muscle injury. Therefore, Asb11 is a gene that marks muscle satellite cell whose function is important for effective regeneration.

MicroRNA regulation of the Hedgehog pathway

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The importance of microRNAs in development is now widely accepted and key roles have been demonstrated in multiple cellular processes including cell fate specification, cell signalling and organogenesis. MicroRNAs are small RNA genes between 19-25 nucleotides in length, which act at the post-transcriptional level to downregulate the expression of target mRNAs. In mammalian species the level of binding between a microRNA and its target mRNA is not 100% and can demonstrate mismatches, gaps and bulges in the alignment. This makes target identification much more complex.

Since their discovery in 1993 the zebrafish model has played a pivotal role in the understanding of this field. However despite extensive investigation little is known concerning the specific targets of individual microRNAs. We approached this problem using the zebrafish as a model system to evaluate the expression, function and interactions between microRNAs and genes involved in the Hedgehog pathway. We identified microRNAs that when altered in expression result in U-shaped somites, reduced embryo size, ventral curvature and mild cyclopia, a phenotype which mimics misregulation of the Hedgehog pathway. Upregulation of the pathway has been indicated by an increase in ptc1 expression and increased numbers of superficial slow-muscle fibres.

MicroRNA control of this critical developmental pathway represents an ideal mechanism to maintain the precise expression gradient of Hh exposure to cells allowing correct specification and signalling. Determination of specific microRNA-mRNA interactions is critical to expand our understanding of this gene family which are emerging as key factors in early embryonic development.

miR-204 is required for vertebrate eye development via *Meis2* targeting and *Pax6* regulation

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The functional role of specific microRNAs in controlling the morphogenetic and cell differentiation events involved in normal eye development in vertebrates is still largely unknown. Here we show that a single microRNA, miR-204, is capable to regulate multiple aspects of eye development in medaka fish. Targeted ablation of miR-204 function by morpholino injections in medaka determined a severe eye phenotype characterized by microphthalmia, aberrant lens formation, incorrect retinal cell differentiation and coloboma. Through a variety of in vitro and in vivo approaches, we found that Meis2 is a key target of miR-204 and plays a pivotal role in the generation of this phenotype via the regulation of the Pax6 pathway. These data demonstrate for the first time that a specific microRNA is involved in the regulation of basic processes underlying eye development and open new avenues on a better comprehension of the pathogenetic mechanisms underlying eye developmental disorders.

Zebrafish Dazl ensures PGC specific gene expression through blocking the miRNA-mediated gene silencing

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Germ cells are the specialized cells that produce next generations. Early in development, primordial germ cells (PGCs) set aside from somatic cells and acquire unique gene-expression program. In many species, maternally supplied mRNAs play significant roles in PGC development. Expression of these mRNAs is confined to PGCs through various mechanisms.
Our previous study showed that *TDRD7* (Tudor-domain-containing protein 7) mRNA is expressed maternally and is restricted to PGCs in zebrafish. In somatic cells, one of the most abundant microRNA (miRNA), miR-430, represses *TDRD7* expression by inducing deadenylation. On the other hand, *TDRD7* escapes from the repression by miR-430 in PGCs, Here we show that Dazl protein relieve the miR-430 mediated repression of *TDRD7* mRNA. Dazl induces polyadenylation through binding to TDRD7 3'UTR and stabilizes the mRNA. We also reveal that this mechanism works on dazl mRNA itself, the other PGC-specific mRNA. These findings indicate that Dazl ensures PGC specific gene expression through blocking miRNA-mediated gene silencing.

Finding miRNAs able to regulate angiogenesis

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MicroRNAs (miRNAs) are short non coding RNAs shown to exert essential roles by regulating the stability and translation of messenger RNAs. They are thought to regulate more than 30% of all protein-coding genes and are frequently misregulated in numerous tumorigenic processes.
Using the zebrafish as animal model, we aimed at discovering some microRNAs that could potentially be involved in regulating angiogenesis. As a first step to our study, we have performed bioinformatic analyses to look for zebrafish miRNA candidates whose mature sequence is conserved in human. We also mainly selected miRNAs that have some predicted angiogenic targets conserved across distant species. The biological function of these miRNAS (mir-204, mir-214, mir-132, mir-365, mir-125b) has been tested *in vivo* by two means: 1) blocking of the miRNA maturation by injection of a morpholino oligonucleotide into fertilized eggs 2) overexpression of the mature form of the miRNA through injection of RNA duplexes at one-cell stage. Mir-125b appeared to be a good angiogenic candidate, as its inhibition leads to an interesting phenotype at moderate doses of morpholino. Although mir-125b has recently been shown to be a negative regulator of p53 (Le *et al*., 2009), our morphant embryos display no obvious apoptosis in the head and look overall good at that particular dose of morpholino. Instead, in mir-125b morphants (or mir-125b-injected duplex embryos), circulation stops and they quickly develop heart edema. In most embryos, intersomitic vessels fail to sprout from the dorsal aorta and/or to migrate properly between the intersomitic boundaries. Tunel assays suggested that there is no increased apoptosis in the tail and trunk of these embryos. The intersomitic vessels rather present supernumerary filopodial extensions that could possibly reflect a lack of proper guidance cues. Interestingly, blocking of mir-125b expression in HUVEC cells also leads to impaired migration of the cells. We are currently trying to extend these results to other angiogenic models and, more importantly, are trying to validate which mir-125 targets, other than p53, could mediate these effects.

A ChIP-seq approach to identify targets of T-box factors in early zebrafish development
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Embryonic development is a process during which the potential fate of individual cells becomes progressively restricted leading to specialized cell and tissue types. Correct fate determination requires the action of general and specific transcription factors, which either activate or repress the expression of specific genes. To understand the biological mechanism by which transcription factors regulate development it is important to identify their targets. We have previously approached this problem using chromatin immunoprecipitation followed by analysis of transcription factor-associated DNA on genomic microarrays (ChIP-chip). Whilst such studies have yielded valuable data they are limited by the coverage of the available microarrays. A superior alternative now available to us is chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq). ChIP-seq data have numerous advantages over microarray data including increased coverage of the genome and no loss of data between genome releases. Here we will present our ChIP-seq methodology, preliminary data for No tail and Eomesodermin genomic binding and a comparison with our ChIP-chip data.

Establishment of medaka full-length cDNA resources -An activity of NBRP Medaka-

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Although medaka genome sequences are now publicly available through Ensembl, USCS genome browser and UT genome browser, the annotation of medaka genome using species specific data such as medaka cDNA and protein is very limited. To overcome this situation, we are now conducting the large scale full-length cDNA sequencing project. We have constructed seven full-length cDNA libraries (early embryogenesis, organogenesis, fry stage, male liver, brain, testis and ovary) with V-capping method (Kato et. al., DNA Res, 12:53-62, 2006). We picked up 24960 clones from each library and sequenced both end of the clones. After mass alignment of all data, we identified over 18000 unique clones with the difference of 3'end. We are now sequencing whole sequences of each representative clone. About 30 % of clones from cDNA libraries of the organogenesis stage and brain are unique in each library. Number of unique sequences in liver cDNA library is only 12.5 %. We observed the variation of transcription start site in the genes, whose transcripts are relatively abundant. All data and clones are searchable and open for public through NBRP Medaka website (http://www.shigen.nig.ac.jp/medaka/).

FISHTRAP: An Insertional Mutagenesis Screen in Zebrafish using the Ac/Ds transposon system

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We are using the *Ac/Ds* transposon to carry out a transposon insertion screen in zebrafish. The transposon system we are using is multifunctional, and contains a gene-trap reporter, an enhancer-trap reporter, and a deleter cassette. In the year 2007-2008, we performed a pilot screen using maize Ac/Ds transposon system, which was recently reported to yield in a high rate of germ-line transmission in zebrafish (Emelyanov, 2006). We tested the efficacy of several Ds insertion cassettes, and identified one which gave a germ-line insertion frequency of ~24%. We find that founders, on average, harbor 3-4 inserts. Expression of the gene trap fluorescent reporter in progeny of injected founders was found in multiple cell types during embryogenesis, including the central nervous system, notochord, circulatory system pronephric ducts, liver, and intestine. F_1 progeny of the *Ds* insertion lines were confirmed by PCR, and flanking sequences from 74 insertion lines obtained by Thermal Asymmetric InterLace (TAIL) PCR. We have performed phenotypic characterization of F_2 fish, and have identified insertion mutants with defects in the digestive organs, CNS, heart etc… Our pilot gene trap insertion screen shows that the maize Ac/Ds can be used successfully to trap genes in zebrafish.
We have now embarked upon a large-scale functional genomics screen as a collaborative effort involving multiple zebrafish laboratories in Singapore. The Ds insertions from the FISHTRAP screen will be made available to the larger research community in due course, and has the potential make a significant impact in the field of zebrafish functional genomics.

Havana and Vega: Providing Manual Annotation for the Zebrafish Community

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The zebrafish genome is being sequenced and analysed in its entirety at the Wellcome Trust Sanger Institute. The manual annotation is provided by the Human and Vertebrate Analysis and Annotation (HAVANA) group and is released at regular intervals via the Vertebrate Genome Annotation (Vega) database (http://vega.sanger.ac.uk). The Vega database is a central repository for high quality, frequently updated, manual annotation of vertebrate finished genomic sequence. Our annotation is completed in close collaboration with the Zebrafish Information Network (ZFIN) (http://zfin.org/), which has enabled us to provide an accurate, dynamic and distinct resource for the zebrafish community as a whole.

Our manual annotation is based on the reference genomic sequence that has been derived from clones meticulously finished in-house to an accuracy of over 99.99%. This method of annotation produces a wealth of reliable information that may not be readily or accurately identified by current automated efforts. Such as, for example, non-coding genes, pseudogenes, complex gene structures, clusters and re-arrangements. All structures are supported by evidence provided by the latest species-specific and cross-species cDNA, EST and/or protein homologies (UniProt) and facilitate the identification of novel splice variants, and poly-A features.

New zebrafish transcriptome sequence from the Illumina (Solexa) Genome Analyzer has recently provided a valuable new source of data. Extremely deep coverage from a range of developmental stages and adult tissues has proven an excellent resource for manual annotation. Gene models have been extended and previously fragmented genes joined. In addition, further alternative splices have been identified and previously un-described genes highlighted.

Full clone annotation of our current finished clone path for chromosomes 2, 4, 5, 8, 9, 10, 13, 18, 19, 20, 22 & 23 is available in Vega. In addition we have annotation for the majority of ZFIN cDNAs that map to our current assembly. These two annotation strategies are on-going and we aim to finish full annotation of the rest of the genome within the next few years.

Elucidation and comparative analysis o fan MHC haplotype in the CHORI-1073double-haplois zebrafish

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The major histocompatibility complex (MHC) comprises a group of genes involved with the adaptive and innate immune systems. Whilst it is known to be present amongst vertebrates, it remains poorly characterised outside of mammals. Its role in disease susceptibility and resistance makes the region a focal point in comparative genomics, hence there is a need for further data from a wider variety of species. The MHC core regions in the zebrafish reference genome currently consist of several haplotypes; this is not conducive for describing a region known to display major variation. We have therefore selected a tile path of BACs from the double haploid (DH) fish allowing the replacement of these mosaic regions with a single haplotype.
The prior sequencing and annotation of the human, pig and dog MHCs has shown that in these cases the MHC exists as a tightly linked cluster, encoding over 200 immune related proteins contained within three sub-regions. The chicken MHC, in contrast, contains just 19 genes. In zebrafish, whilst we have identified a core set of orthologous genes grouped together on chromosome 19, many other members are found scattered across the genome. Similar disparate organisations have been described in other teleosts: in medaka an orthologous core region of class I genes is found on chromosome 11 whereas the class II genes are spread over several chromosomes, and segregation analysis in stickleback has shown class I and class II loci assort independently of each other. This suggests that the different teleost lineages evolved independently following the initial whole genome duplication, via large-scale genomic rearrangements.
As the zebrafish is an excellent model for developmental studies, we anticipate that the elucidation of its MHC combined with genomic comparisons with other lineages will improve the understanding of function of these immunological genes.

Genetic analysis of clonal characteristics of medaka nuclear transplants generated from the somatic cell nuclei transfer to non-enucleated diploidized eggs

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Nuclear transplantation is a key technique for cloning animals using somatic cells. We previously reported a novel method for generating diploid and fertile medaka fish through the transfer of adult somatic cell nuclei to diploidized eggs. Since we used non-enucleated eggs as recipients, we simply accepted that the recipient nuclei would contribute, at least in part, to the formation of nuclear transplants. Interestingly, the resultant transplants have exhibited only the genotypic and phenotypic traits of donor clones to date. To further clarify the nuclear origin of these donor clone-like transplants, we sought to determine whether the transplants had traces of the recipient DNA in their genome.

Populations of Japanese medaka consist of two groups, a northern population and a southern population. HNI-I is one of the representation of northern population and d-rR strain belongs to southern population. Since these two strains are genetically distinct from each other and the numerous DNA polymorphisms known to exist between them have all been well characterized, these polymorphisms could be utilized to effectively identify the origin of the DNA in the transplants. We successfully prepared three adult diploid nuclear transplants using the medaka HNI-I strain as the donor and the d-rR strain as the recipient. We therefore assayed the genotypes of the transplants using 96 polymorphic PCR markers and genomic DNA isolated from the anal fins of all three transplants and from the internal organs (brain, eye, liver, kidney, spleen, muscle) of one of the transplants. The results showed that, at all of the chromosomal locations examined - four sites on each of 24 chromosomes - the polymorphisms obtained showed the transplant genotypes to be identical to HNI-I, indicating that the nuclei of the three transplants originated from the donor. We therefore conclude that the putative medaka clones produced by our new method are true clones of the donor.

Genome-wide functional screen for the enhancers in zebrafish
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In vertebrate genome the enhancers can be scattered over large distances in non-coding sequences. In respect of coding regions they can be located upstream, downstream and in intronic regions. Such scattered distribution makes difficult the identification of enhancers by functional assays. The enhancer trap technique is the effective approach for monitoring gene activity despite on the difficulties of direct identification of regulated gene.
We have previously established several enhancer trap (ET) lines using *Tol2* transposable element from medaka fish and demonstrated that the genomic copy of *Tol2* can be remobilized into a new location after injection of transposase mRNA into a donor line. Using this strategy, we have been able to generate a new collection of 220 ET lines with a different tissue-specific expression of the reporter gene and analyzed a genome-wide distribution of *Tol2* reintegrations. Importantly, in some lines the reporter gene, localized on different chromosomes, formed distinct synexpression groups. We have done the fine mapping and annotation of regions for those ET lines where insertions caused GFP expression in the midline structures, in particularly the roof plate and floor plate. Our data for the first time represent *in vivo* view of genome-wide activity of the tissue-specific enhancers in vertebrates.

Retinal Progenitor Apoptosis and Aberrant Photoreceptor Morphology Characterise The *dying on edge* (*dye*) Mutant

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Aim: To characterise the phenotypes that affect visual function in the *dye* mutant.
Methods: Optokinetic response (OKR) screens of ENU-mutagenised zebrafish identified a family with a recessive mutation disrupting visual function. Retinal morphology was characterised by light microscopy. Cellular lamination and retinal cell type markers were characterised by immunohistochemistry using *zpr1, zpr3, 5E11, Zn5* and *Cralbp antibodies*.
Results: *dye* mutants display a significantly reduced or absent OKR, and are paler and slightly shorter compared to siblings. At 5 dpf *dye* retinas have undergone extensive lamination, although the eyes are smaller. A striking phenotype is the predominant apopotosis restricted to the ciliary marginal zone (CMZ) which is the retinal progenitor cell niche. The retinal pigment epithelium (RPE) is paler and contains numerous vacuoles and the photoreceptor layer appears thinner. Immunohistochemistry indicates that markers of the inner retina (*5E11 (amacrine), Zn5* (ganglion) and *cralbp* (Muller)) stain equivalently in mutants and siblings. However *zpr3* and *zpr1* staining of photoreceptors is significantly reduced in *dye* mutants.
Conclusions: We have identified and partially characterised a novel zebrafish mutant with a visual function defect. Preliminary results suggest that this defect is caused by abnormal photoreceptor morphology and progenitor apoptosis.

Toxicogenomic responses in zebrafish embryos as predictive toxicology models: the effect of azinphos-methyl and acetylcholine esterase inhibitors

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There is a high demand for reliable and ethically acceptable methods to asses the toxicity of industrial chemicals, pesticides, biocides and pharmaceuticals. The zebrafish (*Danio rerio*) embryo test (*Dar*T) has been proposed as model for studying chemical impacts – for the prediction of environmental as well as human health risk assessment. It is considered as alternative for testing of adult animals. However, the assay currently relies on morphological endpoints, which may provide low sensitivity if compared to effects observed during chronic exposures. We hypothesise that the zebrafish embryo test can be used for the prediction of long-term effects of chemicals by analysing gene expression changes. Toxicogenomic approaches would allow identifying responsive genes to predict chronic fish toxicity and the underlying mode of action. Here we used the zebrafish embryo to elucidate sublethal gene expression changes provoked by exposure to azinphos-methyl (APM). APM is an organophosphate that inhibits acetylcholinesterase. We performed microarray analysis and validated the expression changes in a subset of genes by quantitative real-time PCR. Interestingly three genes (*hsbp11*, *pdlim3b* and *socs3a*) were similarly regulated by different acetylcholinesterase inhibitors. Their spatio-temporal expression in exposed embryos suggests a potential role in protecting the development of muscle precursor cells. Furthermore, the increased toxicity of APM in embryos that have been injected with hspb11 antisense morpholinos indicates that hspb11 is involved in adaptation to chemical exposure.

Zf-Models - a large-scale effort to integrate European zebrafish research

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The ZF-MODELS project (Zebrafish Models for Human Development and Disease) was started in 2004 within the European Commission's Sixth Framework Programme, which aimed to overcome fragmentation in European research by encouraging large-scale collaborations.
In this spirit, ZF-MODELS included two major collaborative mutagenesis screens (one with a focus on adult mutations, the other focusing on the brain), a large-scale enhancer-trap screen (leading to the discovery of genomic regulatory blocks), advances in microarray technology and proteomics, the establishment of a TILLING service for the routine production of knock-out zebrafish, and an effort to integrate microarray and mutagenesis results with the Ensembl genome assembly.
In each of these areas, the guiding principle was to make high-throughput resources generated by European labs accessible to researchers studying specific developmental processes or disease models, be it through lab visits, through training, or by providing materials and data.
While the project has been successful in achieving its aims, a major limitation was the lack of a European resource center which would preserve the stocks and other materials generated in the project. This was precluded by the decentralized nature and limited duration of ZF-MODELS. As the project has come to its conclusion in June 2009, establishing such a center is now a priority for the immediate future.

Zebrafish Enhancer Detection (ZED) vector: a new tool to facilitate transgenesis and the functional analysis of cis-regulatory regions in zebrafish

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The identification and characterization of the regulatory activity of genomic sequences is crucial for understanding how the information contained in the genome is translated into cellular function. These cis-regulatory sequences control when, where and how much a gene is transcribed and can activate (enhancers) or repress (silencers) gene expression. Here we describe a novel Tol2 transposon-based vector for assessing enhancer activity in the zebrafish (Danio rerio). This "zebrafish enhancer detector" vector (ZED) harbors several key improvements: 1) a sensitive and specific minimal promoter chosen for optimal enhancer activity detection; 2) insulator sequences to shield the minimal promoter from position effects; 3) a RFP-marker as positive control of transgenesis efficiency in the injected embryos and in the stable transgenic lines; 4) a Flipase excision cassette to remove the positive control of transgenesis in stable transgenic lines if needed, and 5) a Cre-mediated excision cassette to delete the sequence being tested to confirm its enhancer activity. Additionally, we demonstrate that homologous highly conserved non-coding sequences from humans and zebrafish with enhancer activity retain their tissue-specific enhancer activity in this vector. More strikingly, insulators sequences from mouse and chicken, but not conserved in zebrafish, maintain their insulator capacity when tested in this model.

Reverse Genetics in Australia: Fishing for novel zebrafish mutants

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The use of zebrafish as a vertebrate genetic model organism relies mostly on investigating gene function in a forward genetic manner, where identified phenotypes lead to the discovery of novel genes. At the moment, fewer methods are available for reverse genetics, where a gene of interest is knocked out to study its function. There are currently two approaches available for reverse genetics: TILLING and deep sequencing. TILLING (Target Induced Local Lesions In Genome) has initially been established for plants and invertebrates and was shown to be applicable to zebrafish also (Wienholds et al., 2002). Deep sequencing allows the analysis of large groups of DNA samples, thereby detecting mutants with high throughput and low costs.
Requirement for either of those methods is the generation of randomly mutagenised fish that can either be kept alive during the screening process or can be used to set up a frozen sperm / DNA library that allows access to mutants far beyond the time limit of a zebrafish lifespan.
We have treated wild type males with *N*-ethyl-*N*-nitrosourea (ENU) and have subsequently out-crossed the mutagenised males to wild type females. The resulting mutants were used to perform sperm freezing. Simultaneously, tissue for DNA extraction was collected. Amplicons of known genes across the entire genome were sequenced to calculate the mutagenesis rate. The analysis confirmed a high saturation of mutations wit a mutagenesis rate of 1 base-pair exchange every 122 kb. Furthermore, as proof of principle, we resurrected one identified mutant via In Vitro Fertilization from a frozen sperm sample that was stored for 2 years.
The remaining mutant F1 females were crossed into a Tg(acta1:GFP)zf13 background. The resulting F2 families were screened for muscle mutants. This F2 screen resulted in the identification of two fin mutants, two heart mutants, two novel dystrophic and one muscle mutant.
We will now continue to perform state of the art ENU mutagenesis and will collect a total number of 5000 frozen sperm samples with accompanying DNA samples that will allow researchers from the entire community to screen for mutants of their interest. After identification of such, the frozen sperm sample will be used for the resurrection of the mutant through In Vitro Fertilization and the researcher will receive the fish for further analysis.

The Zebrafish Genome: an Update of Mapping and Sequencing Progress

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The Sanger Institute is in the final months of its nine year project to sequence the Zebrafish genome, funded entirely by the Wellcome Trust. At 1.36Gb in size, this is the largest single-centre sequencing project to be taken to HTGS Phase 3 in the world. The strategy used began with libraries of BAC clones, fingerprinted, mapped and sequenced to "gold standard" (at least 99.99% accuracy in euchromatic sequence), with the assembly being aided and informed by the addition of Whole Genome Shotgun (WGS) data. In 2008, a new Heat Shock map was generated by analysis of markers from 48 homozygous fish; this map now forms the basis for final clone selection and gap closure.

Many regions of the genome appear to have high levels of haplotype diversity; BAC and fosmid libraries generated from a single doubled haploid fish (CH-73/1073) have been used to resolve such regions and extend clone coverage. Fluorescence in situ hybridization (FISH) is being used to help anchor contigs, resolve order and estimate gap sizes along chromosomes.

We discuss sequencing progress and present examples of some of the unique challenges faced in finishing this genome, and report results from trials using the new technology platforms. Tools which aid the coordination and analysis of the genome are described.

The latest assembly can be viewed at www.sanger.ac.uk/Projects/D_rerio/wgs.shtml which includes links to pre-Ensembl where the new assembly zv8 can be found.

The sequencing project moves into maintenance phase in January 2010.

The Zebrafish Mutation Resource: Towards a TILLING knockout in every protein coding gene
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Target selected mutagenesis, or TILLING (for targeting induced local lesions in genomes), is a proven method that allows mutations to be identified in virtually any gene. We have previously shown that capillary-sequencing can be used to effectively identify mutations in a high throughput manner. A library of Zebrafish non-sense alleles is being created by the Zebrafish Mutation Resource, each with an associated morphological and molecular phenotype. Phenotypic data will be published via ZFIN (using an adapted version of the Phenote database). Information about the mutations identified by the project can be found on our website: http://www.sanger.ac.uk/Projects/D_rerio/mutres/.
The advent of next generation sequencing technologies has significantly changed the amount of mutations that can be identified and has opened up the possibility of identifying a non-sense allele in every protein coding gene within a reasonable time frame and budget. We have designed a strategy that will allow us to amplify and sequence thousands of exonic fragments on a single lane of the Illumina Solexa sequencing platform. A primer design program has been written that selects 100bp exonic fragments that are most likely to give a non-sense allele by ENU mutagenesis. These fragments (up to 4000 exons per experiment) are amplified across DNA pools from 24 mutagenised zebrafish, and each pool is run on one lane of the Solexa platform. Automated data analysis then searches the sequence for all potential non-sense alleles in the amplified fragments, and putative non-sense alleles are confirmed by capillary sequencing. Using this method the Zebrafish Mutation Resource aims to produce >1000 knockouts over the next 2 years, creating a rich resource for the zebrafish research community.

Integration of heat-shock, mgh and t51 genetic maps into zebrafish genome assembly (zv8)

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Due to the choice of genetic map (T51) on which previous Zebrafish assemblies have been built, there have been many discrepancies between genetic mapping positions and the position of the markers represented in these assemblies. Analysis of the correlation between Zv7 and the three genetic maps (HS, MGH and T51) highlights this problem. Despite the fact that the T51 map was used to anchor physical map fingerprint contigs (FPCs) in Zv7, there is poor correlation between the assembly and the map. Indeed, for Zv7 the average correlation between the three maps and the assembly is only around 0.7. This is an important issue for researches trying to map their mutations as well as for the genome sequencing project, making the task of identifying real gaps between FPCs and resolving them very difficult.

To improve Zv8 quality we have reorganized FPC order and orientation by combining the existing genetic maps: Heat Shock (HS), MGH meiotic maps and the T51 radiation hybrid map. We have reorganized the FPCs, prioritising the meiotic maps HS and MGH for long-range order and chromosome assignment, then using the T51 radiation hybrid map mostly to resolve local order and orientation. In this process we consider an FPC as an indivisible unit. The FPC provides a link between the markers mapped on the FPC and therefore makes an association between the three different maps. We have used this association to integrate map position data derived from the three different maps by sequence alignment between genetic markers and the sequence of each FPC. By using all three maps we increase the coverage and resolution to the maximum possible given available information. We see a striking improvement in the correlation between the Zv8 assembly and each of the genetic maps, which is now at an average of 0.96 for each chromosome. In addition, we find that Chromosome 4 has a significantly increased size now closer to the size predicted by flow cytometry. Assignment of sequence to Chromosome 4, however, needs to be interpreted with caution. The long arm of Chromosome 4 contains very repetitive sequence, which could lead to mis-localisation of FPCs.

The Zebrafish Genome Sequencing Project: Web resources

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The Sanger Institute has recently released a new integrated zebrafish genome assembly, Zv8. This genome assembly shows major improvement compared to its predecessors. It contains a higher precentage of high-quality finished clones and these clones have been ordered and oriented by careful use of the genetic maps HS and MGH and the T51 radiation hybrid map. In this process, we identified further artificial duplications arising from the use of several haplotypes and those have subsequently been removed. Gaps between the finished clones have been filled with contigs from a whole genome shotgun (WGS) assembly with a greater fold coverage than was used for Zv7.

The Zv8 assembly has been fully annotated using the Ensembl gene build pipeline and is accessible in Ensembl (www.ensembl.org). Ensembl now also offers a track for community annotation. Please enter your annotation at http://www.sanger.ac.uk/cgibin/Projects/D_rerio/Annotation/submitAnnotation.pl.

Finished clone sequence only with manual annotation is provided in the Vega browser (vega.sanger.ac.uk). With the sequencing project nearing its completion, we will eventually create a finished clone only assembly and provide integrated manual and automated annotation for it displayed in Ensembl.

We will present the new data to the community and demonstrate the use of the available web resources during the poster sessions and in a one-day workshop (details to be advertised). We will also help with individual problems regarding the use of these resources. Please come and see us!

Meta-analysis of rapamycin modulated expression profiles in the zebrafish and human

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Recent high-throughput gene-expression profiling and transcriptional control studies help deciphering the functional components in the genomes. In this context, large numbers of gene expression profiles under different physiological and pathological conditions are being generated for multiple vertebrate species ranging from zebrafish to humans. Meta-analysis of such multivariate datasets provides unique opportunities for comparative analysis of gene-expression modules. Accordingly, we performed a microarray experiment to decipher the effects of rapamycin on ZF4 transcriptome; and then meta-analyzed this dataset using a series of public zebrafish and human Affymetrix microarray data. We developed a meta-analysis tool in which selected human and zebrafish Affymetrix datasets were made accessible through a web-interface for statistical testing and visual representation using R modules (www.bioconductor.org). Our findings indicated that maternal expression of up- and down-regulated genes with rapamycin was differentially regulated during zebrafish embryogenesis. In addition, zebrafish rapamycin modulated gene expression signature was compared with those obtained from public MCF7 and PC3 datasets. Functional annotation of the commonly altered genes and signaling units were performed using available tools such as DAVID and Pathway Miner. In summary, an orthologous core meta-signature, common to zebrafish and human transcriptomes, could be extracted for defining the molecular contributors of TOR signal inhibition via rapamycin.

Development of estrogen-responsive transgenic zebrafish using a Gal4-UAS system

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To detect oestrogenic endocrine disrupting chemicals (EDCs) in the water we are attempting to develop transgenic zebrafish that are highly responsive to oestrogen. Previous transgenic work for detecting oestrogenic chemicals has used artificially synthesised ERE (estrogen responsive elements) or ERE-containing natural promoters with a Luciferase reporter gene in adult fish, but these systems have limited sensitivity. In an attempt to improve the response sensitivity to oestrogen, we adopted the use of a Gal4-UAS system. We developed a plasmid pERE-TATA-Gal4FF, containing three copies of estrogen response elements (3ERE) that on exposure to estrogen induce expression of Gal4FF, this in turn binds specifically to GAL4-responsive Upstream Activated Sequence (UAS) elements and these drive the expression of a second reporter gene, EGFP (Enhanced Green Fluorescent Protein).

We have examined the response of our construct to estrogen exposure in zebrafish embryos using a transient expression assay. The plasmids (10-20pg) were injected separately into 1cell staged transgenic UAS-GFP (green fluorescent protein) zebrafish embryos, and the embryos were exposed to the natural steroid oestrogen 17ß-oestradiol (E2), the synthetic oestrogen 17α-ethynyloestradiol (EE2), and the weak environmental estrogens Nonylphenol (NP) and Bisphenol A (BPA) and GFP expression examined using a fluorescent microscope. There was no GFP expression detected in embryos in unexposed controls, but specific and mosaic expression of GFP was detected in the lens, skin epithelium and many other cells in both the high (1000 ng/L EE2/E2) and low (100 ngE2/L, 10 ngEE2/L) treated groups (after 24h exposure). For the NP exposures, all embryos died at the highest concentration tested (100µg/L), but GFP expression was observed at an exposure of 10 ug/L. BPA No enhanced GFP expression was seen in any of the BPA exposed groups (concentrations ranging between 10 µg/L and 1 mg/L).

These data have confirmed that the plasmid system produced provides a response to oestrogen when introduced into UAS-GFP zebrafish embryos and suggests that our two step amplification system should produce an "estrogen sensitive" adult transgenic fish, but this has yet to be confirmed. This study is also the first to report on the application of ERE-driving reporter constructs in early life stage embryos to detect exogenous oestrogen exposure.

Antisense morpholino toxicity reveals novel role for apoptotic genes
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Antisense morpholinos (MOs) are the main tool used for knockdown of gene expression in zebrafish. Robu et al. (2007) demonstrated that a large fraction of morpholinos cause non-target related cell death via p53 activation, resulting in loss of embryonic structures and morphological defects. We show that this non-specific induction of p53 activity causes not only loss of gene expression (possibly due to cell loss) but also an intriguing up-regulation and ectopic expression of a subset of genes in the developing central nervous system. We examined the mechanism by which p53 activation produces these changes in gene expression, and surprisingly find that the mitochondrial apoptotic pathway is required. By pharmacologically activating the apoptotic pathway downstream of p53, and conversely blocking it in the presence of active p53, we show that these ectopically expressed genes are not upregulated by direct p53 transcriptional activity, but are instead induced by a Bcl-dependent mechanism. These unexpected non-specific phenotypes involving *gain* of gene expression add to the pitfalls of using MOs. Based on these and other studies, we propose recommendations for the use of MOs to analyze gene function.

Using In Vivo Electroporation for Time-Resolved Genetics in Zebrafish

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Loss-of-function analysis in model organisms has yielded a wealth of information about which genes are important for various aspects of development. In zebrafish, gene loss-of-function can be achieved by microinjection of loss-of-function reagents (e.g. morpholino and RNAi oligonuceotides) at the one cell stage. One caveat to this approach is that the loss-of-function reagent is present throughout development, which can be problematic when attempting to study later developmental events such as development of the nervous system. If the gene of interest is necessary for an earlier in development step, loss-of-function starting at the one cell stage may compromise assessment of neural development. Here we have a characterized a method, *in vivo* electroporation, which can be used to incorporate loss-of-function reagents at specific stages in development. *In vivo* electroporation of GFP and mCherry expression plasmids was used to determine the efficacy, viability, and temporal resolution of the method. We show that electroporation can target multiple neuronal populations of developing neurons including midbrain, hindbrain, cerebellum, and retina. We find that voltages that lead to efficient, reproducible GFP expression are well within the range for which there is very high viability. GFP expression is observed within 6 hours of electroporation, and reaches maximal expression by 24 hours. *In vivo* electroporation is an effective method for delivery of GFP expression plasmids at multiple developmental stages including 24, 48, and 96 hours post-fertilization. This method can also efficiently incorporate two different reagents at the same time – inclusion of both a GFP-expression plasmid and an mCherry expression plasmid in the electoroporation mixture leads to co-expression of GFP and mCherry in 95% of the cells examined. Co-expression of GFP and mCherry was used to determine the efficacy of using *in vivo* electroporation for RNAi-mediated loss-of-function. Inclusion of anti-GFP RNAi oligonucleotides prevented the expression of GFP by at least 80%, as assessed by ratioing GFP (targeted) to mCherry (not-targeted) fluorescence. In conclusion, *in vivo* electroporation can be used to deliver both expression plasmids and RNAi reagents at whatever developmental stage is of interest.

Heat Induction of the Gene in Medaka Embryos and Adult Medaka

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Induction of gene expression in any organ at any time is an important technique for investigation of various biological phenomena. To achieve the purposes, medaka transgenic lines (driver lines) with cre gene under the control of heat shock elements (HSEs) were developed. These lines were crossed to effector lines (loxP[DsRed]-GFP), which allows to express GFP by heat treatment.
We confirmed that the cre-loxP system worked by heat treatment in medaka embryos. After the loxP construct was injected into the embryos of cre lines, the expression of the reporter gene was observed in medaka embryos, following brief heat treatment.
To achieve appropriate conditions of heat treatment in adult medaka, we attempted two means. First, medaka was kept in a hot water (39C or 42C) bath. A day after the treatment, GFP expression was observed in some organs such as gonads, a liver and fins. Secondly, we employed a thin metal probe whose temperature can be controlled and monitored. By inserting this probe into the body cavity, noninvasive heat treatment was possible. This method successfully allowed us to induce gene expression in a tiny area of ovary.

Viral cell transduction in the adult zebrafish brain

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The adult zebrafish brain shows in contrast to mammals an enormous potential to produce new cells. Proliferating zones, visualized so far with the proliferation markers PCNA and MCM5 or by incorporation of the thymidine analog BrdU, can be found in several brain regions along the entire anterior-posterior axis. These germinal zones, mostly located close to the brain ventricle, contain self-renewing progenitor cells that show features of adult mammalian neural stem cells. BrdU tracing experiments showed that cell division can be followed by the formation of postmitotic neurons.

The aim of our study was now to develop a tool to analyze the long-term fate of neural progenitor cells by permanently labelling them.

For this purpose we established virus-mediated transduction of these cells in the adult live zebrafish brain. We chose retrovirus-based systems, which have the advantage that after infection the transgene is stably integrated into the genome of the target cells. We injected MLV- and HIV-based GFP-encoding retroviruses into the brain ventricles of live adult zebrafish. Preliminary results show infection of cells along the ventricular zones with two types of retroviruses and promoters. Further experiments will classify the set of successful technical combinations and reveal the long term fate of infected cells using immunohistological stainings of neuronal and glial markers.

This method will permit the long-term genetic tracing of progenitor cells in the live zebrafish adult brain and will help to further identify the molecular mechanisms controlling stem cell maintenance and differentiation.

Development of living color transgenic medaka for biomonitoring aquatic contamination

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With the advent of GFP reporter gene, it is feasible to apply the living color transgenic fish in monitoring water contamination. Previously, our laboratory has generated a GFP transgenic medaka line under the estrogen-inducible promoter from vitellogenin gene and demonstrated that the Tg*(mvtg1:gfp)* transgenic line faithfully responded to estrogen and other estrogenic compounds by displaying green fluorescence color and thus provided a convenient biomonitoring tool amendable for online surveillance of estrogenic compounds (Zeng et al., Eng. Sci. Tech. 39:9001). In the current presentation, we report the generation of second biomonitoring transgenic medaka line using a stress-inducible promoter, *hsp70*, with the designation of Tg*(hsp70:gfp)*. The transgenic line was generated with the aid of maize Ac/Ds transposon system. Out of 12 founders screened, we obtained germline transmission of the transgene from 10 of them, indicating a high efficiency of the Ac/Ds system to aid transgene integration. All of the transgenic lines we obtained show strong ubiquitous GFP induction within 4 hours of heat shock treatment at 37°C for two hours in 2 dpf or older embryos. The expression of GFP is consistent with that of endogenous *hsp70* mRNA after heat shock. In addition, the transgenic line also responded to several heavy metals including mercury, arsenic and cadmium. Thus the newly developed transgenic line may be used for monitoring stressed caused by heavy metals and potentially other chemicals to be determined. Besides Tg(*hsp70:gfp*), we are also interested in developing medaka GFP line using xenobiotic inducible *cyp1a1* promoter. Transcript level of *cyp1a1* was elevated in liver of 6 dpf embryo when exposed to benzo[a]pyrene (BaP) compared to vehicle control. By injection of a GFP construct under the *cyp1a1* promoter into medaka embryos, we also found that GFP expression was elevated by xenobiotic compounds such as BaP and 2,3,7,8-Tetrachlorodibenzo-p-dioxin, indicating the potential to develop stable Tg(*cyp1a1:gfp*) line for monitoring such pollutants. Currently, screening of founders for Tg(*cyp1a1:gfp*) is in progress and we hope to characterize this line with various categories of persistent organic pollutants (POP) including polyhalogenated biphenyls, pesticides and etc.

Combining bac recombineering & I-SCEI transgenesis to assess oscillatory gene expression
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Over the last decade the segmentation clock has emerged as an interesting model to study oscillatory processes at the molecular level. Cyclic genes are components of this clock and controlled at the transcriptional level. However, little is known about their enhancer architecture. Our aim is to identify and characterize regulatory elements that confer cyclic gene expression and test whether different cyclic genes share a conserved enhancer architecture.
To test this hypothesis we chose the zebrafish *her1/7* locus as starting point. Both cyclic genes are temporally and spatially coexpressed and located in a head-to-head orientation, implying that they might share regulatory elements within their intergenic region (~ 12 kb). To narrow these down, we have isolated and sequenced orthologous BAC clones of the closely-related goldfish and carried out phylogenetic footprinting of both *her1/7* loci. This *in silico* approach revealed highly conserved, intergenic elements. In order to test the requirement for and the sufficiency of these putative regulatory elements *in vivo*, we have introduced a new logic to generate reliable transcriptional reporters of cyclic gene expression. Conventional transgene constructs fail the challenging task to recapitulate very dynamic expression patterns such as cyclic gene expression because of prolonged reporter stability on both the mRNA and protein level. To overcome this problem we have employed BAC recombineering to manipulate the endogenous *her1/7* locus without size limitations. We have tagged either *her1* or *her7* with Venus, subcloned the desired size of the modified *her1/7* loci and introduced *I-SceI* sites for enhanced transgenesis frequencies.
Based on this approach we created multiple transgenic lines for her1::Venus and her7::Venus, whose reporter activity is indistinguishable from the endogenous mRNA patterns. Additionally these lines allow us for the first time to track protein oscillations *in vivo*. Based on this proof of principle experiment we are currently generating deletions of putative regulatory elements and test our *in silico* predictions *in vivo*. In parallel we carry out a similar approach for other cyclic genes such as *dlC,* which could eventually shed light on a common enhancer architecture shared between different cyclic genes.

Exposure effects on zebrafish of natural POP mixtures

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In the present study, developmental and reproductive effects of lifelong exposure to environmental relevant concentrations of 2 natural mixtures of persistent organic pollutants (POPs) were investigated using classical and molecular methods in a controlled zebrafish model. The mixtures used were extracted from burbot (*Lota lota*) liver originating from freshwater systems in Norway: one mixture with high levels of polybrominated diphenyl ethers (PBDEs) and one mixture with background levels of PBDE. Both mixtures had low, but substantial levels of PCBs and DDTs. The concentration of POPs measured in the zebrafish ranged from levels detected in wild fish from Lake Mjøsa, to concentrations reported in human and wildlife populations.

Phenotypic effects observed in both exposure groups include earlier onset of puberty, increased male/female sex ratio and increased body weight at 5 months of age. The exposed liver and gonad transcriptomes revealed changes in the expression of genes involved in endocrine signalling and growth in accordance with the observed phenotypic effects. Networks predicted by Ingenunity Pathway Analysis (IPA) indicate similar effects on puberty and weight gain by both mixtures, suggesting the PBDEs not having a main role in those events. The effects observed in the experimental zebrafish model raise the question of whether chemical pollution represents a risk to the reproductive health of the wild fish inhabitating the freshwater system.

Screening for small molecule modulators of disease processes and developmental mechanisms using the zebrafish

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Several groups within the MRC Centre for Developmental and Biomedical Genetics have established disease models that provide a basis for high content molecular screens. More recently, we have established a facility for medium-throughput screening using such high content biological assays with the dual aim of identifying novel reagents for continued research and the establishment of systems to speed the identification and validation of lead compounds for drug discovery. In contrast to conventional drug screening programs, where efforts are focused on a relatively small number of disease-relevant target pathways and proteins, the whole organismal nature of our models provides the potential for identification and validation of reagents affecting multiple pathways in the pathogenic process.
To take full advantage of the different screening methods that are available when using a whole organism, embryos will be screened for morphological, enzymatic and fluorescent output using a combination of automated and manual methods.

Optogenetic dissection of behaviour identifies spinal cerebrospinal fluid contacting neurons that drive locomotion

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Locomotion relies on neural networks called central pattern generators (CPGs) that generate periodic motor commands for rhythmic movements[1]. Little is known about the spinal inputs to the CPG that drive spontaneous locomotion. Here we identified such an input using a combination of intersectional gene expression and optogenetics[2] in zebrafish larvae. The photo-stimulation of one specific cell type was sufficient to induce a symmetrical tail beating sequence that mimics spontaneous slow forward swimming. The responsible neuron is the Kolmer-Agduhr (KA) cell[3], which extends cilia into the central canal of the spinal cord with an ipsilateral ascending axon that terminates in a series of consecutive segments[4]. Genetically silencing KA cells reduced the frequency of spontaneous free swimming, indicating that KA cell activity provides necessary tone for spontaneous forward swimming. Ablation of descending brain connections had no effect on the KA-induced behaviour, demonstrating that the KA circuit operates within the spinal cord. Our results suggest that spinal cerebrospinal fluid contacting neurons provide a positive drive for the locomotory CPG in early development and that this system may be conserved in vertebrates.

The zebrafish embryo as a model for studying extracellular matrix dynamics
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The extracellular matrix (ECM) is comprised of a biochemically diverse array of proteins and other macromolecules that provide tissues with essential mechanical and functional properties. As a non-living component of multicellular tissues, remodeling of the ECM during development and wound healing necessitates the secretion of proteases into the matrix and subsequent re-synthesis of ECM molecules in a new configuration. A complex family of zinc-dependent proteases - the matrix metalloproteases (MMPs) - are largely responsible for ECM remodeling in vertebrates. Misregulation of these proteases is central to a plethora of pathologies, including tumor metastasis, heart disease and arthritis. As such, a great deal of research has been focused on understanding the biochemistry and cell biology of MMPs and their regulation, however, largely due to the complexity of their post-translational regulation, much less work has been done in the context of a whole organism. We propose that the zebrafish embryo presents an excellent model system in which to study these phenomena. We have identified 23 MMP homologes in the zebrafish genome and have developed several techniques and reagents to assay the distribution, abundance, and activity patterns of these ECM remodeling effectors in the zebrafish embryo. In conjunction with classical embryological, biochemical and molecular techniques, opportunities for investigating the regulation of ECM remodeling in the zebrafish embryo are now unparalleled in vertebrate embryos.

Gene Targeting in Zebrafish by Homologous Recombination
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Zebrafish is an important model organism but the lack of the ability to modify the genome by homologous recombination limits its utility.
We aim to establish a gene targeting, by homologous recombination, system in zebrafish using an approach similar to that described in *Drosophila* by Rong and Golic (2000). Although this approach is time consuming to establish, it is generic in nature and if successful can be easily applied to many loci and possibly even other teleosts.
As a model system we aim to correct the *GolB*^{1} allele of the *Golden* SLC24A5 locus which is characterised by delayed and reduced development of melanin pigmentation.
Our method makes use of a Tol2 transposon based system to establish golden transgenic lines with either:

1. An insertion targeting construct containing an 18Kb section of the *Golden* locus with the wild type nucleotide in exon 5 and an I-SceI site, surrounded by attachment sites for ϕC31 integrase.
2. An excision construct, which inducibly directs the expression of I-SceI endonuclease and ϕC31 integrase using a heatshock promoter (HSP70).

Upon crossing and heatshock, unidirectional site specific recombination mediated by the ϕC31 integrase releases and circularises the targeting construct. I-SceI will then linearise the DNA by introducing a double strand break (DSB). In principal, the DSB will initiate homologous recombination within the target locus and result in the correction of the *GolB*^{1} mutation and reversion to wild type at the golden locus.
We have shown that the targeting construct can be inducibly and easily excised from the germ line genome in both males and females. We are currently measuring the rate of insertion of the excised targeting construct into the genome and the rate of reversion to the wild type phenotype.
It is practical to screen as many as 10,000 embryos for targeting, even in a small fish facility, suggesting that this approach has a reasonable chance of success.

What a tiny fish can do – From toxicity screens to disease model with the zebrafish embryo

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Early embryogenesis is the most susceptible period in vertebrate development. For this reason, particular attention is paid to the assessment of teratogenic effects in the regulatory context for the evaluation of environmental and human health risks. Governmental authorities and industries alike are urged to adequately assess possible adverse effects of chemicals, like pharmaceuticals, on the development of humans or animals. An exceptionally promising candidate for developmental toxicity testing is the zebrafish embryo assay. This assay proves a very versatile platform and also shows excellent potential for medium to large scale-screening applications. Besides its use as a non-target screen in human and environmental toxicology, the zebrafish embryo test is also a smart tool for target screening in drug discovery. For screening applications, the zebrafish' outstanding track record as a vertebrate development and genetic model outweighs by far its few disadvantages compared to e.g. the rodent model. The project UNIFISH takes advantage of these assets to develop universally applicable and automated assays, based on the zebrafish embryo test. UNIFISH pursues a comprehensive approach and combines microscopically performed morphometric analyses with, among other things, genomics (initially transcriptome analysis). All data are integrated in a database, establishing an extensive compound-specific mode-of-action (MOA) and toxicology data library. The library will form the core of a multifactorial knowledge-base for active compounds. Such a knowledge-base can be of great benefit and value for pharmaceutical, chemical or even food companies. The knowledge-base will contain zebrafish embryo toxicology and MOA-related data for a vast array of compounds, thus providing key information which is indispensible for the establishment of systematic relationships or simulations of effect of unknown compounds. Compound-specific gene expression patterns and biomarkers can easily be extracted from the data, and follow-up studies will add information on gene function, obtained e.g. from loss-of-function experiments with morpholino knockdowns, *in-situ* hybridisation screens with wildtype or mutant zebrafish embryos or from studies involving transgenic lines. The knowledge-base can flexibly be extended by including additional effect endpoints, like metabolomics data e.g., to meet individual customer or user needs.
Hence, UNIFISH aims to develop a technology platform that, on the one hand, suits legal authorities' and industry requirements to reduce the number of animals used for testing, and on the other hand delivers a versatile tool for drug discovery.

Increased anxiety-like behavior in zebrafish with point mutation in the glucocorticoid receptor

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Hyperactivation of the hypothalamo-pituitary-adrenal (HPA) system is one of the most prominent neurobiological findings in patients with stress-related psychiatric disorderss. Altered negative feedback control of the HPA, promoted by deficit in glucocorticoid receptor function, often denotes heritable predisposition for major depression following stressful life events. Here we show that adult zebrafish with a point mutation in the glucocorticoid receptor (GR^{s357}) are more susceptible to the detrimental effects of stress and thus offer an excellent model system in which to study the involvement of dysfunctional GR in the etiology of stress-induced anxiety disorders. In vitro transient transfection assay reveals that R443C substitution in the DNA-binding domain of GR abolishes all transactivation and transrepression activity. Dysfunctional GR^{s357} protein and impaired negative feedback may account for the observed increase in hypothalamic corticotropin-releasing-hormone (CRH) and proopiomelanocortin (POMC) mRNA as well as plasma cortisol in basal, non-stressed state. Behavioral testing reveals significant increase in freezing behavior of the homozygote fish in isolated arena following repeated exposures to similar conditions. This behavior is inhibited by acute (Diazepam) and chronic (Fluoxetine) anxiolytic and antidepressants treatments. Accordingly, changes in serotonin transporter mRNA levels before and following SSRI treatment, point toward a potential role for brainstem serotonergic system in these behaviors. Finally, mutant fish that are allowed visual interaction with other fish do not display the freezing behavior, suggesting social buffering effect. The result presented in this study emphasizes the uniformity in symptoms and machinery of stress-induced anxiety disorders across vertebrates and validates the use of zebrafish GR^{s357} mutant for exploring the neural basis of certain aspect of human disorder.

zebrafishbrain.org: an online neuroanatomical resource

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A complete description of the connections of all the neurons of the zebrafish nervous system, ie. the zebrafish connectome, is some years from being a reality. For this epic task to be completed, some key technologies remain to be developed. However, the developing zebrafish is likely to be a candidate for the first 'complete' first-draft vertebrate connectome because of its relative simplicity and accessibility. We are taking what might be considered the first tentative steps towards this enticing goal through the development of zebrafishbrain.org. This is a web-based neuroanatomical resource we aim to be a repository for all neuroanatomical knowledge about the zebrafish. The data contained within should provide a spring-board for the next steps toward the more distant goal of the connectome.

Currently, the resource consists of annotated image data derived from in-house high-resolution confocal imaging of a selection of transgenic lines (originally from many sources) which have specific expression patterns in one or more neuroanatomical structures. Embryonic or larval specimens are fixed and usually counter-stained, incorporating one of several brain-specific neuroanatomical antibodies for orientation, the specimens are then mounted and imaged at high resolution using scanning confocal microscopes. The stacks are processed using volocity software (Improvision) and projections are loaded into our database where they are annotated. These annotated data are then web-browseable by neuroanatomical structure. Detailed descriptions or 'tutorials' about specific brain structures have also been written, these use a selection of the image data to describe in detail the structure in question. To ensure interoperability we are maintaining parity with the ZFIN anatomical ontology in our lists of neuroanatomical structures for annotating and tutorial production.

The project is at an early stage: we have used data from about 30 transgenic lines and have produced a handful of fully-fledged tutorials to the level of detail we want to be standard for all structures. We are currently trying to secure funding both for the further development of the neuroinformatic side of the project (particularly to be able to present data in 3D space), to gather together more brain-specific transgenic imaging to populate the database, to write more tutorials and to extend detail to the level of individual neuronal morphologies, perhaps by using brainbow-type technologies.

We are interested in obtaining data from transgenic lines, or in obtaining the lines themselves, where expression clearly demarcates specific brain structures or classes of neurones or glia. Please contact us if you can provide such data or if you are interested in writing tutorials on your areas of expertise.

This project is part of ZF-MODELS, an EU-wide FP6 consortium.

DARENET: A novel technological platform to promote the use of zebrafish model

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Initially, the zebrafish (*Danio rerio*) model was used as a model in developmental biology. However, in more recent years this model organism has come to the attention of the international scientific community in many other applicable areas in both basic and applied research. Even though the areas of application and the scientific objectives of the different research centres and companies are quite varied, they all use the same model for research and development. Common procedures and methodologies should therefore be standardised, making it essential to introduce standard operating procedures (SOPs) in order to consolidate this model organism as an alternative method to other vertebrate model organisms such as mice, which have a higher cost in economic and ethical terms. In this regard, the European Union is promoting the use of alternative vertebrate model organisms in all applicable areas. This is why one of the objectives of this Spanish Technological Platform is to promote the implementation of SOPs that ensure animal welfare for this model according to the "3Rs": Reduction, Refinement and Replacement. These include the development of protocols to evaluate the animal's welfare and to control its health, to establish microbiological and genetic standards for the animal and standardise anaesthesia, analgesia endpoint criteria etc. Likewise, another important objective of this platform is to increase research that uses this model organism in all priority areas such as health, biotechnology and environment and eventually, promoting the presence of this platform in private and public research centres, and especially in large and small companies.

Towards a Mechanistic Understanding of Apical Detachment during Neurogenesis in the Zebrafish Retina

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During retinal ganglion cell (RGC) differentiation, post-mitotic neurons are generated at the apical surface of the retinal epithelium. Their cell bodies then migrate basally, while their apical and basal processes remain attached to the retinal membranes. Subsequently, apical processes are retracted and an axon is extended from the basal surface of each cell. While the initial outgrowth of the retinal axon from the basal process has been extensively studied, the mechanism underlying apical process detachment remains unknown.
This study focuses on the molecular basis of apical process retraction. So far, we discovered that the repellent guidance molecule, Slit1b, is involved in apical process retraction. We first checked the expression of *slit1b* mRNA during the onset of RGCs differentiation in zebrafish and found that it is expressed at the apical surface of the retina. Furthermore, using time-lapse approaches, we observe that during zebrafish retinal development *slit1b* morphants show delayed apical retraction. In these morphants, RGCs extend axons at similar stages as in wildtype but do so while still retaining an unretracted apical process.
Interestingly, a previous report has demonstrated that Slit can induce downregulation of the cell surface adhesion molecule, N-cadherin, through Robo receptor activation. Studies are under way to test if a similar mechanism applies to apical retraction during RGCs differentiation. To do this, we use a combination of immunocytochemistry and *in vivo* live-imaging approaches. Our study will shed light on the molecular events underlying apical retraction in RGCs and might uncover general mechanisms during neuronal differentiation in other model systems.

Live Imaging of Developing Connectivity in the Spinal Circuit of the Zebrafish Embryo
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Before sensory systems provide input to the nervous system, networks such as the spinal cord, retina and hippocampus display spontaneous, rhythmic bursts of action potentials. Global patterns of this activity have been shown to be locomotor-like in vertebrate models such as the rat and chick, with synchronization in ipsilateral regions of the spinal cord and alternation between the left and right side of the cord. We are interested in how these highly-coordinated activity patterns arise in development. The genetic accessibility and transparency of zebrafish allow us to apply optogenetic techniques to monitor the activity of confined populations of spinal cord neurons in an intact animal and through developmental time.
Using the UAS:GAL4 system, we are targeting the genetically-encoded calcium indicator GCaMP to motoneurons to observe their activity patterns during different stages of development. At 20 hours post-fertilization (hpf), three hours after the onset of spontaneous activity in the spinal cord, calcium transients in ipsilateral motoneurons are tightly synchronized and alternation is present between the left and right side. Two hours earlier (18hpf), however, spontaneous events in individual motoneurons are independent of each other, and neither synchronization nor alternation is observed. To further understand how the network progresses from sporadic to coordinated activity, we are using time-lapse calcium imaging to follow the development of correlations between individual neurons during this developmental period. An analysis of when and where correlated and anti-correlated groups appear will provide insight into the steps taken during the maturation of the underlying circuit.

In-Vivo quantification of cellular processes by high-resolution microscopy
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Cells have to carry out numerous functions that range from replication and energy conversion to molecule transport. In-vivo there are distinct cell types with different morphological properties and consequently variable functions. Currently there is no tool to identify, track and measure physical parameters of cells and nuclei, during vertebrate development allowing to correlate them to cell function. Basal parameters like volume and shape of organelles should be linkable with cellular processes. We want to develop a computer-based method that automatically recognizes and analyzes various cell identities in-vivo by their cytoplasmic/nucleic volume ratio and shape descriptions in living zebrafish embryos. Therefore, we want to generate a double-labeled fishline, to examine and analyze distinct cell types and automate their correlation with the imaging softwares Image J and Volocity. This method could be a basic tool for the identification and quantification of cellular processes like migration or cell division in-vivo.
To reach these aims, we already constructed a multicistronic vector that contains minitol2-integration sites to generate transgenic fishlines. With this construct we are able to label the membranes and nuclei of the fish embryos with different fluorescent proteins. Up to now, we injected this construct into embryos and were able to preliminarily analyze these embryos by confocal microscopy. We are able to identify single cells of living embryos in these confocal stacks, concluding that the live-imaging with this multicistronic construct works as expected. Consequently, it should be possible to expand this method by using a third fluorescent protein to visualize cellular processes and organelles.

In toto imaging of somitogenesis and muscle formation in FlipTrap zebrafish embryos

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Somitogenesis and myogenesis are highly conserved mechanisms in vertebrate species that give rise to skeletal formations. It is therefore important to observe these processes with high resolution to understand how muscles form, are maintained and degenerate throughout life. To that end, we combined two powerful methods in living zebrafish embryos to study muscle formation and degeneration with high-resolution and non-invasively: 1) in toto single-cell imaging using "embryo arrays", individual cell labeling and confocal microscopy, and 2) a FlipTrap genetic screen to isolate lines with citrine fusion protein expression in muscles and to create cre-lox conditional mutants. This novel screening approach has so far identified 10 lines with functional fluorescent protein fusions to endogenous muscle proteins and is used to assess in vivo the specific function of the trapped genes, before and after disrupting their function with cre-lox recombination. We show that the trapped proteins recapitulate endogeneous protein expression using immunostaining, and we compare the distribution of the specific mRNAs by combining imaging of the trapped protein with in situ hybridization. Time=lapse microscopy of the fluorescent protein allows us to watch the process of muscle formation and the distribution of the trapped proteins with unprecedented resolution in living zebrafish embryos. Together, these two approaches enable us to gain more insights into the roles played by specific proteins in muscle formation and maintenance.

Identification and Functional Analysis of the Gene Regulatory Network that Regulate a Primary Sensory Neuron Lineage in the Developing Zebrafish Spinal Cord using In toto Imaging and FlipTrap Screen

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The zebrafish spinal cord is a powerful model system to watch developmental circuits function *in vivo*. Early in development, the spinal cord emerges from a sheet of neuroepithelial cells that converge toward the midline to form a neural tube. In zebrafish and other anamniotes such as the frog, the boundary between the neuroectodermal sheet and non-neural ectoderm, the neural plate border (NPB), has been shown to give rise to both neural crest (NC) and Rohon-Beard (RB) neurons, which are early-born mechanosensory neurons essential for the embryonic and larval escape response. Loss of transcription factors that are expressed in the NPB, such as Prdm1a and Olig3, were shown previously to reduce the number of NC and RB cells. Thus, an "equivalence group" was proposed to give rise to both cell types. However, the complete cell lineage of RB neurons and the gene regulatory network (GRN) that induce, specify, and determine this lineage are unknown. We are using *in toto* imaging technology to elucidate the complete cell lineage of RB neurons. First, we are doing *in vivo* time-lapse confocal and two-photon imaging of zebrafish embryos wherein all the cell nuclei and membranes are tagged with different fluorescent proteins. Then, we are analyzing our imaging data sets to generate the RB lineage tree using GoFigure2, an open source image analysis and segmentation software suite being developed in our lab. Recently, a candidate gene approach had been used to characterize proposed components of the RB lineage GRN. To define novel components of the RB lineage GRN, we are screening for genes that are expressed in the RB lineage using the FlipTrap, a novel conditional gene trap mutagenesis approach also developed in our lab. FlipTraps generate a tissue-specific and functional endogenous fusion with yellow fluorescent protein. In the presence of Cre recombinase, truncated and mutant versions of the FlipTrap fusion are tagged with red fluorescent protein. Together, these studies will enable us to generate a mechanistic synthesis of how a biological program is executed in a developing embryo.

The Par/aPKC Complex Controls The Vectorial Migration Of Medaka Macrophages In Vivo

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The establishment and maintenance of cell polarity is a requirement for leukocyte migratory response to inflammatory cues. A conserved polarity complex consisting of Par3, Par6 and atypical PKC (aPKC) in conjunction with small GTPases of the Rho Family temporally and spatially controls polarization in several cell types. We used a Medaka (*Oryzias latipes*) transgenic line as a model system to explore the functional role of this signaling complex *in vivo* during wound-triggered macrophage polarization and directed migration. By using a transient transgenesis approach, we overexpressed specifically in macrophages the orl_Par3 dominant-negative deletion mutant known to interfere with Par3/aPKC binding. Compared with control cells, orl_Par3 mutant-expressing macrophages display a prolonged "sensing time" with non-polarized status and, as an outcome, a delayed response to the injury. Closer inspection of cell morphologies reveals increased number of unpolarized pseudopodes and elongated phenotypes in mutant-expressing cells. Accordingly, blocking PKCξ signaling with a myr-PKCξ pseudosubstrate inhibitor impairs macrophage directional motility when compared with untreated cells. RhoA signaling is contributing as well to stabilize macrophage asymmetry as abnormal cell elongation and concomitant reduction in cell speed occurs in the presence of a Rho Kinase pharmacological inhibitor. Collectively, our data support the hypothesis that the Par/aPKC complex and its effector GTPases are involved in regulating the directional migration of myeloid cells responding to inflammatory cues in vivo.

Potential role of mesoderm and extracellular matrix in organising polarization and morphogenesis of zebrafish neuroepithelium

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The development of the zebrafish neuroepithelium is a highly coordinated morphogenetic process in both time and space. This involves the convergence of neuroepithelial cells towards the dorsal midline and the generation of the apico-basal cell polarity. One of the key tissues that might actively contribute to co-ordination of this process is the adjacent mesoderm. Analyses by time-lapse microscopy have revealed that during the initial stages of neurulation, neural plate and mesoderm move in a tightly co-ordinated way. In addition we are analysing neural morphogenesis in a series of mesoderm mutant embryos. For example, in embryos with defective mesoderm convergence due to the reduction of Has2 function, we find that neural plate convergence is severely affected. Furthermore embryos that completely lack head mesendoderm due to loss of Nodal signalling, develop a neural primordium with chaotic apico-basal organisation. Time-lapse microscopy demonstrates that the normal highly co-ordinated movements of neurulation are severely disrupted in these mesoderm-less embryos. Although, Nodal mutant embryos show defective neural tube morphology, our experiments suggest that lack of mesoderm rather that Nodal signalling itself is required for neural tube development. In addition, we demonstrate that apico-basal organisation and neural tube morphogenesis can be rescued in mesoderm-less mutant embryos by replacing mesoderm. Although, we do not know the molecular nature of such tissue-interaction, components of the extracellular matrix (ECM), which are expressed between these two embryonic tissues may to have an important role in this process. We are currently testing whether ECM and mesoderm together can regulate the co-ordination of neural plate cell movements and organise their polarity.

Regulation of planar cell polarity signalling by the prenylation pathway

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During vertebrate gastrulation, the body axis is established by a variety of co-ordinated and directed movements of cells. One of these movements is convergence and extension (CE), which is regulated by a non-canonical Wnt/planar cell polarity (PCP) pathway. From our forward genetic screen, we have identified 3-hydroxy-3-methyglutaryl-Coenzyme A reductase 1b (hmgcr1b) gene as a dominant enhancer of the *silberblick (slb)/wnt11* CE phenotype. *hmgcr1b* mutant embryos exhibit only very mild CE phenotype during gastrulation while showing a thicker yolk extension at pharyngula stages. Notably, abrogation of *hmgcr1b* also enhances the CE defects of other core PCP mutants/morphants. The prenylation pathway is one of branches downstream of HMGCR, and has been implicated for lipid modification at the C-terminus of proteins. To test the possibility that the prenylation pathway regulates activities of the PCP pathway, we abrogated farnesyl transferase (FT) or geranylgeranyl transferase (GGT) function using morpholinos on PCP mutant/morphant backgrounds. Consistent with the notion that FT preferentially performs lipid modification on to proteins with the CAAX motif including the core PCP protein Prickle (Pk), abrogation of FT, but not GGT, enhances the *pk1a* or *pk1b* morphant CE phenotype, suggesting the specificity for targets of the prenylation enzymes.

4D time lapse analysis of zebrafish eye development

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To understand the development of tissues, biologists have commonly used sectioning or SEM to visualize their morphology. However, these methods require fixation, preventing analysis of different time points during the development of the same animal, and thus obscuring the dynamic aspects of morphogenesis. To circumvent this problem during our study of zebrafish eye morphogenesis, we carry out live 4D imaging of the developing eye primordium from 12 to 24 hpf, collecting complete z-stacks every 3.5 min after labeling chromatin and cell membranes with histone-mCherry and EGFP-CAAX, respectively.
Here we describe several analysis methods that we have developed for these 4D datasets. We analyzed lens and retina morphology over time using manual segmentation in Amira. We developed custom software for manual 4D cell tracking. The LongTrack application allows us to track individual cells either forwards or backwards through time, and which can export XYZ coordinates or volume images of the tracked cells. Tracks of individual cells can then be combined with the eye segmentation as polygon meshes, allowing us to visualize changing eye morphology and cell movements within this developing tissue.
We have also begun to analyze the relative contributions of cell division and cell migration to eye morphogenesis. We measure cell numbers from the histone-mCherry data using custom 3D cell counting software, and manually identify mitotic figures to generate a 4D map of mitoses, plotted topographically on the retina and lens using eye segmentation. Through these comprehensive analyses, we aim to generate a 4D atlas of normal eye development in zebrafish.

Ugly duckling, SANT domain protein interacts with Wnt/PCP pathway to regulate convergence and extension movements

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During vertebrate gastrulation, the germ layers form by internalization and undergo various types of movements such as epiboly, convergence and extension. Wnt/PCP pathway regulates convergence and extension (C&E) movements. In zebrafish, mutations in components of Wnt/PCP pathway such as *knypek/glypican4* and *trilobite/vangl2* cause a C&E defect. However, the molecular mechanisms that control C&E movement are not fully understood.

To identify additional genes involved in C&E movements, we performed a genetic screen for mutations that modify the *knypek (kny)* phenotype. We identified *vu66* mutant as a recessive enhancer of the *kny* mutant phenotype. *vu66* is a recessive lethal mutation and the homozygous embryos show shortened body axis, degenerated tail, smaller head, heart malformation and increased cell death. Interestingly, *vu66;kny* double mutant embryos exhibit more severely shortened tail than either single mutant. Furthermore, *vu66* also interacts genetically with *trilobite*. Gene expression analysis in *vu66;kny* compound mutant suggests that the general patterning of the embryos is largely normal. The expected *vu66* mutation point is located very close to *ugly duckling* (*udu*) mutant locus in chromosome 16. *udu* was initially identified in the Tubingen genetic screen as a mutation affecting morphogenesis (Hafter et al., 1996) and subsequently reported as a primitive erythroid defect mutant by Liu et al. (2007). We sequenced RT-PCR products of *udu* gene amplified from *vu66* homozygous embryos and discovered a nonsense mutation that generates a truncated protein. Moreover, *vu66* mutant does not complement *udu*^{sg1} mutant allele. Thus we conclude that *vu66* is a new allele of *udu* locus. *udu* encodes 2,055 amino acid protein of largely unknown function. It contains some conserved domains including putative SANT domain in the C-terminal region and this domain is essential for the function of Udu protein (Liu et al., 2007). *udu* is expressed maternally and then ubiquitously during gastrulation (Liu et al., 2007) suggesting that Udu zygotic function is compensated by the maternal products. To determine the function of Udu during development, we generated maternal-zygotic (MZ) *udu* mutant by using germ-line replacement (Ciruna et al., 2002). MZ*udu* embryos show severe morphogenetic abnormalities including C&E defect. To analyze the interaction of Udu with Wnt/PCP pathway, we also generate MZ*udu* and *kny* double mutant. The double mutant shows enhanced C&E defects. Moreover, gene expression analyses suggest that Wnt/PCP pathway genes are normally expressed in *udu* mutant embryos. Taken together, we hypothesize that *udu* regulates C&E movements acting largely in parallel to Wnt/PCP signaling.

Control of cell migration in the development of the lateral line
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The formation of the posterior lateral line of teleosts depends on the migration of a primordium that originates near the otic vesicle and moves to the tip of the tail. Groups of cells at the trailing edge of the primordium slow down at regular intervals of time and eventually settle to differentiate as sense organs. We have shown that the migration of the primordium is driven by the chemokine SDF1, which is present along the path of migration, and by its two receptors CXCR4 and CXCR7. *cxcr4b* is expressed in the leading cells but not in the trailing cells of the primordium, and *cxcr7* is expressed in the reciprocal pattern (highly in the trailing cells and not at all in the leading cells). Our results suggest that CXCR4 is required for the migration of the primordium and that CXCR7 provides the directionality. Recent work by others have shown that the WNT/FGF system also plays a role in driving migration (Nechiporuk and Raible, 2008, Lecaudey et al, 2008, Aman and Piotrowski, 2009).
We have identified yet other components of the network that regulates migration of the primordium and we will report their interactions with the two systems, CXCR4/CXCR7 and WNT/FGF.

Timely differentiation of rod photoreceptors depends on a feedback regulatory loop between NeuroD and Six6

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Timely generation of distinct neural cell types in appropriate numbers is fundamental for the generation of a functional retina. In vertebrates *Six6*, a transcription factor of the *Six/sine oculis* family, is initially expressed in multipotent retina progenitors and then becomes restricted to differentiated retinal ganglion and amacrine cells as well as to the proliferative ciliary marginal zone. How *Six6* expression in the retina is controlled and what are its precise functions is still unclear. To begin to address this issue we have used bioinformatic searches and transgenic approaches in medaka fish (*Oryzias latipes*), identifying a highly conserved regulatory enhancer in the *Six6* locus responsible for its expression in differentiating and adult retina. Search for transcription factor binding sites, luciferase and ChIP assays together with gain-of-function studies indicated that NeuroD, a bHLH transcription factor, directly binds an "E-box" sequence present in this enhancer, regulating Six6 expression in the retina. NeuroD-induced Six6 over-expression in medaka fish embryos promotes unorganized retinal progenitor proliferation, in agreement with previous Six6 gain-of-function studies. Notably, as the retina differentiates, NeuroD-mediated Six6 over-activation impairs photoreceptor differentiation, with no apparent changes in other retinal cell types. Interestingly gain- and loss-of function of Six6 induces changes in NeuroD expression levels and alterations in the expression and localization of the photoreceptor differentiaion marker, rhodopsin. Together these results indicate that NeuroD is a direct regulator of *Six6* expression in the eye development and suggest that the appropriate generation of photoreceptor precursors and their subsequent differentiation requires a NeuroD-Six6 feedback regulatory loop.

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olSox2-mediated activation of *olSix3.2* promotes telencephalic versus eye field specification
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Vertebrate forebrain derivatives are specified at early stages of gastrulation in the anterior neural plate by the overlapping expression of several transcription factors including Six3, a member of the *Six/sine* oculis family of homeobox containing transcription factors. The medaka fish (*Oryzias latipes*) genome contains two *Six3* paralogs: *olSix3.1* and *olSix3.2*. The combined expression pattern of these two genes recapitulates that of the mammalian or avian *Six3*, with a preponderant expression of *olSix3.1* in the eye and of *olSix3.2* in the telencephalic and thalamic regions, suggesting a possible regulatory and functional sub-specialization of the two genes as consequence of their independent evolution after genome duplication. To begin to test this possibility and decipher the logic of *Six* gene regulation, we have identified the precise regulatory code necessary for proper *olSix3.2* expression. This code comprises a series of enhancers, silencers and silencer blockers (Conte and Bovolenta, Genome Biol. 2007; 8(7):R137). On the basis of searches for transcription factor binding sites, expression analysis, luciferase reporter and chromatin-protein interaction assays, we have further determined that Sox2 and Pax6 directly bind and activate the enhancers responsible for early and late expression of *olSix3.2* in the forebrain and its derivates. Consistent with these results, over-expression of Sox2 in medaka fish embryos, up-regulates *olSix3.2* levels. Notably, this up-regulation causes severe microphthalmia associated with an expansion of the telencephalic territory. Because previous studies have shown that interference with *olSix3.1* expression affects predominantly eye development, we propose that in medaka fish, *Six3* functions have been segregated between two paralogs, where *olSix3.2* is mostly responsible for telencephalic specification.

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Induced early expression of mrf4 but not myogenin rescues myogenesis in the myod/myf5 double morphant zebrafish embryo

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Muscle regulatory factors activate myogenesis in all vertebrates, but their role has been studied in great detail only in the mouse embryo, where all but myogenin – Myod, Myf5 and Mrf4 – are sufficient to activate (albeit not completely) skeletal myogenesis. In the zebrafish embryo, *myod* and *myf5* are required for induction of myogenesis because their simultaneous ablation prevents muscle development. Here we show that *mrf4* but not *myog* can fully rescue myogenesis in the *myod/myf5* double morphant via a selective and robust activation of *myod*, in keeping with its chromatin-remodelling function in vitro. Rescue does not happen spontaneously, because the gene, unlike that in the mouse embryo, is expressed only at the onset of muscle differentiation. Moreover, because of the transient nature of morpholino inhibition, we were able to investigate how myogenesis occurs in the absence of a myotome. We report that in the complete absence of a myotome, subsequent myogenesis is abolished, whereas myogenesis does proceed, albeit abnormally, when the morpholino inhibition was not complete. Therefore our data also show that the early myotome is essential for subsequent skeletal muscle differentiation and patterning in the zebrafish.

Ethanol alters the establishment of the eye field during development

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Ethanol impairs ventralization of the central nervous system and disrupts cellular organization. Within the anterior neural plate, ethanol has been reported to avoid the evagination of the optic vesicles, resulting in cyclopia, a condition where the optic vesicles are partially or totally fused. The mechanisms by which ethanol affects the morphogenesis of the optic vesicles are largely unclear.

The aim of this work is the characterization of the retinal precursors derived from the eye field. We have analyzed the cytoarchitecture of these domains using semithin sections and immunohistochemistry against β-catenin. *In situ* hybridization was carried out to determine the expression patterns of genes required for eye formation, such as *rx1*, *rx3* and *six3*. We have used AB zebrafish untreated embryos as control and embryos exposed to 2.4% ethylic solution from dome stage to tailbud as experimental group. The stages analyzed are 3 somites (prior to evagination), 6 somites (mid-evagination) and 10 somites (post-evagination).

In control animals the eye field is distinguishable from the 3-somites stage, and at 10-somites stage two optic vesicles are observed. The cellular morphology in control animals changes from circular (3 somites-stage) to fusiform (10 somites-stage). After the exposition to ethanol, the separation between cells is wider than in control animals, changes in cellular morphology are not evident and several pyknotic nuclei can be observed. The expression pattern of *rx3* is altered after exposition to ethanol, indicating an incorrect evagination of the optic vesicles. Moreover, while in control animals, at 10 somites-stage, *rx1* expression is not present in the area between optic vesicles, it is preserved after ethanol treatment. *Six3*missense mutations has been reported in holoprosencephaly and cyclopia, we have observed a reduction in the expression of this gene after the ethanol treatment.

Our results indicate that, during early embryonic development, ethanol may disrupt the cytoarchitecture of the eye field and impair the key changes in cellular shape that produce the evagination of the optic vesicles. The alteration in the expression of visual field patterning genes may affect the signalling pathways required for this phenomenon.

All the procedures and experimental protocols were in accordance with the guidelines of the European Communities Directives (86/609/EEC and 2003/65/EC), the current Spanish legislation for the use care of animals in research (RD 1201/2005, BOE 252/34367-91, 2005) and conformed to NIH guidelines.

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Expression analysis and functional characterization of the *dro1/cl2* gene during zebrafish somitogenesis

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The recently cloned human DRO1/CL2 gene is expressed in almost all adult tissues and is downregulated in several cancer cell lines and solid tumors. DRO1/CL2 is indeed considered an oncosuppressor gene.

To understand the physiological role of DRO1/CL2, we cloned its zebrafish homolog and analysed its function during embryonic development. The *dro1/cl2* zebrafish gene shares high homology with the mammalian counterpart and the translated protein (867 aa) presents the same structure, showing the contemporary presence of a signal peptide and multiple nuclear localization signals.

The spatio-temporal analyses evidence a maternal and zygotic *dro1/cl2* expression. Its expression level starts increasing from the initial steps of segmentation up to late somitogenesis. Whole mount *in situ* hybridization experiments show a diffuse *dro1/cl2* expression during cleavage and epiboly. Interestingly, during somitogenesis the *dro1/cl2* expression becomes mainly restricted to the notochord. In later developmental stages it becomes detectable in the muscles of both trunk and head.

To investigate the functional role of the *dro1/cl2* gene, we performed loss- and gain-of-function experiments by injecting a specific *dro1/cl2* MO and the full length mRNA, respectively. Morphants showed abnormally shaped somites in the early somitogenesis, while the *dro1/cl2* overexpressed embryos were not affected.

Therefore, we investigated possible alterations of markers expressed during different phases of somitogenesis. The myogenic markers *myod* and *myogenin* resulted heavily disrupted in the paraxial mesoderm, while were unaltered in the adaxial one. The segmentation clock marker *her1*, and the segment polarization markers *mesp-a* and *mesp-b* showed no significant alterations. Interestingly, while the expression pattern of *gadd45*ß2 in the anterior presomitic mesoderm was almost unchanged, its expression disappeared in the already formed somites. The injection of *gadd45*ß2 mRNA in *dro1/cl2* MO injected embryos rescued their phenotype, confirming the specificity of the *dro1/cl2* knocking-down effect.

This *dro1/cl2* loss-of-function effect on the somites could be due to a disruption of nothocordal signals. However, *no tail* and *sonic hedgehog* were unaffected in *dro1/cl2* morphants. By converse, the inhibition of Hh signalling strictly downregulated *dro1/cl2* expression in the notochord, indicating *dro1/cl2* to be a potential target of the Hh pathway.

Our data suggest that the *dro1/cl2* notochordal signal may play a role in lateral somitic maturation through *gadd45*ß2.

Constructing a synthetic segmentation clock in yeast

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During zebrafish embryogenesis distinct structures called somites are formed sequentially and at regular intervals from the unsegmented presomitic mesoderm (PSM) tissue. A segmentation clock, which was first described in the "clock and wavefront model", tightly regulates this complex system and accounts for the timing and positioning of new somites. More specifically, the segmentation clock in the PSM relies on two important principles: functional intrinsic oscillators in individual cells and communication between cells that allows for synchronization of oscillators at the tissue level. The existence of a wavefront and clock in zebrafish was discovered in the form of signaling gradients in the PSM as well as cyclic expressions of PSM genes, such as *her1*, *her7* and *delC*. Mutations in these genes often result in abnormal segmentation patterns, altered segment size or failure of proper boundary formation. At this stage, experimental data of mutant phenotypes in combination with molecular interaction models suggest the role of individual clock components. However, it is difficult to verify the function of each component within the zebrafish embryo and thus the exact molecular mechanism of these oscillators is not well understood. Therefore, a synthetic approach will be implemented by rebuilding the segmentation clock piece by piece in *S. cerevisiae* (budding yeast). In conjunction with de novo synthetic oscillator principles, this method can elucidate the function and dynamics of individual clock components within the segmentation clock network, thereby furthering our understanding of somitogenisis in zebrafish.

Conditional activation of Notch signaling during zebrafish somitogenesis

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Repeated compartments are formed during vertebrate somitogenesis driven by the segmentation clock in the presomitic mesoderm (PSM), which consists of core components including cyclic genes (with oscillating expression patterns) and non-cyclic genes. In zebrafish, it was proposed that Delta-Notch interaction across PSM cells targets downstream *hairy* and *enhancer of split-related* (*her*) family genes, synchronizing oscillation and causing a feedback-loop to Delta-Notch signaling. However, the detailed roles of particular Delta-Notch combinations that are involved in particular regulation or the coordination mechanism of the segmentation clock are still unclear. Furthermore, there are still some controversies whether the Delta-Notch paralogs (e.g. Notch1a/Notch2 and DeltaC/DeltaD) function redundantly in PSM or not. Blocking Notch signaling with DAPT, a γ-secretase inhibitor, can achieve temporal control of Notch signaling. Nevertheless, there is no agonistic chemical that can stimulate Notch signaling. Heterologous fusion with the hormone-binding domain (HBD) of steroid receptors can establish a hormonal inducible system, which has rapid kinetics after activation and tunable induction level. A mutated HBD of human estrogen receptor (ER^T), which binds to tamoxifen but not estrogen, was first developed by Chambon et al. In the present study, over-expression constructs of the Notch intracellular domain (NICD) or Delta ICD fused to $FP:ER^T$ will be introduced into the corresponding genetic deficiency background. By analyzing and comparing the subsequent signal effects and somite phenotypes, the specific functions of particular Delta-Notch genes during zebrafish somitogenesis could be elucidated.

WNT signaling in zebrafish somitogenesis as a model system to study gradient formation in vertebrate development and disease

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Wnt signaling molecules control many processes in pattern formation and proliferation during normal development, as well as regulating stem cells and cancer progression during pathogenesis. Wnts usually signal at a distance from their site of synthesis, but how these molecules build signaling gradients is not well understood. In this project we aim to understand mechanisms of Wnt gradient formation using zebrafish somitogenesis as a model system. Somitogenesis is the process in which the vertebrate embryo body is sequentially subdivided into somites, epithelial clusters of cells that give rise to the segmented axial bone, muscle and skin of the adult. The periodic formation of somites from the presomitic mesoderm is thought to result from interaction of a molecular clock with extra-cellular gradients of Wnt glycoproteins secreted from the tailbud, but the mechanism of Wnt movement and gradient formation in this tissue is completely unknown. Candidate Wnt proteins are Wnt3l and Wnt8, which are locally expressed in the tailbud of the zebrafish embryo. We will first determine Wnt distribution in the zebrafish presomitic mesoderm and will analyse candidate cellular receptors contributing to Wnt gradient formation. Unusually for secreted signaling molecules, some Wnts have been shown to be lipid modified, but the role of lipids in Wnt signaling is not clear. Thus, we will determine whether Wnt signaling molecules are lipid modified in zebrafish embryos. Next, we will determine whether secreted Wnt proteins are found in multi-component complexes in the zebrafish embryo. Finally, we will determine the functions of lipid modifications of Wnt proteins in gradient formation in the living zebrafish embryo. The lessons learnt from this developmental system will serve as a model for understanding Wnt gradient formation and signaling range in vertebrate development and disease.

Analysis of cell movements in the presomitic mesoderm of the zebrafish embryo

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Segmentation in the vertebrate embryo is evident in the paraxial mesoderm in the form of somites, which are repeated structures that give rise to the vertebrae and the muscle of the trunk and tail. Segmentation occurs in an anterior-to-posterior sequence, where somites appear sequentially as the embryo grows from the posterior unsegmented tissue of the embryo, the presomitic mesoderm (PSM). In the zebrafish, one new pair of somites is formed about every 25 minutes. This process is controlled by an assembly of cellular oscillators whose locally coordinated behavior is controlled by intercellular Notch signaling. These oscillations are arrested at an anterior wavefront at different phases of the oscillation cycle, translating the temporal periodicity into striped spatial patterns of static gene expression. Although several components of this segmentation clock have been identified, complete understanding of the collective behavior of the genetic oscillations in the PSM is far from achieved. To explain and examine the relationship between temporal program and spatial pattern, there is a general need to have a quantitative measurement of the properties of the system, in particular, the role of cellular movements. It is well known from theoretical studies that the characteristics of relative motion of oscillators affect their synchronization properties and the patterns the oscillations form. In this work, we would like to obtain the trajectories of pre-somitic mesoderm cells and quantitatively characterize the cellular movements based on velocity, acceleration, mean square displacement, and correlation functions. Recently, Keller *et. al.* published digital datasets for the movements of all the nuclei of the cells of a single zebrafish embryo. Based on cell movement properties extracted from this data, we would like to derive quantitative cell motility models in order to understand the dynamics of cell movements, and how this affects the synchronization of the oscillating PSM cells.

Role of terra in vertebrates

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When looking from the outside, the vertebrate body axis is symmetric between the left and right sides. Nevertheless, when looking inside we see a different scenario with the internal organs acquiring an asymmetric distribution. Early in development, a complex genetic cascade is responsible for the transfer of information from regions adjacent to the embryonic node to the lateral plate mesoderm (LPM), a territory that will contribute to several organs. At the same time, so that the axial skeleton and skeletal muscles form in a correct way, somites must maintain a bilateral symmetric position between both sides of the embryonic axis. This symmetric patterning of embryos relies on the synchronized segmentation of the presomitic mesoderm (PSM).
We have shown that *terra/dmrt2*, a zinc finger-like transcription factor, is required to create left-right asymmetries in the LPM but it is also necessary to promote left-right symmetry in the PSM. It has already been described that *terra/dmrt2* knockout in the mouse leads to defects in late somite differentiation. Nevertheless, since nothing is known about the effect on left-right patterning and segmentation clock synchronization we are investigating the role of Terra/Dmrt2 on these embryonic processes. Strikingly, our results seem to indicate that the role of *terra/dmrt2* in the left-right patterning is not conserved during mouse development possibly to the loss of its expression at the level of the node.

Deciphering the zebrafish segmentation clock: unanswered questions about the *her1/7* feedback loop

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During somitogenesis a set of genes, mainly coding for Notch pathway components, shows oscillating expression in the presomitic mesoderm (PSM) at the tail end of the embryo, behaving as a segmentation "clock", which leaves its trace in the periodic spatial pattern of formed somites. The core clock components in zebrafish are proposed to be a pair of Notch target genes, *her1* and *her7*. Both code for inhibitory bHLH transcription factors and show oscillating expression in the PSM. Mathematical analysis demonstrates that direct autoinhibition of the expression of these genes by their own protein products can, dependent on certain conditions, give rise to oscillations within each cell of the PSM.
Notch signalling between adjacent cells is then proposed to keep their individual clocks ticking in synchrony, thanks to oscillating expression of the Notch ligand DeltaC, driven by Her1/7.
Measurements of several key parameters of the system have supported the model quantitatively and qualitatively. But it is far from proven that a simple *her1/7* feedback loop, operating in the way that we have proposed, really is the ultimate pacemaker of the clock. Other *her* genes also oscillate in the PSM, and their relative importance remains to be clarified. Many other important features of the *her1/7* control loop also remain to be analysed, including the nature of the key time delays, the functional dependence of the rate of initiation of *her1/7* transcription on the concentrations of Her1, Her7 and NotchICD proteins, the cross-regulatory interactions between *her1*, *her7* and the other oscillatory *her* genes, the sources of noise in the gene-expression oscillator, and the mechanism that keeps the clock going for the appropriate number of cycles. We will present our efforts to tackle some of these questions quantitatively.

Ptf1a determines inhibitory cell fates in the zebrafish retina at the expense of all excitatory cell fates

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During neurogenesis of the central nervous system (CNS), a multitude of different neuronal types form from multipotent progenitor cells. This process is highly co-ordinated via the control of gene expression. We study mechanisms of cell fate determination during neurogenesis in the zebrafish retina, an accessible and highly organised part of the CNS.
The pancreas transcription factor 1a (Ptf1a) was implicated in the determination of inhibitory cell fates throughout different areas of the nervous system, including the retina. Using a transgenic ptf1a:GFP line we show that Ptf1a is expressed in differentiating horizontal and amacrine cells – the two inhibitory neuronal types of the retina. We provide a morphometric and immunohistochemical classification scheme of Ptf1a expressing amacrine cells in the zebrafish system, which give insights into subtype specific synaptic connectivity and visual pathways.
Time-lapse imaging reveals that Ptf1a expression commences primarily in postmitotic cells before cells move to their specific layers to differentiate. Ptf1a is necessary to determine these fates as revealed by knock down, in which cells transdifferentiate into all of the glutamatergic neuron cell types of the retina (photoreceptors, bipolar, ganglion cells) in the expected histogenic order, i.e. early cells with Ptf1a knockdown transdifferentiate into the early cell fates and *vice versa*. Ptf1a is also sufficient to drive the inhibitory fates at the expense of all glutamatergic neuron cell types as shown in our overexpression studies, in which we drive Ptf1a expression using the GAL4/UAS system.
Using *in vivo* timelapse imaging, we can show that Ptf1a in the wild type condition is switched on in different excitatory lineages arising from Vsx2 (bipolar cell) and Ath5 (ganglion cell) positive cells. This suggests that the inhibitory fates are determined sequentially upon lineages, which are themselves restricted to forming subsets of excitatory lineages.

Studying the activity of the proneural genes *ngn1* and *ascl1a* in zebrafish

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Proneural genes which are necessary to the formation of neurons code for bHLH transcription factors. The two families of proneural genes: *achaete-scute* and *atonal* are conserved from *Drosophila* to vertebrates. These two families have divergent activities in the formation of neurons both in *Drosophila* and mouse. The transgenic lines, allowing the misexpression of *neurogenin1* (*ngn1*) or *achaete-scute-like1a* (*ascl1a*) after heat-shock induction, show that proneural genes of the *achaete-scute* and *atonal* family also have divergent activity in zebrafish. Moreover, rescue experiment of the Mauthner neuron in *ngn1-/-* and *spg-/-* mutants embryo, show that Ngn1 and Ascl1a differ in their abilities to save mutants. Proneural genes are transcription factor, making it likely that divergent activity between *atonal* and *achaete-scute* family comes from transcriptional regulation of different targets genes. In an attempt to understand these divergences, we have begun a study of *deltaA* gene regulation, a potential target of Ngn1 and Ascl1a in zebrafish. Both gain and loss of function experiment suggest that *deltaA* is a target of Ngn1 and Ascl1a. The functional analysis of the *deltaA* promoter led us to identify a fragment of 470pb which is necessary for a regulation by Ngn1. In this fragment, there is a cluster of three potential Ngn1 binding sites (E-Box) which appear to act in a cooperative manner to allow the regulation of *deltaA* by Ngn1. Moreover, EMSA experiment and transgenic lines show that Ngn1 can bind on this E-Box and that they are necessary to allow the regulation of *deltaA* by Ngn1.
The study of this module will lead us to gain further insight in the molecular basis explaining divergences of activity between proneural genes of the *achaete-scute* and *atonal* families.

Zebrafish as a Model Organism to Screen New Drugs Potentially Able to Modulate the Sirtuins Expression

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Sirtuins comprise a unique class of evolutionary conserved NAD^+-dependent deacetylases that are key regulators of many physiological processes. They appear as a potential druggable set of enzymes for treatments of age-associated diseases, such as diabetes, cancer, and neurodegenerative disorders. They attracted so much interest in many research areas that resulted in a firestorm of chemical and cellular investigations in order to understand the sirtuins, and to discover ligands that may modulate their activity. For molecule screenings a cost-effective, easy manipulated and consolidate model organism is needed, and zebrafish fits perfectly these requirements. Thus, here we report, for the very first time, identification of all sirtuin-related genes and their expression pattern in nine different tissues of adult zebrafish, in order to give a complete scenario for testing potential modulators in a tissue-specific manner. We identified the zebrafish sirtuin members, determinate their phylogenetic relationship and their expression pattern through semi-quantitative RT-PCR. The analysis resulted in eight sirtuin-related genes identified, and their phylogenetic analysis resulted on seven well-resolved terminal clades, corresponding to each one of the sirtuins (SIRT1,2,4-7) and two SIRT3 paralogues. Each gene showed a unique expression profile, illustrating a wide tissue distribution of sirtuin family members in zebrafish. Only *SIRT1*, *SIRT3*, *SIRT5,* and *SIRT6* related genes were expressed in all tissue analyzed, and *SIRT1* is the member with the higher level of expression in all nine organs. With this panorama, new drugs based on sirtuin modulators may be tested, and in the future, could lead us into more selective and powerful therapies to human disorders associated with aging process.

Role of Hif-1alphain the Neural Crest Cells Migration

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Hypoxia-inducible factors (HIFs) are a key regulator of the gene expression in response to hypoxia and are involved in several developmental processes, including angiogenesis, vasculogenesis, heart and central nervous system development. In this study we have designed two morpholinos (MOATG-*hif-1 alpha* and MOSS-*hif-1 alpha*) to generate the loss of function of *hif*-1 *alpha* and mRNA microinjections to rescue the morphant phenotypes. The loss of function of Hif-1 alpha was analyzed by *in situ* hybridizations to neural crest cells induction and migration markers. We have observed that the loss of function of Hif-1 alpha do not induce a variation in the expression of neural crest induction markers as *foxD3* and *Ap2* at the 1-somite to 3-somites stages, however, the expression of *crestin*, a neural crest cells migration marker, is affected at 8 and 20 somites. Later in the embryonic development we have observed morphological defects in pharyngeal cartilage; a neural crest cells derivate, using *Alcian blue* stain. The defects observed were rescued, in the 80% of the total embryos, co-injecting the morpholino MOATG-*hif-1 alpha* with both *hif-1 alpha* and *snail1b* mRNAs. These findings show that Hif-1alpha plays a key role in neural crest cells migration, but not in neural crest cells induction process. Furthermore these data suggest that Hif-1alphacould be an upstream regulator of *snail1b* in the neural crest migration.

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17β-ESTRADIOL RECEPTORS EXPRESSED IN EMBRYONIC AND ADULT ZEBRAFISH IN ABSENCE AND PRESENCE OF LIGAND

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The nuclear receptors encompass a group of regulatory proteins involved in a number of physiological processes. The estrogen receptors (ERs), of which one alpha and one beta isoform have been identified in mammals function as transcription factors in response to 17β-estradiol. In zebrafish there are three isoforms of estrogen receptors and they are denoted *esr1* (ERalpha), *esr2a* (ERbeta2) and *esr2b* (ERbeta1). Total RNA of embryos (<3, 6, 12, 24, 48, 72, 96 and 120 hours post fertilization) and of several organs (liver, intestine, eye, heart, brain, ovary, testis, gill, swim bladder and kidney) of not exposed or ligand exposed adult fish were isolated. Using specific primers for each of the three zebrafish ERs the expression levels were quantified using real time PCR. Data from individual fish show that all three estrogen receptors were expressed in organs of adult fish. Two of the three receptor genes, *esr1* and *esr2b*, but not *esr2a* were expressed during the embryonic stages and in presence of 17β-estradiol the mRNA levels of the two active ERs accumulated. In adult fish, the *esr2b* expression was not regulated in response to ligand in the organs tested (except in heart) while the expression of *esr2a* was altered in liver, heart, intestine, brain and testis. The conclusions are that i) estrogen receptors in absence of ligand are expressed in embryos and in organs of adult zebrafish; ii) altered expression of *esr* genes in response to 17-βestradiol is restricted to the specific cellular context; iii) the synthetic estrogenic ligand 4-nonylphenol acts as a 17β-estradiol agonist during development and as agonist or antagonist in adult fish. Current understanding of *esr* gene function in development and in the early and adult life will help to understand mechanisms of mimicking endocrine chemicals.

Effect of Liver-X-Receptor (*lxr*) knock down during development in zebrafish

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The Liver-X-receptors (LXRs) alpha and beta are activated by oxysterols. LXRs are implicated in cholesterol metabolism and are potential drug targets for treatment of atherosclerosis. They also regulate lipid and glucose metabolism. Moreover, recent studies have shown the importance of LXR beta for prevention of motor neuron degeneration, and for maintenance of testicular function. Effects of targeted deletion of LXRs in mice are generally observed at adulthood. However, considering the importance of cholesterol during development, a role of LXR during this period, possibly not manifested until adult age, is reasonable to suggest. For these reasons, we choose to explore LXR function during embryonic development in the zebrafish model. The zebrafish Lxr protein shares high sequence similarity with mammalian LXR alpha and we have shown that it has transcriptional activity in response to classical LXR-ligands. In adult fish all examined organs express *lxr*. Treatment of zebrafish at adult stage or during development with the synthetic LXR ligand, GW3965, induces the expression of genes involved in lipid and cholesterol pathways, suggesting a conserved metabolic role of Lxr in zebrafish. We observe a ubiquitous expression of *lxr* mRNA during zebrafish development that is particularly pronounced in the liver at 3 dpf. In addition to a metabolic role of Lxr the ubiquitous and temporal expression pattern suggests a developmental role. Indeed, the knock down of Lxr expression by morpholino anti-sense oligonucleotids induces developmental defects in zebrafish embryos such as impairment of general lipid distribution, reduced yolk utilization and cartilage defects.

Abnormal neurogenesis, angiogenesis and haematopoiesis in zebrafish lacking group 4 paralog of Hox genes

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The vertebrate central nervous system contains many classes of neurons with specialized properties. In the developing hindbrain, the functional loss of individual and combinations of Hox genes has revealed their role in specifying rhombomere identity and neuronal development. However, the interaction of Hox genes with transcriptional program to specify distinct neuronal lineages within the rhombomeres remains to be elucidated. Haematopoiesis in the vertebrate is characterized by the induction of ventral mesoderm to form haematopoietic stem cells and the eventual differentiation of these progenitors to form the peripheral blood lineages. Several genes have been implicated in the differentiation and development of haematopoietic and vascular progenitor cells. The first haematopoietic and endothelial progenitors are derived from a common embryonic precursor, termed the haemangioblast. The genetic cascades that regulate the differentiation of the haemangioblast to haematopoietic and endothelial cells are largely unknown. In general, much of embryonic development is coordinately regulated by temporal and spatial expression of transcription factors, such as the Hox gene family.

Here, through the morpholino knock down of group 4 paralog of Hox genes, we have identified an early defect in neurogenesis, angiogenesis and haematopoiesis. We demonstrate that single and combined knockdown of 4th group of Hox genes results in a loss of olig2, nkx 2.2 and islet-1 expressing progenitors that gives rise to somatic motoneurons and branchio motoneurons. Alcian blue staining showed that the pharyngeal cartilages in the morphants were malformed or abolished.

The differentiation of endothelial cells is tightly connected with the formation of blood vessels during development. Vasculature was examined in Tg Fli:EGFP embryos, where the *fli1* promoter drives expression of enhanced green fluorescent. Loss of hoxc4a and d4a function results in profound alterations in vascular development and angiogenesis, including atrophic trunk dorsal aorta and interruption of anterior aortic bifurcation, failure in intersomitic vessel (ISV) development and sprouting, and lack of blood circulation. Remarkably, morphants are characterized by the loss of arterial endothelial cell identity in dorsal aorta, as shown by the lack of expression of the arterial markers such as *flk-4, flk1* and *fli-1* and the vein marker *flt4*. Also blood pooling was observed in the cardiac region, eye and in yolk. Taken together, these results demonstrate for first time that paralog group 4 genes play a significant role in haematopoiesis and angiogenesis.

Transcriptional Regulation of the Stem Cell/Progenitor Gene c-myb

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The transcription factor c-Myb plays an essential role in stem/progenitor cell proliferation and differentiation in the blood, the brain and the intestine. It is also known to be expressed in a number of other tissues, including the neural retina and the olfactory placode. Little is known about the transcriptional regulation of *c-myb*. This is why we are investigating the transcriptional regulation of *c-myb* in zebrafish embryos. In zebrafish embryos, *c-myb* expression is detected in the retina, the intestine, the olfactory placode, the branchial arches, the brain and in haematopoietic tissues. To find regulatory elements controlling *c-myb* expression in zebrafish we have used three different approaches. Firstly, we have tested sequences upstream of the zebrafish *c-myb* locus. Upstream sequences were amplified by PCR and cloned in front of a *gfp* reporter gene in a *Tol2* transposon vector. The resulting constructs were injected into zebrafish embryos and the transposon sequences were used to facilitate genome integration. Embryos that displayed transient GFP expression were grown up. Transgenic founders were identified in crosses with wild-type fish. Secondly, we have searched for regulatory elements outside the promoter proximal region. Assuming that such elements may be evolutionally conserved we searched for conserved noncoding elements in interspecies genomic comparisons using the sequence alignment programme MLAGAN. These elements were individually cloned in front of a basal promoter of *c-myb* in the *Tol2* reporter vector and tested in transgenic zebrafish. Finally, we have modified a BAC which contains 176kb of genomic sequence around the *c-myb* locus by introducing an *rfp* reporter gene into the *c-myb* locus. The modified BAC has been injected into zebrafish embryos. At the meeting, we will give a progress report.

Nonsense-mediated mRNA decay effectors are essential for zebrafish embryonic development and survival

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The nonsense-mediated mRNA decay pathway (NMD) is a conserved mRNA quality-control mechanism that ensures the fidelity of gene expression by detecting and degrading mRNAs containing nonsense mutations. In doing so, the NMD pathway protects eukaryotic cells from potentially dominant-negative effects resulting from the accumulation of truncated proteins. In addition to this protective function, NMD regulates 1–10% of wild-type transcripts, thereby influencing a broad range of biological processes including development, signal transduction and cell cycle progression. In *metazoa,* seven genes (*upf1–3*, *smg1* and *smg5–7*) have been identified as essential for NMD; here we show that the zebrafish genome encodes orthologs of *upf1*, *upf2*, *smg1*, *smg5–7* and two *upf3* paralogs, which are provided maternally and are ubiquitously expressed throughout early zebrafish embryogenesis.
Across species, the importance of NMD effectors varies. Indeed, NMD effectors are not essential in *S. cerevisiae* or *C. elegans*. In contrast, *upf1* is an essential gene in *D. melanogaster*. In *A. thaliana*, the *UPF1* and *SMG7* genes are essential for embryonic viability, and loss-of-function mutants in the *UPF3* gene result in strong phenotypes. *Upf1* and *upf2* are also essential for early embryonic development in the mouse. Remarkably, phenotypes associated with the depletion of Smg1, Smg5–7 have not been described in vertebrates. The lack of information on the importance of these additional NMD effectors in the context of a vertebrate organism raises the question of whether the phenotypes seen in *upf1* and *upf2* knockouts can be ascribed to the inhibition of the NMD pathway or to the inhibition of an unknown function that Upf1 or Upf2 acquired over the course of evolution.
To gain further insight on the evolution and physiological relevance of NMD we investigated this pathway in a basal vertebrate, the zebrafish *Danio rerio* and show, that Upf1 is required for degradation of PTC-containing mRNAs in zebrafish embryos using the *golden*^{b1} mutant. Moreover, its depletion has a severe impact on embryonic development, early patterning and viability. Similar phenotypes are observed in Upf2-, Smg5- or Smg6-depleted embryos, suggesting that zebrafish embryogenesis requires an active NMD pathway. Using cultured cells we demonstrate that the ability of a PTC to trigger NMD is strongly stimulated by downstream exon-exon boundaries. Thus, as in mammals and plants, but in contrast to invertebrates and fungi, NMD is coupled to splicing in zebrafish. Our results, together with previous studies show that NMD effectors are essential for vertebrate embryogenesis and suggest that the coupling of splicing and NMD has been maintained in vertebrates, but lost in fungi and invertebrates.

Regulation of SIRT1, PGC-1 α and PPARγ by Resveratrol in Liver of *Zebrafish*

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Calorie restriction (CR) slows the appearence of age-associated diseases and extends life-span in a wide variety of model organisms. One of the mechanisms proposed is the modulation of sirtuin (SIRT), a conserved family of enzymes with NAD+-dependent deacetilase activity, found in all domains of life. SIRT 1 has become the most well-studied protein, an important nuclear deacetilase, whose substrates include proteins primarily involved in transcriptional regulation, such as the transcriptions factors PGC-1 α and PPARγ. The molecule of *trans*-resveratrol is known to modulate SIRT1 and has been proposed to partially mimic CR by this mechanism.. *Zebrafish* (*Danio rerio*) is an used model organism because it shows a balance between simplicity and complexity, easy manipulated, but actually there is one work about SIRT1 modulation. The aim of this work was to demonstrate the gene expression of the SIRT1 and its two targets PGC-1 α and PPARγ by resveratrol in the *zebrafish* liver. For the treatments, animals were introduced into test aquariums (2 L) containing resveratrol solution (5mg/L dissolved in 100 µL ethanol), during 15, 30 or 60 min. Total RNA was isolated from adults zebrafish liver (n=5) and cDNA species were synthesized through semi-quantitative RT-PCR. SIRT1, PGC1-α, PPAR-γ and β-actin primers were designed, and PCR optimal conditions were determined. Reactions were performed and analyzed on 1.0% agarose gel with GelRed®. The relative mRNA abundance of each gene versus β-actin was determined by optical densitometry using ImageJ1.37. Results were expressed as mean ± S.D and statistically compared by one-way ANOVA, followed by Tukey test, considering $P \leq 0.05$ as significant. The means of expression of SIRT1 in liver of zebrafish was 0,75 (SD ± 0,08), 0,36 (SD ± 0,02), 0,56 (SD ± 0,07) and 0,67 (SD ± 0,09) in control, 15, 30 and 60 minutes, respectively. The decrease of gene expression of SIRT1 in 15 and 30 minutes are statistically significant versus control ($p<0,01$ and $p<0,05$). The expression means of PGC1-α was not significant different than the control in 15 and 30 min treatments, but was observed an significant increase of expression in 60 minutes (1,01 SD ± 0,03 $p<0,05$). The similar results are observed in PPAR-γ gene expression, but the increase was not significant. Regarding the targets gene expression PGC1-α and PPAR-γ our results are in agreement with literature. However, the SIRT1 gene expression, that is tissue-specific, we saw a decrease in 15 and 30 minutes returning to the control level, in 60 minutes. Similar event was saw in mice subjects a CR. We can conclude that our work is the first that consolidate the zebrafish as model of SIRT1 expression by *trans*-resveratrol.

The Spatio-temporal Expression of Sarcomeric Myosin Heavy Chain Genes during Muscle Development of Medaka *Oryzias latipes* Are Regulated by Their 5′-flanking Region as Revealed by Transgenic Constructs

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Class II myosin is a major contractile protein in muscle fibers and composed of two heavy chains (MYHs) and four light chains, with the former heavy chain functioning as a motor protein by splitting ATP when bound to actin. It is well known that vertebrate muscles contain different MYH genes (*MYH*s) which show spatio-temporally different expression patterns.
Here, we analyzed the expression of sarcomeric *MYH*s and their transcriptional regulation during muscle development in medaka *Oryzias latipes* to investigate their relation to muscle formation. First, we randomly cloned *MYH*s from embryos and larvae and analyzed their expression patterns by RT-PCR and *in situ* hybridization at several developmental stages. As a result, three embryonic fast skeletal muscle type *MYH*s were predominantly expressed during muscle development: one was expressed specifically in horizontal myoseptum from 2 days post-fertilization (dpf), whereas the other two in whole myotome from 3 dpf and intensely expressed at hatching stage. Then, we analyzed the transcriptional regulation by the 5′-flanking region of each *MYH*. We prepared several constructs in which the 5′-flanking region of *MYH* was fused to the marker genes encoding green fluorescent protein (GFP) and red fluorescent protein (RFP) and introduced them into fertilized eggs. The spatio-temporally specific expression patterns of GFP and RFP in transgenic fish recapitulated those of endogenous *MYH*s. Furthermore, these constructs were expressed in zebrafish embryos in a similar manner as found in medaka, suggesting common regulatory functions of the 5′-flanking region of fish *MYH*s in expression during development.

The *hmgb* gene family in zebrafish: six members with overlapping expression patterns

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HMGB (High Mobility Group Box) proteins are very abundant chromatin binding factors that contact the minor groove of the DNA with very limited sequence specificity and sharply bend it, imparting structural and functional plasticity to the chromatin. HMGBs regulate numerous nuclear processes including transcription, replication and repair and have also key roles as secreted proteins.
In mammals the HMGB family comprises three highly conserved members: HMGB1, HMGB2 and HMGB3, all characterized by two homologous tandem DNA binding domains (HMG box A and B) and by an acidic C-terminal tail. The main differences of HMGB family members lie in the length of the acidic tail, which is longer in HMGB1 and shorter in HMGB3, and in their expression patterns: in fact, HMGB1 is almost ubiquitous while HMGB2 and HMGB3 are largely expressed during embryogenesis but have a very restricted expression pattern in adults.
Through bioinformatic analyses, we identified six zebrafish cDNA/genomic sequences homologous to mammalian *HMGB* genes. We found two co-orthologs for each mammalian gene and named them *hmgb1a*, *hmgb1b, hmgb2a, hmgb2b, hmgb3a* and *hmgb3b*, on the basis of phylogenetic trees and syntheny analysis. The six zebrafish proteins display the canonical structure of HMGB factors, i.e. the two HMG boxes and the acidic tail, and are highly related to the mammalian homologs. z*fhmgb* genes also possess the typical *HMGB* intron-exon structure: in fact, they are interrupted by four introns and contain five exons of which the first one is not translated.
We analyzed the expression of the *hmgb* family genes in zebrafish embryos. By *in situ* hybridization and Real-Time RT-PCR we show that all *hmgb* transcripts share a maternal origin, but they differ in expression levels. Until 1 dpf, all z*fhmgb* genes display largely overlapping expression domains although their expression levels are differentially modulated. Later in the development *hmgb1a/1b*, *hmgb2a/2b* and *hmgb3a* are restricted to specific regions of the embryos, mainly in the central nervous system while *hmgb3b* is barely detectable. Our data support the hypothesis that the co-orthologous genes are mainly expressed in the same cell types and therefore are probably implicated in the same biological functions.

Long-term alcohol treatment increases SIRT1 gene expression in zebrafish liver

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Sirtuin 1 (SIRT1) belongs to a unique class of evolutionary conserved NAD^+-dependent deacetylases that play important physiological roles in aging and metabolism. Recently, is emerging as a metabolic master regulator by either direct modifying histones or regulating the activity of several transcriptions regulators, as PGC-1α (peroxisome proliferator-activated receptor- γ coactivator1 α) and PPAR-γ (peroxisome proliferator-activated receptor-γ). Previous data indicate that chronic ethanol feeding develops to alcohol liver diseases, including steatosis, necrosis and fibrosis. Furthermore, accumulating evidence indicates that the alcohol-induced changes in SIRT1 protein level were regulated at the transcription and translational levels since both mRNA and protein levels were affected by chronic alcohol consumption. Since sirtuin family appears as a druggble set of enzymes, new therapeutic strategies could be studied with these targets, specially using cost-effective, easy manipulated and consolidate model organisms, as the zebrafish. This work aimed to study the role of chronic alcohol consumption during 7, 14 and 28 days in the gene expression of SIRT1, PGC-1α and PPAR-γ related-genes in zebrafish liver. Animals were treated with ethanol 0.5% during 7, 14 and 28 days. Immediately after exposure, the fish were euthanized and their livers frozen in liquid nitrogen. Total RNA was isolated from adults zebrafish liver (n=5) and cDNA species synthesized through semi-quantitative RT-PCR. SIRT1, PGC-1α, PPAR-γ and β-actin primers were designed, and PCR optimal conditions were determined. Performed reactions were analyzed on 1% agarose gel with GelRed®. The relative mRNA abundance of each gene versus β-actin was determined by optical densitometry using ImageJ1.37. Results (mean±S.D) were statistically compared by one-way ANOVA, followed by Tukey test, considering $P \leq 0.05$ as significant (*). Gene expression patterns showed activation of SIRT1 gene in all treatments (0.7842 ± 0.07; 0.9849 ± 0.07*; 0.9917 ± 0.01*; 0.8885 ± 0.03 - control, 7, 14 and 28 days of exposure, respectively). The expression of SIRT1 target genes, PGC-1α (0.8736 ± 0.03; 0.8130 ± 0.01*; 0.8763 ± 0.02; 0.4244 ± 0.04) and PPAR-γ (0.6196 ± 0.05; 0.7670 ± 0.08*; 0.7663 ± 0.05*; 0.4855 ± 0.05) demonstrated a similar pattern, with a decreased expression at 28 days treatment. Our results suggest that the increase in SIRT1 mRNA levels could be mediated by metabolic changes due to alcohol metabolism in zebrafish. Since SIRT1 gene has a tissue-specific expression pathern, other organs could help to elucidate the effect of chronic alcohol consumption over both sirtuins and sirtuins target genes in this model organism.

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Signaling mechanisms regulating lower jaw development

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Cranial neural crest cells (NCC) populate the pharyngeal arches and eventually form the bone, cartilage and connective tissue of the jaw, with defects in NCC development leading to craniofacial abnormalities. Development of the mandibular portion of the first pharyngeal arch into a lower jaw relies on multiple signaling cascades, including one mediated by signaling from the endothelin-A receptor (Ednra). Loss of the Ednra-Dlx5/6-Hand2 pathway results in loss of NCC identity within the mandibular arch and severe defects in lower jaw development. While Hand2, a basic-Helix-Loop-Helix (bHLH) transcription factor expressed in the mandibular arch, appears to be a primary mediator of the Ednra/Dlx signaling pathway, little is known about its activity. In order to better understand these pathways in zebrafish, we have begun to determine whether Hand2 acts as a transcriptional activator or repressor and whether this function requires bHLH dimerization or DNA binding. For our analysis of *hand2* as an activator/repressor, we created super-activator (VP16)- and super-repressor (Engrailed - EnR)-*hand2* constructs. To examine whether Hand2 action requires DNA binding or HLH dimerization, we created basic (ΔR100D)- and HLH (ΔF110P)-*hand2* mutant constructs. Capped mRNA generated from each of these constructs was injected into wild type and *han*^{s6} zebrafish embryos at the 1-2 cell stage. Embryos were fixed at 5 days post fertilization and stained with alcian blue to analyze cartilage development. While our analysis is ongoing, we have thus far found that injection of all of our constructs into wild type embryos leads to mispatterning of the ceratohyal, while *hand2*$^{\Delta R100D}$ also results in loss of the ceratobranchials. When injected into *han*^{s6} mutants, we find that *hand2*$^{\Delta R100D}$ rescues all ventral cartilages, while *hand2*$^{\Delta F110P}$ only rescues the anterio-ventral cartilages. Injection of *VP16-hand2* also only rescues the anterio-ventral cartilages, while injection of *EnR-hand2* rescues both the anterio- and posterio-ventral cartilages. Based on this preliminary data, it appears that Hand2 functions differently in the development of the anterior and posterior pharyngeal skeleton and that some of these functions occur independently of DNA binding.

Ribosomal protein L11 (RPL11) deficiency in zebrafish leads to a selective upregulation of P53-modulatory nucleolar proteins

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Ribosome biogenesis is a tightly controlled multi-step process that begins in the nucleolus and ends with the formation of mature ribosome in the cytoplasm. Several nucleolar proteins play critical role in pre-RNA processing and pre-ribosomal assembly within the nucleolus. Recent findings indicate that impairment of ribosome biogenesis induced by the decrease of an RP or mutations in nucleolar proteins activate the p53 pathway presumably through a nucleolar stress or ribosomal stress. Studies in cell lines have identified RPL11 as a major inhibitor of the p53-MDM2 regulatory loop. However, our previous data demonstrate that knockdown of RPL11 activates the p53 pathway in zebrafish, which is contrary to the cell line-based results. In an effort to investigate how an RP deficiency activates the p53 response, we examined the expression of several nucleolar protein genes in L11-deficient embryos (morphants). A total of nine nucleolar proteins (wdr36, wdr55, bap28, rrs1, bxdc1, ncl, gnl3, pes, and bop1), already identified for their role in ribosomal assembly, were analyzed in this study. Quantitative and semi-quantitative RT-PCR revealed a selective upregulation of *gnl3* and *bop1* transcripts in the morphants whereas the expression of other nucleolar protein genes was similar to that in wild-type embryos. *In situ* hybridization showed enrichment of *gnl3* and *bop1* in the head region of the morphants that displayed morphological abnormalities and an increased p53 localization. Furthermore, a time-course analysis at various stages post fertilization indicated that overexpression of *gnl3* and *bop1* coincided with the activation of p53 response in the morphants. Interestingly, aberrant expression of Nucleostemin (the human homolog of gnl3), and Bop1 activates p53 in human and mouse cells respectively. These results suggest that an activated p53 response in L11-deficient embryos could be due to an overexpression of nucleolar proteins involved in the p53 pathway.

prdm genes in zebrafish craniofacial development

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The zebrafish *prdm* gene family is comprised of a set of 17 genes that all contain an N-terminal PR/SET domain to mediate protein interactions, and a variable number of DNA-binding zinc finger domains. *prdm1a*, a member of the *prdm* gene family, exhibits craniofacial defects including missing posterior ceratobranchial cartilages. The potential roles of other members of this gene family in zebrafish craniofacial development are currently unknown. We hypothesize that multiple *prdm* genes are involved in the patterning and development of the zebrafish face. Gene expression analysis of candidate *prdm* genes identified three novel targets – *prdm3*, *prdm5* and *prdm16*. These genes are expressed in temporal-spatial patterns consistent with putative involvement in craniofacial development. *prdm3* and *prdm16* are expressed in specific neural and branchial arch domains at both 24 and 48 hours post fertilization (hpf), while *prdm5* is expressed at low levels ubiquitously throughout the embryo at similar stages. Loss of function (LOF) analysis was carried out either by knockdown analysis through antisense oligonucleotide morpholino (MO) injections or mutant embryo analysis. Injections of translation-blocking MOs targeted to *prdm3* and *prdm16* did not result in craniofacial cartilage defects. However, injections of splice-blocking MO to inhibit translation of the DNA binding zinc fingers of *prdm16* result in a shortened neurocranium through mispatterning of the trabeculae cranii and ethmoid plate. *prdm5*^{hi61} mutant embryos also exhibit neurocranial shortening, in addition to reduction or loss of the branchiostegal rays. We are currently examining markers for neural crest specification, patterning and migration, in addition to bone differentiation.

Mutational analysis of ptf1a function and transcriptional regulation
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The heterotrimeric transcription factor complex PTF1 is a critical determinant of cell fate of the exocrine pancreas and the GABAergic inhibitory neurons of the cerebellum, dorsal spinal cord and retina. The PTF1a component of the PTF1 complex is also expressed in precursors of endocrine tissue of the pancreas and hindbrain glutamatergic neurons, although its requirement in these domains is less well understood. A conserved non–coding sequence upstream of mouse Ptf1a partially recapitulates the endogenous Ptf1a expression; it directs transgene expression to neural and acinar tissue, and significantly to multipotent pancreatic progenitor domains. The enhancer's activity requires two PTF1 binding sites, which are thought to mediate autoregulation. We have identified a non–coding sequence upstream of zebrafish *ptf1a*, conserved among anamniotes, that contains three putative PTF1 binding sites. We have demonstrated that the sequence acts as a transcriptional enhancer, and in transgenic zebrafish recapitulates part of the *ptf1a* endogenous expression. We are using transgenic enhancer analysis to dissect the functional modules of this element and zinc finger nuclease technology to alter the endogenous locus. We anticipate that such an approach will prove informative on the requirement of *ptf1a* autoregulation and expression levels in different spatiotemporal settings.

Functionally conserved cis-regulatory elements of COL18A1 identified through zebrafish transgenesis

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The completion of multiple vertebrate genome sequences has presented an important challenge to understand and predict function from primary DNA sequence, particularly for non–coding sequence. It is commonly hypothesized that evolutionary conservation predicts functional DNA, although sequence conservation has proven an imperfect predictor of enhancer function. Type XVIII collagen is a component of most basement membranes; mutations in the *COL18A1* gene lead to Knobloch Syndrome, an autosomal recessive disease characterized by vitreoretinal and macular degeneration and occipital encephalocele. *COL18A1* has 43 exons; three isoforms are derived by transcription from two different promoters and alternative splicing. The three isoforms display distinct and complex patterns of tissue-specific expression, and are found in kidney, lung, brain, and retina. Expression levels of *COL18A1* are thought to be clinically important in vasculogenesis, and in predisposition to hepatocarcinoma and diabetes type 2. Therefore, identification of the regulatory regions will provide insight into normal and pathogenic regulation of *COL18A1* expression. We have employed an efficient system of transgenesis in the zebrafish to functionally evaluate potential enhancer elements regulating *COL18A1* transcription. Using the VISTA and PhastCons algorithms, twenty conserved non-coding sequences (CNSs) were selected *in silico* from the interval encompassing *COL18A1* and tested for their ability to regulate specific transcription in transgenic zebrafish. We identified four elements that control transcription consistent with endogenous *col18a1*, in tissues including retina, pronephric duct and pronephros, blood vessels, gut, cartilage and liver. Significantly, VISTA and PhastCons identified largely different sequences; only three pairs of CNSs, partially overlapping, were found with both algorithms. In one pair, only the shorter sequence was an active enhancer, suggesting a negative regulatory element in the longer sequence. Importantly, it demonstrates the risk of relying on a single approach for comparative sequence analysis. Although the algorithms we used did not detect non–coding conservation from human to teleosts at the *COL18A1* locus, the human sequences functioned appropriately in zebrafish transgenics. Additional post–hoc computational analysis on positive enhancer sequences revealed alignments between mammalian and teleost sequences, which we hypothesized predict the corresponding zebrafish enhancers; for one of these, we demonstrated functional overlap with the orthologous human enhancer sequence. Our results provide important insight into the biological function and regulation of *COL18A1*, and point to additional sequences that may contribute to complex diseases involving *COL18A1*. More generally, we show that combining functional data with targeted analyses for phylogenetic conservation can reveal conserved cis–regulatory elements in the large number of cases where computational alignment alone falls short.

Deciphering the *pax6* Transcription Network

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The *pax6* gene has been defined as a selector gene, responsible for the determination of multiple cell fates in different developing tissues: the eye, central nervous system and pancreas. This multi-tasking is achieved by complex spatiotemporal and quantitative regulation of *pax6* expression at the transcriptional level. P*ax6* transcripts encode proteins that are highly conserved across evolution. Furthermore, P*ax6* cis-regulatory sequences are also well conserved across vertebrates.
Using comparative genomics, we are investigating transcriptional regulation by pax6. We have developed a bioinformatic procedure to identify genes that are transcriptionally regulated by any given DNA binding transcription factors (TFs). This procedure makes use of combined data and tools, such as bibliographic knowledge of transcription factor binding site (TFBS), genome sequences and probabilistic models to predict evolutionarily conserved sequences (ECRs) and the target genes under direct regulation.
We have identified more than one hundred vertebrate putative pax6 target genes and have validated some experimentally, in zebrafish, using methodologies such as morpholino induced knock downs, *in situ* hybridization and ChIP. This set of loci is highly enriched for genes that encode proteins with DNA-binding or transcription factor activity, reinforcing the idea of Pax6 as a major transcriptional modulator. Furthermore, we are starting to investigate how specific pax6 functions are achieved through some of the targets.

Characterization of the *Hoxd4* neural enhancer

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Hox genes encode homeodomain-containing transcription factors, required for correct patterning of the anterior-posterior axis, genital tubercle and limbs, organogenesis, morphogenesis, hematopoiesis, hair shaft production and mammary gland development. They are highly conserved throughout the animal kingdom and their expression is tightly regulated.
The Featherstone lab aims to understand how *Hox* expression is controlled through the study of the vertebrate *Hoxd4* neural enhancer. The *Hoxd4* neural enhancer is required for setting the anterior border of *Hoxd4* expression between transiently divided segments (rhombomeres) 6 and 7 in the developing hindbrain. Sequence comparison between mouse and zebrafish *Hoxd4*/*hoxd4a* neural enhancers revealed eight blocks of homology, denoted as sites A-G and a retinoic acid response element (RARE). Sites D-F and the RARE have been previously characterized. To complete this phase of my studies, I will characterize the remaining sites A, B, C and G and identify the transcription factors acting through the neural enhancer.
Site-directed mutagenesis of the mouse enhancer and functional tests in differentiating P19 cells showed that mutations in the conserved sites A, B, C, G, individually and together, had no effect on enhancer strength, but compound mutation in sites A-G except RARE delayed *Hoxd4* gene expression. These mutants will next be assessed in zebrafish embryos at ≥16 hours post-fertilization (hpf) when rhombomeres are clearly visible. A similar analysis of the zebrafish *hoxd4a* neural enhancer will be carried out in zebrafish embryos.
Genetic evidence suggests that a member of the maf family transcription factor (Kreisler in the mouse and valentino in zebrafish) is a negative regulator of *Hoxd4* in the embryonic hindbrain. Sequence alignment has shown that conserved sites F and G show some homology to the consensus maf recognition sequence (TGCTGANT/CCNGNN). Preliminary results from electrophoretic mobility shift assays (EMSA) show that Kreisler binds to sites F and G. This hypothesis will next be verified by chromatin immunoprecipitation (ChIP) and genetic knockdown in zebrafish.

The relationship between number of tandem repeat in medaka mitochondrial DNA and cold adaptation

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Medaka *Oryzias latipes* is very hardy and tolerate a wide range of salinities and temperatures (10–40°C) and has a large geographic distribution and a distinct local genetic variation. We compared the whole mitochondrial DNA (mtDNA) sequences of Medaka among 8 local stocks and 4 inbred strains. Phylogenetic trees based on the mtDNA-encoded genes indicated that the nucleotide substitutions in the Northern Japanese group were slightly more frequent in tRNA and 12S rRNA gene trees than other genes. Among tRNA genes, the most divergent one was the $tRNA_{Thr}$ gene as reported in humans previously. The number of tandemly repeated 11 nucleotide units (TR) in mtDNA control region was highly varied, while the two other *Oryzias* species, inhabiting tropical regions, had no repeats. Comprehensive comparisons between the TR number and meteorological data in corresponding habitat indicated that the TR number correlated to the certain data related to the cold environment and the seasonal temperature variation. In cold (5°C) acclimated fish, mRNA levels varied among mitochondria coding genes. The expression of the cytochrome oxidase subunit I gene in some local population was induced by cold temperature and seemed to be correlated with the number of repeated sequence in CR.

Genome wide expression analysis of transcription factors in zebrafish embryos

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The knowledge of gene regulation both in space and time provides essential information for the construction of gene regulatory networks and understanding of embryonic development and genetic diseases. This is especially true in the case of transcription factors (TFs), which are critical regulators of gene expression and control many aspects of cellular behaviour from proliferation to cell fate determination and differentiation. Surprisingly, the expression pattern of TFs known in zebrafish is relatively small (only 52% of TFs have an annotation in ZFin, ZFishv7 assembly) and overall the in situ databases produced so far in many vertebrates are of limited use regarding stage specificity, resolution and coherence of annotations and expression patterns. The objective of this project is the establishment of a collection of full-length cDNAs of all zebrafish TF genes in order to generate in situ expression data and provide cDNAs for functional studies.
We first established a repertoire of transcriptional regulators based on InterPro domains to map the zebrafish genome (ZFishv7). The coverage of this virtual set of genes includes 1939 DNA binding TFs, 546 genes implicated in chromatin remodelling and 142 general transcription factors. This set was shown not only to be comprehensive but also to include 668 genes with clear DNA binding domains, which were missed in precedent genome-wide TF screens. We then isolated full-length cDNA of 900 genes with transcriptional regulatory activity from 2 different libraries (pool of 16hpf to 36hpf, adult brain). The grouping of expression data by hierarchical clustering allowed us to define several groups of genes co-expressed in different organs including somites, diencephalon and blood at 24 hours post fertilization.

The proximal promoter of the zebrafish GSDF gene drives transgene expression specifically in fish testis

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The Gonadal Soma Derived Factor (GSDF) is a new member of the Transforming Growth Factor (TGF)-β superfamily recently identified in trout Sertoli and Granulosa cells (*Sawatari et al., 2007, Dev Biol, 301:266*). This factor increases the proliferation of the primordial germ cells and early spermatogonia. We have carried out a phylogenetic analysis of the GSDF genes in vertebrates and found orthologous genes in teleost species but not in tetrapods. The identity, order and orientation of the genes surrounding the GSDF counterparts are well conserved among the teleost species indicating that the GSDF gene belongs to a syntheny. In addition, we demonstrate that the orthologous GSDF genes are expressed in the male gonad of both zebrafish and medaka.

In order to determine whether the zebrafish proximal promoter of the GSDF gene was sufficient to drive gene expression specifically in the Sertoli cell of the testis, we have produced two independent transgenic medaka lines carrying a 2kb promoter fragment of the GSDF gene inserted upstream to the open reading frame of the green fluorescent protein (DrGSDF:GFP). A mendelian segregation of the transgene was observed indicating that both transgenic lines harbour a single transgene insertion site. No GFP expression was detectable using macroscopic observation techniques during the early development. However, the use of the quantitative RT-PCR allowed us to detect the expression of the transgene in the adult male medaka. Transgene expression was detected mainly in the testis and was barely detectable in the other tissues examined. In addition, no transgene expression was detectable in the female tissues including the ovary. A comparative analysis of the GSDF proximal promoter sequences collected from zebrafish, medaka, stickleback, fugu and trout genomes showed that three complex DNA regions are conserved within the first 600 bp. These regions contain transcription factors binding motifs, some of them are known to be involved in specific gene expression in the Sertoli cells according to studies in mammals.

In conclusion, the short GSDF promoter fragment presented in this study will be a valuable tool 1) to express transgenes specifically in the male gonad, 2) to discover new *cis* DNA regulatory elements and cognate binding proteins involved in testis-specific gene expression.

FinTRIMs: a highly diversified gene family of teleost fish with the hallmarks of antiviral restriction factors

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In mammals, the members of the tripartite motif (TRIM) protein family are involved in various cellular processes including intrinsic immunity against viral infection; the best known example being the TRIM5alpha protein which confers resistance to HIV to many primates. Viruses exert strong selective pressures on the defense system, and antiviral TRIMs show signs of diversification through gene expansion and positive selection. We describe a large new subfamily of TRIMs in teleosts, called finTRIMs, identified first in rainbow trout as virus-induced transcripts. FinTRIMs are formed of nearly identical RING/B-box regions and C-termini of variable length; the long variants include a B30.2 domain. The zebrafish genome harbors a striking diversity of finTRIMs, with 84 genes distributed in clusters on different chromosomes, and displaying strong polymorphism. A phylogenetic analysis revealed different subsets suggesting lineage-specific diversification events. Accordingly, the number of fintrim genes varies greatly among fish species. The closest mammalian relatives are trim16 and trim25, but they are not true orthologs. The B30.2 domain of zebrafish finTRIMs evolved under strong positive selection. The positions under positive selection are remarkably congruent in finTRIMs and in mammalian TRIM5alpha, concentrated within a viral recognition motif in mammals. The B30.2 domains most closely related to finTRIM are found among NOD-like receptors (NLR), indicating that the evolution of TRIMs and NLRs was intertwined by exon shuffling. The diversity, evolution, and features of finTRIMs suggest an important role in fish innate immunity; this would make them the first TRIMs involved in immunity identified outside mammals.

The role of *hemicentin* in fin development

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We have studied a group of non-allelic zebrafish mutants with blistering in the skin of embryonic fins. Applying positional cloning, we have identified two genes as *fras1*, *frem2* that encode structurally related basement membrane proteins. The orthologues of those genes, in human, are related to a recessive multisystem disorder known as Fraser syndrome. The patients present cryptophthalmos, syndactyly and around 45% of the newborns die as a consequence of lung or renal dysfunctions. During development Fras1 and Frem2 work together with other basement membrane proteins to stabilize dermis-epidermis structure. As in mice, zebrafish Frem2 seems to interact with Fras1 and maintain the stability of the protein complex in the basement membrane. This is fundamental for proper fin morphology. A third gene was identified in the group of zebrafish mutants and encodes a novel potential basement membrane protein, *hemicentin1* (*hmcn1*). *nagel (hmcn1)* mutants, *frem2 (blasen)* and *fras1 (pinfin)* have similar blister formation around the same stages of development. Vertebrates have two orthologue. Zebrafish *hmcn1* is expressed in epidermal cells, while *hmcn2* is primarily expressed in mesenchymal cells of the fin fold. *nagel* mutants analysis indicates that in contrast to Frem2, Hmcn1 is not required for Fras1 protein stability within the basement membrane. However, this result does not exclude the possibility that the proteins interact in an indirect manner. The multidomain protein structure of Hmcn1 indicates that it can bind to nidogens and other basement membrane proteins that have been shown to interact directly with Fras/Frem proteins.

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