



UNIVERSITÀ
DEGLI STUDI
DI BRESCIA

Dottorato di Ricerca in
Genetica Molecolare, Biotecnologie e Medicina Sperimentale

Settore scientifico disciplinare BIO/18 – Genetica

CICLO XXXVI

**Generation and characterization
of zebrafish mutant lines
for *ap1s1*, *ap1s2* and *slc7a6os*,
three genes of biomedical relevance**

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Anno Accademico 2022/2023

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*A Gelmo,
chissà cosa mi avresti detto
se fossi stato qui...*

ABBREVIATIONS

AJ: adherens junctions
AMO: antisense oligo morpholino
BD: bipolar disorder
BLAT: Basic Local Alignment Search Tool-Like Alignment Tool
Cas9: CRISPR-associated proteins
CCP: clathrin coated pits
CDD: congenital diarrheal disorders
CFD: cerebral folate deficiency
CNS: central nervous system
COX: Cytochrome-c-oxidase
CRISPR: clustered regularly interspaced short palindromic repeats
CSF: cerebrospinal fluid
EEG: electroencephalogram
EKV3: Erythrokeratoderma Variabilis type 3
ENU: ethylnitrosourea
EtOH: ethanol
FRG: filaggrin
GRCz11: Genome Reference Consortium Zebrafish build 11
GRPR: gastrin-releasing peptide receptor
GWAS: genome wide association study
HAR: human accelerated regions
HDR: homologous directed repair
HM: hybridization mix
HotSHOT: hot sodium hydroxide and Tris
HR: homologous recombination
ID: intellectual disability
IF: immunofluorescence
ISH: *in situ* hybridization
KO: knockout
LBs: lamellar bodies
LOF: loss of function
MCS: multiple cloning site
MeDOC: Mendelian disorders of cornification

MEF: mouse embryonic fibroblast
MERRF: myoclonus epilepsy with ragged-red fibers
MetOH: methanol
MR: mental retardation
MRI: magnetic resonance imaging
NAT: natural antisense transcript
NES: nuclear export signal
NHEJ: non-homologous end joining
NLS: nuclear localization signal
nORFs: novel open reading frames
NS: Netherton syndrome
OPBA: organismo preposto per il benessere animale
ORF: open reading frames
pc: post coitus
PCR: polymerase chain reaction
PH3: phosphorylated-histone-H3
PME: progressive myoclonus epilepsies
PMG: polymyography
SCZ: schizophrenia
SIB: self-injurious behaviour
TALENs: Transcription activator-like effector nucleases
TBP: TATA-binding protein
TE: transposable elements
TEER: trans-endothelial electrical resistance
TGN: *trans*-Golgi network
TILLING: Targeted Induced Local Lesions IN Genomes
TMS: tricaine methanesulfonate
TPM: transcript per million
TTD: trichothiodystrophy
ULD: Unverricht-Lundborg disease
VLCFAs: very long chain fatty acids
WT: wild-type
ZFN: Zinc Finger Nucleases
ZO-1: zonula occludens 1

1 ABSTRACT (ENGLISH VERSION)

The goal of this PhD thesis was the characterization of three genes involved in rare human Mendelian disorders using zebrafish animal model. In particular, CRISPR/Cas9 genome editing technology enabled the introduction of loss-of-function mutations in *ap1s1*, *ap1s2* and *slc7a6os* genes in *Danio rerio*. The first line of research was focused on the integration of previous knowledge on the adaptor protein 1 (AP-1) complex. Adaptor proteins assemble on the cytosolic face of cellular membranes to promote cargo sorting. AP-1 is usually involved in vesicular trafficking between the *trans*-Golgi network, the early or recycling endosomes and the plasma membrane and it is composed of four subunits (γ 1, β 1, μ 1 and σ 1). Although deficiency for the ubiquitous subunits γ 1 and μ 1A is lethal, knockout mice for *Ap1s2* (encoding for the σ 1B subunit) are viable, but reveal impaired tissue functions. Mutations in *AP1S1*, encoding for σ 1A, are responsible for the autosomal recessive and multisystemic MEDNIK syndrome (MIM # 609313). Conversely, mutations in *AP1S2* are causative of an X-linked mental retardation, the Pettigrew syndrome (MIM # 304340). Our CRISPR/Cas9 approach allowed the introduction of different frameshift deletions in *ap1s1* and *ap1s2* genes in *Danio rerio* and the presence of viable and fertile adult $-/-$ individuals confirmed that deficiency of σ 1A/ σ 1B subunit is not lethal in zebrafish. A preliminary characterization of the neurogenesis showed subtle alterations in key neural marker expression in *ap1s1*^{del4/del4} and *ap1s2*^{del5/del5} mutants, while preliminary behavioural data on larvae suggested that *ap1s1*^{del4/del4} and *ap1s2*^{del5/del5} mutants are characterized by faster movements, during dark periods, when compared to wild-type individuals. It has been shown that *AP1S1* has an important role in copper homeostasis; thus, *ap1s1* zebrafish mutants were treated with high doses of copper solutions (500-1100 μ g/L of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ dissolved in common medium water) from 0 hpf to 5 dpf. Preliminary data suggested that administration of copper to *ap1s1* mutants affects survival rate in the first 5 days post fertilization and could alter the hatching capability in the same period. Further analyses will have to be performed to clarify the role of *ap1s1* both in copper metabolism and in the onset of the complex phenotype of MEDNIK patients. The second line of research was focused on the characterization of *slc7a6os* gene, whose putative orthologue in *S. cerevisiae* (*IWR1*) has a role in the nuclear import of RNA polymerase II. Mutations in human *SLC7A6OS* gene are causative of a rare form of a progressive myoclonic epilepsy (MIM # 619191). Crosses between heterozygotes of the del11 frameshift mutation of the *slc7a6os* gene in zebrafish produce viable progeny in the Mendelian genotypic ratios until 1.5 mpf, when we assist to a decrease in $-/-$ individuals due to the death of female offspring. A few *slc7a6os*^{-/-} males survive until adulthood and are characterized by morphological defects. Preliminary investigation on neurogenesis showed subtle alterations of *neurod1* expression at 24 hpf and 30 hpf and in the outer region of the eye at 72 hpf of $-/-$ individuals. In particular, abnormalities in the marginal zone and in the inner nuclear layer

of Tg(*neurod1*:EGFP) *slc7a6os*^{del11/del11} retinas were observed through fluorescence light sheet microscopy. Preliminary behavioural tests indicated that -/- larvae (5 dpf) show a generally high average distance travelled in dark/light phase. Therefore, adults (6 mpf) subjected to the “novel tank test” showed that -/- individuals spend more time at the bottom of the tank, in contrast to +/- and control fish, which explore all the three different zones of the tank during the test session. These behavioural changes will need to be supported by additional tests and could be related to the severe morphological defects that specifically affect -/- individuals.

2 ABSTRACT (ITALIAN VERSION)

L'obiettivo di questa tesi è stato la caratterizzazione di tre geni coinvolti in rare patologie mendeliane, attraverso il modello animale zebrafish. In particolare, la tecnologia di editing CRISPR/Cas9 ha permesso l'introduzione di mutazioni loss-of-function in *ap1s1*, *ap1s2* e *slc7a6os* di *Danio rerio*. La prima linea di ricerca aveva come scopo l'integrazione di conoscenze pregresse sul complesso adattatore AP-1: le proteine adattatrici si assemblano sul lato citosolico delle membrane cellulari per riconoscere le molecole da trasportare. AP-1 è solitamente coinvolto nel traffico vescicolare tra il *trans*-Golgi, gli endosomi precoci/di riciclo e la membrana plasmatica ed è composto da quattro subunità ($\gamma 1$, $\beta 1$, $\mu 1$ e $\sigma 1$). Sebbene il deficit di $\gamma 1$ o $\mu 1A$ sia letale, topi knockout per *Ap1s2* (codificante $\sigma 1B$) sopravvivono all'età adulta, ma presentano alterate funzioni tissutali. Mutazioni in *AP1S1*, codificante $\sigma 1A$, sono responsabili della sindrome autosomica recessiva multisistemica MEDNIK (MIM # 609313). Diversamente, mutazioni in *AP1S2* causano un ritardo mentale legato al cromosoma X, la sindrome di Pettigrew (MIM # 304340). CRISPR/Cas9 ha permesso l'introduzione di delezioni frameshift nei geni *ap1s1* e *ap1s2* in *Danio rerio* e la presenza di individui -/- adulti, vitali e fertili, ha confermato che la carenza di $\sigma 1A/\sigma 1B$ non è letale in zebrafish. Una caratterizzazione preliminare della neurogenesi ha mostrato lievi alterazioni nell'espressione di marcatori specifici nei mutanti *ap1s1*^{del4/del4} e *ap1s2*^{del5/del5} a diversi stadi di sviluppo, mentre i risultati preliminari sul comportamento delle larve hanno suggerito che i mutanti *ap1s1*^{del4/del4} e *ap1s2*^{del5/del5} mostrano movimenti più rapidi, durante i periodi di buio, rispetto agli wild-type. È stato dimostrato che *AP1S1* ha un ruolo importante nell'omeostasi del rame; i mutanti zebrafish di *ap1s1* sono stati trattati quindi con dosi elevate di soluzioni di rame (500-1100 $\mu\text{g/L}$ di $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ disciolti in acqua pesci) da 0 hpf a 5 dpf. I dati preliminari suggeriscono che la somministrazione di rame ai mutanti *ap1s1* possa influire sul tasso di sopravvivenza nei primi 5 giorni dopo la fecondazione e potrebbe alterare la capacità di hatching nello stesso periodo. Ulteriori analisi saranno finalizzate a chiarire il ruolo di *ap1s1* nel metabolismo del rame e nell'insorgenza del complesso fenotipo dei

pazienti MEDNIK. La seconda linea di ricerca aveva come scopo la caratterizzazione del gene *slc7a6os*, il cui ortologo in *S. cerevisiae* (*IWR1*) ha un ruolo nell'importazione nucleare della RNA polimerasi II. Mutazioni a carico del gene *SLC7A6OS* umano causano una rara forma di epilessia mioclonica progressiva (MIM # 619191). Incroci tra eterozigoti del11 di *slc7a6os* in zebrafish generano una progenie vitale nei rapporti genotipici mendeliani fino a 1,5 mpf, quando assistiamo ad un calo nel numero di -/- per la morte dei mutanti di sesso femminile. Alcuni maschi *slc7a6os*^{-/-} sopravvivono all'età adulta ma presentano difetti morfologici. Indagini preliminari sulla neurogenesi hanno mostrato lievi alterazioni nell'espressione di *neurod1* a 24 hpf e 30 hpf e nella regione esterna dell'occhio di -/- analizzati a 72 hpf. In particolare, anomalie nella zona marginale e nello strato nucleare interno della retina di individui *slc7a6os*^{del11/del11} Tg(*neurod1*:EGFP) sono state riscontrate mediante microscopia light sheet a fluorescenza. Esperimenti comportamentali preliminari hanno mostrato larve -/- (5 dpf) caratterizzate da una distanza media percorsa più elevata nelle fasi di buio/luce. Gli adulti (6 mpf) sottoposti al “novel tank test” hanno mostrato individui -/- che trascorrono più tempo sul fondo della vasca, rispetto ai +/- e ai controlli, che esplorano le tre diverse zone. Tali alterazioni comportamentali dovranno essere supportate da ulteriori test e potrebbero correlarsi ai difetti morfologici caratterizzanti specificamente gli individui -/-.

3 THE AIM OF THE WORK

During this three year-PhD, my research was focused on the characterization of genes at high biomedical relevance in *Danio rerio*. In particular, the human orthologs of the investigated genes are involved in rare neurodevelopmental or neuromuscular disorders. Loss of function mutations can be easily introduced in the fish using the CRISPR/Cas9 genome editing technology. The use of zebrafish as a model organism facilitates the study of gene function during the embryonic development, which would otherwise be difficult in other models such as mice. Moreover, the relatively ease in handling and versatility of this animal allows to perform the functional characterization at various levels, including the use of tests for the study of behavioural phenotypes both in larvae and adults.

The first line of research was aimed at creating zebrafish knockout models in order to deepen the knowledge of the adaptor protein 1 (AP-1), started years ago by our group (Zizioli, et al., 2010) (Zizioli, et al., 2017) (Mignani, et al., 2023). Several animal models have already been generated with mutations in different AP-1 subunits, suggesting that deficiency for the ubiquitously expressed AP-1 adaptins, $\gamma 1$ or $\mu 1A$, is embryonic lethal both in zebrafish and mouse (Zizioli, et al., 1999) (Meyer, et al., 2000) (Zizioli, et al., 2017). Furthermore, gene knockdown in zebrafish for the ubiquitous $\sigma 1A$ subunit have been performed, resulting in perturbation in skin formation, reduced pigmentation, and severe motility deficits due to impaired neural network development, which was therefore consistent with the presentations of patients with MEDNIK syndrome (Montpetit, et al., 2008). Conversely, knockout mice for *Ap1s2*, the gene encoding the $\sigma 1B$ subunit, resulted to be viable but show reduced motor coordination and severely impaired long-term spatial memory (Glyvuk, et al., 2010) (Baltes, et al., 2014), resembling the clinical features of Pettigrew syndrome, a human X-linked disorder due to loss of function (LOF) mutations in AP1S2 (Tarpey, et al., 2006). The aim of this study was the generation and *in vivo* characterization of *ap1s1* and *ap1s2* zebrafish line hopefully representing novel vertebrate models for MEDNIK and Pettigrew syndrome, respectively.

The second line of research was focused on the characterization of *slc7a6os* gene. A new natural antisense transcript (NAT) of the *SLC7A6* gene has been previously identified by our research team on members of the SLC7 family of amino acid transporters in humans. Such NAT is encoded by *SLC7A6OS*, a highly conserved gene in vertebrates and homologous sequences have been identified in many eukaryotic organisms. The only information available at the molecular level comes from the study of the putative yeast orthologous *IWR1*, which plays a fundamental role in the import of RNA Polymerase II into the nucleus and in the dynamics of transcription of the holoenzyme. Our group has contributed to the identification and characterization of a splicing mutation in human *SLC7A6OS* gene, which has been associated with a rare form of progressive myoclonic epilepsy (PME), characterized by tonic-clonic, ataxia, attention disorders and visual perception deficits, with very

early onset of symptoms. The *slc7a6os* gene was previously studied in zebrafish in our laboratory: the spatial-temporal expression of the gene was analysed and, through oligonucleotides antisense morpholino, it was possible to temporarily inactivate its functionality in zebrafish embryos, which showed defects in neurogenesis and somitogenesis (Benini, et al., 2015). The goal of this project aimed at the creation and functional characterization of zebrafish lines with inactivating mutations in the coding region of the *slc7a6os* to better understand the biological function and relevance of the gene in vertebrates. *slc7a6os*^{+/-} fish, similarly to PME patients, express a reduced amount of the wt transcript and could present some features useful for the study of the pathogenesis of the human disease, while the study of *slc7a6os*^{-/-} individuals would determine the functional consequences of the absence of the gene. This study will help to improve our knowledge on the biological role of this human gene-disease, both during embryonic development and in the adult individual, and will lay the foundation for the creation of a more faithful zebrafish model of a rare form of human progressive myoclonic epilepsy.

4 INTRODUCTION TO *AP1S1* AND *AP1S2*

4.1 GENERAL FEATURES OF PROTEIN COATS AND VESICULAR TRAFFICKING

The inside of a eukaryotic cell is wisely structured in membrane-bound organelles to allow the compartmentalization of the wide range of molecules involved in biological functions. Various secretory, endocytic, and intracellular transport routes move the different molecular cargos through this system. One of the most common mechanisms at different stage of the endomembrane pathway involves protein coats (also referred to as vesicle coats or membrane coats). The coat is made up of supramolecular assemblies of proteins, which are recruited onto a particular membrane compartment (the donor) where they shape the membrane into a vesicle and collect the cargo. After the vesicle buds, it sheds its coat and goes on to fuse with a different membrane compartment (the acceptor). Among the different types of coated pits, the coatamer complexes COPI and COPII facilitate trafficking between the ER and the Golgi, while many of the post-Golgi trafficking pathways involves adaptor protein complexes (APs). Most of the transport vesicles in the periphery of the cell, including those originating from the plasma membrane, utilize a coat containing the three-legged triskelion scaffold protein called clathrin (Fig. 1A; 3A), that does not appear to bind directly membrane or cargo. Instead, adaptor proteins (APs) are required to couple them together by the interaction with sorting signals present in the cytosolic tails of transmembrane cargos at one side and with the clathrin at the opposite one (Fig. 1B) (Robinson & Bonifacino, Adaptor-related proteins, 2001) (Traub & Bonifacino, 2013) (Robinson, Forty Years of Clathrin-coated Vesicles, 2015) (Dell'Angelica & Bonifacino, 2019).

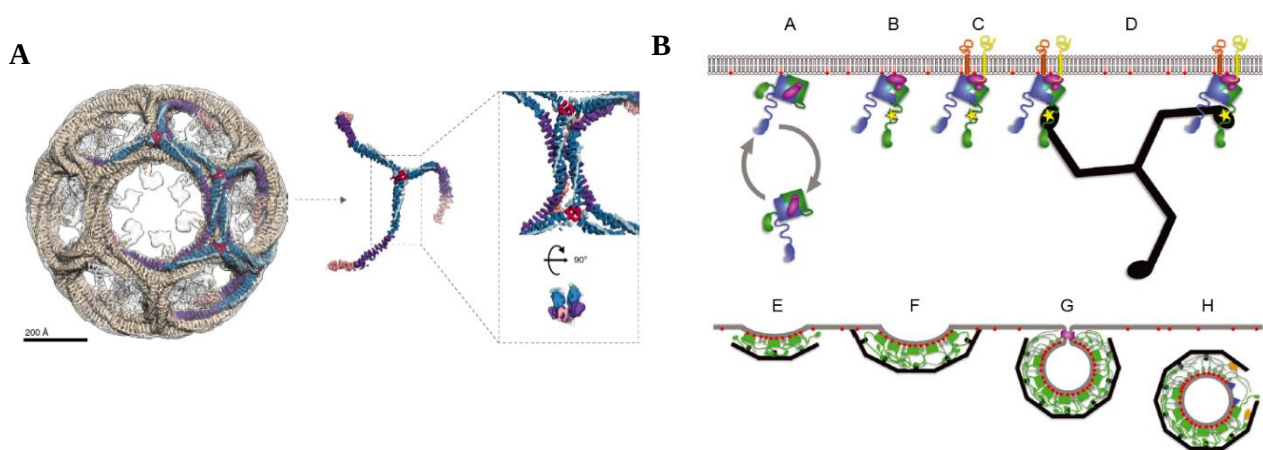


Figure 1. (A) Cryo-EM map of a clathrin coat architecture. A triskelion is shown in isolation (middle) and in the context of neighbouring heavy chains (right) (Morris, et al., 2019). **(B) Steps in the formation of an endocytic CCV** (Robinson, Forty Years of Clathrin-coated Vesicles, 2015).

Protein coats are found in all eukaryotes and were already present in the last eukaryotic common ancestor (LECA) 1-1.5 billion years ago. COPI and five AP complexes (AP-1 to AP-5) are structurally related and thought to have evolved from a single ancestral complex (Fig. 2A) (Robinson, 2015). Adaptor protein complexes (APs) show a distinct subcellular localisation, according to their specific function (Fig. 2B). First it was discovered that AP-1 is associated with the *trans*-Golgi network (TGN) and the tubular endosomes, whereas AP-2 is located at the plasma membrane and involved in clathrin-mediated endocytosis (Robinson & Bonifacino, 2001) (Robinson, 2015). Additional adaptor proteins, AP-3 and AP-4, were found in the 1990s on the basis of their homology to AP-1 and AP-2, leading to the localisation of AP-3 at endosomes, where it traffics cargo from tubular endosomes to late endosomes, lysosomes and related organelles (Dell'Angelica & Bonifacino, 2019), while AP-4 is primarily located at the level of TGN, where it is involved in anterograde trafficking from the TGN to endosomes (Matsuda & Yuzaki, 2008). The recently described AP-5 complex is a non-clathrin coat localized at the level of late endosomes and/or lysosomes and related organelles.

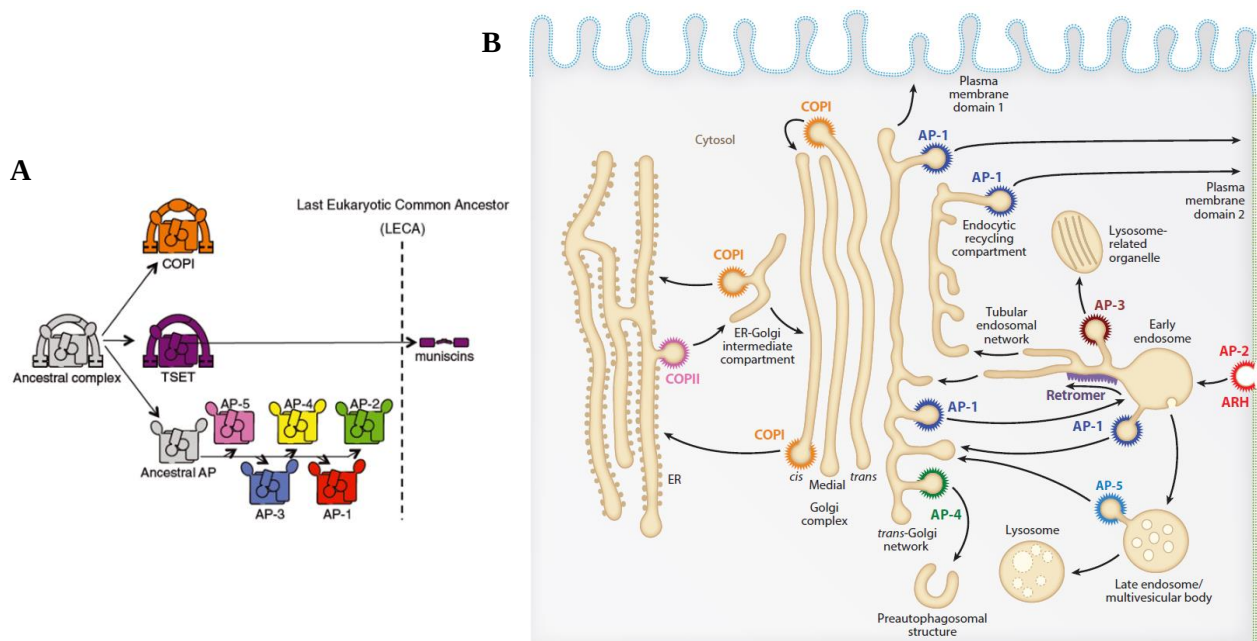


Figure 2. (A) Proposed evolution of the seven related heterotetramers: the five AP complexes, COPI and TSET. This complex is associated with the plasma membrane, although it is slightly more closely related to the early-Golgi COPI complex than to the post-Golgi AP complexes. It has been lost from animals and fungi (Robinson, 2015). **(B) Schematic representation of the different protein coats occurring in the endomembrane system of a eukaryotic cell** (Dell'Angelica & Bonifacino, 2019).

The heterotetrameric AP complexes share a conserved structural organization which is composed of four subunits: two large subunits (~100-110 kDa; α - ϵ / β 1-4), one medium (~50 kDa; μ 1-4), and one small subunit (~17 kDa; σ 1-4) (Boehm & Bonifacino, 2002). The N-terminal domain of the large

subunits, together with the medium and small subunits, form a compact core, while the C-terminal domains of the large subunits form appendages that connect to the core by flexible linkers (Fig. 3B). The large subunits (α , γ , δ or ϵ , and $\beta 1-4$) mediate binding to the target membrane and clathrin recruitment. The small subunits are part of the AP complex core and have been suggested to contribute to the stabilization of the complex, in conjunction with the medium subunit μ , which is primarily involved in protein cargo sorting. The large adaptin subunits form a rectangular “head,” with each large subunit contributing an “ear” on either side. The β subunit interacts with the μ subunit and the α - ϵ subunit interacts with the σ subunit. Interestingly, several subunits of AP complexes 1–3 occur in different cell-type specific isoforms (*e.g.* AP-1 has two γ , two μ , and three σ isoforms), generating considerable complexity of assembly (Martinelli & Dionisi-Vici, 2014).

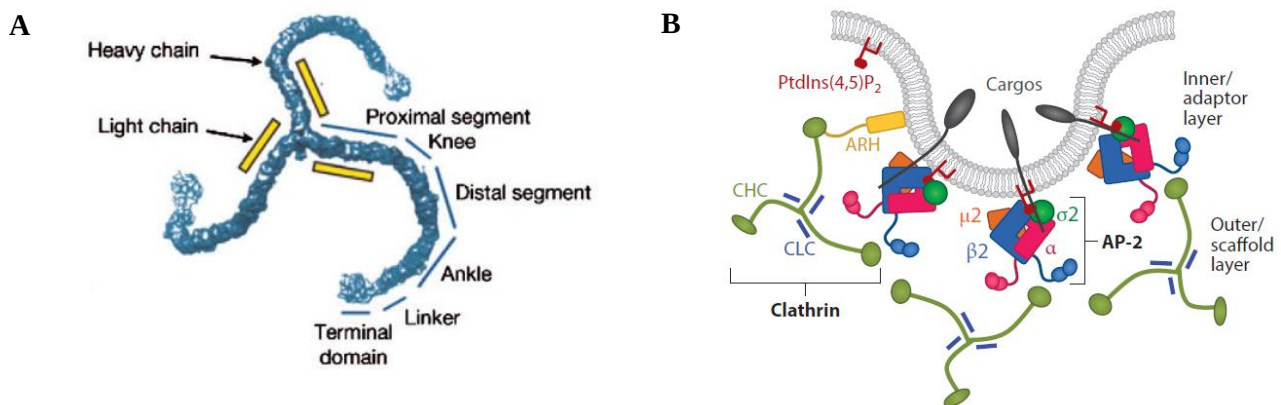


Figure 3. (A) Clathrin triskelion, showing the various domains (Robinson, 2015). (B) Schematic representation of the AP-2 coat assembled on the membrane bud (Dell'Angelica & Bonifacino, 2019).

4.2 GENETIC DISORDERS OF PROTEIN COATS: THE ADAPTINOPATHIES

In humans, different mutations in subunits of all the coats have recently been shown to cause various genetic disorders of protein coats that are collectively referred to as “coatopathies”. The inheritance of these conditions can be autosomal recessive, autosomal dominant, or X-linked. Defects in coats such as COPI, COPII, AP-1, and AP-2 impair specific transport pathways, compromising the function and viability of the cells (Dell'Angelica & Bonifacino, 2019). Several genetic defects in which the causative mutation is localized in an AP subunit gene have been identified (Table 1) and are commonly defined as “adaptinopathies”. This results in membrane proteins failing to get packaged correctly into vesicles, so that they end up in the wrong part of the cell. Furthermore, patients with a wide range of disorders, particularly involving the nervous system, have been identified with mutations in each of the AP complexes (Sanger, Hirst, Davies, & Robinson, 2019). Mutations in the gene encoding the sigma 1A subunit ($\sigma 1A$) are responsible for the complex and multifactorial

MEDNIK syndrome (Mental retardation, Enteropathy, Deafness, peripheral Neuropathy, Ichthyosis, and Keratoderma, MIM # 609313) described below. Conversely, mutations in the gene encoding an alternative sigma subunit, the subunit sigma 2 (also called $\sigma 1B$), have been identified in patients with an X-related mental retardation (Borck, et al., Clinical, cellular, and neuropathological consequences of AP1S2 mutations: further delineation of a recognizable X-linked mental retardation syndrome, 2008) (Tarpey, et al., 2006), the so called Pettigrew syndrome (PGS, MIM # 304340), also described below.

Mutations in the gene encoding the third isoform of the sigma subunit are involved in an autosomal dominant disease named Pustular psoriasis (MIM # 616106), whose clinical features affect primarily the integumentary system (skin, nails and hair) through the presence of pustules and dystrophic nails where distribution of lesions may be generalized, palmoplantar or acral.

Gene affected	Disease phenotype	Phenotype MIM number
AP1S1	MEDNIK syndrome	609313
AP1S2	X-linked intellectual disability	304340
AP1S3	Pustular psoriasis	616106
AP2S1	Hypocalciuric hypercalcemia, type III	600740
AP2M1	Developmental and epileptic encephalopathy	601024
AP3B1	Hermansky-Pudlak syndrome 2	617050
AP3B2	Early infantile epileptic encephalopathy 48	617276
AP3D1	Hermansky-Pudlak syndrome 10	608233
AP4B1	Spastic paraplegia 47	614066
AP4E1	Spastic paraplegia 51	613744
AP4M1	Spastic paraplegia 50	612936
AP4S1	Spastic paraplegia 52	614067
AP5Z1	Spastic paraplegia 48	613647

Table 1. Genetic disorders associated with AP complexes: gene, phenotype and MIM number are reported for each AP complex (Sanger, Hirst, Davies, & Robinson, 2019).

4.3 ADAPTOR PROTEIN 1 (AP-1): STRUCTURAL OVERVIEW AND STUDIES

The first discovered adaptor complexes were AP-1 and AP-2 (Fig. 4), as abundant components of CCVs. Adaptor protein complex 1 (AP-1) is found at the cytoplasmic face of coated vesicles located at the Golgi complex, where it mediates both the recruitment of clathrin to the membrane and the recognition of sorting signals within the cytosolic tails of transmembrane receptors. In general, AP-1 functions in bidirectional transport between the TGN and endosomes. However, in polarized cells such as epithelial cells and neurons, it plays a more specific role in sorting to the basolateral and somatodendritic domains of the plasma membrane, respectively.

In 2004, Heldwein et al. proposed the structure of the clathrin adaptor protein 1 core that comprises N-terminal fragments (~70 kDa) of the two large chains, $\beta 1$ and γ , plus the intact medium and small chains, $\mu 1$ (~50 kDa) and $\sigma 1$ (~20 kDa) with extended hinge and ear regions comprising the C-terminal portions of γ - and $\beta 1$ -adaplin (Fig. 4A). Its molecular architecture closely resembles that of the core of AP-2, the plasma-membrane-specific adaptor, for which a structure has been determined (Collins, McCoy, Kent, Evans, & Owen, 2002). Both structures represent an “inactive” conformation

with respect of cargo with a tyrosine-based sorting signal. AP-1 is recruited to membranes by the interaction with phosphatidylinositol-4-phosphate (PI-4-P) and the GTP-bound form of ARF GTPases (in particular ARF1), which binds to two sites near the N-termini of γ - and β 1-adaptin, plus a third site on the central trunk region of γ -adaptin. ARF1 binding triggers a conformational change from a cytosolic, locked conformation to a membrane-bound, unlocked conformation (Heldwein, et al., 2004). The unlocking of AP-1 exposes binding sites for at least two types of sorting signals fitting the consensus motifs Yxx Φ (i.e., tyrosine-based signals) and [DE]xxxL[LI] (i.e., dileucine-based signals). Whereas Yxx Φ signals bind to a site on the C-terminal domain of μ 1 (Ohno, et al., 1995) (Mattera, Farías, Mardones, & Bonifacino, 2014), [DE]xxxL[LI] signals bind to a site on σ 1, with some contribution from γ -adaptin (Janvier, et al., 2003) (Mattera, Boehm, Chaudhuri, Prabhu, & Bonifacino, 2011). Fig. 4 (B-C) shows the structure of the adaptor protein 1 complex core that includes the subunits described above (The PyMOL Molecular Graphics System, Version 2.3.4 Schrödinger, LLC; amino acid length and isolation species of the crystalized subunits are reported):

- γ 1 (613 aa, mouse) in blue
- β 1 (584 aa, rat) in red
- μ 1 (423 aa, mouse): μ 1N in green, μ 1C in yellow-orange
- σ 1A (158 aa, mouse) in magenta

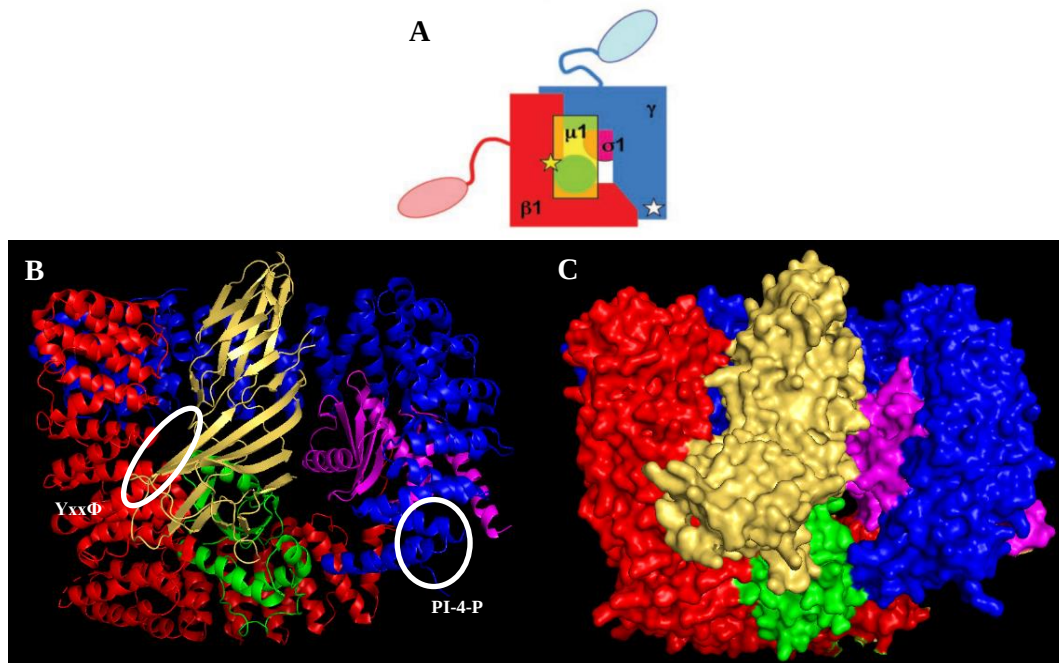


Figure 4. Structure of the AP-1 core. (A) Diagram of AP-1. It comprises the large-chain trunks (γ , blue; μ 1, red), the medium chain (μ 1N, yellow–green; μ 1C, yellow–orange), and the small chain (σ 1, magenta). Appendages of the large chains are linked to the trunks by extended flexible connectors. Yellow star, binding site for tyrosine-based sorting signal; white star, binding site for inositol-phosphate head group (Heldwein, et al., 2004). **Molecular reconstruction (The PyMOL Molecular Graphics System, Version 2.3.4 Schrödinger, LLC) (B) Ribbon representation.** Disordered segments are dotted lines of appropriate colour. Locations of the proposed PI-4-P-binding site on γ and of the Yxx Φ site on μ 1 are shown by white ellipses **(C) Molecular surface representation.**

Some of the compositional subunits occur as multiple isoforms encoded by different genes, so combinatorial assembly of different subunit isoforms gives rise to different AP heterotetramers, which may display tissue-specific expression and function. AP-1 complexes containing μ 1A or μ 1B have been referred to as AP-1A or AP-1B, notwithstanding the differences in the other subunits. AP-1A is ubiquitously expressed, whereas AP-1B is epithelial-specific (Park & Guo, 2014). Most polarized epithelial cells co-express the epithelial cell-specific AP-1B complex in addition to the ubiquitously expressed AP-1 (now named AP-1A). Despite their close homology (Ohno, et al., 1995), AP-1A and AP-1B have distinct functions in endosomal/lysosomal sorting or basolateral membrane targeting, respectively. Analysis of the zebrafish genome revealed the existence of a single β 1-adaptin gene, whereas two genes are present for the γ -subunit (*ap1g1* and *ap1g2*). The β -1 subunit is involved in several processes, including detection of mechanical stimulus in sensory perception and sensory organ development. Auditory/vestibular *ap1b1* mutants in *Danio rerio* exhibit progressive signs of degeneration in the sensory epithelium, in addition to decreased expression of the Na^+/K^+ ATPase pump in the basolateral membrane and its mislocalization to apical bundles in hair cells (Clemens Grisham, Kindt, Finger-Baier, Schmid, & Nicolson, 2013). In zebrafish AP-1/ γ 2 and AP-1/ γ 1 fulfil different and essential functions, particularly involving the neurodevelopment: γ 2-adaptin is homologous to γ 1, but it is only present in vertebrates while γ 1 is found in all eukaryotes (Zizioli, et al., 2017). Moreover, three μ 1-adaptin genes exist in the teleost, encoding μ 1A, μ 1B and the μ 1C isoform, which is not found in mammals. The μ 1C expression pattern largely overlaps with that of μ 1A, while μ 1B is expressed in epithelial cells. Gene knockdown studies demonstrated that each of these μ 1 adaptins is essential for zebrafish embryogenesis, with μ 1A and μ 1C being involved in central nervous system development and μ 1B in kidney, gut and liver formation (Zizioli, et al., 2010) (Gariano, et al., 2014). Finally, four different genes, *ap1s1*, *ap1s2*, *ap1s3a* and *ap1s3b* encode for the three different isoforms of the small AP-1 σ subunit in *Danio rerio*, called σ 1A, σ 1B, and σ 1C. The *ap1s1* gene is involved in extracellular matrix organization and nervous system development and it represents the orthologue of the human *AP1S1* gene implicated in MEDNIK syndrome. To validate the pathogenic effect of the first splice mutation described in human, *ap1s1* knockdown had been performed, resulting in a phenotype which consisted of perturbation in skin formation, reduced pigmentation, and severe motility deficits due to impaired neural network development (Montpetit, et al., 2008). Conversely, *ap1s2* and *ap1s3* are the putative orthologues of the human *AP1S2* and *AP1S3* genes, mutated in Pettigrew syndrome and in Pustular psoriasis, respectively. To date no studies have been published either for subunit σ 2 or σ 3 (3a-3b) in zebrafish: they are predicted to localize to intracellular-membrane organelle, with *ap1s3b* expressed in olfactory placode and peripheral olfactory organ.

4.4 GENETIC MODELS FOR ADAPTOR PROTEIN 1 SUBUNIT SIGMA

In the past, generation of knockout and knockdown animal models highlighted the important roles of AP subunits in vesicular traffic and their indispensable functions during development of vertebrates, their various tissues and the immune system. More in general, the lack of any subunit either for AP-1 or AP-2 complexes renders the entire complex dysfunctional and, more specifically, deficiency for the ubiquitously expressed AP-1 large adaptins, $\gamma 1$ or $\mu 1A$, is embryonic lethal. $\gamma 1$ -deficient murine embryos cease development at blastocyst stage (embryonic day 3.5 pc), whereas $\mu 1A$ -deficient embryos cease development at mid-organogenesis (day 13.5 pc). The observation that $\mu 1A$ -deficient embryos reach organogenesis is probably due to the presence of the homologous $\mu 1B$, which is expressed exclusively in polarized epithelial cells (Meyer, et al., 2000). In contrast, the ubiquitous $\gamma 2$ cannot complement $\gamma 1$ deficiency (Zizioli, et al., 1999). Moreover, injected embryos with splice-blocking morpholino (MO) for both adaptins $\gamma 1$ and $\gamma 2$ show lethal impairment of CNS in *Danio rerio*, in addition to an impaired development of somites and of the anterior-posterior axis. Almost the 70% of the morphants embryos die before 72 hpf, while the remainder survive for a longer period and are characterized by a delayed hatching time, but die at 6 dpf (Zizioli, et al., 2017). A study describing the generation of a CRISPR/Cas9 knockout model for the *ap1g1* gene in zebrafish has recently been published (Mignani, et al., 2023). Similarly to what is observed in mice, $\gamma 1$ mutant fish cease their development during the transition between the blastula and gastrula stage. Moreover, it is interesting to note that heterozygous females and males have reduced fertility, due to impaired oocyte and sperm maturation, and showed morphological alterations in the brain, gonads and intestinal epithelium. More in detail, they showed impaired cell adhesion in epithelial tissues, such as cadherin protein sorting and adherent junction (AJ) formation.

With particular attention to the smaller adaptin of the AP-1 complex, the σ subunit, three encoding genes have been identified in mammals and it has been demonstrated that loss of any one of them is non-lethal. This suggest whether small subunits can contribute to the stabilization of the complex rather than the assembly process of the same. As already mentioned (Table 1), human Mendelian diseases have been associated with mutations in each of the three genes encoding for the σ subunits of adaptor protein complex 1. Researchers hypothesized that the relevance of $\sigma 1A$ and $\sigma 1B$ in the nervous system as opposed to $\sigma 1C$ based on their levels of expression in many cell types. Studies on HeLa cells have shown that $\sigma 1A$ is the most abundant, followed by $\sigma 1C$ and $\sigma 1B$ (Itzhak, Tyanova, Cox, & Borner, 2016). In contrast $\sigma 1A$ is twice as abundant in mouse neurons as $\sigma 1B$, while $\sigma 1C$ is undetectable (Itzhak, et al., 2017), confirming the analysis of Northern blotting and RT-PCR on mouse tissues that indicates how $\sigma 1A$ and $\sigma 1B$ are strongly expressed in the brain compared to $\sigma 1C$ (Glyvuk, et al., 2010). Different $\sigma 1$ isoforms may have different cargo preferences, leading several

membrane proteins to exhibit aberrant localisations or expression levels in cells that are deficient in one of the $\sigma 1$ genes. With regards to zebrafish, Montpetit et al. (2008) verified that *ap1s1* knockdown models with morpholino die in embryonic stages subsequent to 48 hpf. No characterization of *ap1s2* in zebrafish are currently available, even if studies on $\sigma 1$ B-deficient human cells (Borck, et al., 2008) and mice (Glyvuk, et al., 2010) (Baltes, et al., 2014) demonstrated that lack of $\sigma 1$ B subunit is non-lethal, although impairment in vesicle recycling and adipogenesis are observed.

4.5 MEDNIK SYNDROME IS DUE TO MUTATIONS IN *AP1S1* GENE

4.5.1 INTRODUCTION AND CLINICAL FEATURES

MEDNIK syndrome (MIM # 609313) is defined as a rare disorder of copper metabolism caused by mutations in *AP1S1* and characterized by intellectual deficit, enteropathy, sensorineural hearing loss, peripheral neuropathy, lamellar and erythrodermic ichthyosis, and keratoderma (<https://www.orpha.net>). Mutated forms of *AP1S1* therefore result in abnormal lipid transport and secretion, and consequently to an altered formation of lamellar bodies (LBs), which are specialized keratinocyte organelles responsible for the delivery of lipids to the extracellular space. More in general, MEDNIK syndrome is also included in the major group of Mendelian disorders of cornification (MeDOC), usually defined as “inherited ichthyoses”, which are characterized by visible scaling and/or hyperkeratosis of most or all of the skin (Fig. 5). This etiologically and phenotypically heterogeneous group of conditions is caused by mutations in various different genes important for keratinocyte differentiation and epidermal barrier function. To date, therapy of MeDOC is symptomatic, rarely is type-specific or corrective, and it is commonly based on patient’s experience rather than disease pathogenesis (Schmuth, et al., 2012).

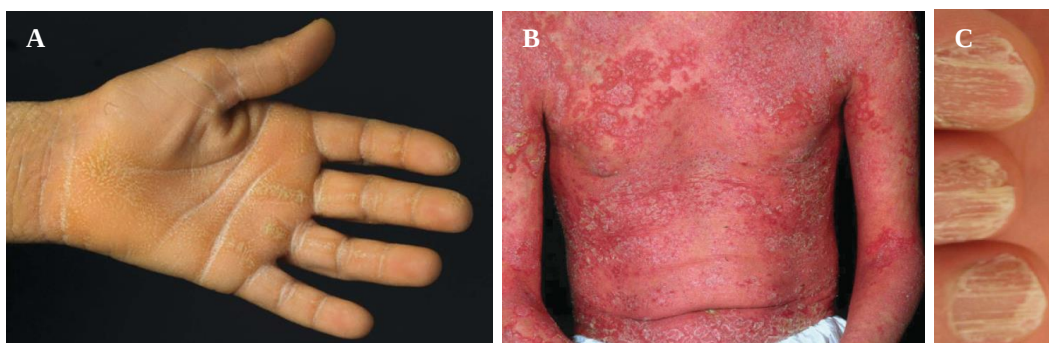


Figure 5. Variety of phenotypical and histological features and other dermatologic findings in different MeDOC (Schmuth, et al., 2012). (A) Fine scaling phenotype in ichthyosis vulgaris (IV) (filaggrin (FLG)). (B) Generalized fine scaling phenotype with concomitant severe erythema in Netherton syndrome (NS, serine protease inhibitor, kazal-type 5 (SPINK5)). (C) Onychoschisis of fingernails in trichothiodystrophy (TTD).

Patients affected by MEDNIK usually live in a relatively isolated population descended from a limited number of ancestors; the disease was initially identified in five children of three families from the Kamouraska region (Quebec, Canada) and it was previously named Erythrokeratoderma Variabilis type 3 (EKV3) (Saba, et al., 2004), characterized by similar skin lesions as observed for EKV, including congenital hyperkeratosis and red patches of variable sizes, shapes, and duration. Thus, it is a pathological condition similar to a previously described disease (Beare, Nevin, Froggatt, Kernohan, & Allen, 1972). MEDNIK is also characterized by multisystem involvement, including congenital chronic diarrhoea, and an elevation of very long chain fatty acids (VLCFAs).

The gene responsible for this autosomal recessive syndrome was mapped by identifying a common homozygous region, corresponding to a few Mb segment of chromosome 7p harbouring about 100 genes including *AP1S1*. A splice mutation in human *AP1S1* was described in four families with MEDNIK syndrome. Sequencing revealed that the founder mutation was located in the acceptor splice site (A to G) of exon 3 (IVS2-2A>G) and it is predicted to cause the skipping of exon 3, leading to a premature stop codon at the beginning of exon 4 (Montpetit, et al., 2008).

Recently, a novel mutation in *AP1S1* has been identified in a Turkish child with ichthyosis and intellectual disability with MEDNIK syndrome (Incecik, Bisgin, & Yılmaz, 2018). Sequencing analyses showed a homozygous insertion c.364dupG (NM_001283.4), which is predicted to cause a frameshift of the reading frame (p.D122Gfs*18) in exon 4 to 5 and position 74 of 138 (coding) of *AP1S1* gene. Cerebral magnetic resonance imaging (MRI) showed brain atrophy, while blood tests revealed low levels of ceruloplasmin and high concentrations of serum copper and very long fatty acids (C26:0). The *AP1S1* gene codes for the $\sigma 1A$ subunit of adaptor protein complex 1 (AP-1) that directs intracellular trafficking of copper pumps (ATP7A and ATP7B); thus, MEDNIK syndrome represents a multisystem disease that combines clinical and biochemical signs of both Menkes and Wilson's diseases (Martinelli & Dionisi-Vici, 2014), in which liver copper overload is treatable using zinc acetate therapy, a recommended drug to reduce copper intestinal absorption. Tentatively zinc sulphate¹ was started to the patient to treat the copper metabolism defect due to this homozygote c.364dupG mutation in *AP1S1* gene. The approach was successful in reducing copper overload and cholestasis, with clear improvement of the patient's clinical and neurological conditions.

Another clinical study was published in 2020, identifying two novel *AP1S1* mutations, c.269T>C (p.Leu90Pro) and c.346G>A (p.Glu116Lys), as causative of an intractable diarrhoea in 2 consanguineous families (Klee, et al., 2020). Normally, early-onset diarrhoea is characteristic of a group of > 50 monogenic entities defined as congenital diarrheal disorders (CDD), including defects

¹ Despite the fact that zinc acetate is the best treatment option for MEDNIK syndrome, treatment with zinc sulfate was started in this study in order to be an alternative treatment option, because zinc acetate is more expensive and harder to get than zinc sulphate.

in nutrient and electrolyte absorption, enterocyte polarization, enteroendocrine cell differentiation, and epithelial integrity, but also immunodeficiencies, congenital disorders of glycosylation, and some defects of the vesicular sorting and transporting machinery. Klee and her group reported the identification of two novel *AP1S1* mutations in 3 patients with isolated and non-syndromic congenital intestinal failure. To better define the consequences of *AP1S1* dysfunction, the authors of the study observed that knockout of *AP1S1* in a genome edited human intestinal epithelial cell line (CaCo2) disrupted epithelial barrier function, revealing altered localization of tight-junction proteins ZO-1 and claudin 3, decreased transepithelial electrical resistance and an increased dextran permeability of the CaCo2-*AP1S1*-KO monolayer. In addition to that, lumen formation in 3D cultures of these cells was abnormal. Re-expression of wild-type *AP1S1* in CaCo2-*AP1S1*-KO cells reverted these abnormalities, while expression of *AP1S1* containing either missense mutation did not.

4.5.2 MEDNIK ASSOCIATED WITH DEFECTIVE COPPER METABOLISM

In humans, mitochondrial respiration, antioxidant defence, neurotransmitter synthesis, connective tissue formation, and skin pigmentation are some of the cellular processes in which copper is required. To now, only few inherited disorders of copper metabolism have been described; the most commonly known, Menkes disease (MIM # 309400) and Wilson's disease (MIM # 277900) are caused by mutations in two closely related copper ATPases: ATP7A, mediating copper uptake in the gastrointestinal tract and copper delivery to the brain, and ATP7B, mediating copper excretion by the liver into bile (Kaler, 2013). Another defect associated with copper metabolism abnormalities has been described, the Huppke-Brendel syndrome, caused by mutation in *SLC33A1*, a gene required for acetylation and secretion of ceruloplasmin (Huppke, et al., 2012). Along with this disorder, a novel homozygous mutation in *AP1S1* was identified in a clinical study of a Sephardic-Jewish patient with MEDNIK syndrome, who showed severe perturbation of copper homeostasis with hypocupremia, hypoceruloplasminemia, liver copper accumulation and intrahepatic cholestasis (Martinelli, et al., 2013). DNA sequencing of the gene revealed the presence of a homozygous single G insertion in a stretch of eight consecutive Gs between nucleotides 356 and 365 in exon 4 (Fig. 6), which therefore was predicted to cause a frameshift with a premature stop codon, 17 amino acids downstream of the mutation site (p.Asp322GlyfsX17).

Patients with MEDNIK syndrome are therefore a puzzling picture which combines the clinical and biochemical signs of both Menkes and Wilson diseases. Zinc acetate treatment was attempted, resulting in a marked improvement of behavioural disturbances and of cognitive function, along with a clear relief from itching. Also biochemical parameters, such as serum transaminases, plasma

ceruloplasmin and serum free copper level normalized, with a striking reduction of plasma total bile acids levels and liver copper overload.

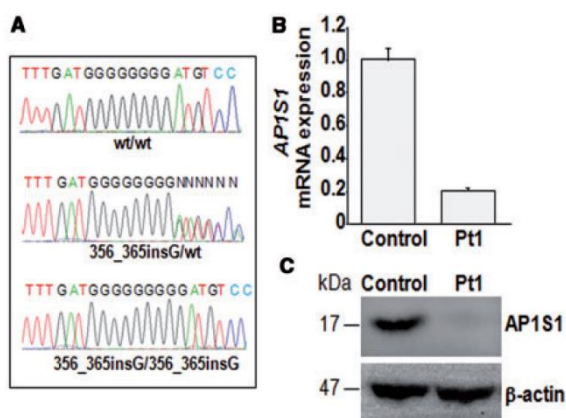


Figure 6. Mutation analysis and AP1S1 expression in a Sephardic-Jewish patient (Martinelli, et al., 2013). (A) Sequence analysis of genomic DNA showing homozygous G insertion in *AP1S1* between nucleotides 356 and 365 in affected patient (A, lower); proband's parents and her healthy sister carry the mutation in a heterozygous state (A, middle); wild-type (wt) sequence is given for comparison (A, upper). (B) Reduced expression of *AP1S1* mRNA in skin fibroblasts of the patient when compared with normal control subjects (quantitative PCR analysis). (C) Western blot analysis shows absence of AP1S1 protein in fibroblasts from the patient relative to fibroblasts from control subjects (normalization using b-actin as internal control).

To confirm the pathogenic link between *AP1S1* mutation and copper-related abnormalities observed in the patient, Martinelli et al. reviewed the whole French-Canadian series of original MEDNIK patients (Montpetit, et al., 2008), confirming the presence of both brain atrophy and liver disease as distinctive characteristics. In particular, investigation of the expression level of different copper-dependent enzymes in fibroblasts of affected patients showed marked reduction of COX-II and -IV subunits and superoxide dismutase, whereas the copper unrelated proteins SDH-70 and porin were normally expressed; in accordance with this severe reduction of COX activity was observed, along with a reduction of PMP70 activity, a protein involved in the transport of long- and branched-chain acyl-CoAs in peroxisomal membrane (Fig. 7).

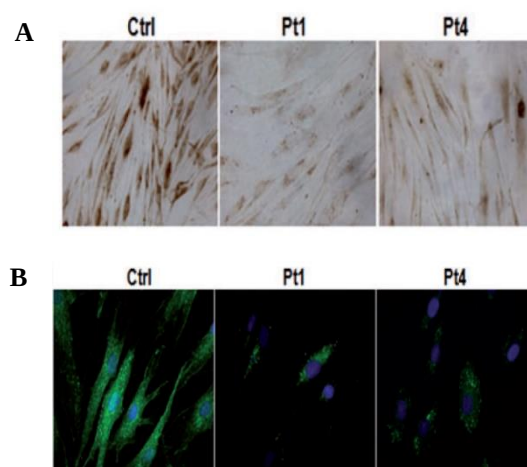


Figure 7. Expression analysis Cu-dependent enzymes in primary skin fibroblasts (Martinelli, et al., 2013). (A) Cytochrome-c-oxidase (COX) immunocytochemistry shows a severely reduced activity in all fibroblasts from Patients 1 and 4, with absent signal in some cells. (C) Immunofluorescence analysis of patients' fibroblasts stained with anti-PMP70 antibodies, showing a marked reduction in protein levels compared with control subjects. All images taken with a 40X magnification objective.

In this study subcellular localization of ATP7A pump was also investigated in fibroblasts, both in basal conditions and after increasing copper concentration in culture medium. While under basal

copper concentration, ATP7A in control fibroblasts showed an extensive co-localization pattern with the *trans*-Golgi marker p230 (yellow merge signal), copper cell loading led to ATP7A translocation to the plasma membrane (Fig. 8A). On the contrary, in *AP1S1* mutant fibroblasts, ATP7A protein was mainly localized to the cell periphery and linked to cytoskeletal filaments both in baseline conditions and after increasing the copper concentration (Fig. 8B). Rescue experiment was obtained by overexpressing wild-type *AP1S1* in mutant fibroblasts, thus restoring the correct co-localization of ATP7A within the *trans*-Golgi network (yellow merge signal) under basal copper concentration, as well as the physiological transfer to the cytoskeleton and plasma membrane after copper exposure.

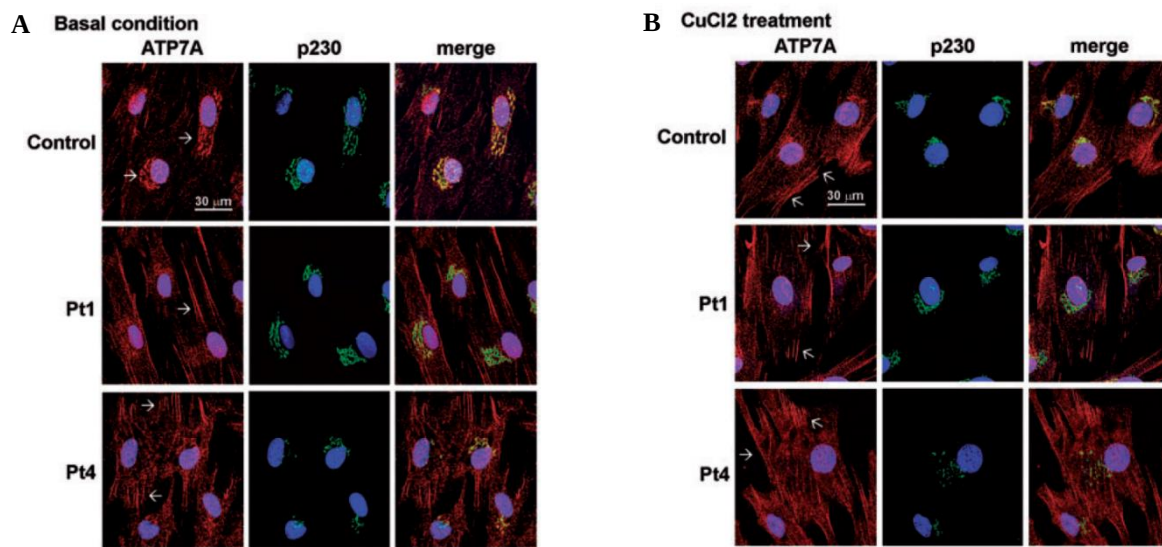


Figure 8. Subcellular localization of ATP7A in dermal fibroblasts under normal conditions or after treatment with copper solution (Martinelli, et al., 2013). Cells were co-stained with antibodies against ATP7A (red) and p230, the *trans*-Golgi network marker (green), and with Hoechst nuclear counterstain (blue). (A) Under normal copper concentrations, ATP7A and p230 colocalize in control fibroblasts, whereas reduced *trans*-Golgi localization and diffuse ATP7A signal (red) throughout the cells (arrows) is observed in *AP1S1* mutant fibroblasts. (B) Increasing copper concentration (200 μM) lead to an increase in ATP7A distribution for control cells (arrows) and a reduced co-localization between ATP7A and p230 (green signal, merge). Fibroblasts of patients showed a clear ATP7A cytoskeletal distribution that did not overlap with the *trans*-Golgi network (green signal, merge).

A role of *AP1S1* protein in regulating intracellular trafficking of copper ATPases was suggested, as the σ subunit of AP-1 is considered to interact, in combination with the large γ subunit, with dileucine-based recognition signals, which are characteristic of both ATP7A and ATP7B C-terminals. *AP1S1* mutation causes abnormal intracellular copper trafficking with consequent deficiency of copper-dependent enzymes, demonstrating that the pathogenic mechanism of MEDNIK syndrome is due to a qualitative abnormality of intracellular copper metabolism. Moreover, structural similarities of ATP7B with ATP7A (Lutsenko, Barnes, Bartee, & Dmitriev, 2007) suggest that impairment of a clathrin-dependent intracellular trafficking may also affect the function of the Wilson's disease protein, explaining liver copper accumulation and Wilson's-like phenotype in MEDNIK syndrome.

4.6 GENETIC MODELS FOR ADAPTOR PROTEIN 1 SUBUNIT SIGMA 1

4.6.1 σ 1A CHARACTERIZATION IN *ap1s1* ZEBRAFISH KNOCKDOWN

Up to now, little is known about the σ subunit role in AP complex formation and function *in vivo*. The first characterization in zebrafish animal model was obtained in 2008 by the studies of Montpetit et al., using the morpholino knockdown (KD) strategy. In particular, *ap1s1* mRNA translation was inhibited using an antisense oligo morpholino (AMO) targeting its start codon (Fig. 9).

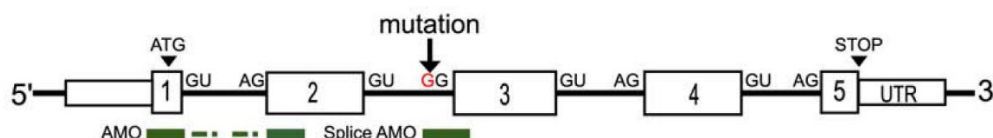


Figure 9. Schematic representation of the Human *AP1S1* gene (Montpetit, et al., 2008): location of the two different morpholinos used to knockdown *ap1s1* in zebrafish, targeting either ATG or exon 3 acceptor splice site, are shown in green.

Morphological defects were observed in 48 hpf KD larvae, with smaller size, reduced pigmentation and skin disorganization when compared to wild-type individuals (Fig. 10-11). In addition to that, prominent changes in the skin organization were detected: in contrast to the well-defined, fan-like, ray structure of the WT caudal fin, the fin of the KD larvae was disorganized with rounded-up cells. Western blotting and immunolabelling in whole mount larvae were performed to verify the specificity of the AMO: a decrease in the intensity of the Ap1s1-specific labelling in KD larvae compared to the WT was observed. Also, larvae injected with a control AMO (5 mispaired bases) did not show significant differences compared to the WT (Fig. 10). The pathogenicity of the splice mutation identified in the French/Canadian patients was validated by designing a morpholino targeting the *ap1s1* intron 2 acceptor splice site: the same abnormalities in skin and fin morphology were assessed, although phenotype was less penetrant.

Skin malformations were investigated by using immunolabelling for p63, which is a specific marker of basal keratinocytes nuclei and, interestingly, p63-positive keratinocytes were present both in WT and KD larvae (Fig. 11 A-B). Immunolabelling for phosphorylated-histone-H3 (PH3) was likewise performed in order to verify the presence of changes in the population of proliferating cells, but no obvious differences were observed in the same groups.

Specific abnormalities of keratinocytes were later explored by immunolabelling larvae for laminins and cadherins. Several differences were detected, including a more diffuse signal of laminin (Fig. 11 C-D), along with an abnormal localization, and a less obvious cadherin signal at the cell membrane of keratinocytes doubly-labelled with cadherin (green) and p63 (orange) in KD larvae (Fig. 11 E-F).

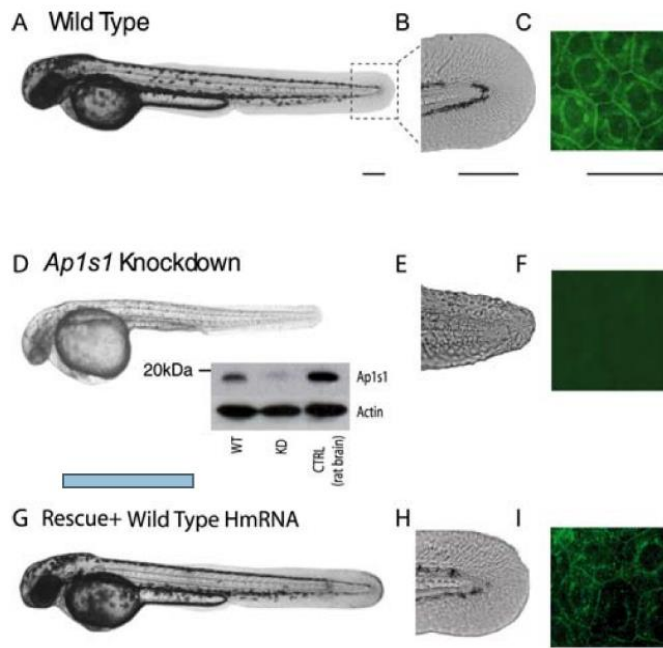


Figure 10. Morphological phenotype of *ap1s1* knockdown zebrafish (Montpetit, et al., 2008).

Transmitted light images of 48 hpf larvae are reported, showing smaller size, reduced pigmentation (D) and skin disorganization (E) of KD larvae compared to WT (A, B) and rescued larvae (G, H). Whole mount immunofluorescence using anti-Ap1s1 antibody showed diffuse staining in KD (F) when compared to distinct and polygonal localization in both normal (C) and rescued (I) individuals. Western blotting analysis indicates nearly complete knockdown of Ap1s1 protein (CTRL = control rat brain proteins; actin was used to normalize). Scale bars in (A, D, G) = 100 μ m, (B, C, E, F, H, I-Ciii) = 50 μ m

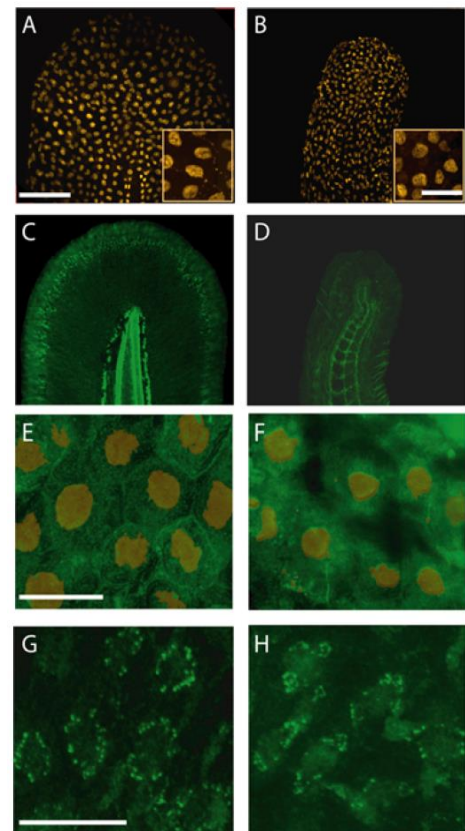


Figure 11. Laminin and cadherin immunostainings in *ap1s1* knockdown larvae (Montpetit, et al., 2008).

Differences between WT (left side) and KD larvae (right side) can be appreciated for immunolabelling: p63-labelled nuclei (orange) of basal keratinocytes (A-B); laminin (C-D); p63 and cadherin (E-F); cytokeratin distribution (G-H). Scale bars: 10 mm, Insets in A, B, = 20 μ m.

Important investigations were also performed through behavioural analyses by subjecting 48 hpf larvae to the so called “touch-evoke” test to assess muscle performance and function (Sztal, Ruparelia, Williams, & Bryson-Richardson, 2016). At that developmental stage, wild-type larvae normally respond by swimming, which is characterized by alternating tail movements with a beat frequency of about 30 Hz (Saint-Amant & Drapeau, 1998) (Buss & Drapeau, 2001). Conversely, the well-formed 48 hpf *ap1s1* KD larvae showed a severe behavioural phenotype which consists of reacting to the stimulus by tail coils, an embryonic motility pattern that usually disappears around 24-30 hpf (Fig. 12 A, F). Thus, investigations in the spinal cord neural organization was performed and anti-acetylated tubulin staining revealed a reduction in axonal processes in the spinal cord of KD animals compared to WT (Fig. 12 B, G). 48 hpf larvae were labelled using anti-HU (an RNA binding protein is found in neuronal cells leaving the mitotic cycle), revealing that the number of newly born

neurons in KD larvae (Fig. 12H) significantly decreased to 51% of control, WT, levels (Fig. 12C). Subsequently, interneurons and motoneurons were stained by using anti-Pax2, which labels a large subset of early differentiating interneurons and anti-HB9, a homeobox gene necessary for motoneuron differentiation. It was interesting to note that, whereas the number of motoneurons was unchanged, a 46 % reduction in the number of interneurons in *ap1s1* KD larvae (Fig. 12 E, J) compared to the WT (Fig. 12 D, I).

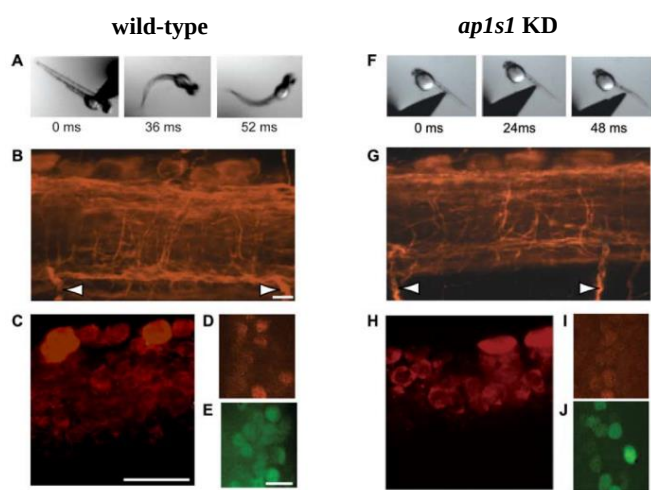


Figure 12. Abnormal behavioral phenotype and impaired development of spinal neural network of *ap1s1* knockdown zebrafish (Montpetit, et al., 2008). Consecutive images illustrating response to touch in 48 hpf larvae: while WT larvae reacted to touch by swimming away (A), KD larvae exhibit slow and impaired reaction (F). Immunostaining in wholemount 48 hpf WT (B–E) and *ap1s1* KD larvae (G–J) illustrating the reduced axonal labeling (anti-acetylated tubulin; B and G red, the arrows point to the ventral roots), the reduced number of newly born cells (anti-HU antibody; red in C and H), and the number of interneurons (anti-Pax2 antibody; E and J green) in the KD larvae. The number of motoneurons (anti-HB9 antibody; D and I, red) was similar both in WT and *Ap1s1* KD. Scale bars: 25 μ m.

4.6.2 σ 1A CHARACTERIZATION IN A KNOCKOUT INTESTINAL CELL LINE

In 2020, the clinical and genetic spectrum of *AP1S1*-associated diseases was extended by Klee et al., with the identification of 2 novel *AP1S1* missense mutations in 3 patients who died within the first 3 months of life due to an intractable diarrhoea, but did not present any other symptoms of MEDNIK syndrome. The pathogenicity of the missense mutation and, more importantly, the effect of *AP1S1* loss-of-function, were investigated by generating an *in vitro* model by deleting *AP1S1* from the intestinal cell line CaCo2 (CaCo2-*AP1S1*-KO) using CRISPR/Cas9 genome editing technology (Sanjana, Shalem, & Zhang, 2014). The driving hypothesis was that trafficking of tight-junction and or related proteins to the basolateral plasma membrane could be disrupted upon loss of *AP1S1*. Although general morphology of 2D filter-grown CaCo2-*AP1S1*-KO monolayers was unaffected, tight-junction protein zonula occludens 1 (ZO-1) showed rarefication at the apical circumference, marked accumulation at cellular contact sites and aberrant basal localization (Fig. 13a). This aberrant phenotype could be reverted by the stable reintroduction of *AP1S1* wild-type protein, but not by expression of *AP1S1* missense mutation (Fig. 13b). The findings obtained for ZO-1 were recapitulated with intestinal tight-junction marker claudin 3 that showed interruption of its normally marked circumferential localization (Fig. 14). Moving to 3D cultures of CaCo2 cells devoid of *AP1S1* or expressing the *AP1S1* missense mutants, cyst formation and polarity were abnormal.

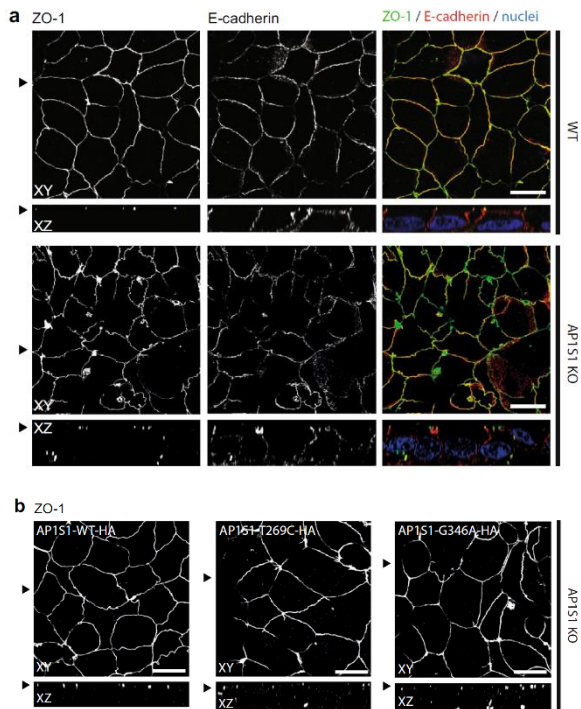


Figure 13. Laser-scanning immunofluorescence micrographs of CaCo2 WT and AP1S1 KO cells (Klee, et al., 2020). (a) Tight-junction protein ZO-1 shows aberrant localization upon *AP1S1* deletion and in cells expressing patient mutations (*AP1S1*-T269C and *AP1S1*-G346A) but not *AP1S1*-WT (b). Basolateral E-cadherin staining is not affected by *AP1S1* depletion. Scale bar 10 μ m, arrowheads indicate respective plane in XY and XZ.

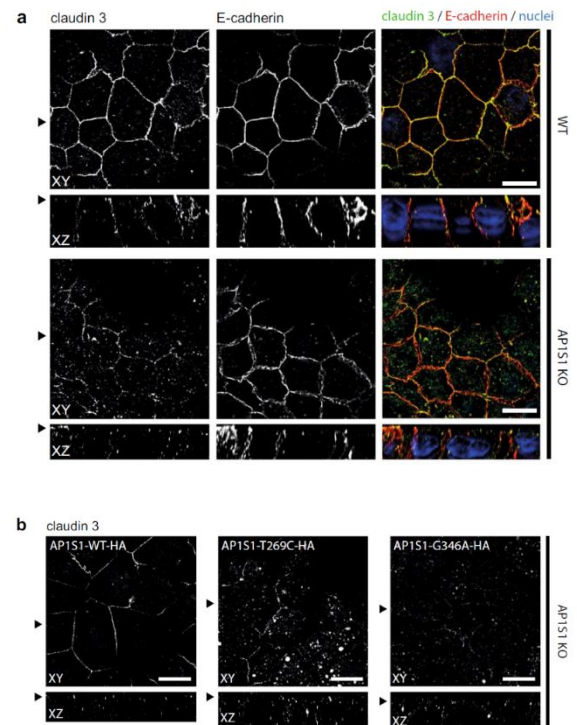


Figure 14. Laser-scanning immunofluorescence micrographs of CaCo2 WT and AP1S1 KO cells (Klee, et al., 2020). (a) Tight-junction protein claudin 3 shows decreased signal at the apical circumference and increased intracellular localization upon *AP1S1* depletion and in cells expressing patient mutations (*AP1S1*-T269C and *AP1S1*-G346A) but not *AP1S1*-WT (b). Basolateral E-cadherin staining is not affected by *AP1S1* depletion. Scale bar 10 μ m, arrowheads indicate respective plane in XY and XZ.

According to these findings, barrier function was investigated by TEER measurement (trans-endothelial electrical resistance) in filter-grown monolayers and subsequently by determining dextran permeability. In the first case, TEER was significantly reduced in CaCo2-*AP1S1*-KO monolayers compared with the parental cells, and could be reconstituted with reintroduction of WT, but not mutant *AP1S1*, as expected. Moreover, significant increases in dextran permeability were observed for *AP1S1* KO cells, and those expressing patient mutations. Taken together, these evidences indicated that tight-junction formation was disrupted upon loss or mutations of *AP1S1* and, therefore, aberrant epithelial barrier function might underlie or contribute to congenital intestinal failure in patients with *AP1S1* loss-of-function mutations.

4.7 PETTIGREW SYNDROME IS DUE TO MUTATIONS IN *AP1S2* GENE

4.7.1 INTRODUCTION AND CLINICAL FEATURES OF PETTIGREW SYNDROME

Mental retardation (MR) is defined as a disability characterized by “significant limitations both in intellectual functioning and in adaptive behaviour as expressed in conceptual, social and practical adaptive skills” with onset before the age of 18 years (WHO, 1992). Generally, intellectual disability (ID) results from generalized neurodevelopmental disorder (IQ below 70) that can originate from environmental causes and/or genetic anomalies, and its incidence in children is estimated to be of 1-3% (Piton, Redin, & Mandel, 2013). It is more common in males because of the high incidence of mutations in genes located on the X chromosome that together explain 10-12% of MR in males (Ballarati, et al., 2012). The most common cause of XLMR is an expansion of an unstable trinucleotide repeat in the 5' UTR of the *FMR1* gene in patients with fragile X syndrome (Verkerk, et al., 1991). Although it is estimated that mutations in 80 other genes have also been implicated in XLMR, there remains a substantial proportion of families with a clear causative mutation yet to be found. According to the clinical symptoms, radiological or metabolic features, this disorder is categorized into syndromic ID and non-syndromic ID. The extent of the genetic heterogeneity associated with non-syndromic XLMR usually compromises the search for additional underlying genes because linkage information from different families cannot be combined with confidence (Tarpey, et al., 2006).

Pettigrew syndrome (PGS, MIM # 304340), initially reported as Fried-type syndromic mental retardation, is a rare X-linked mental retardation (XLMR) caused by *AP1S2* mutations. PGS patients show from moderate to severe ID and other highly variable clinical features such as choreoathetosis, hydrocephalus, Dandy Walker malformation, seizures and iron or calcium deposition in the brain (Huo, Teng, Wang, & Liu, 2019); female carriers of *AP1S2* mutations have showed random X-inactivation and normal phenotypes (Cacciagli, et al., 2014). In 1972, Fried was the first to report a Scottish pedigree with X-linked mental retardation (Fig. 15A) and/or hydrocephalus. The family included six affected males with IQs falling within the range of 20-50. Two patients had hydrocephalus, one of which was suspected to be the result of stenosis of the aqueduct of Sylvius. All patients had delayed motor development and difficulty in walking as adults. Subsequent studies (Pettigrew, Jackson, & Ledbetter, 1991) (Cowles, Furman, & Wilkins, 1993) (Carpenter, Brown, Qu, & Keenan, 1991) (Wakeling, Jolly, Fisk, Gannon, & Holder, 2002) (Turner G, 2003) (Saillour, et al., 2007) described other families affected by distinct X-linked forms of mental retardation with key manifestations, including severe or nonspecific ID, early hypotonia with progression to spasticity and contractures, choreoathetosis, seizures and a long, narrow face with coarse features, cystic enlargement of the fourth ventricle with cerebellar hypoplasia (Dandy-Walker malformation) and

iron accumulation in the basal ganglia with neuroaxonal dystrophy similar to that in neurodegeneration with brain iron accumulation-1 (NBIA1). Turner et al. (2003) specifically described an X-linked recessive form of MR characterized by profound ID, delayed walking, and in the adults, difficult and aggressive behaviour. There was moderate reduction both in the occipitofrontal circumference and in height, and a similar facial appearance: triangular in shape with high forehead, prominent ears, and a small pointed chin (Fig. 15B).

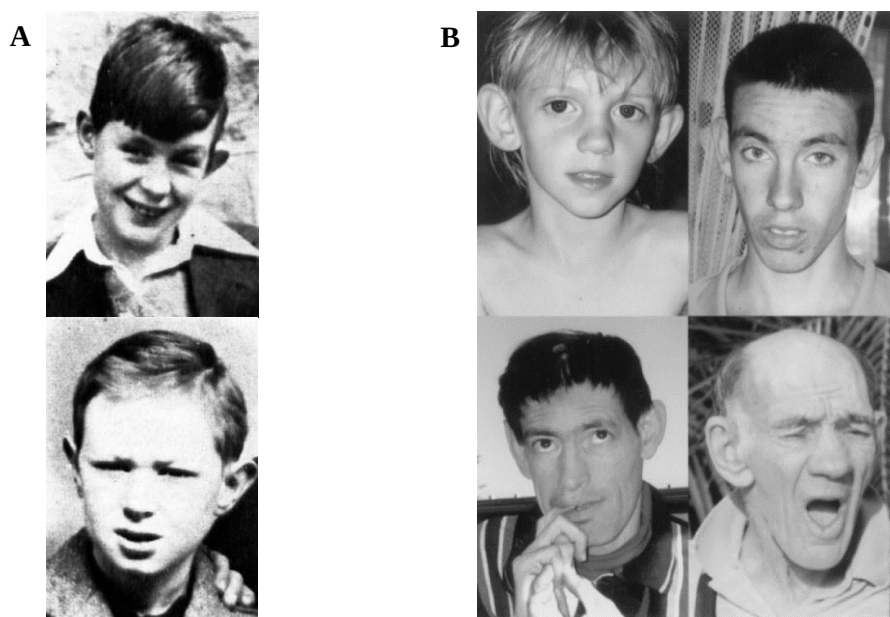


Figure 15. Facies of individuals affected by Pettigrew syndrome. (A) Mentally retarded males as boys described by Fried, K. (1972). From the top: III-13 and III-24. (B) Affected individuals of different ages described by Turner, G. et al. (2003). Top row: IV-23 at four years, IV-18 at 15 years. Bottom row: III-12 at 38 years, II-4 at 70 years. Note the similar facial appearance, triangular in shape high forehead, long nose and face, small pointed jaw and prominent ears.

In 2012, a clinical case was reported by Leuzzi et al., describing a 6.5-year-old Italian boy with a new clinical variant of cerebral folate deficiency (CFD), associated with microcephaly, delayed growth, ligamentous laxity and mild muscular hypotrophy and hypotonia, along with severe intellectual disability and recurrent fierce and compulsive self-injurious behaviour (SIB). Since his social responsiveness and language comprehension were relatively preserved, clinical features of the patients were not compatible with the diagnosis of autistic spectrum disorder (Leuzzi, Mastrangelo, Celato, Carducci, & Carducci, 2012). After a six year follow-up, the patient did not show any clinical improvements and remained without a molecular diagnosis until 2019, when whole-exome sequencing analysis revealed a novel nonsense mutation in exon 3 of the adaptor protein 1 sigma 2 (*AP1S2*) gene on the X chromosome. Moreover, clinical features overlapped with those described in

AP1S2-related disorders. Since elevated cerebrospinal fluid (CSF) protein levels have been reported in two patients bearing *AP1S2* mutations, but no further studies on CSF have been performed, the hypothesis was whether cerebral deficiency could be a recurrent abnormality in *AP1S2*-related phenotypes. Indeed, the folate receptor is a glycosylphosphatidylinositol-anchored protein sorting from the *trans*-Golgi network to the plasma membrane. As folate receptor cycles to the cell surface by endosomal trafficking, it is possible that an *AP1S2* defect could negatively affect the processing and trafficking of the folate receptor in the analysed patient, resulting in the cerebral folate deficiency detected. It is interesting to note that folate deficiency is known to be associated with neural tube defects and hydrocephalus and *AP1S2* mutations have been reported in patients with congenital hydrocephalus (Shaheen, et al., 2017).

4.7.2 MOLECULAR GENETICS OF PETTIGREW SYNDROME

The *AP1S2* gene maps to chromosome Xp22.2, it is composed of five exons and encodes for a main protein product of 157 amino acids. To date, several disease-causing mutations have been reported for *AP1S2*, including a microdeletion and eight-point mutations summarized by Huo et al. in 2019, most of those being either nonsense or splice changes scattered throughout the *AP1S2* protein (Fig. 16). Patients with splice site mutations were more likely to be affected by seizures; by contrast, patients with nonsense variants are more susceptible to microcephaly. Interestingly, all pathogenic variations reported in ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) for *AP1S2* gene in Pettigrew syndrome patients are loss of function mutation (frameshift, nonsense or splice site).

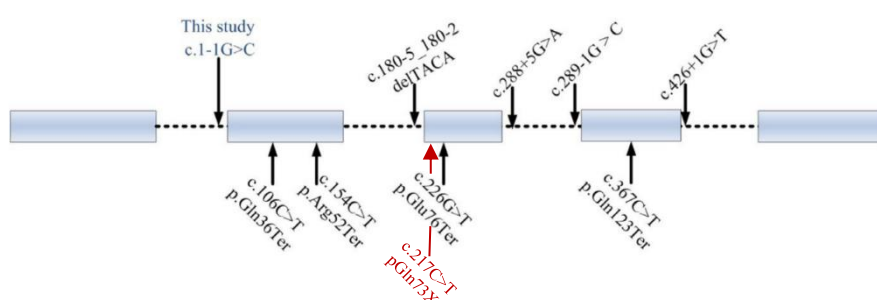


Figure 16. Summary of *AP1S2* mutations (Huo, Teng, Wang, & Liu, 2019). A recent identified mutation (Cappuccio, et al., 2019) is reported in magenta (deletion identified by Ballarati et al. in not reported).

4.8 GENETIC MODELS FOR ADAPTOR PROTEIN 1 SUBUNIT SIGMA 2

4.8.1 $\sigma 1B$ CHARACTERIZATION FROM PATIENT MUTATED FIBROBLASTS

In 2008, Borck et al. described four patients in two unrelated families, affected by XLMR, in which *AP1S2* nonsense and splice mutations segregated. Three males in two generations of the first family were genotyped and a c.154C>T mutation in exon 2 of the *AP1S2* gene was detected, confirming the previously identified by Carpenter et al. (1999) and Tarpey et al. (2006). The alteration affects a hypermutable CpG dinucleotide and is predicted to lead to a p.R52X nonsense substitution. Considering that inactivation of various AP-1 subunits leads to destabilization of the remaining subunits of the complex in model organisms, the consequences of this genetic alteration on the integrity of the AP-1 complex have been investigated in patient fibroblasts (Borck, et al., 2008), first by immunofluorescence (IF) using an anti-AP-1 γ -adaptin antibody. As a control, the distribution of the AP-2 complex was tested in the same cells by IF using an anti-AP-2 α -adaptin antibody. In patient fibroblasts there is no evident alteration of the subcellular localization, stability or function of the AP-1 complex. Both in control and patient fibroblasts, AP-1 and AP-2 complexes showed staining patterns corresponding to the *trans*-Golgi network and the plasma membrane clathrin-coated pits (CCPs), respectively (Fig. 17 A-B). The data are consistent with Western blot analyses where γ and $\mu 1A$ subunits resulted to be expressed at similar levels in patient and control fibroblasts (Fig. 17C). Moreover, western blot experiments using anti- $\sigma 1A$ antibodies revealed similar expression levels of AP1S1/ $\sigma 1A$ and the colocalization of AP1S1/ $\sigma 1A$ with γ -adaptin either in control and patients fibroblasts (Fig. 17D), suggesting that the structural role of AP1S2/ $\sigma 1B$ can be compensated by functional AP-1 complexes containing $\sigma 1A$ subunit. The localization of the known AP-1 cargo cation-dependent mannose-6-phosphate receptor (CD-M6PR), previously proved to be mislocalized in mice deficient for the AP-1 $\mu 1A$ subunit (Meyer, et al., 2000), has been investigated. Distribution of CD-M6PR was similar in patient and control fibroblasts and it colocalized with AP-1 at the *trans*-Golgi network, indicating that trafficking was not affected in *AP1S2*/ $\sigma 1B$ mutant fibroblasts (Fig. 17E).

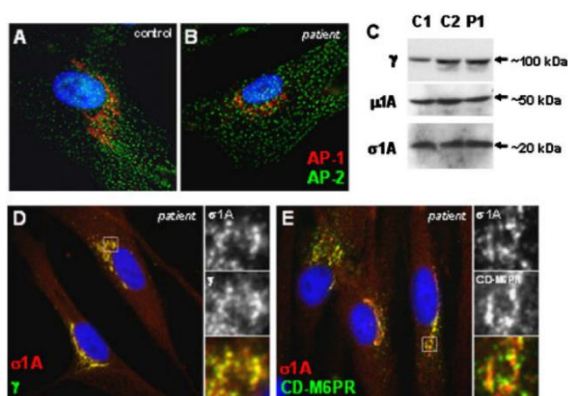


Figure 17. AP-1 complex in *AP1S2*/ $\sigma 1B$ deficient cells.

Control (A) and patient fibroblasts (B) staining for AP-1 (γ subunit, red) and AP-2 (α subunit, green). (C) Western blot on control and patient fibroblasts lysates for AP-1 subunits using anti- γ , - $\mu 1A$, and - $\sigma 1A$ subunit antibodies (indicated on the left). Molecular weights are reported on the right. C, control; P1, Patient 1. (D) Distribution of AP-1 complex subunits γ (green) and $\sigma 1A$ (red). (E) Distribution of AP-1 ($\sigma 1A$, red) and cation-dependent mannose 6 phosphate receptor (CD-M6PR, green). A representative area (white squares) was enlarged (3X) to show colocalization of the markers (D and E, right column). Nuclei were stained with DAPI (blue) (Borck, et al., 2008).

4.8.2 $\sigma 1B$ CHARACTERIZATION IN *Ap1s2* DEFICIENT MICE

In 2010, Glyvuk et al. found that $\sigma 1B$ -adapin plays an important role in synaptic vesicle (SV) recycling through endosomal intermediates in small hippocampal boutons, leading to the description of a novel function of AP-1 and the first *in-vivo* characterization of a $\sigma 1$ isoform in a targeted knockout mouse of the X-chromosomal $\sigma 1B$ -adapin (Glyvuk, et al., 2010). $\sigma 1B$ -deficient mice are hypoactive, display deficits in neuromotor learning and spatial learning and memory tasks. They are calm and have problems in finding the water source, but growth and development result normal. Together, these finding support genetic screens in humans for *AP1S2* as the causative gene of the Pettigrew syndrome (Tarpey, et al., 2006): PGS patients learn to walk only at 5-6 years of age, rarely develop an intelligible language and often require lifelong comprehensive care. In Glyvuk's study, tissue-specific mRNA expression of $\sigma 1$ isoforms was analysed by Northern blot and RT-PCR. The $\sigma 1A$ mRNA is ubiquitously expressed in all tissues, with a significantly higher expression in the brain, whereas expression levels of $\sigma 1B$ and $\sigma 1C$ are highly variable and tissue-specific, most tissues expressing $\sigma 1A$ and only one of the other two isoforms (Fig. 18 A-C).

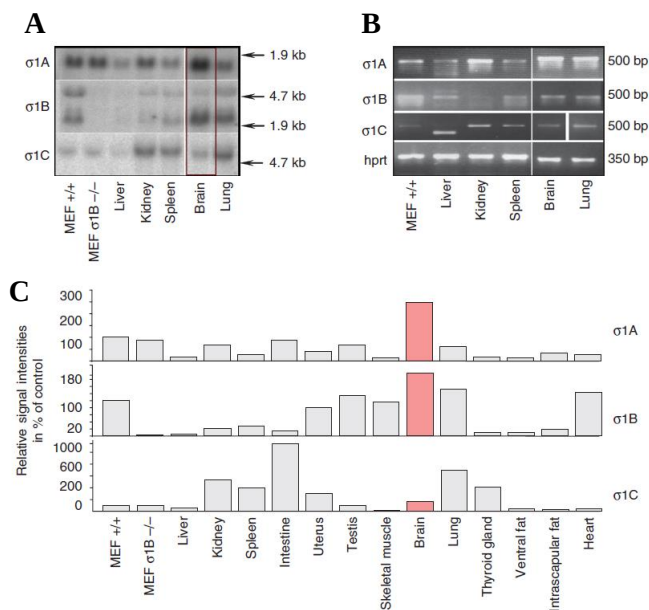


Figure 18. AP-1/ $\sigma 1$ tissue-specific expression pattern. (A) Northern blot. $\sigma 1A$ and $\sigma 1B$ are not upregulated in the $\sigma 1B$ -knockout and are highest in the brain. (B) RT-PCR. (C) Expression levels in all tissues tested. Northern blot signal intensities were normalized relative to the signal in mouse embryonic fibroblasts (MEFs) (Glyvuk, et al., 2010).

The high expression levels of $\sigma 1B$ and $\sigma 1A$ in the brain confirm earlier studies on human tissue samples (Takatsu, Sakurai, Shin, Murakami, & Nakayama, 1998) and the distribution of $\gamma 1$ -adapin as documented in Allen Mouse Brain Atlas (Seattle (WA): Allen Institute for Brain Science, 2009; available on <http://mouse.brain-map.org>), with high expression in hippocampus. In particular, AP-1 complex is located to the pre-synapses. A reduction in the number of SVs at rest in $\sigma 1B^{-/-}$ small hippocampal boutons and its marked decrease after turnover of the entire SV pool (Fig. 19A) are

accompanied by the appearance of large organelles, indicating σ 1B-dependent recycling of synaptic vesicles through endosomes. Deficiency in vesicle biogenesis is excluded as SVs are efficiently targeted to the active zone (Fig. 19B).

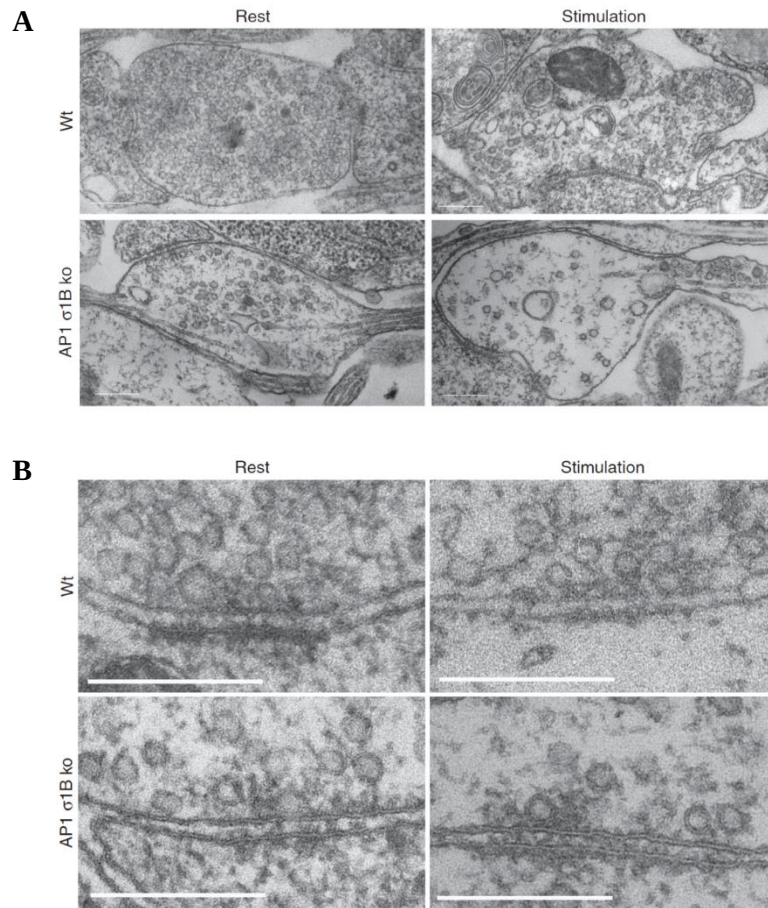


Figure 19. Electron microscopic images of hippocampal boutons (A) and active zone (AZs) synapses (B) from control and σ 1B^{-/-} animals, taken at rest and after stimulation (900 AP/10 Hz). Scale bar: 200 nm (Glyvuk, et al., 2010).

Expression levels of AP-1, AP-2 and clathrin were investigated both in mouse embryonic fibroblasts (MEFs) (Fig. 20B) and brain homogenates (Fig. 20C) by Western blots using different antibodies: anti- γ 1, anti- α and anti-clathrin antibodies, respectively. AP-1 adaptins were also analysed in control and knockout fibroblasts (Fig. 20A). The σ 1B deficiency does not alter protein levels of γ 2- or μ 1A-adaptin and clathrin in fibroblasts, whereas γ 1 is reduced by 20%. Viability of σ 1B-deficient mice and expression pattern demonstrate that σ 1B is required for tissue-specific AP-1 functions rather than ubiquitous “house-keeping” functions. However, deletion of the σ 1B subunit leads to an increase in α -adaptin expression (25%) in the brain homogenates and MEFs. In the brain, γ 1 expression is normal

in $\sigma 1B$ deficient animals (Fig. 20C), demonstrating that in this tissue $\sigma 1$ -adaptin expression is not limiting for complex formation unlike the case in embryonic fibroblasts.

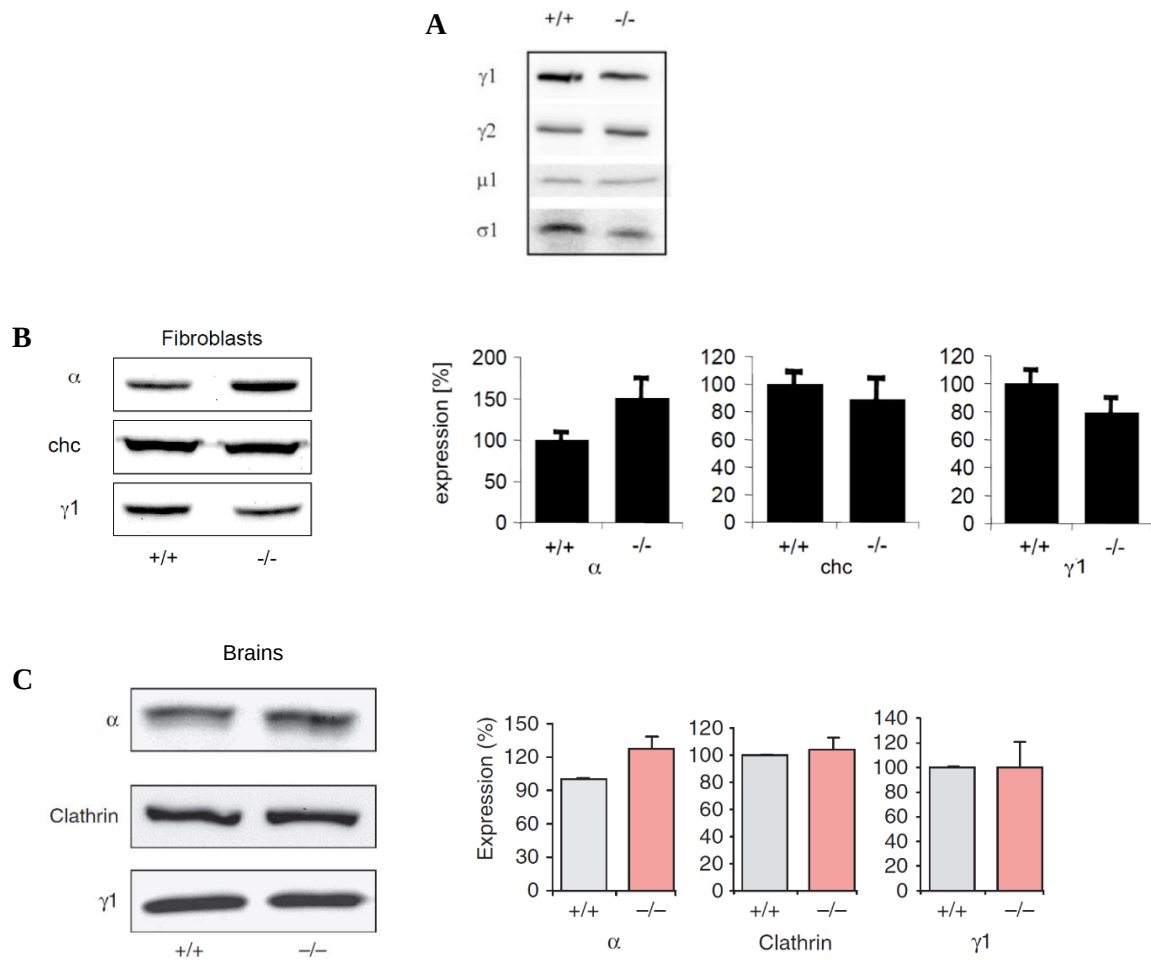


Figure 20. Expression of related adaptins and clathrin. (A) Western blot analysis of AP-1 adaptins in control and $\sigma 1B$ knockout mouse embryonic fibroblasts (MEFs), demonstrating reduced $\sigma 1$ -adaptin expression and unaltered expression of $\mu 1A$ and $\gamma 2$. Expression analysis of α - and $\gamma 1$ -adaptins and clathrin in wild-type (+/+) and knockout (-/-) on MEFs (B) and brain homogenates (three brains per genotype, (C) by Western blotting (Glyvuk, et al., 2010).

In 2014, Baltes et al. described altered sorting of sortilin in murine adipocytes deficient for the AP-1/ $\sigma 1B$ subunit, resulting in the inhibition of adipogenesis (Baltes, et al., 2014). Sortilin is a type I membrane glycoprotein in the vacuolar protein sorting 10 protein (Vps10p) family of sorting receptors. While it is ubiquitously expressed in many tissues, sortilin is most abundant in the central nervous system (CNS). Sortilin functions in protein transport between the Golgi apparatus, endosome, lysosome, and plasma membrane, leading to its involvement in multiple biological processes such as glucose and lipid metabolism as well as neural development and cell death (Nielsen, et al., 2001). The altered distribution of sortilin allowed to enhance the knowledge about the functional role of the *Ap1s2* gene in *M. musculus*, with $\sigma 1B$ -deficient mice displaying impaired recycling of synaptic

vesicles and lipodystrophy. So, a novel molecular mechanism regulating adipocyte precursor cell differentiation was described, involving the AP-1/ σ 1B complex and sortilin. AP-1-dependent sortilin sorting is mediated by two motifs, one of which binds specifically to σ 1B. Scientists observed that σ 1B-deficient animals appeared to have a hump, caused by a severe lipodystrophy of subcutaneous adipose tissues, showing the complete loss of inter-scapulae adipose tissue in some individuals (Fig. 21). These observations were consistent with quantifications showing 50% reduction in the amount of epididymal adipose tissue in both male and female σ 1B^{-/-} animals. In addition to that, adult σ 1B^{-/-} animals (5 months) were 15% lighter than the wild-type controls, indicating that the mass of adipocyte in general is reduced.

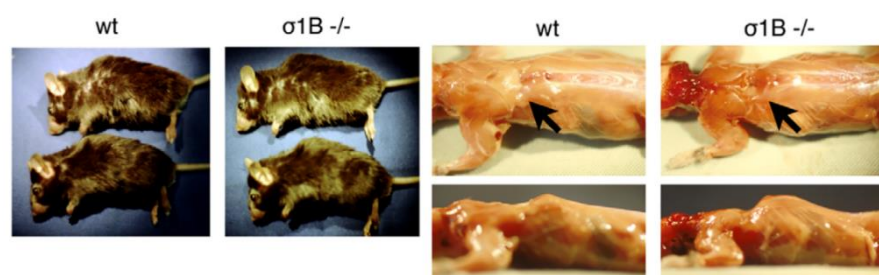


Figure 21. Lipodystrophy in wild-type and σ 1B-deficient mice. A litter of two wild-type (wt) and two σ 1B^{-/-} animals (left panels) and the subcutaneous tissues of one wild-type and one σ 1B^{-/-} mouse (right panels) are shown. σ 1B^{-/-} animals appear to have a hump due to the loss of inter-scapula (arrows) and other subcutaneous adipose tissues (Baltes, et al., 2014).

Growth rates analyses between 4 and 14 weeks of ages were similar in wild-type and knockout animals, showing that σ 1B^{-/-} individuals do store lipids and that their adipocytes increase in volume like wild-type cells. Among other relevant features that have been investigated, breeding success of σ 1B^{-/-} animals was normal (indicating that mammary gland was not impaired, as it happens in many breast tumours, where σ 1B is overexpressed) and glucose metabolism or insulin regulation appeared to be unaffected by σ 1B deficiency.

Sortilin expression was increased in σ 1B^{-/-} adipose tissue, whereas its expression in brain, the tissue with highest sortilin and AP-1/ σ 1B expression, was not altered, demonstrating the existence of adipocyte-specific σ 1B-dependent sortilin trafficking routes that are involved in the regulation of adipogenesis and tissue mass (Fig. 22 A-C). Moreover, sortilin was colocalized and able to form a complex with the adipogenesis-inhibiting DLK1 receptor (Fig. 22 F), thus preventing DLK1 downregulation (Fig. 22 D-E). The resulting DLK1 overexpression led to enhanced inhibition of adipogenesis.

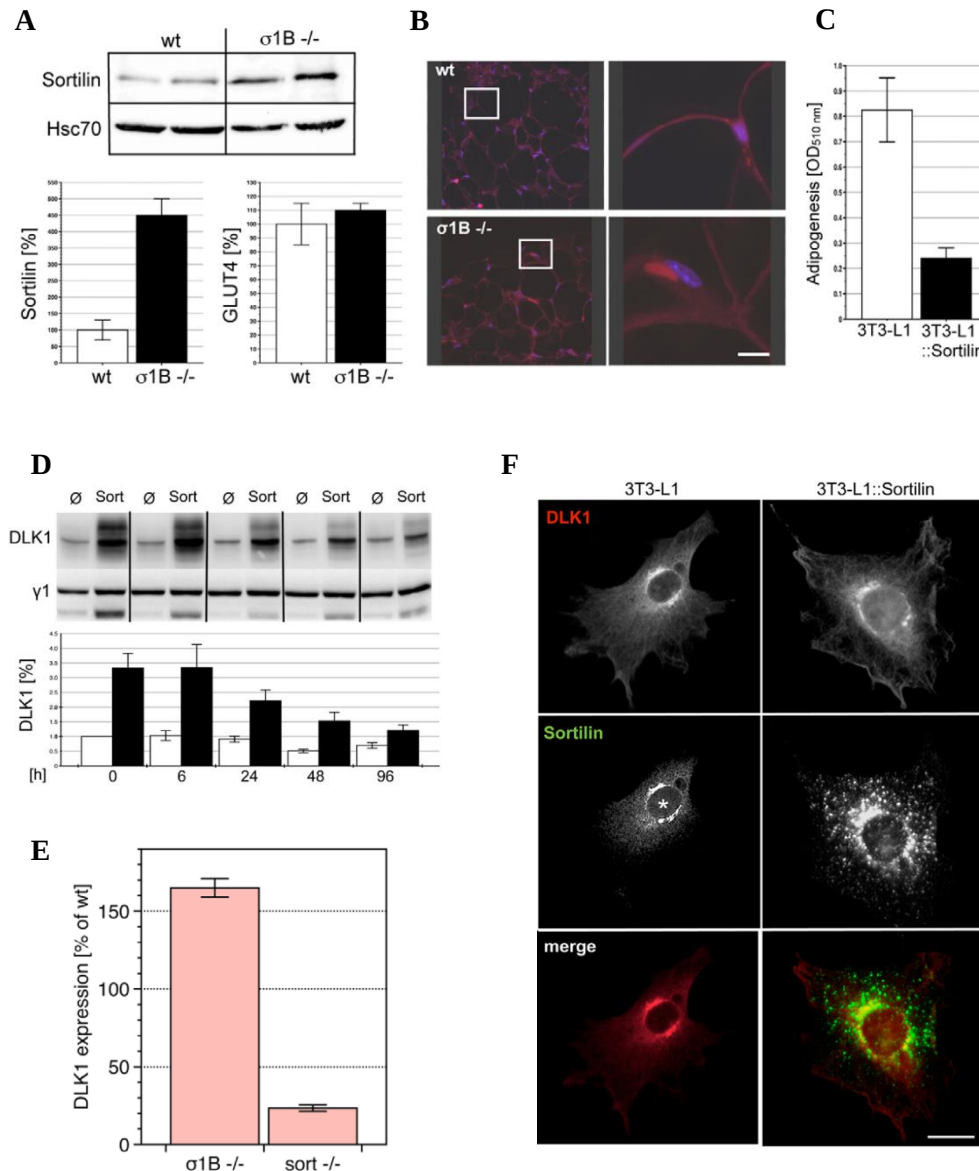


Figure 22. (A) Sortilin and GLUT4 expression in wild-type and $\sigma 1B^{-/-}$ adipocytes was investigated by Western blot (upper panel) and quantified by normalization to Hsc70 (lower panel). (B) Immunofluorescence microscopy was performed to examine the expression of sortilin (red) in cryosections of epididymal adipocytes. Nuclei were stained with DAPI (blue). The boxed area in the left image is shown at a higher magnification in the image on the right. Scale bar: 20 mm. (C) Sortilin-overexpressing 3T3-L1 cell lines show reduced adipogenesis, as determined by staining with Oil Red. (D) DLK1 expression during the differentiation of wild-type ($\emptyset$) and sortilin-overexpressing (Sort) 3T3-L1 cells was investigated by Western blot (upper panel). Quantification of DLK1 expression was performed and is presented in the lower panel. (E) DLK1 expression in adipose tissues from $\sigma 1B^{-/-}$ and sortilin $^{-/-}$ mice was determined and is shown relative to DLK1 expression in wild-type mice (n = 52 per genotype). (F) The distribution of sortilin and DLK1 in 3T3-L1 cells was investigated by immunofluorescence microscopy. Sortilin expression is low in non-transfected 3T3-L1 cells so the signal was enhanced post image recording. Merged image signals were not modified. Scale bar: 20 mm (Baltes, et al., 2014).

Among the AP-1-adaptins binding motifs that are present in the sortilin cytoplasmic domain, the membrane-proximal YxxΦ (YSVL), which binds to μ1A, and the distal dileucine-based sortilin motif (SxxxLL), where the serine is essential for σ1B binding (Fig. 23A); this serine is part of a consensus motif, which would allow the regulation of sortilin sorting by AP-1-σ1B. Finally, Baltes et al. described the mechanism by which sortilin missorting (due to σ1B deficiency) leads to sortilin and DLK1 overexpression. First, sortilin does not accumulate in a specific compartment, thus only its transport kinetics can be altered, which is in line with the reduced rate (but not complete block) of synaptic vesicle recycling in σ1B^{-/-} synapses (Glyvuk, et al., 2010).

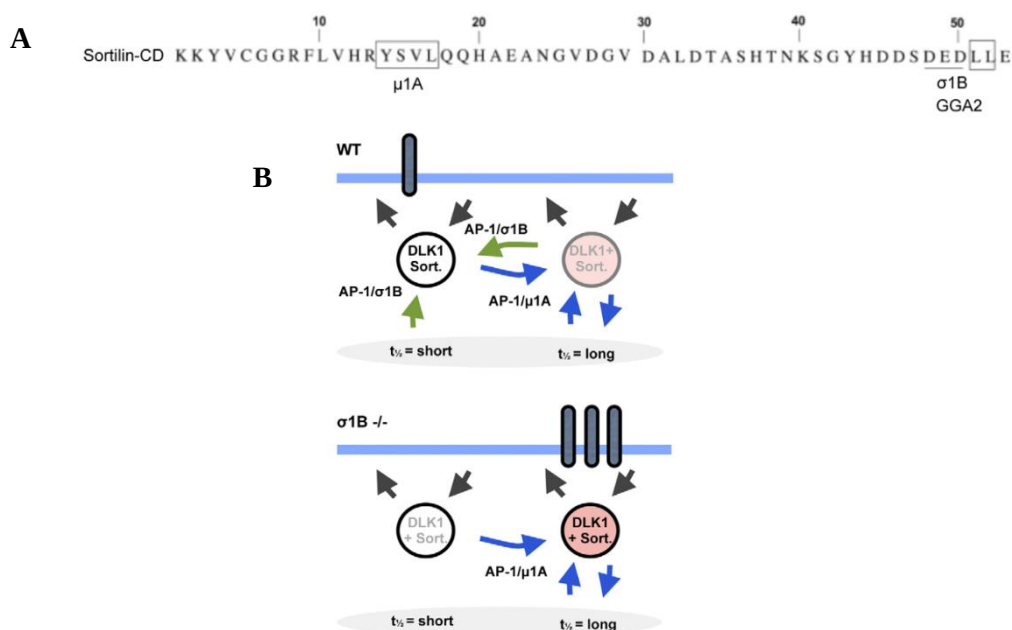


Figure 23. (A) Binding motifs in the sortilin cytoplasmic domain. (B) A model for $\sigma 1B$ - and $\mu 1A$ -dependent sortilin and DLK1 in adipocytes. AP-1 provides to sortilin and DLK1 (dark blue bar) sorting between the TGN (grey) and the plasma membrane (blue bar). Upper panel: in wild-type (wt) cells DLK1–sortilin complexes are sorted by AP-1- $\sigma 1B$ complexes (green arrows, dileucine based sorting) into endosomes that are associated with short protein half-lives ($t_{1/2}$) or by AP-1- $\mu 1A$ complexes (blue arrows, YxxØ-based sorting) into endosomes that are associated with longer protein half-lives. Lower panel: in $\sigma 1B^{-/-}$ cells, only sorting by AP-1- $\mu 1A$ into endosomes associated with longer half-lives takes place, thus leading to increased DLK1 expression (Baltes, et al., 2014).

5 INTRODUCTION TO *SLC7A6OS*

5.1 MUTATIONS IN *SLC7A6OS* ARE A NOVEL GENETIC CAUSE OF PROGRESSIVE MYOCLONUS EPILEPSIES (PMEs)

5.1.1 PROGRESSIVE MYOCLONUS EPILEPSIES: INTRODUCTION AND CLINICAL FEATURES

Progressive myoclonus epilepsies (PMEs) are a group of rare Mendelian disorders defined by the combination of action and reflex myoclonus, other types of epileptic seizures, and progressive neurological deterioration, including dementia and ataxia. Myoclonus refers to sudden, brief, involuntary movements and its clinical characteristics in all the specific causes of the PME syndrome are very similar. In patients with moderate to severe action myoclonus is therefore difficult to determine the presence of cerebellar signs, unless the myoclonus can be controlled by drug therapy (Marseille, 1990). PMEs usually begin in late childhood or adolescence and, unless progression is invariable, prognosis and longevity may vary considerably according to the aetiology. Indeed, the specific disorders that cause PME are diagnosed by recognition of the characteristic ages of onset of the disorders, the associated clinical signs and the clinical course. Usually, the fundamental examination for the identification of the myoclonus-epileptic syndrome is the electroencephalogram (EEG) associated with the polymyography (EMG). This allows to highlight the presence of the typical pattern of pathological muscle activation that characterizes the myoclonus.

Treatment of PMEs remains essentially symptomatic of seizures and myoclonus, together with palliative, supportive, and rehabilitative measures. The response to therapy may initially be relatively favourable, afterwards however, seizures may become more frequent, and progressive neurologic decline occurs (Orsini, et al., 2019).

5.1.2 MOLECULAR GENETICS OF PME

In 1989, a consensus statement published after a Marseille workshop (Marseille, 1990) allowed to define the various types of PME known at that time. Five different conditions have been described as a consequence of gene defects: Unverricht-Lundborg disease, Lafora disease, several forms of neuronal ceroid lipofuscinoses, myoclonus epilepsy with ragged-red fibers [MERRF], and type 1 and 2 sialidoses. Overall, this classification includes over 20 known genetic causes that can be broadly divided in two clinical groups: the first includes those associated with significant cognitive decline such as Lafora disease, while the second is characterized by a relatively preserved cognition and largely exemplified by Unverricht-Lundborg disease (ULD). ULD is considered the most common, less severe form of PME, with autosomal recessive inheritance and onset in late childhood or

adolescence. It has been associated with mutations in the cystatin B (*CTSB*) gene, which protects cells from endogenous proteases. Deficiency of *CSTB* is the cause for hyperexcitability and impaired neuronal function of cortical networks. An unstable minisatellite repeat expansion of a dodecamer (CCCCGCCCCGCG) in the 5' UTR of the gene could be the most likely source of the disorder (Orsini, et al., 2019).

5.1.3 A FORM OF PROGRESSIVE MYOCLONUS EPILEPSY CAUSED BY MUTATION IN *SLC7A6OS*

Recently, a homozygous splicing variant in *SLC7A6OS* has been identified in two unrelated families (Portuguese and Turkish, respectively), where multiple relatives were affected with a “ULD-like” PME phenotype (Mazzola, et al., 2021). A single genome-wide significant linkage peak was found for both families on chromosome 16q22.1 where the two families share a common haplotype of ~ 880 kb in length, consistent with a founder effect of the pathogenic mutation. Clinical features of the six investigated patients were similar, including tonic-clonic seizures, ataxia, disorders in attention and visual perception. EEG recordings show relative preservation of the background rhythms and generalized polyspike, polyspike-waves and sometimes spike-wave discharges. Moreover, magnetic resonance imaging (MRI) showed deterioration to marked cerebellar and cerebral atrophy in several individuals of Family 2.

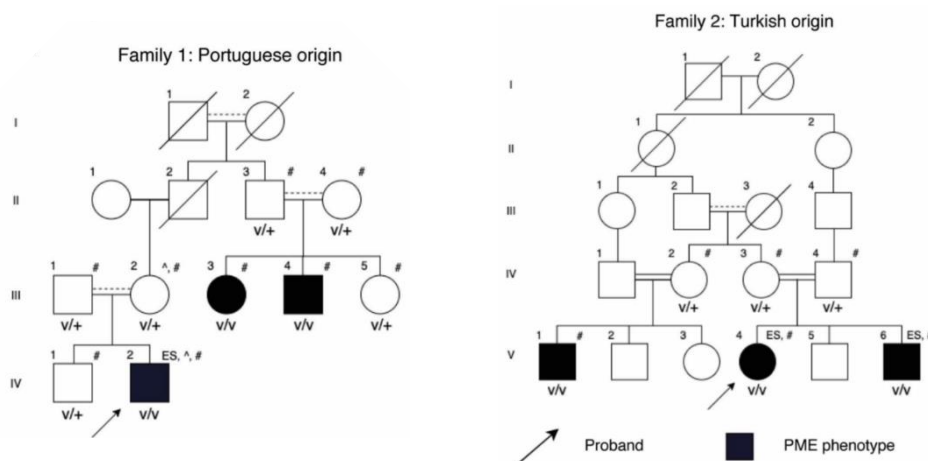


Figure 24. Family pedigree showing *SLC7A6OS* variant segregation in Family 1 and 2 (Mazzola, et al., 2021). Affected individuals are reported in black. Consanguineous parental unions are indicated with two connecting lines (even if consanguinity was not reported for the members of the first family, individuals came from the same rural area and a common ancestor has been hypothesized).

Pedigree structure suggested autosomal recessive inheritance, supported by consanguinity in both families (Fig. 24). Whole exome analysis led to the homozygous *SLC7A6OS* NM_032178.2:

c.191A>G substitution being identified as the most likely pathogenic variant in both families. The mutation affects the second last nucleotide of the first exon, altering the classic consensus sequence of the splicing donor site (AGgt to GGg) and may result in anomalous processing of the RNA encoded by the mutated allele, causing intron 1 sequence retention (121 bp) in the mature mRNA. The evaluation of the effect mutation on the splicing of the transcript was performed by RT-PCR analyses on cDNA amplicons, from exon 1 to exon 3, of different individuals: affected subject IV-2 (Family 1), his mother, III-2, unaffected and a healthy control (Fig. 25). A single expected product of 455 bp was observed in control; in the patient and his mother, the normal 455 bp amplicon was decreased in amount and there was an additional product of 576 bp.

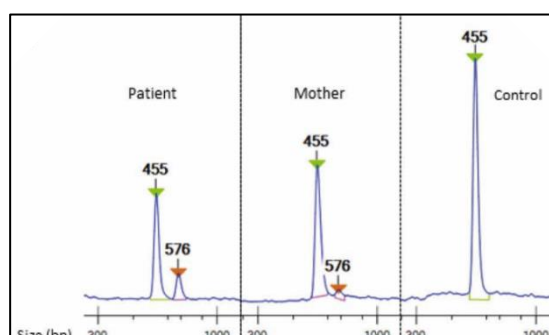


Figure 25. Analysis by capillary electrophoresis of RT-PCR products obtained by amplification with primers located on exons 1 to 3 (Mazzola, et al., 2021).

The 567 bp RT-PCR product was sequenced by Sanger's enzymatic method, revealing the presence of the 121 bp intron 1 sequence leading to the production of an abnormal isoform that does not allow protein synthesis. This indicates that the *SLC7A6OS* c.191A>G mutation is able to interfere with proper splicing of the intron 1. Moreover, the study demonstrated that even in patients with the homozygous variant there is a significant percentage of properly processed mRNA in the mature transcript (Fig. 26A), along with the “anomalous” transcript. Quantification of *SLC7A6OS* transcripts were performed by RT-qPCR in patient IV-2, showing expression in blood of 60% and in lymphoblastoid cells 15% of that in controls. Western blot analysis showed a decrease in the abundance of *SLC7A6OS* protein in patient III-2 (Family 1) compared to controls (Fig. 26B).

Mutation c.191A>G, besides being able to interfere with the correct splicing of the mRNA, also represents a missense mutation, involving the substitution of glutamine in position 64 with an arginine. Therefore the PME patients studied not only produce a reduced amount of *SLC7A6OS* protein of the expected length (309 aa), but this polypeptide has the p.Q64R mutation that could be detrimental to its functionality. Although according to the recent AlphaMissense predictions, the

p.Q64R is likely a benign substitution with a 0.117 pathogenicity score, the amino acid residue encoded by the mutation is quite preserved in vertebrates, including zebrafish.

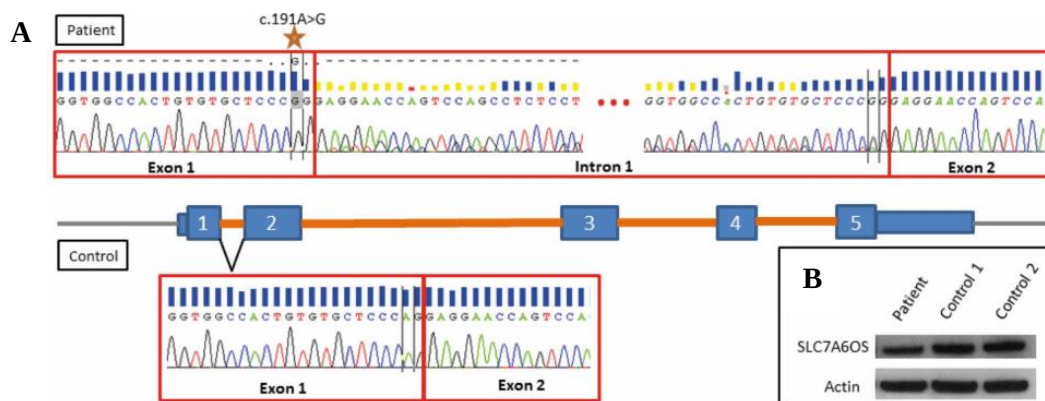


Figure 26. (A) Sanger sequencing of both the 455 and 576 bp amplicons. (B) Western Blot analysis of SLC7A6OS protein. The analysis was performed in duplicates with two deposits each time. Mean estimations with a ChemiDoc imager were about 50% of the expression in controls (Mazzola, et al., 2021).

5.2 INTRODUCTION TO SLC7A6OS STUDIES

5.2.1 THE SLC7A6OS GENE

SLC7A6OS, acronym for Solute Carrier Family 7 Member 6 Opposite Strand Transcript, has been identified for the first time in studies from our research group, concerning the SLC7 amino acid transporter family in *Homo sapiens*. The gene encodes for a protein coding natural antisense transcript (NAT) whose last exon overlaps with the long 3' UTR of the human *SLC7A6* gene. *SLC7A6OS* is highly conserved in vertebrates and homologous sequences can be found in eukaryotic model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. In humans, *SLC7A6OS* encodes for a soluble protein of 309 amino acids with predicted nuclear import and export signals and no significant sequence identity to any other human polypeptide. Its function in vertebrates is still largely unknown.

5.2.2 IWR1 AND RNA POLYMERASE II NUCLEAR IMPORT

The main clues about SLC7A6OS biological function come from studies on the putative *S. cerevisiae* ortholog, named *IWR1*. Mass spectrometry studies led to the identification of a new factor that binds to the RNA polymerase II (RNPII) complex, the so called Iwr1 (“interacts with RNA polymerase II”) (Gavin, et al., 2002). Pol II is a conserved 12-subunit enzyme that transcribes mRNA from protein-

coding genes in the nucleus of eukaryotic cells; its structure and function has been extensively studied, but its biogenesis is not completely understood. The holoenzyme is composed of 12 polypeptide subunits (Rbp1-12) organized into three large subgroups around the three main subunits (Rbp1-3). It has been shown that the formation and aggregation of the subunits takes place in the cytoplasm and precedes nuclear import (Boulon, et al., 2010). The holoenzyme performs its function at the nuclear level where it is responsible for the transcription of protein coding genes of eukaryotic cells. Thus, the complete and functional enzyme must be able to translocate from the cytoplasm, where it is assembled, to the nucleus. Since Pol II subunit sequences lack a known nuclear localization signal (NLS), Pol II likely binds an additional NLS-containing factor that mediates nuclear import. Analysis of the protein interactome in the yeast *Saccharomyces cerevisiae* showed that Pol II subunits copurify with the protein Iwr1 (Czeko, Seizl, Augsberger, Mielke, & Cramer, 2011). Bioinformatic analysis of the Iwr1 sequence (Kosugi, Hasebe, Tomita, & Yanagawa, 2009) revealed a putative bipartite NLS in the N-terminal protein region (Fig. 27) along with a nuclear export signal (NES) in the central portion of the protein.

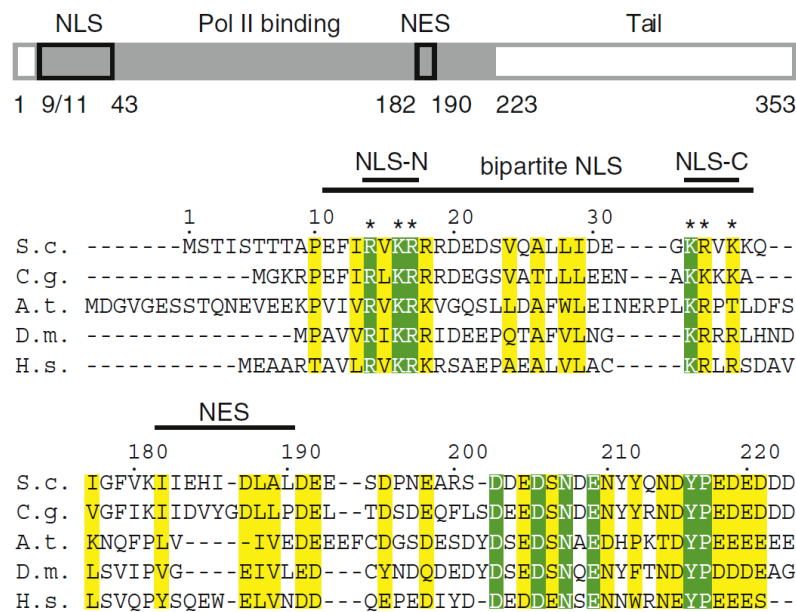


Figure 27. Schematic representation of yeast (S.c.), Iwr1, and amino acid sequence conservation. The Pol II-binding region is indicated in gray, boxes delineate NLS and NES sequences. Invariant residues are highlighted in green, residues with ≥ 80% conservation in yellow. Asterisks indicate mutations to alanine in the mutant variants. S.c., *Saccharomyces cerevisiae* (Q07532); C.g., *Candida glabrata* (Q6FR91); A.t., *Arabidopsis thaliana* (NM_128581); D.m., *Drosophila melanogaster* (NP_524940); H.s., *Homo sapiens* (BC013778); NCBI accession numbers are indicated in brackets.

To explain the mechanism of action, a cyclic model for Iwr1 function in Pol II nuclear import has been proposed: Iwr1 binds Pol II that is assembled in the cytoplasm and then uses its bipartite NLS

to direct Pol II nuclear import. Iwr1 binding between the large Pol II subunits (Rbp1 and Rbp2) may sense complete polymerase assembly and limit nuclear import to functional Pol II. After the formation of the Iwr1-RNA Pol II complex, NLS directs the nuclear import of functional Pol II by interacting with the NLS Kap60/95 receptor. Once in the nucleus, Iwr1-RNA Pol II is disassembled: Iwr1 is displaced from the Pol II active centre cleft during formation of the transcription initiation complex on promoter DNA and by transcription initiation factors including TBP and TFIIB. This event determines the release of the nuclear export signal (NES) present on Iwr1, which allows the export in the cytoplasm. In the free cytoplasm Iwr1 is "recycled": it can bind and import another complex Pol II into the nucleus, closing the cycle (Fig. 28). Cyclic Iwr1 function is apparently conserved throughout eukaryotes, since the putative human Iwr1 orthologue (now named SLC7A6OS) can partially substitute the yeast protein in cells. Functional conservation is also suggested by the high level of identity of amino acid sequences in functionally relevant Pol II-binding regions.

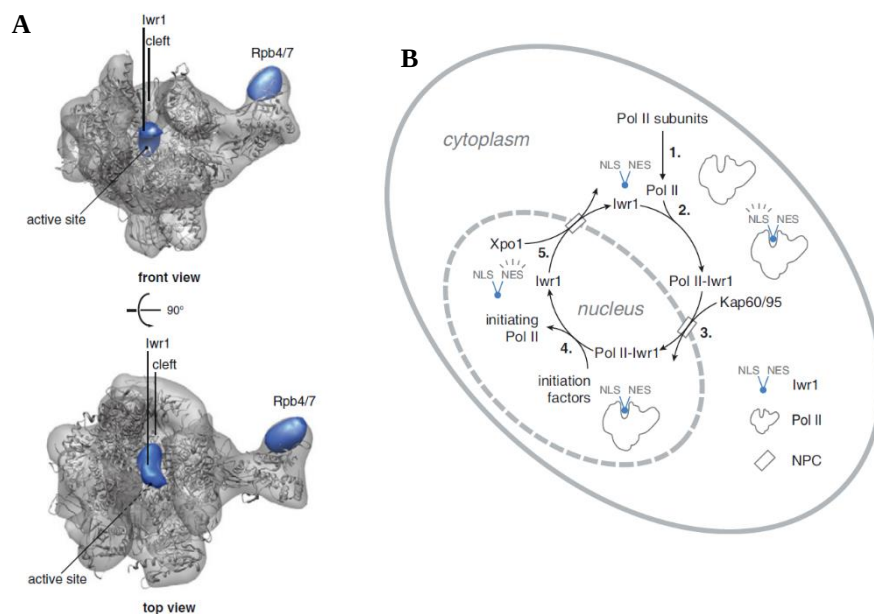


Figure 28. (A) Cryo-EM map of the Pol II-Iwr1 complex. (B) Cyclic model for Iwr1-directed Pol II nuclear import. Pol II assembles from its subunits in the cytoplasm (1). Iwr1 senses complete assembly by binding in the Pol II cleft (2). The Iwr1 NLS directs Pol II import through the nuclear pore complex via Kap60/95 (NPC, 3). Assembly of Pol II with initiation factors on promoter DNA releases Iwr1 (4). The Iwr1 NES facilitates nuclear export via Xpo1, completing the cycle (5) (Czeko, Seizl, Augsberger, Mielke, & Cramer, 2011).

It seems that alternative (and less efficient) molecular mechanisms could mediate the RNA Pol II nuclear import: the $\Delta iwr1$ strain is very sick, but viable, and thus a fraction of Pol II must be imported into the nucleus in an Iwr1-independent manner. Such inefficient, fractional Pol II import may be mediated by any Pol II-binding factor that contains an NLS, such as the general transcription factors

TFIIB and TFIIF (Hodges, et al., 2005) (Süel & Chook, 2009). This is consistent with the observation that strains expressing Iwr1 variants that contain mutations in NLS-N grow more slowly than strains that do not express Iwr1 at all. In these cases, functionally deficient Iwr1 binds Pol II and likely blocks binding of alternative NLS-containing factors. In addition to its interaction with Pol II, Iwr1 seems to play a key role in regulating all three RNA polymerases. Even if Iwr1 does not affect the nuclear import of Pol I and Pol III, it may be involved in the nuclear transport of the TATA-binding protein (TBP), an essential component of the transcription initiation complex of all the RNA polymerases. More recently, genetic and molecular evidence has shown that Iwr1 interacts with RNPII also during the transcriptional elongation phase and is involved in disassembling the enzyme from chromatin. Moreover, the absence of Iwr1 may cause genome instability which is particularly evident in yeast mutants with defects in DNA repair (Gómez-Navarro, Peiró-Chova, & Estruch, 2017).

5.3 GENETIC MODELS FOR *SLC7A6OS* GENE

5.3.1 *slc7a6os* CHARACTERIZATION IN ZEBRAFISH KNOCKDOWN

In silico analyses allowed to determine that *SLC7A6OS* is highly conserved in vertebrates and that homologous sequences can be found in several eukaryotic model organisms. Thus, in 2015 our group decided to investigate the functional role of *SLC7A6OS* in vertebrates using *Danio rerio* as model organism (Benini, et al., 2015). The analysis of the genomic regions surrounding the *slc7a6os* gene allowed to identify conserved synteny between human chromosome 16 and *Danio rerio* chromosome 7 (Synteny Database). Noteworthy, the zebrafish *slc7a6os* gene, like its human counterpart, it is flanked on the 3' side by the *slc7a6* gene (Fig. 29).

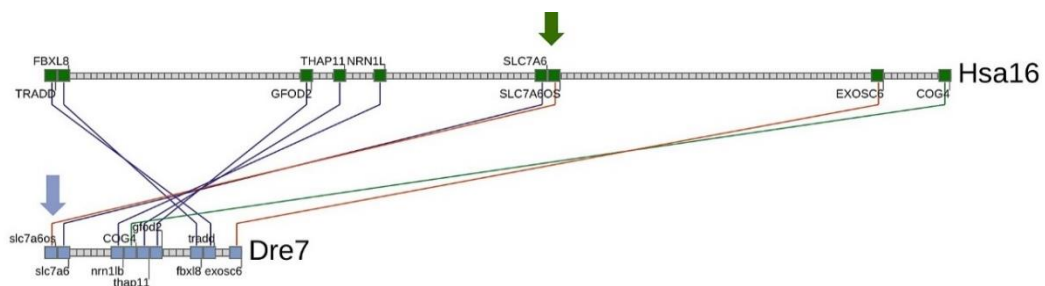


Figure 29. Graphical representation (Synteny Database) of conserved synteny around the *SLC7A6OS* locus between *Homo sapiens* chromosome 16 and *D. rerio* chromosome 7 (Benini, et al., 2015). Genes are reported as squares, with their order but not their physical location preserved. Coloured squares are members of the cluster while grey squares represent genes in the interval but that do not have orthologues or paralogs in the other segment. Lines connecting squares between the two clusters represent orthologous or paralogous gene pairs. The *SLC7A6OS* gene is indicated by either a green (*Homo sapiens*) or light blue (*Danio rerio*) arrow. The analysis was carried out based on the *Homo sapiens* Genome Reference Consortium build 37 and *Danio rerio* Zv9 genome assemblies.

Zebrafish *slc7a6os* gene encodes for a protein of 326 amino acids, with a 46% identity to human SLC7A6OS and 14% to *Saccharomyces cerevisiae* Iwr1. Similarly to the yeast counterpart, the Slc7a6os in *Danio rerio* presents a putative bipartite nuclear localization signal (NLS) in the N-terminal region as well as a nuclear export signal (NES) in the central portion of the polypeptide sequence. The degree of conservation of amino acid sequence and the presence of functionally relevant motifs such as NLS and NES (Fig. 30) suggest that Slc7a6os has a biological role similar to Iwr1 at the cellular level.

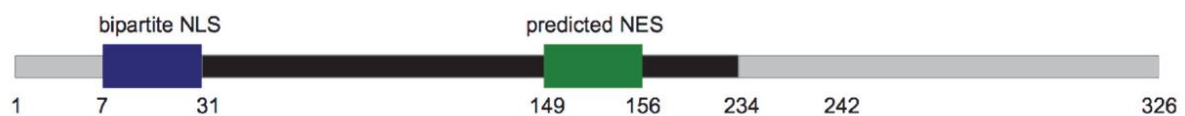


Figure 30. Schematic representation of the position of nuclear localization signal (NLS), nuclear export signal (NES) and the putative RNA polymerase binding domain (black bar) along the length of the *Danio rerio* Slc7a6os protein (gray bar) (Benini, et al., 2015).

Expression analyses through RNA-Seq generated by the Wellcome Trust Sanger Institute and available through the Ensemble Genome Browser demonstrated that the gene is expressed throughout development, confirming Real Time PCR assays on cDNA obtained from different zebrafish developmental stages, as shown in Fig. 31A (Benini, et al., 2015). Semi-quantitative RT-PCR experiments performed to evaluate *slc7a6os* expression in organs dissected from adult zebrafish suggest that the gene is differentially transcribed in the tissues analysed (Fig. 31B).

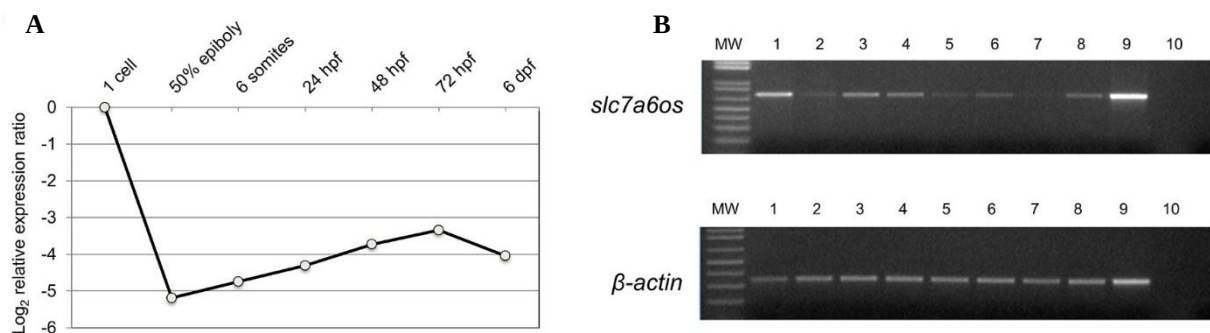


Figure 31. (A) Real-Time PCR expression analysis of *slc7a6os* throughout *Danio rerio* development. All reactions were run in triplicate. The relative expression levels, represented as the mean $\pm$ SEM in log₂ scale, were determined with respect to the 1-cell stage and normalized to elongation factor 1 α (ef1 α). The gene has a maternal and zygotic expression; the expression decreases rapidly in the first day post fertilization and progressively increases during development **(B) RT-PCR expression analysis of *slc7a6os* in adult zebrafish tissues.** β -actin was amplified as housekeeping gene. 1: brain; 2: intestine; 3: eye; 4: heart; 5: kidney; 6: swim bladder; 7: branchias; 8: testis; 9: ovary; 10: negative control.

Additional experiments were performed to study the spatial and tissue-specific patterns of *slc7a6os* expression. In particular whole-mount *in situ* hybridization (WISH) were performed on zebrafish embryos from 0.2 hpf to 48 hpf with a specific RNA antisense probe (Fig. 32): ubiquitous expression from the zygote stage until the early stages of development (cleavage and epiboly) demonstrated that *slc7a6os* is a maternal gene. At later embryonic stages, the hybridization signal becomes more specific, with defined expression pattern in the rostral part, at the level of the central nervous system during somitogenesis and in defined structures, such as hindbrain, midbrain, cerebellum, telencephalon, rhombomeres and spinal cord neurons, at 22 hpf, and diencephalon at 24 hpf.

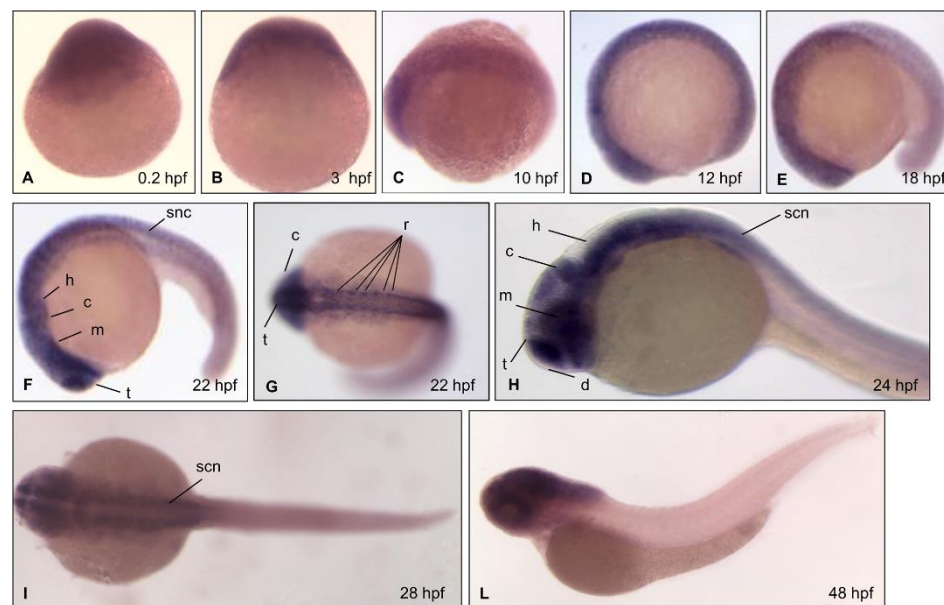


Figure 32. Analysis of *slc7a6os* expression by whole-mount *in situ* hybridization at different stages during zebrafish development (Benini, et al., 2015). Embryos at (A) 0.2 hpf (1–2 cell stage); (B) 3 hpf, (high stage); (C) 10 hpf (bud stage); (D) 12 hpf (6 somites), (E) 18 hpf (18 somites), (F, G) 22 hpf (26 somites), (H) 24 hpf, (I) 28 hpf; (L) 48 hpf were examined by whole-mount *in situ* hybridization with an antisense *slc7a6os* riboprobe. Abbreviations: d, diencephalon; m, midbrain; h, hindbrain; c, cerebellum; t, telencephalon; s, somites; scn, spinal cord neurons; r, rhombomeres.

The overall expression of *slc7a6os* gene is mostly localized in the CNS, indicating that it may be required in specific neuronal pathways, which include differentiation and organogenesis of the developing brain. To determine the functional role of the gene in zebrafish development, a knockdown experiment was performed by the injection of a splicing morpholino (*slc7a6os*-MOspl1) into one cell stage AB embryos. The interface between hindbrain and midbrain appears not well defined in morphants when compared to non-injected embryos at 24 hpf (Fig. 33). A schematic representation of the specifically designed morpholino is reported in Fig. 33 (left side).

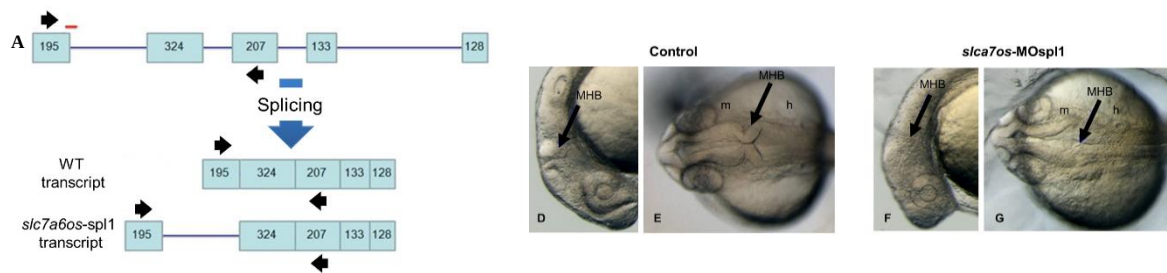


Figure 33. (A) Schematic representation of the *slc7a6os*-MOsp1 splice blocking morpholino and the effect on *slc7a6os* transcript. The splice blocking morpholino was synthesized targeting the exon1-intron1 boundary (A, red bar). RT-PCR experiments were performed on RNA extracted from *slc7a6os*-MOsp1-injected and control embryos with *slc7a6os* oligonucleotides on exon 1 and 3 (black arrows in A). **(D to G) Phenotypic effects of *slc7a6os* loss-of-function at 24 hpf.** *slc7a6os* MO injected embryos exhibit CNS malformations with unclear boundaries between developing brain regions, especially at midbrain-hindbrain and hindbrain-midbrain boundaries (F, lateral view; G, dorsal view). The arrowheads indicate the midbrain-hindbrain boundary. Abbreviations: h, hindbrain; m, midbrain, mhb, midbrain-hindbrain boundary (Benini, et al., 2015).

At 28 hpf the majority of *slc7a6os*-MOsp1 injected embryos (93%, n = 114) showed an altered somite morphology and appeared partially or completely immotile, but no alterations in the expression of *myod* myogenic marker was observed. In addition to that, the central nervous system is severely affected in *slc7a6os*-MOsp1 injected embryos, with compromised midbrain, hindbrain and cerebellum (Fig. 34).

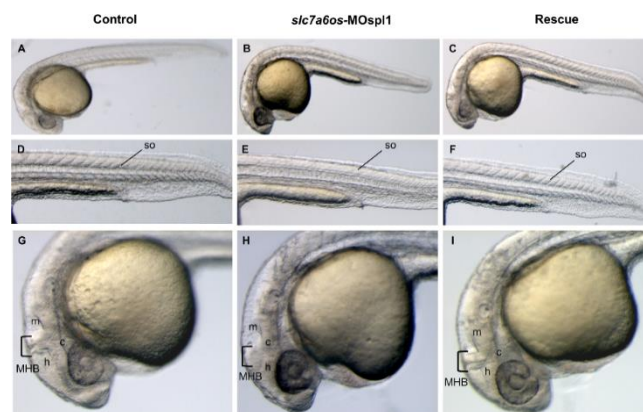


Figure 34. Morphological analysis of *slc7a6os* morphants and phenotypic rescue at 28 hpf (Benini, et al., 2015). MO injected embryos (6 ng/embryo) exhibit alterations in somite structure and brain malformations (B, E, H) when compared to control embryos (A, D, G). Undefined boundaries between brain subregions, at hindbrain and midbrain regions (H) are visible in magnifications of lateral views. Injection of synthetic mRNA rescues the morphological differences that are observed in morphants (C, F, I). Abbreviations: h, hindbrain; c, cerebellum; m, midbrain; mhb, midbrain-hindbrain boundary; so, somites.

Impairments of the midbrain-hindbrain boundary were more pronounced at 48 hpf, along with abnormalities in the cerebellum region, telencephalon, dorsal diencephalon and an overall disorganized structure of somites (Fig. 35).

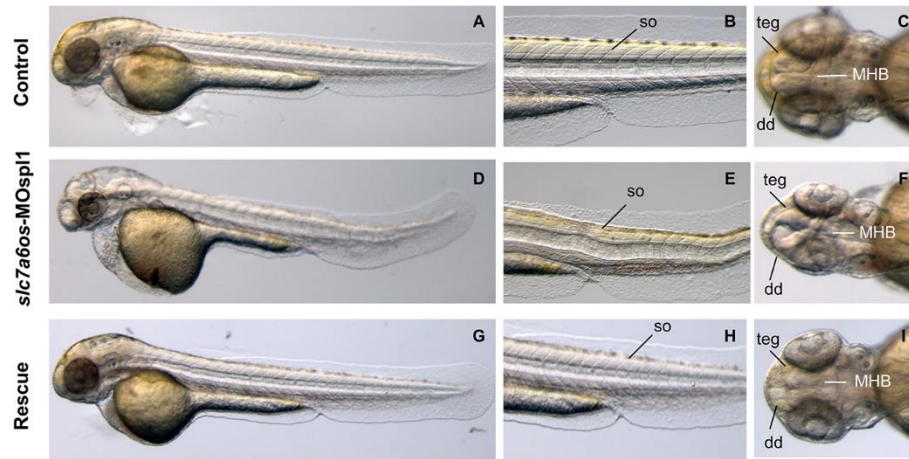


Figure 35. Morphological analysis of *slc7a6os* morphants and phenotypic rescue at 48 hpf (Benini, et al., 2015). MO injected embryos have an evident pericardial and yolk-sac edema and the somite structures are not well-defined (D-E) when compared to controls (A, D). Dorsal view of morphants shows that the CNS abnormalities are more pronounced and reveals a severe disorganization of the midbrain–hindbrain boundary, the dorsal diencephalon and the tegmentum (F). The expression of synthetic *slc7a6os* mRNA rescues the phenotype in morphants (G-I). Abbreviations: so, somites; teg, tegmentum; dd, dorsal diencephalon; mhb, midbrain–hindbrain boundary.

Moreover, at 72 hpf 91% of morphants exhibited a severe pericardial and yolk-sac edema. Phenotypic/functional rescue experiments were successfully performed by co-injecting each embryo with 6 ng of *slc7a6os*-MOsp11 and 400 pg of synthetic *slc7a6os* mRNA (Fig. 36).



Figure 36. Morphological analysis of *slc7a6os* morphants and phenotypic rescue at 48 hpf (Benini, et al., 2015). MO injected embryos are characterized by more pronounced pericardial and yolk-sac edema, CNS malformations and general morphology alteration (B). Again, the morphological differences seen in morphants are rescued by the coinjection of synthetic *slc7a6os* mRNA (C).

To deepen the role of *slc7a6os* during CNS development, the expression of two transcription factors, *pax2a* and *neurod1*, was investigated through whole mount *in situ* hybridization at different embryonic stages. While *pax2a* is one of the earliest genes activated during development of midbrain and midbrain-hindbrain boundary (mhb) and required for the development and organization of hindbrain, spinal cord interneurons and optic stalk, *neurod1* is specifically expressed during central nervous system formation and it represents a key differentiation factor for neurogenesis (it is also involved in the determination of the subtypes in the ganglia). From 24 hpf the expression of *neurod1* is more restricted to the forebrain (telencephalon), the symmetric primordial of the trigeminal ganglia, olfactory placode and is caudally expressed in spinal cord (Benini, et al., 2015). At 16 hpf the expression of *pax2a* in morphants was reduced, showing a strong reduction of the signal in the mhb compared to controls that persisted at 28 hpf, when a less defined development of the telencephalon was also detected (Fig. 37).

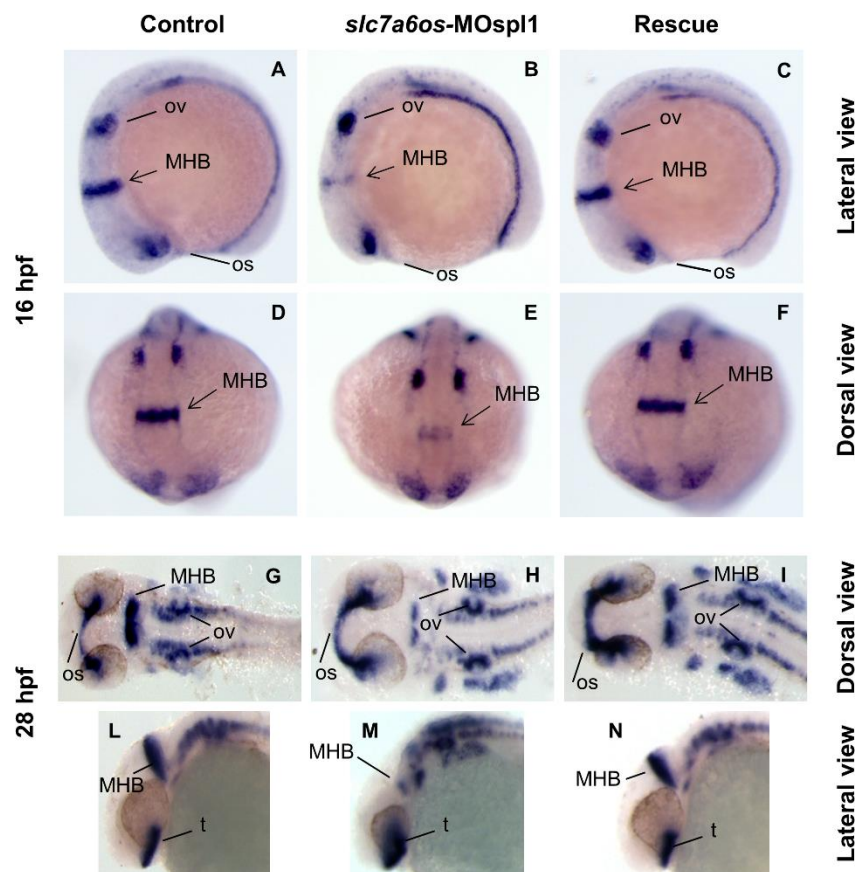


Figure 37. Analysis of *pax2a* expression in *slc7a6os*-MOSpl1 injected embryos (Benini, et al., 2015). Abbreviations: mhb, midbrain-hindbrain boundary; op, optic stalk; ov, otic vesicles; t, telencephalon.

The vast majority of *slc7a6os* morphants showed altered *neurod1* expression pattern when compared to controls. At 24 hpf *neurod1* expression was not detectable in the lateral line ganglia (anterior and posterior); a strong reduction was observed also in the dorsal diencephalon, telencephalon and octave statoacoustic ganglia (Fig. 38). The lateral line in fish is a sensory system closely related to our auditory system, comprising a set of discrete sense organs, and a corresponding set of neurons that extend their axons in the hindbrain. Since the anterior lateral line (ALL) and the posterior lateral line (PLL) ganglia forms at the level of the hindbrain, just anterior and posterior to the otic placode, these data suggest that in the lack of Slc7a6os leads to impairment in the differentiation or specification of ALL and PLL ganglia.

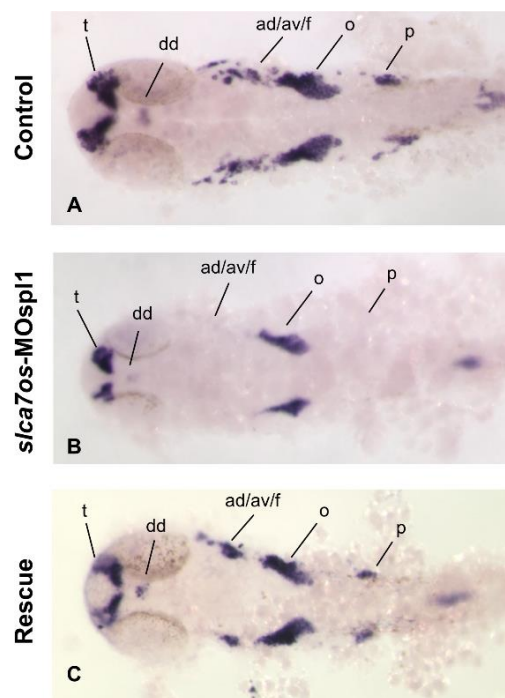


Figure 38. Analysis of *neurod1* expression in *slc7a6os*-MOSpl1 injected embryos (Benini, et al., 2015).

Abbreviations: ad/av/f, anterodorsal/anteroventral lateral line/ facial placodes/ ganglia; dd, dorsal diencephalon; o, octave statoacoustic ganglia; p, posterior lateral line ganglia; t, telencephalon.

5.4 THE ZEBRAFISH ANIMAL MODEL

Zebrafish (*Danio rerio*), is a small freshwater fish native of South Asia (Gange) characterized by short times between two successive generations, small size and easy maintenance. It represents the vertebrate animal model on which this project has been developed. Initially selected because its transparent embryo and fast reproduction, is an excellent system in which to study development and has now become the second most used animal in laboratories worldwide. Thus, the current applications of zebrafish studies are now highly diversified in neurobiology, immunology, adult physiology, oncology, and regenerative medicine, exploiting its advantages for *in vivo* approaches and imaging (Jaenisch, Zhang, & Gage, 2017). Zebrafish is the most advanced aquatic specie for the use of CRISPR/Cas9 genome editing technology in laboratories.

Danio rerio genome has been completely sequenced in 2013 at the Wellcome Trust Sanger Institute. Zebrafish genome size is about 1.5 Gb and presents about 26000 protein coding genes, located on 25 different chromosomes. About 71.40% of human genes have at least one orthologue in zebrafish and 69% of fish genes have at least one orthologue on the human genome (Howe, et al., 2014). For a given human gene frequently two or more putative co-orthologues in zebrafish exist (*e.g.* two putative co-orthologues of the human *AP1S2* exist on zebrafish DNA, located on chromosome 11 and 23, respectively). This is often attributed to a specific event in the evolutionary history known as the teleost-specific genome duplication although in several cases the presence of many paralogues is likely due to more complex evolutionary events.

Information about zebrafish genomic, phenotypic and developmental data are available in the Zebrafish Model Organism Database (ZFIN), which integrates comprehensive data involving zebrafish genes, mutants, transgenic lines, phenotypes, genotypes, gene expression, morpholinos, antibodies, anatomical structures and publications (Howe, et al., 2013).

6 MATERIALS AND METHODS

6.1 MANTEINANCE AND HANDLING OF ZEBRAFISH LINES

All experiments were performed in accordance with the Italian and European Legislations (Directive 2010/63/EU) and with permission for animal experimentation from the Local Committee for Animal Health (Organismo Preposto per il Benessere Animale, OPBA) of the University of Brescia and the Italian Ministry of Health (Authorization Number 287/2018-PR; 856/2020-PR). *Danio rerio* (zebrafish) were maintained in proper cabinets/aquariums (TECNIPLAST, Buguggiate, VA) located at the Department of Molecular and Translational Medicine (DMMT) in the University of Brescia (Zebrafish Facility). Each cabinet is composed of water tanks with a capacity of 3-5 L, where zebrafish are housed (~15 individuals each). Optimal environmental conditions require a water pH value between 6.5 and 8.0, conductivity between 500 and 1000 μ S, temperature between 20 and 30 °C and constantly monitored levels of nitrite and ammonia. As *D. rerio* is a photoperiodic animal, aquarium are located in a timed light-dark cycle-room to respect the teleost circadian rhythm, which is composed of 14 hours of light and 10 hours of dark (Aleström, et al., 2020). Matings are set up in organizing tanks and upon fertilization the eggs are usually collected in Petri dishes with “Fish water”, incubated at 28 °C, where development is correctly reached, according to published studies (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Starting from 5 dpf, zebrafish are able to feed independently and they are normally supplied with fine feed granular (GM 75); subsequently, food is replaced with larger feed granular (GM 150) according to the developmental stage of the animals, until they reach the adult size (4-5 cm), when they start eating GM 300 small granular food. Administration of dry food is alternated with artemias once a day, allowing the fish to maintain a balanced diet and to develop the predatory ability. The wild-type AB line employed included the AB strain (KIT Institute - Karlsruhe-Germany) and a transgenic line Tg(*neurod1*:EGFP) (Thomas, Ochocinska, Hitchcock, & Thummel, 2012).

6.2 SOLUTIONS

Fish water

The common “medium water” for fertilized eggs and developing embryos was thus composed:

Instant Ocean Sea Salt	0.1 g
Calcium sulphate dihydrate	0.1 g
Sodium bicarbonate	0.19 g
Methylene blue (2000X)	0.5 mL
Deionized H ₂ O	1 L

Tricaine methanesulfonate (TMS)

Tricaine methanesulfonate (TMS, Sigma-Aldrich, St. Louis, MO, USA) is a power commonly used for anaesthesia, sedation and euthanasia of fish as it acts as a muscle relaxant that operates by preventing action potentials. The optimum concentration may vary with the size and species of the fish. It is easily soluble in water but it needs to be prepared freshly each time because TMS is light-sensitive and toxic products may form upon exposure to light.

Stock solution as composed as reported (50X):

Tricaine methanesulfonate (TMS)	400 mg
Fish water	100 mL

N-Phenylthiourea (PTU)

N-Phenylthiourea (P-7629, Sigma-Aldrich, St. Louis, MO, USA) is a member of the class of thioureas in which one of the hydrogens is replaced by a phenyl group. It is commonly employed in research to inhibit pigmentation; this allows to have completely transparent embryos.

Composition is described below:

Fish water:	500 mL
PTU:	0.3 g

Paraformaldehyde (PFA) 4%

Paraformaldehyde (PFA, P-6148, Sigma-Aldrich, St. Louis, MO, USA) is the polymeric form of formaldehyde; it acts as a cross-linker of proteins allowing to maintain cellular and subcellular structures. It is commonly used to fix embryos at different stages before hybridization analyses.

Preparation (100 mL) requires the following steps:

- Weigh 4 g of formaldehyde under a chemical hood.
- Heat 90 mL of H₂O to 70 °C, add 100 µL of NaOH 2M and mix. Dissolve powder. Add 10 mL of PBS 10X and 100 µL of HCl. Mix and filter the solution with syringes.
- Aliquot in 15 mL Falcon tubes and store at -20 °C.

6.3 GENERATION OF KNOCKOUT LINES USING CRISPR/Cas9 TECHNOLOGY

6.3.1 GENE TARGET IDENTIFICATION

Microinjection was performed to generate mutated lines for *ap1s1*, *ap1s2* and *slc7a6os* genes in zebrafish, respectively. The optimal target sequence was identified using different bioinformatics tools, such as “CRISPR Design Tool” by SYNTHGO (<https://www.synthego.com/products/bioinformatics/crispr-design-tool>), for the first two genes, and “CHOPCHOP” (<https://chopchop.cbu.uib.no/>), for *slc7s6os* gene.

A forward and a reverse primer for PCR-based genotyping were selected by the software or properly designed using “Primer3” designing tool (<https://primer3.org/>). Specific positions of the sgRNAs on the genomic sequence are reported below; in particular, sgRNAs designed for *ap1s1* and *ap1s2* both have their target site on the second coding exon of the gene, while guide RNA target site on *slc7a6os* is located on the third coding exon (Fig. 39).

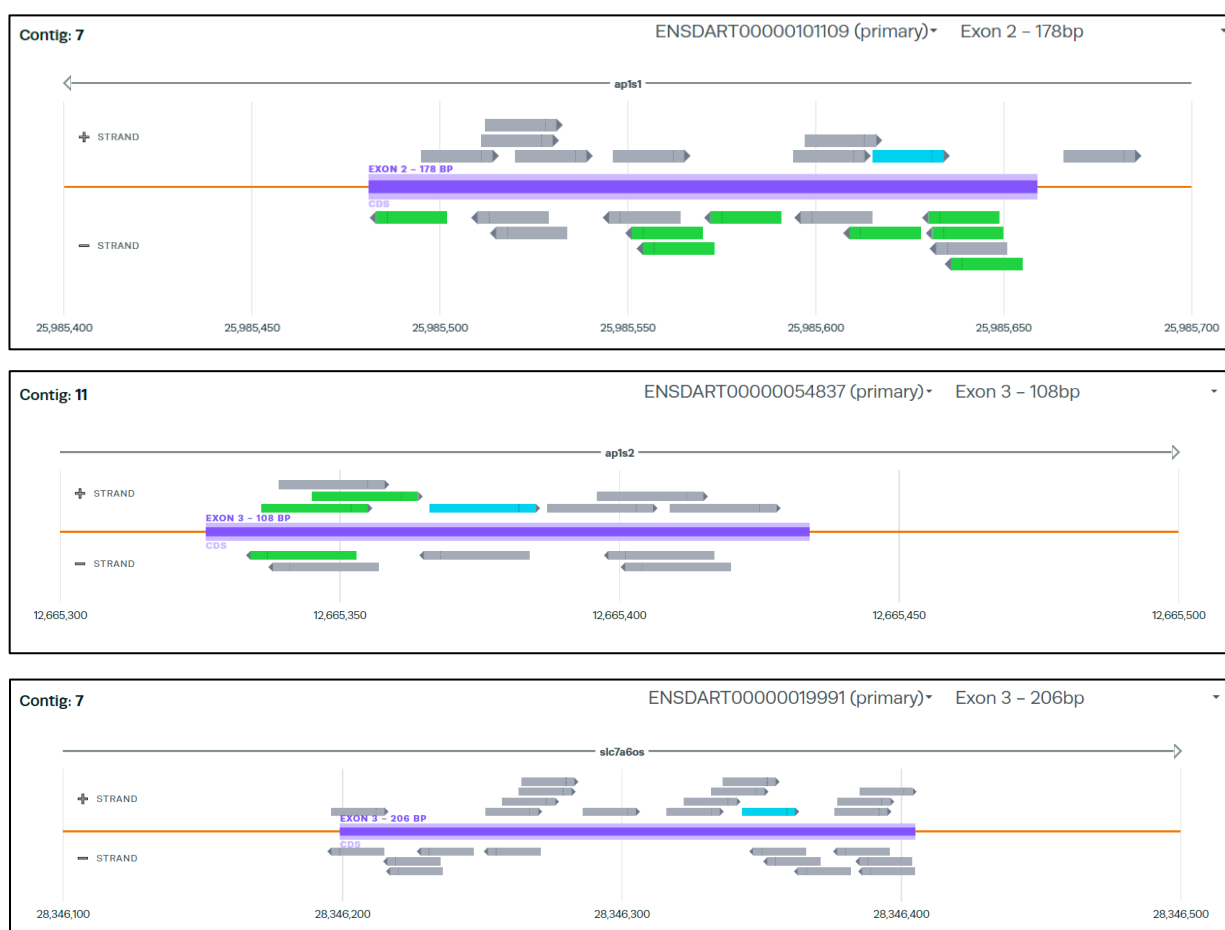


Figure 39. sgRNA localization on genomic DNA. The target sites of the selected sgRNAs are indicated in light blue together with additional target sites (in green and grey). First panel: position of the sgRNA target site on the second coding exon of *ap1s1* (ENSDART00000101109). Second panel: position of the sgRNA target site on the second coding exon of *ap1s2* (ENSDART00000054837). Third panel: position of the sgRNA target site on the third coding exon of *slc7a6os* (ENSDART00000019991).

6.3.2 TEMPLATE GENERATION FOR sgRNA SYNTHESIS THROUGH INDEPENDENT CLONING TRANSCRIPTION PROTOCOL

In vitro synthesis of sgRNA for *slc7a6os* gene was performed according to previously published guidelines (Gagnon, et al., 2014), using a 60 bp specific oligonucleotide (containing the promoter for SP6 polymerase, the target sequence of *slc7a6os* and a sequence complementary to the constant oligonucleotide) and a 80 bp constant oligonucleotide (“tracrRNA”).

The steps required for the synthesis protocol are as follows:

- Pairing between specific and constant oligonucleotides (100 µM): 95 °C for 5 minutes; 95 °C to 85 °C (- 2 °C/s); 85 °C to 25 °C (- 0.1 °C/s); hold stage: 4 °C.
- Addition of T4 DNA polymerase: 37 °C for 10 min.
- Column purification process (Wizard® PCR Clean-Up System – Promega, Madison, WI, USA). Elution was performed with 25 µL of RNase free water. NanoDrop quantification and gel electrophoresis of the product were performed.
- Transcription (Ambion MEGAscript™ SP6 Transcription Kit – ThermoFisher Scientific, Waltham, MA, USA): incubation at 37 °C for 6 hours, addition of 1 µL of TurboDNase and incubate at 37 °C for 15 min.
- Purification of the sgRNA by precipitation by adding 21 µL of ammonium acetate 5M and 60 µL of ethanol 100%. Mix and incubate O/N at -80 °C.

6.3.3 MICROINJECTION

Microinjections were performed using a manipulator (M275, Leica, Wetzlar, Germany) and a micro injector (InjectMan N12, FemtoJet – Eppendorf, Hamburg, Germany). Zebrafish embryos were collected and injected at one cell stage with 4 nL of a microinjection solution. Proper needles obtained from borosilicate glass capillaries (Harvard Apparatus, Holliston, Massachusetts, USA) are suitably prepared using a specific “microelectrode puller” (Puller PN-30 - Narishige, London, UK). Generation of mutant lines have been obtained using the CRISPR/Cas9 technology, introducing an injection mixture composed by the sgRNA (200-500 ng) and the Cas9 (EnGen™ Cas9 NLS; *S. pyogenes* - New England Biolabs, Ipswich, MA). The sgRNA concentration does not represent a fixed quantity, indeed every experimental procedure may require different concentrations; specific information about the concentrations of the sgRNAs are reported in “Results”. Morpholino (Gene Tools, LLC, Summerton Way, Philomath, OR, USA) mixtures were prepared according to the desired concentrations, heated at 65 °C for 10 min and cooled at 4 °C before being injected. Approximately

two hours after the microinjection, the embryos were observed and those in which the presence of phenol red is verified were collected into a Petri dish containing “Fish water” and incubated at 28 °C.

6.3.4 GENERATION OF MUTANT LINES IN *D. rerio*

Microinjection was performed on wild-type AB embryos at one-cell stage and Cas9-induced mutations were verified by screening the genomic DNA from larvae (5 dpf) or adult fish tails (3 mpf), as described later. The potential founders were selected as presenting a high level of “mosaicism”, confirmed by Sanger sequencing. They have been crossed with wild-type AB individuals to obtain the F1 generation, which is characterized by the presence of both heterozygotes fish for the different mutations induced by the Cas9 and wild-type individuals. Once reached the sexual maturity, F1 heterozygotes have been crossed to obtain the F2 generation and thus the three different genotypic classes (likely $+/+$, $+/-$ and $-/-$ ²), on which the characterization experiments have been conducted. Fig. 40 shows the generic scheme of crossings for the generation of zebrafish mutant lines; a wider description, for each investigated gene, is reported in “Results” section of this thesis.

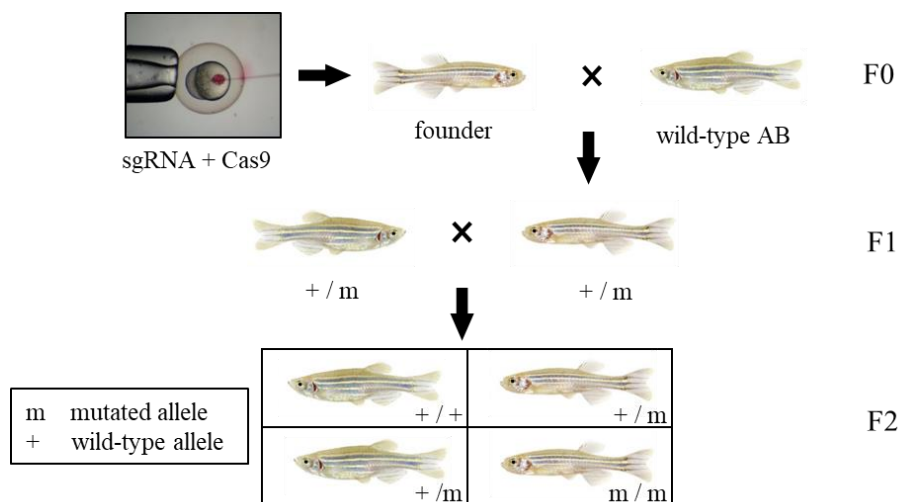


Figure 40. Schematic representation of the crossings for the generation of stable mutant lines in *D. rerio*.

² Symbols + and – are used when referring to wild-type or mutated alleles, respectively, within the investigated zebrafish genes.

6.4 LYSIS AND DNA EXTRACTION

Cas9-induced mutations were identified starting from genomic DNA extracted from embryos or larvae/adult fish tails. Microinjected eggs were incubated at 28 °C and partially destined for embryo genotyping, while remaining specimens were transferred to the appropriate aquarium system to achieve sexual maturity (~3 months), when the adult genotyping was performed.

6.4.1 LYSIS AND DNA EXTRACTION FROM WHOLE MOUNT LARVAE

Larvae genotyping was performed at 5 dpf using the HotSHOT (hot sodium hydroxide and Tris) protocol by adding NaOH (50 mM) to the individual larvae, incubating at 95 °C for 20 min and by adding Tris HCl pH 7.5 after cooling the samples to 4 °C.

6.4.2 LYSIS AND DNA EXTRACTION FROM TAILS

An individual fish was recovered through the use a proper net and its transfer to a tricaine solution (TMS, Sigma-Aldrich, St. Louis, MO, USA). Once anaesthetized, the fish was transferred to a Petri dish and the tail was cut with the help of a scalpel. The fish was then placed in a separated tank (few minutes are required for the fish to resume swimming ability), while the tail was collected. DNA extraction from tails was performed using the same protocol used for larvae. When necessary, genotyping was also carried out through fin clipping from larvae, following the guidelines published in the literature (Kosuta, et al., 2018).

6.5 PCR FOR IDENTIFICATION OF GENE MUTATIONS

Extracted DNA was amplified through PCR, organized as follows:

Amplification of *ap1s1* and *ap1s2* genes: denaturation: 95 °C for 5 min; 35 cycles (denaturation: 95 °C for 15 sec; annealing: 62 °C for 30 sec; elongation: 72 °C for 30 sec); final cycle: 72 °C for 5 min; hold step: 4 °C.

Amplification of *slc7a6os* genes: denaturation: 95 °C for 5 min; 30 cycles (denaturation: 95 °C for 15 sec; annealing: 61 °C for 30 sec; elongation: 72 °C for 30 sec); final cycle: 72 °C for 5 min; hold step: 4 °C.

Primers for genotyping

Primers for *ap1s1* genotyping were designed using Primer3, while those for *ap1s2* were selected through “CRISPR Design Tool” by SYNTHGO. In both cases, the company provided for the synthesis of the selected sgRNAs (target sequence is shown in red). The nucleotide sequences of the primers for genotyping and the selected sgRNA are reported below.

ap1s1 sgRNA AND PRIMERS

EX2 sgRNA **GGAGGCGCAGCUUCCCCUGC** + SYNTHGO modified scaffold RNA sequence

Forward primer: **GTGCTTTCCGTCTCTCACTCTC**

Reverse primer: **CAATGAACATTCTGTCTCAGCGT**

A 256 bp amplicon was expected after the PCR amplification of a wild-type gDNA.

ap1s2 sgRNA AND PRIMERS

EX2 sgRNA: **GGAGAUCAUCCACCGAUACG** + SYNTHGO modified scaffold RNA sequence

Forward primer: **CAGATATGCCAGCCTGTACTTTT**

Reverse primer: **ACTGCCAAAATACTTGTCCAGC**

A 112 bp amplicon was expected after the PCR amplification of a wild-type gDNA.

Two additional primers were selected, using Primer3 tool, for the genotyping of *zgc:162858*, *ap1s2* paralogue. We used the same PCR program used for *ap1s2* amplification (a 302 bp amplification product was expected for *zgc:162858*).

For_*zgc:162858*: ATGTTAAACCGGGCCTGAAT

Rev_*zgc:162858*: CCTGTAGACACATTTATTCAGTTCTCA

Primers for *slc7a6os* genotyping were designed using Primer3 tool. The nucleotide sequences of the primers for genotyping and the selected sgRNA are reported below.

slc7a6os sgRNA AND PRIMERS

EX 3 sgRNA: ATTTAGGTGACACTATAGGCCACAC**AGGCTGGATCC**GTTTTAGAGCTAGAAATAGCAAG

Forward primer: **CGAGAGAAAGAGGATGATTACG**

Reverse primer: **AGCTCTCCCTCTTGCGAGT**

A 110 bp amplicon was expected after the PCR amplification of a wild-type gDNA.

6.6 GENOTYPING

6.6.1 NON-DENATURING POLYACRYLAMIDE GEL

The analysis on agarose gel was followed by non-denaturing polyacrylamide (12%) gel electrophoresis. PCR products containing only perfectly complementary DNA molecules (either wild-type or mutated) migrate as a single band, whereas in the presence of DNA species that are not 100% identical (*e.g.* a wild-type and a mutated one) heteroduplex complexes are formed that migrate slower than homoduplex on the native polyacrylamide gel.

6.6.2 DNA SEQUENCING AND INTERPRETATION

Sanger DNA sequencing was performed on Applied Biosystems capillary instrument (ThermoFisher Scientific, Waltham, MA, USA). PCR products are processed using EXOSAP treatment to hydrolyse excess primers and nucleotides. 1.0 µL of EXO and 1.0 µL SAP were added to 3.0 µL of the amplification product. An incubation at 37°C for 15 minutes was followed by an enzymatic inactivation at 80°C for 15 minutes. Samples were cooled to 4 °C. 1.0 µL of sequencing primer was added to 2.0 µL of the DNA sample (10 µM concentration), followed by 10 minutes of incubation at 60 °C, leaving Eppendorf tubes lids opened to dehydrate by evaporation the samples to be sequenced.

6.6.3 BIOINFORMATICS AND PREDICTIVE ANALYSES

The DNA sequences from individuals obtained by CRISPR/Cas9 genome editing were analysed using Poly Peak Parser (<http://yosttools.genetics.utah.edu/PolyPeakParser/>), a free online tool that compares and aligns the chromatograms of a sample sequence and a reference provided by the operator and generates a chromatogram in which regions with significant sequence differences are indicated by light blue bars. Despite the program has been developed for the identification of indel alterations in heterozygotes, even in “mosaic” individuals it could provide information about the presence of CRISPR/Cas9-induced mutations in F0 individuals which show a high level of mosaicism (potential founders).

Prediction of the protein sequence encoded by a mutated allele has been performed using both DNA Strider and ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>), a tool provided by NIH that searches for open reading frames (ORFs) in the DNA sequence of interest.

6.7 RNA EXTRACTION AND RETROTRANSCRIPTION

RNA was extracted using the common TRI Reagent® (Trizol - Sigma-Aldrich, St. Louis, MO, USA) protocol. Reverse transcription reaction was performed on the total extracted RNA using M-MLV RT reverse transcriptase (Promega, Madison, WI, USA) and according to manufacturer instructions.

6.8 RT-PCR ANALYSIS

Amplification of a cDNA portion has been amplified by RT-PCR reaction using specific primers. The products were investigated by electrophoresis on agarose (1.5%) gel. Analyses of the migration pattern was useful to investigate the impact of the mutations induced by the Cas9 on the transcripts of the interested genes. Amplification profiles were organized as follows: 95 °C for 5 min; 35 cycles (denaturation: 95 °C for 15 sec; annealing: 58-61 °C for 30 sec; elongation: 72 °C for 30 sec); final cycle: 72 °C for 5 min; hold step: 4 °C). Primers are reported in the next section (qPCR).

6.9 QUANTITATIVE PCR (qPCR)

Reverse transcribed products (cDNAs) were amplified by qRT-PCR using the system ViiA 7 (Applied Biosystems, Waltham, Massachusetts, USA). The targets were detected with the SYBR green approach, using SYBR Green Master Mix (Biorad, Hercules, CA, USA). Zebrafish *rpl13a* was used as the housekeeping gene to normalize the quantification cycle (C_q) values of the other genes. DNA amplifications were performed on a thermal cycler (QuantStudio™ - Life Technologies, Carlsbad, California, USA) as follows: hold stage: 50 °C for 2 min; denaturation: 95 °C for 10 min; denaturation and annealing (x 40 cycles): 95°C for 15 sec and 60 °C for 1 min; melt curve stage: 95 °C for 15 sec; 60 °C for 10 min; 95 °C for 15 sec. Relative expression of the target genes was determined using the $2^{-\Delta\Delta C_t}$ method, as the fold increase compared with the controls. Data are presented as means $\pm$ SEM of the $2^{-\Delta\Delta C_q}$ values (normalized to *rpl13a* of three replicates). Primers sequences are reported below.

<i>ap1s1</i>	For <i>ap1s1</i>: GGTGGAGGAGCAGGACAAT Rev <i>ap1s1</i>: CCCCATAAGAACTCGTCCA
<i>ap1s2</i>	For <i>ap1s2</i>: AGAAAATCTCCCGTGAGGTCA Rev <i>ap1s2</i>: CTCATTTTCCTGGTCCTCCACA
<i>zgc:162858</i>	For <i>zgc:162858</i>: GAAGATCACGCGGGAGCTGG Rev <i>zgc:162858</i>: CCAGCGTGATCAGCTCGTTG
<i>rpl13a</i>	For <i>rpl13a</i>: TCTGGAGGACTGTAAGAGGTATGC Rev <i>rpl13a</i>: AGACGCACAATCTTGAGAGCA

6.10 *IN SITU* HYBRIDIZATION

In situ hybridization (ISH) technique allows specific nucleic acid sequences to be detected in morphologically preserved cells, tissues or embryos, relating microscopic topological information to gene activity at the DNA or mRNA level. Preparation of the embryos, synthesis of the probes, and ISH protocol were performed on whole-mount zebrafish embryos, according to published guidelines in literature (Thisse & Thisse, 2008).

6.10.1 PREPARATION OF THE EMBRYOS

Eggs were collected in a Petri dish, cleaned and unfertilized eggs were discarded. Embryos were allowed to develop in regular fish water. In embryos older than 24 hpf, pigmentation was prevented at the end of the gastrulation (10 hpf) by adding a solution of 1-phenyl-2-thiourea (PTU, P-7629, Sigma Aldrich, St. Louis, MO, USA) at the proper concentration. Chorions were removed manually prior or after fixation, which was performed in 4% paraformaldehyde (PFA, P-6148, Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at room temperature or O/N at 4 °C. They were subsequently dehydrated up to 100% Methanol (MeOH) before proceeding with *in situ* hybridization experiments.

6.10.2 SYNTHESIS OF THE PROBE FOR *ap1s2*

A fragment of the *ap1s2* gene (550 bp) was generated through PCR on gDNA from wild-type AB fish, using primers localized on the last coding exon of the gene (**left primer:** GAAGCCGAGACACCACGTA; **right primer:** TCCCTTAAAAGTCTAAGTGTTTCAGC). This regions of *ap1s2* do not show any evidences of repeated sequences in zebrafish genome and is rather divergent from *zgc:162858*. Amplification profile was: denaturation: 95 °C for 5 min; 35 cycles (denaturation: 95 °C for 15 sec; annealing: 53 °C for 30 sec; elongation: 72 °C for 30 sec); final cycle: 72 °C for 5 min; hold step: 4 °C. Part of the reaction volume was purified using the “GenElute™ PCR Clean-Up Kit” (Sigma-Aldrich, St. Louis, MO, USA) and quantified. Purified PCR has been inserted into pGEM T-Easy vector using T4 DNA Ligase (Promega, Madison, WI, USA) according to manufacturer’s instructions (O/N at 4 °C) and then transformed into JM-109 competent *E. coli*. Bacterial cells were harvested in selective medium (Ampicillin); DNA was extracted from positive clones using the “GenElute™ Plasmid Miniprep Kit” (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. Orientation of the inserted fragment (Fig. 41) was investigated through different approaches on the extracted bacterial DNA:

- **PCR** (T7 **forward:** TAATACGACTCACTATAGGG; **reverse:** TCCCTTAAAAGTCTAAGTGTTTCAGC). Amplification profile was: 95 °C for 5 min;

35 cycles (denaturation: 95 °C for 15 sec; annealing: 53 °C for 30 sec; elongation: 72 °C for 30 sec); final cycle: 72 °C for 5 min; hold step: 4 °C.

- **Restriction enzyme digestion:** DraIII (or AdeI) was selected for its capability to produce a single cut in the plasmid and a one within the 550 bp *ap1s2* insert, respectively, leading to two different couples of fragments, according to the orientation of the insert in the vector. Digestions were performed at 37 °C for 1 hour.
- **Sanger DNA sequencing:** linearized plasmid (with EcoRI) has been sequenced using the T7 primer (TAATACGACTCACTATAGGG).

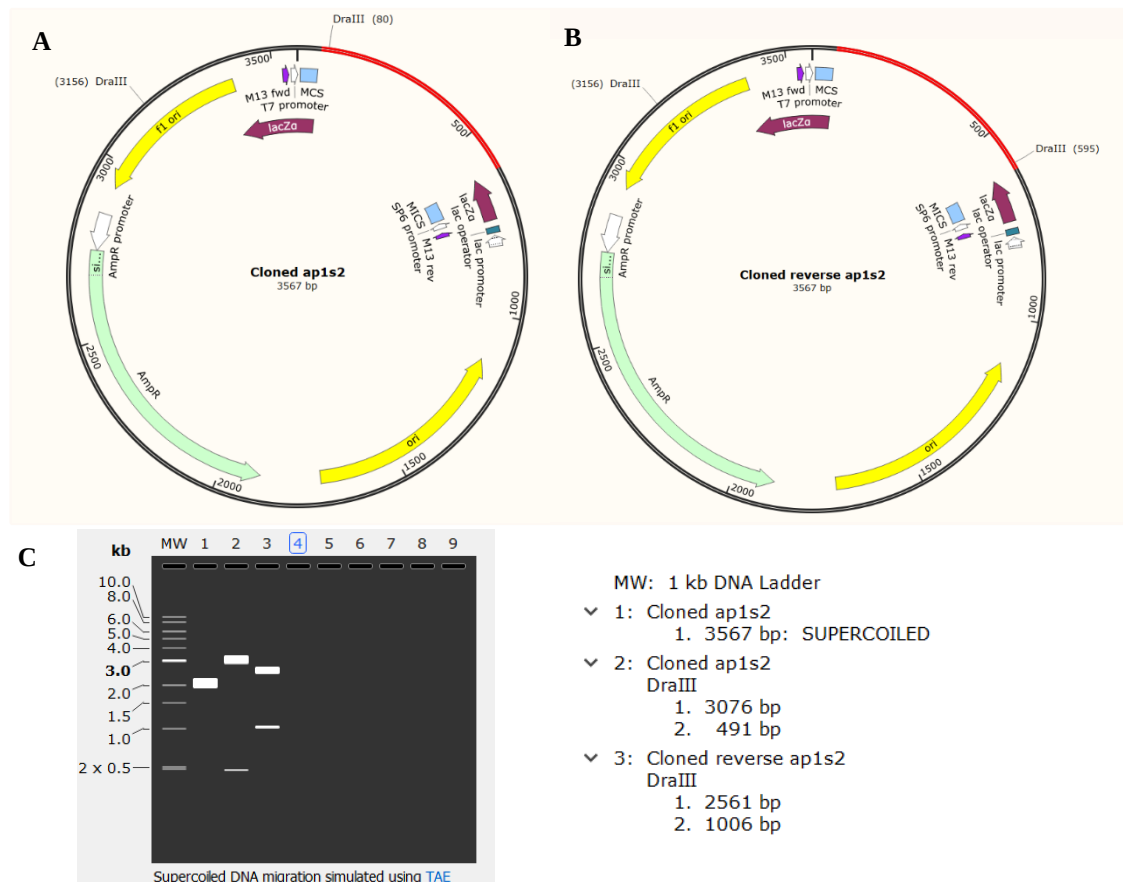


Figure 41. Ligation between pGEM T-Easy vector (Promega) and the 550 bp *ap1s2* fragment (SnapGene viewer). The cloned fragment is reported in two different orientations, according to the proximity of the 5' to the T7 (A) or the SP6 (B) promoter, respectively. (C) Agarose gel electrophoresis of the digestions simulation. From the left: DNA ladder 1 kb; undigested pGEM T-Easy vector + 550 bp fragment; pGEM T-Easy vector + 550 bp fragment in “T7” orientation, digested with DraIII; pGEM T-Easy vector + 550 bp fragment in “SP6” orientation, digested with DraIII.

Orientation of the *ap1s2* cloned fragment, with its 5' in proximity to the T7 promoter, was confirmed and two enzymes were selected:

- *Apal* produces a single cut after the sequence of the T7 promoter, allowing the synthesis of the antisense probe using the SP6 polymerase.
- *PstI* produces a single cut after the sequence of the SP6 promoter, allowing the synthesis of the sense probe using the T7 polymerase.

Linearization of the plasmid DNA (37 °C for 2 hours) was followed by purification on column (GenElute™ PCR Clean-Up Kit, Sigma-Aldrich, St. Louis, MO, USA). Gel electrophoresis analysis and quantification at NanoDrop 1000 were performed. Antisense and sense RNA probes were obtained by *in vitro* transcription of plasmid DNA with T7 or SP6 RNA polymerase (Roche, Basel, Switzerland), using a digoxigenin labelling mixture according to manufacturer's protocol (Roche, Basel, Switzerland).

6.10.3 WHOLE MOUNT *IN SITU* PROTOCOL

Whole mount *in situ* hybridizations (WISH) was performed according to Thisse, B and Thisse, C (2008) protocol. After treatment with proteinase K (10 µg/mL, Roche, Basel, Switzerland), the embryos were hybridized overnight at 68° C with DIG-labelled antisense or sense RNA probes (400 ng/µL). Probe staining was performed with NBT/BCIP (blue staining solution, Roche) alkaline phosphatase substrates. WISH images were taken with the ZEISS AxioZoom.V16 microscope (ZEISS, Oberkochen, Baden-Württemberg, Germany) at different magnifications (112X, Plan-NEOFLUAR Z 1.0x/0.25 FWD 56mm; 200.1X, objective: Plan-NEOFLUAR Z 2.3x/0.57 FWD 10.6mm) and processed using Zen Pro software from ZEISS.

6.10.4 SPATIAL-TEMPORAL ANALYSIS OF KEY NEUROGENESIS MARKERS IN MUTANTS

The following is a list of the neural markers, analysed using RNA antisense probes (<https://zfinfo.org/>), with a description of the biological role and the stages selected for the hybridization experiments:

- ***pax2a***: it is one of the first and crucial genes to be specifically expressed during the development of the midbrain and the midbrain hindbrain boundary. This gene is necessary for the development and organization of hindbrain, midbrain hindbrain boundary (mhb), interneurons of the spinal cord and optical peduncle; it is specifically expressed during the gastrulation and somitogenesis phase. The expression of *pax2a* was evaluated at 16 hpf and 24 hpf in the following structures (Thisse, et al., 2001):
 - **16 hpf**: immature eye, midbrain hindbrain boundary, neural tube and otic placode.
 - **24 hpf**: hindbrain, mesoderm, midbrain hindbrain boundary, neural tube, optic stalk, otic vesicle, pronephric duct, spinal cord.
 - **30 hpf**: hindbrain, midbrain hindbrain boundary, otic vesicle, pharyngeal arch 3-7 skeleton, pronephric duct, spinal cord, cloaca.
- ***neurog1***: it codifies for a transcriptional regulator that activates transcription by binding to the E box-containing promoters and it is involved in neuronal differentiation. It is

required for the development of Rohon-Beard spinal sensory neurons and dorsal root ganglion neurons, but not for primary motoneurons or autonomic neurons. It is required for development of all cranial ganglia but not associated glial cells. It regulates epiphyseal neurogenesis, and it is required for the development of basal forebrain dopaminergic neurons; in particular, it is involved in the specification of dopaminergic progenitor cells. The expression of this gene was evaluated at 30 hpf, in these structures (Thisse, et al., 2001): alar plate midbrain region, basal plate midbrain region, cranial ganglion, diencephalon, forebrain, hindbrain, hypothalamus, midbrain, neural tube, optic tectum, primary neuron, proliferative region, rhombomeres, spinal cord, tegmentum, telencephalon.

- ***neurod1***: it codifies for a transcriptional factor that assumes a key role in the neuronal differentiation process; in particular, it is expressed in mitotic and post-mitotic neurons (20-48 hpf). The expression of this gene begins at the embryonic stage of epiboly (5 hpf) and increases progressively up to 5 dpf, with a high expression between the “pharyngula prim-15” phase (24 hpf) and “hatching long pec” (48 hpf). The maximum expression is observed between the “protruding mouth” and the 5 dpf. Analysis of *neurod* expression was analysed in the following structures (Rauch, et al., 2003):
 - **24 hpf**: ganglion of the anterior lateral line, dorsal diencephalus, posterior lateral line, statoacoustic ganglion (VIII) and telencephalon.
 - **48 hpf**: brain, cranial ganglion, diencephalon, epiphysis, eye ganglion, hindbrain, pancreas, rhombomeres, telencephalon.
 - **72 hpf**: diencephalus, epiphysis, eyes, retina, midbrain, hindbrain, rhombomers, telencephalon and spinal cord.
- ***dlx2a***: it is predicted to have DNA-binding transcription factor activity and RNA polymerase II cis-regulatory region sequence-specific DNA binding activity. It is involved in several processes, including neural crest cell development; pharyngeal system development and skeletal system development. Its expression is located in several structures, including brain; cranial cartilage; neural crest; pectoral fin bud and pharyngeal arch. Analysis of *dlx2a* expression was evaluated at 48 hpf, in the following structures (Rauch, et al., 2003): hypothalamus, telencephalon/diencephalon ventricular zone, pharyngeal arches, AER pectoral fin.

6.10.5 SPATIAL-TEMPORAL ANALYSIS OF *myod* GENE IN MUTANTS

myod is a helix–loop–helix muscle-specific transcription factor that has long been recognized for its crucial role in myoblast proliferation and differentiation, which acts upstream of or within pharyngeal muscle development, skeletal muscle tissue development and striated cell development. Early muscle development was analysed by hybridizations of embryos at 16.5 hpf (20 somites). At this developmental stage, *myod* is expressed in the following structures (Thisse & Thisse, 2004): paraxial mesoderm, segmental plate and somites.

6.10.6 F-ACTIN STAINING

Phalloidin is a bicyclic eptapeptide able to bind with high affinity the actin monomers; its conjugation with fluorescent molecules allows the observation of the actin fibres using a classic fluorescence microscope. 48 hpf embryos were fixed with 4% PFA at 4 °C overnight. The embryos were then washed using PBS/0.1% Tween 20 (PBST) three times for 10 min, and permeabilized in 2% TritonX-100 in PBS for 2 hours at room temperature. Embryos were incubated in blocking solution (10% sheep serum, 2% BSA, 0.5% TritonX-100 in PBS) for 3 hours at room temperature. The molecular probe was prepared by diluting phalloidin (Alexa Fluor 488, Sigma Aldrich, St. Louis, MO, USA) 1:400 in blocking solution. Embryos were incubated in staining solution for 3 hours in the dark at room temperature. Several washes with PBST were performed at 4 °C overnight and 2 hours at room temperature the following day. Images were taken with the ZEISS AxioZoom.V16 microscope (ZEISS, Oberkochen, Baden-Württemberg, Germany), equipped with ZEISS Axiocam 503 colour digital camera in 16 bit, at different magnifications (40X and 80X, objective: Plan-NEOFLUAR Z 1.0x/0.25 FWD 56mm).

6.11 OIL RED O (ORO) STAINING AND QUANTIFICATION

Oil Red O (ORO) staining allows to identify the deposition of neutral lipids and triglycerides (Nishio, et al., 2012). Staining procedure was performed according to published guidelines (Chen, et al., 2015) (Schlombs, Wagner, & Scheel, 2003): briefly, following anaesthesia with MS-222 (200 ppm), zebrafish were fixed in 4% paraformaldehyde for 12 h at 4°C (or 2 hours at room temperature) and washed three times in 1X phosphate-buffered saline (PBS). Fish were subsequently pre-incubated in 60% isopropanol for 30 min and dyed with fresh 0.3% Oil Red O for 3 h. A single experimental setting included of 12-13 larvae for a total of 5 replicates for each genotype group. Samples were ready for microscopic observation following three washes with 60% isopropanol and a washout in PBS 1X. AxioZoom.V16 microscope (ZEISS, Oberkochen, Baden-Württemberg, Germany) was used for observations and capturing images (32X, objective: Plan-NEOFLUAR Z 1.0x/0.25 FWD

56mm). Oil Red O staining in zebrafish is only a qualitative measure, in which lipid deposition can only be assessed visually. Hence, the main limitation in using ORO staining is that there is a lack of efficient and dependable methods that allows for the quantification of ORO staining. Quantification of the stained embryos for these preliminary data were adapted to a novel technique for zebrafish embryogenesis that allows researchers to rapidly quantify the amount of ORO stain detected in the zebrafish embryo (Yoganantharjah, Byreddy, Fraher, Puri, & Gibert, 2017). Stained samples were dissected to evaluate three different parts of the larvae: the head, the central body and the tail. Fig. 42 is representative of the reference points used for dissection.

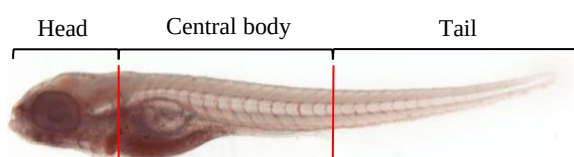


Figure 42. Anatomical references for dissection of ORO stained larvae.

Sample sizes of 12-13 embryos were pooled together per Eppendorf tube to extract adequate volume of ORO stain. Replicates for each treatment group was not less than 5 Eppendorf tubes. After removing PBS, 250 μ L of 4% v/v Nonidet P-40 (Octylphenoxy poly(ethyleneoxy)ethanol) made up in 100% isopropanol was added to each Eppendorf tube. Samples were briefly mixed in a vortex machine and incubated at room temperature for 2 hours until the zebrafish bodies were lacking any ORO staining (tissue becomes visibly white), indicating all the ORO stain has been completely extracted into solution. 200 μ L of the solution that contained the extracted ORO stain was then pipetted into respective wells of a 96-well plate and OD (absorbance) was read at 495 nm at the EnSight Multimode Plate Reader (Revity, Waltham, Massachusetts, U.S.); data were exported using Exported with Kaleido Software 2.0. OD measurements of ORO stained embryos were then normalised to OD measurements of non-stained zebrafish embryos.

6.12 MORPHOLOGICAL ANALYSIS ON LARVAE AND ADULTS

Morphological analysis on embryos and larvae have been performed through ZEISS Axio Zoom.V16 (10X - 25X; objective: Plan-NEOFLUAR Z 1.0x/0.25 FWD 56mm) after anaesthetization with the correct amount of tricaine methanesulfonate (TMS, Sigma-Aldrich, St. Louis, MO, USA). Observational studies were performed on adult anaesthetised or sacrificed individuals using Leica Zoom 2000 Stereo Microscope (Leica, Wetzlar, Germany) and ZEISS Axio Zoom.V16 microscope (objective: Plan-NEOFLUAR Z 1.0x/0.25 FWD 56mm; ZEISS, Oberkochen, Baden-Württemberg, Germany).

6.13 LIGHTSHEET FLUORESCENCE MICROSCOPY

For Light Sheet image analysis Tg(*neurod1*:EGFP) embryos were fixed and included in a low melting agarose matrix 0.5% (Top Vision Low Melting Point Agarose, ThermoFisher Scientific, Waltham, MA, USA), then put in the instrument holder. Images were acquired using ZEISS LightSheet microscope V1 (ZEISS, Oberkochen, Baden-Württemberg, Germany) supported by ZenPro software using a 488–30 nm laser and 505–545 nm filter. A Z-stack was performed to acquire the whole central nervous system and Maximum Intensity Projection was obtained. Images from the same experiment were taken with the same laser intensity and exposure time to generate comparable images. After the acquisition, 3D images were generated and manipulated using Arivis Vision 4D (ZEISS, Oberkochen, Baden-Württemberg, Germany) 3D.

6.14 HISTOLOGICAL ANALYSES

6.14.1 HISTOLOGICAL SECTIONS ON EMBRYOS

Embryos at 3-4 dpf for histological sections have been agarose-embedded, placed in 70% Ethanol (EtOH) and processed with the purpose of substitute water with paraffin, adapting published guidelines (Sabaliauskas, et al., 2006). Histological sections of 6 µm were performed on 3-4 dpf larvae using Leica rm2155 microtome and observed under Leica Zoom 2000 stereo microscope (Leica, Wetzlar, Germany). Haematoxylin and eosin staining was performed (30 sec haematoxylin; 20-30 sec alcoholic eosin). Subsequently, stained sections have been mounted and images have been acquired with ZEISS Axio Observer at 20X (ZEISS, Oberkochen, Baden-Württemberg, Germany).

6.14.2 HISTOLOGICAL SECTIONS ON ADULTS

Adult fish were fixed for 24 h in Bouin's solution (picric acid, acetic acid and formaldehyde) at room temperature O/N, washed several times with 70% EtOH- NH₃ (Ethanol added with NH₃) and conserved in 70% Ethanol. The samples were embedded in paraffin using an automatic processor Donatello series 2 (Diapath S.p.A., Bergamo, Italy). Serial paraffin sections (5 µm thick) of each sample were cut with a semiautomatic microtome Galileo semi-series 2 (Diapath S.p.A., Bergamo, Italy). Alternate sections were deparaffinized, and rehydrated, according to standard procedures, and stained with haematoxylin–eosin stain (automatic stainer Giotto; Diapath S.p.A., Bergamo, Italy) for general morphology. The micrographs were performed using an optical microscope (Olympus BX50 microscope, Hamburg, Germany, equipped with a camera and an image analysis system (Image-Pro Premier 9.1; Media Cybernetics; Maryland, USA).

6.15 TREATMENTS OF EMBRYOS WITH COPPER SOLUTIONS

Wild-type AB and *ap1s1* mutants have been treated with high doses of copper solutions (500-1100 µg/L of CuSO₄ · 5 H₂O (209198 - Sigma Aldrich, St. Louis, MO, USA) dissolved in fish water solution) from 0 hpf to 5 hpf, adapting published guidelines (Johnson, Carew, & Sloman, 2007) for 24-well plates, with a total treatment volume of 1 mL per condition. At least 6 replicates with 10-13 embryos for each experimental condition were analysed. Treated embryos have been observed each day under Leica Zoom 2000 stereo microscope (Leica, Wetzlar, Germany). Characterization has been performed by analysing the mortality and the hatching ability of the embryos from 48 hpf to 72 hpf. Moreover, preliminary studies on cell death mechanisms have been analysed through acridine orange staining at 72 hpf, while locomotor behaviour has been investigated at 5 dpf (behavioural test is described later in the text). Fig. 43 summarizes the timeline of the entire experimental protocol (movements at 30 hpf have been recorded, even if not discussed in this thesis).

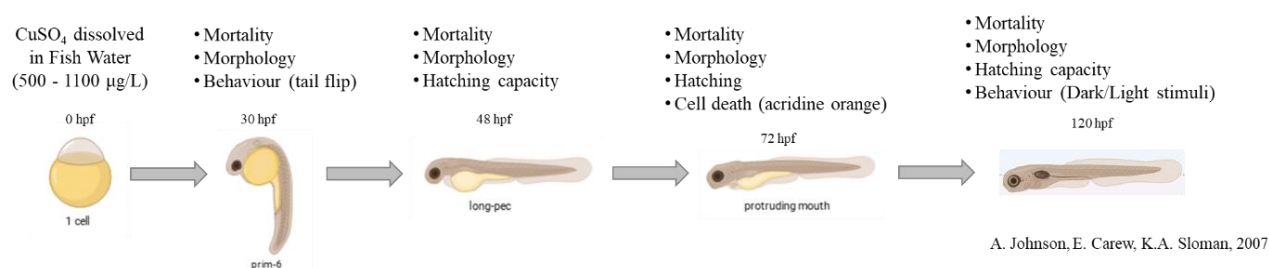


Figure 43. Experimental setting for treatments with copper solutions.

6.15.1 MORTALITY AND HATCHING

During the 72 h test period, mortality and hatching of both wild-type controls and *ap1s1* mutants for each concentration was recorded at 2, 3, 5, 24, 26, 48 and 72 hpf, where time points were based on preliminary studies and known developmental stages (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Death in early embryos was classified as those exhibiting a marked loss in translucency, change in colouration or degree of fungal infection and a cessation in heartbeat was the criteria for more developed embryos and larvae (Johnson, Carew, & Sloman, 2007).

6.15.2 ACRIDINE ORANGE STAINING

To analyse the level of cell death, acridine orange staining was performed adapting published protocols (Perkins, Nicholas, Baye, Link, & Dowling, 2005). Embryos at 48 hpf were dechorionated and incubated for 45 min in acridine orange staining solution 2.5 µM (Sigma-Aldrich, St. Louis, MO, USA; concentration was assessed to have no toxic effect that allowed an efficient staining), then rinsed three times using fish water. Pictures of single embryos were taken with the same

magnification (32X), laser intensity and exposition using ZEISS Axio Zoom.V16 (ZEISS, Oberkochen, Baden-Württemberg, Germany) equipped with ZEISS AxioCam 503 color digital camera in 16 bit (objective: Plan-NEOFLUAR Z 1.0x/0.25 FWD 56mm). Images were exported in tiff format and converted in 8 bit using ImageJ program (Schneider, Rasband, & Eliceiri, 2012) for dead cell counting.

6.16 BEHAVIOURAL ANALYSES

6.16.1 ANALYSIS OF BEHAVIOUR IN LARVAE

Behavioural tests on larvae were performed using DanioVision (Noldus, Wageningen, the Netherlands) instrument. 5 dpf larvae were displaced individually in a 96 square and then subjected to light stimulus: habituation in light for 30 min, three cycles of dark/light stimulus for 10 min each have been performed (Basnet, Zizioli, Taweedet, Finazzi, & Memo, 2019). During the test, each larva was recorded and parameters such as velocity and travelled distance have been calculated and analysed using EthoVision XT software (Noldus, Wageningen, the Netherlands).

6.16.2 ANALYSIS OF BEHAVIOUR IN ADULTS

Adult individuals were subjected to a simple analysis called as “novel tank test”, which uses vertical distribution in a new environment as a validated behavioural test to evaluate “anxiety-like” behaviours in adult zebrafish. The experimental protocol (Haghani, Karia, Cheng, & Mathuru, 2019) requires the transfer of an adult individual from the housing tank to a new observation tank and subsequent tracking of free movements lasting ten minutes (EthoVision XT, Noldus, Wageningen, the Netherlands). For recording purposes, a USB webcam was used, and the tank was backlit with a white acrylic screen to provide enough contrast for detection. The swimming trajectories were analysed using EthoVision XT software (Noldus, Wageningen, the Netherlands). The novel tank diving test was divided into three sections (top, middle, bottom) and average distance and latency were measured. Latency in each section has been evaluated by generating heatmaps.

6.17 STATISTICAL ANALYSES

Reported data are a representation of two or more independent experiments with similar results. We performed the statistical analysis using GraphPad Prism 6 software (Boston, MA, USA) for Windows. The significance between various groups was determined by ANOVA, corrected for multiple comparison, by two-tailed unpaired Student’s t-test, or by chi-square analysis as indicated in the legend of each figure, while survival curves were analysed using log-rank (Mantel-Cox) statistic test.

7 RESULTS FOR *ap1s1* KNOCKOUT LINE

7.1 *IN SILICO* ANALYSIS OF *ap1s1* GENE

The aim of this thesis was the integration of previous knowledge on AP-1 complex, deepening the role of σ 1A-coding gene in zebrafish.

In silico analysis was performed using several bioinformatics tools and public genome sequence databases, revealing the presence of a single putative orthologous zebrafish gene, *ap1s1*, mapping on chromosome 7 (<https://zfin.org/ZDB-GENE-040426-1119>). Briefly, a sequence homology search using as a query the human AP1S1 protein (NM_001283.5) was performed on *D. rerio* genome assembly (Genome Reference Consortium Zebrafish build 11 - GRCz11) using BLAT (Basic Local Alignment Search Tool-Like Alignment Tool) from the UCSC Genome Browser (<https://genome.ucsc.edu/cgi-bin/hgBlat>). In addition to a single entry for the *ap1s1* gene, located on chromosome 7, additional entries corresponding to paralog gene products have been identified. The multiple sequence alignment among different Ap1s family members is shown and discussed in the *ap1s2* section of this thesis. A very high level of sequence identity (91.72%), is present between the human and the zebrafish AP1S1 polypeptides (which are both 158 aa long), indicating a high conservation of these gene products during evolution.

Genomic organization of *ap1s1* in *D. rerio* was investigated using the University of California, Santa Cruz (UCSC) – Genome Browser, showing the localization of the *ap1s1* gene on chromosome 7 and its length in base pairs. In *D. rerio* the gene is composed of 5 exons and 4 introns, with a RefSeq transcript of 1728 bp (NM_001328014.1) encoding for a protein of 158 amino acids (NP_001314943). Besides the former RefSeq, additional isoforms have been annotated and reported, all protein-coding, and likely originated either from events of alternative splicing or the usage of alternative promoter, as emerged mainly from RNA-Seq studies (Fig. 44).

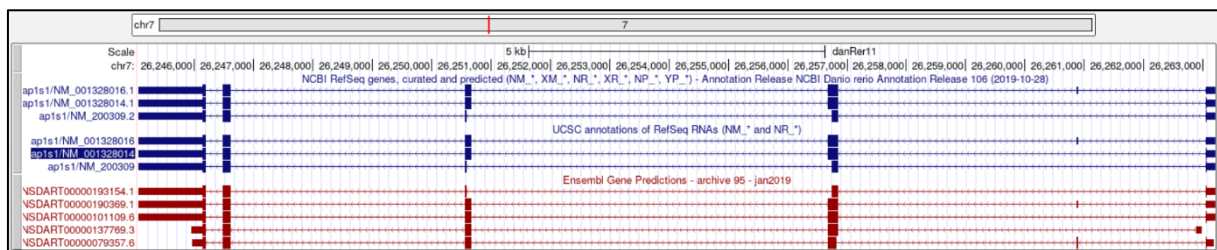


Figure 44. Genomic organization of *ap1s1* within zebrafish chromosome 7 (UCSC Genome Browser).

A synteny analysis of the *AP1S1* locus was performed. In genetics, “synteny” describes the physical colocalization of genetic loci on the same chromosome within an individual or species. The analysis performed with Genomicus web server (<https://www.genomicus.bio.ens.psl.eu>) showed a partial conservation of the *ap1s1* flanking genes in zebrafish, mouse rat and man (Fig. 45).

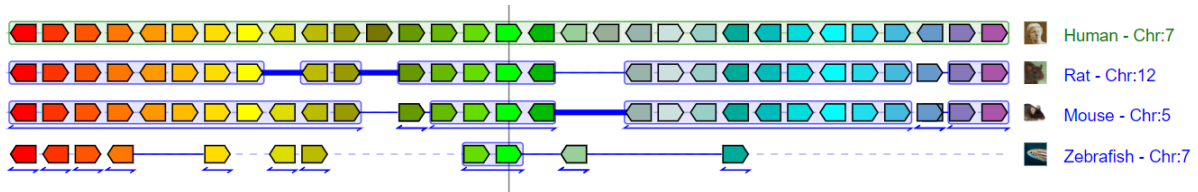


Figure 45. Synteny analysis for *AP1S1* in *H. sapiens*, *R. norvegicus*, *M. musculus* and *D. rerio* performed using Genomicus web server. The *AP1S1* gene in the species analysed is shown in light green in the middle of the figure as indicated by a vertical dotted line.

7.2 EXPRESSION ANALYSES AT DIFFERENT DEVELOPMENTAL STAGES

According to published results (White, et al., 2017) concerning RNA-Seq analysis of a wide pattern of genes (up to 23,643), *ap1s1* (ENSDARG00000056803) expression was relatively low in all embryonal stages. Data are reported in the graph below (Fig. 46) and expressed in TPM (transcript per million). TPM are calculated from the number of reads that mapped to each particular gene sequence taking into account the gene length and the sequencing depth. Higher values at the earlier developmental stages (from one zygote to gastrula – 75% epiboly) indicate the presence of maternal *ap1s1* mRNA accumulation. The expression of the gene then decreases until it settles to values similar to each other around the second and third day of fertilization. This expression profile is comparable to that obtained for the same gene as part of a systematic study of the zebrafish transcriptome using RNA-Seq (Yang, et al., 2013).

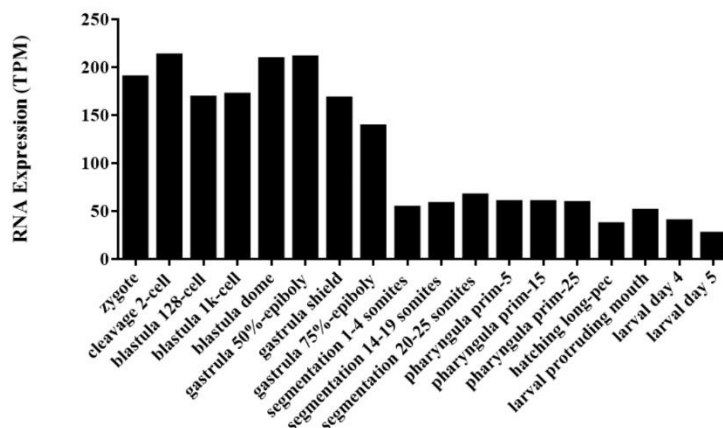


Figure 46. Expression levels of *ap1s1* (ENSDARG00000056803) transcript, expressed at different zebrafish embryonal stages (White, et al., 2017).

7.3 GENERATION OF *ap1s1* KNOCKOUT LINE USING CRISPR/Cas9

The experimental study began with the generation of loss of function (LOF) mutations in the coding region of *ap1s1* gene using CRISPR/Cas9 technology (for more details about the experimental design and strategy see “Materials and Methods”). After microinjection of a synthetic sgRNA (targeting the second coding exon of *ap1s1* and thus referred to as *ap1s1*-EX2 sgRNA) and Cas9 protein, zebrafish embryos have been incubated at 28 °C and allowed to grow to the 5 dpf developmental stage, when they were transferred to a proper tank in the aquarium system and allowed to grow until adulthood. Several microinjections have been performed, using two different sgRNA concentration (250 pg/embryo and 500 pg/embryo), but only 500 pg/embryo injection dose gave rise to a population of individuals with evidence of mosaicism. Relevant information about microinjection experiments is indicated in Table 2.

DATE	sgRNA	MICROINJECTED EMBRYOS	MORTALITY 24 hpf	GENOTYPING
17/12/2018	<i>ap1s1</i> -EX2 sgRNA (SYNTHEGO) 500 pg/embryo	95	24/95 (25.26%)	16 adults

Table 2. Scheme of *ap1s1*-EX2 injection in wild-type AB embryos at one-cell stage.

Part of the injected embryos who reached the adulthood (16) have been subjected to genotyping through electrophoresis on polyacrylamide (12%) gel of the PCR products from tails (Fig. 47). All the individuals showed evidence of mosaicism, with an electrophoretic migration pattern characterized by the presence of the expected amplification product (256 bp) and several bands at higher molecular weight, likely corresponding to the presence of heteroduplexes between wt and DNA molecules that acquired mutations by the action of Cas9 endonuclease.

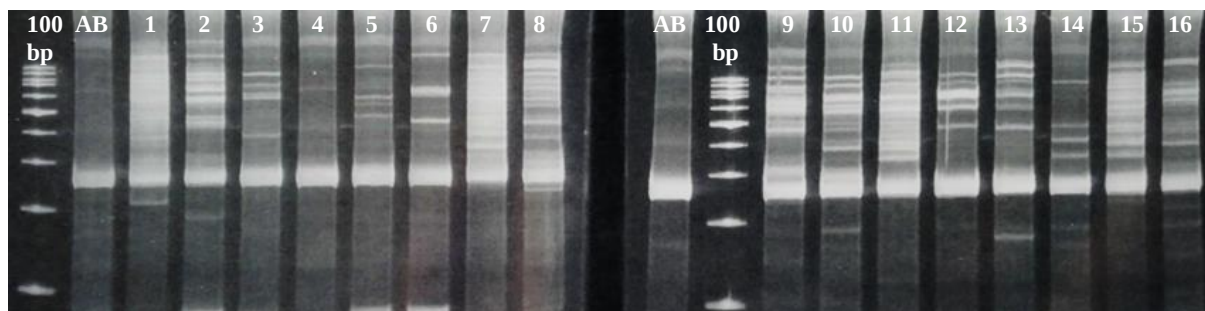


Figure 47. Electrophoresis on non-denaturing polyacrylamide (12%) gel of genotyping PCR products from F0 generation adult fish. Abbreviations AB: wild-type AB.

Since all the fish analysed could represent putative mosaic individuals, it was decided to proceed with systematic crosses with wild-type AB adults of the opposite sex, then subjecting the offspring to PCR genotyping. A part of the generated population from each mating was screened to identify the heterozygotes for mutations induced by Cas9 endonuclease. More in detail, only a single male gave rise to a promising F1 generation; the generated larvae have been let to grow until adulthood (~3 mpf), when genotyping on non-denaturing polyacrylamide (12%) gel has been performed on DNA extracted from adult tails. Only three individuals out of 43 fish (~ 6.98%) showed an electrophoretic migration pattern characterized by the presence of only two bands with higher molecular weight than the PCR product expected for the wild-type allele (256 bp). Sanger DNA sequencing revealed the presence of a single mutation in heterozygosis, corresponding to a deletion of four nucleotides (Fig. 48). The amplicon relative to the mutated allele, on the other hand, is not visible to eye, as the number of bases interested in the mutation do not involve a sufficiently wide region to be discriminated with a 12% polyacrylamide gel.

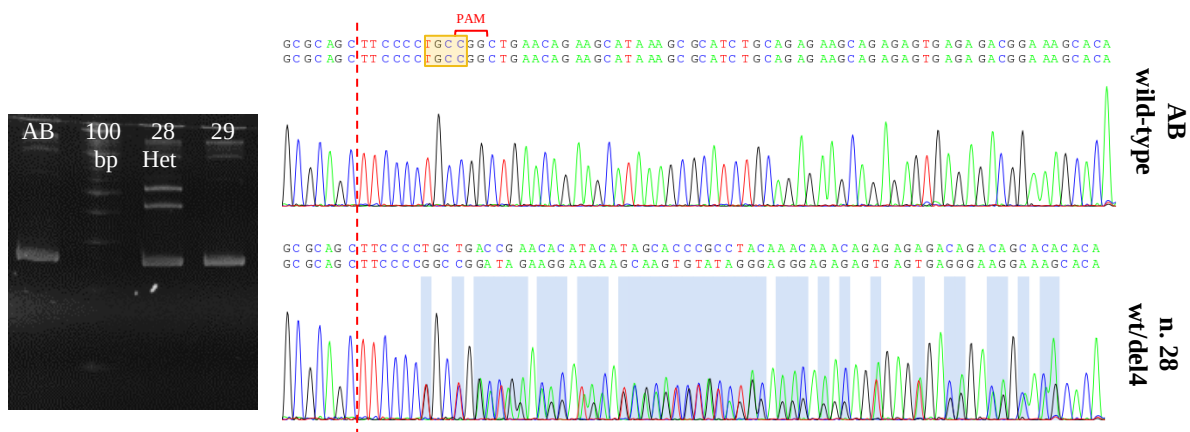


Figure 48. Left side: electrophoresis on non-denaturing polyacrylamide (12%) gel of genotyping PCR products from F1 generation adult fish. Electrophoresis shows the migration patterns corresponding to a putative heterozygote (28) and a wild-type F1 fish (29). **Right side: sequencing chromatograms of genotyping PCR products from F1 adults.** From the top: AB (wild-type AB adult); an illustrative heterozygote chromatogram (n. 28). The number of the F1 individual analysed is indicated on the right, along with the genotype. The PAM sequence adjacent the sgRNA target site is shown in red. Nucleotides deleted by the mutations are reported in yellow.

Unfortunately, the selected individuals resulted to be all males; thus, we set up a further cross with wild-type AB fish. The individuals who reached the sexual maturity were then genotyped to select the heterozygotes for the del4 mutation. Among the 34 larvae that were placed in the aquarium system, 23 reached the adulthood (67.65%) and 10 out of these (43.48%) resulted to be heterozygotes for the selected mutation (*ap1s1^{wt/del4}*). Genotyping has been performed by analysing migration pattern on native polyacrylamide (12%) gel of the PCR products obtained by amplification of the genomic DNA extracted from the tail (data not reported). Subsequent incross between *ap1s1^{wt/del4}*

allowed to obtain the three genotypic classes, *ap1s1*^{wt/del4} (6/13, 46.15%), *ap1s1*^{wt/wt} (4/13, 30.77%) and *ap1s1*^{del4/del4} (3/13, 23.08%), on which the phenotype characterization experiments were performed.

The del4 is a frameshift mutation, leading to the formation of a truncated (53 aa) Ap1s1 protein (Fig. 49), as predicted by the ORF Finder, a tool provided by NCBI. Most likely the aberrant transcript is degraded by the nonsense-mediated decay (NMD) mechanism.

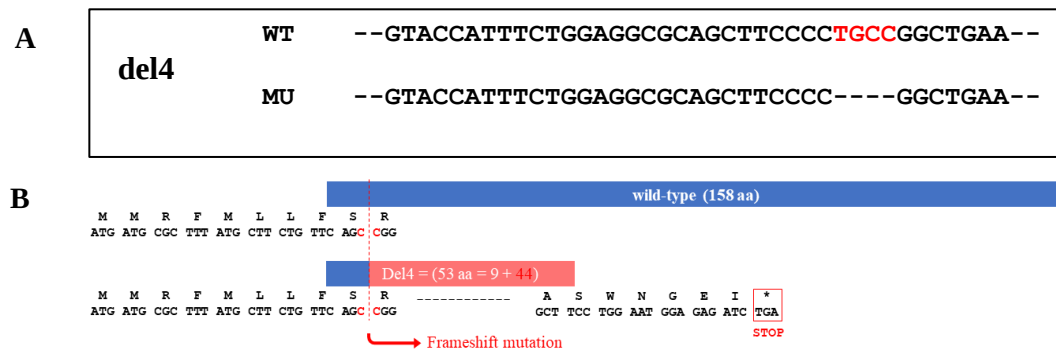


Figure 49. (A) Sequence reconstruction of the mutated allele identified. (B) ORF prediction for the *ap1s1* mutant allele isolated. The protein encoded by each allele is shown. In blue is schematically indicated the portion corresponding to the wt Ap1s1 protein, in red the polypeptide originating by frameshift mutations in del4 allele.

7.4 GENOTYPE SEGREGATION ANALYSIS

Segregation of the three genotypic classes for the del4 mutation was analysed on adult tails (~3 mpf) by screening PCR products on native acrylamide (12%) gel. The evaluation was performed comparing the distributions of the individuals in the different genotypes and the typical Mendelian ratios, using chi-square statistic test (Fig. 50A). The analysed data showed a discrepancy between the observed distributions and the expected results ($p = 0.0209$), with a lower number of *ap1s1*^{del4/del4} individuals at 3 mpf (20.47%). Analyses with larger numbers of individuals could provide more information in order to properly elucidate the statistical significance of these data. A preliminary investigation was conducted to assess the survival rate of *ap1s1* mutants, compared to controls, in the first month of life. In particular, larvae were incubated at 28 °C in Petri dishes until 5 dpf, when a total number of 48 individuals for each group has been placed in separated tanks within the aquarium system, counting and recording every day the number of deaths. The analysis of the data collected showed a tendency of *ap1s1*^{del4/del4} individuals to die after the fifth day of development, with a significant different survival curve when compared to that of control individuals (Fig. 50B), according to log-rank (Mantel-Cox) statistic test ($p < 0.0001$). This could partially justify the presence of a lower

number of homozygous individuals for the del4 mutation who reach the adulthood in F2 generation (Fig. 50A). Nevertheless, this represent an extremely preliminary result from a single experiment that should be repeated in the future.

From a first morphological analysis of the larvae generated by crossing between heterozygous individuals for del4 mutation it was not possible to identify obvious and visible differences between the individuals of the three different genotypic classes. This was later confirmed by more in-depth analysis of morphology, conducted on 5 dpf larvae generated by the crossing of *ap1s1*^{del4/del4} individuals.

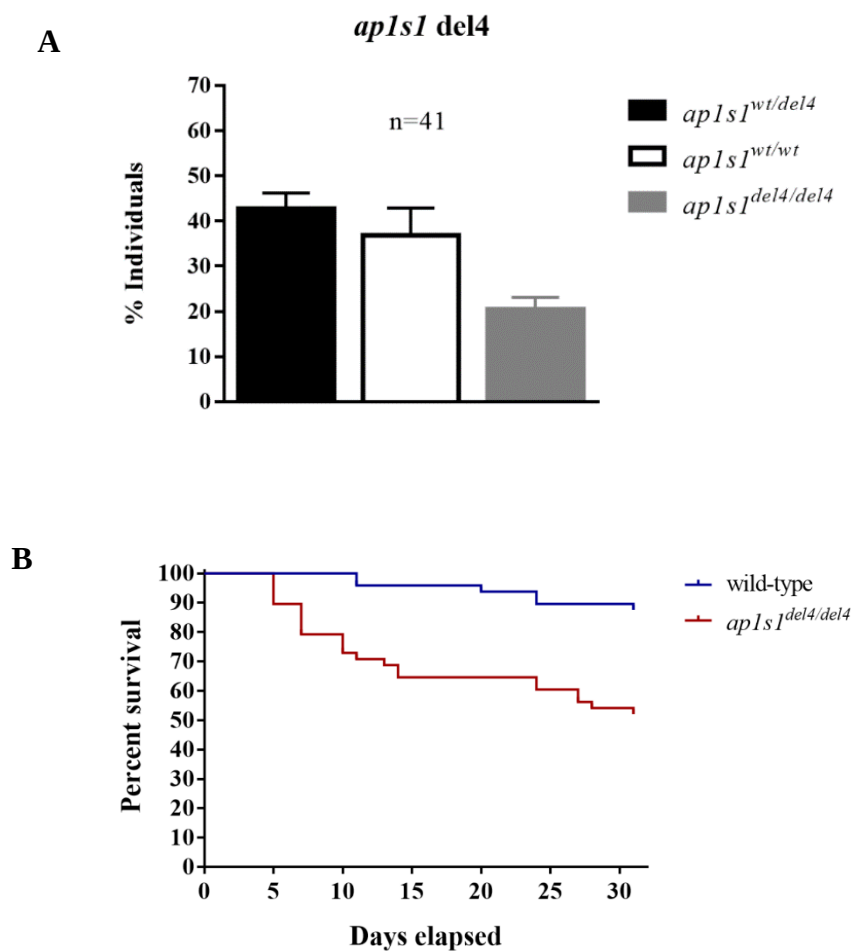


Figure 50. (A) Analysis of the genotypic segregation on populations derived from crosses of heterozygous individuals for del4 mutation at 3 mpf. Results were obtained from two different experiments, for a total number of 41 individuals. Statistical analysis has been performed using chi-square statistic test ($p = 0.0209$), comparing the mean distributions of *ap1s1* F2 genotypes and the expected Mendelian ratios. **(B) Survival curves for *ap1s1*^{del4/del4} individuals compared to controls.** Results were obtained from a single preliminary experiment on $n = 48$ individuals, both for mutants and wild-type line. Statistical analysis has been performed using the log-rank (Mantel-Cox) test (****: $p < 0.0001$). Abbreviations: wt: wild-type; del4: deletion of 4 nucleotides; dpf: days post fertilization; mpf: months post fertilization.

7.5 MORPHOLOGICAL ANALYSIS ON LARVAE

Development of zebrafish larvae has been investigated by comparing the morphological presentation of *ap1s1*^{del4/del4} individuals (n = 170) at 5 dpf and wild-type AB controls (n = 179) at the same developmental stages. Observed phenotypes have been divided into three different classes, indicated as “wild-type like”, “deflated swim bladder” and “severe”; they have been annotated and reported in Fig. 51. Distributions of the morphological phenotypes, with respect to the different experimental groups, did not show any significant difference according to a chi-square statistic test, thus suggesting that *ap1s1* mutants are able to develop properly until the larval stage, likewise the control larvae.

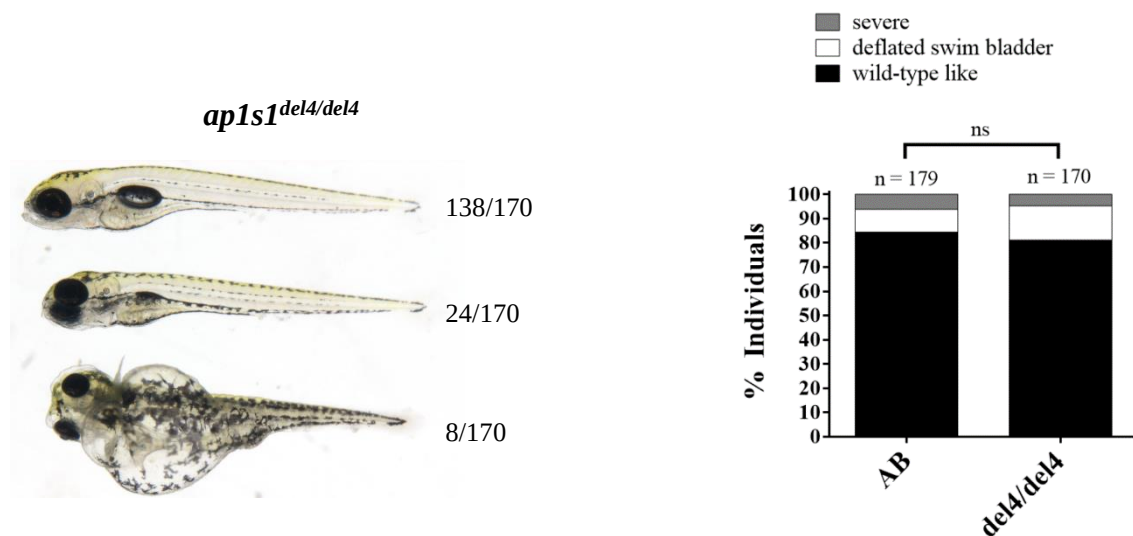


Figure 51. Morphological classes observed for *ap1s1* mutant individuals (left side). Represented larvae were generated by crossings between *ap1s1*^{del4/del4} individuals and compared to wild-type AB. For each class, the number of individuals in the total population analysed is shown alongside the representative image of the different morphologies. **Distribution of the observed morphological phenotypes in 5 dpf larvae (right side).** References to the fish line and genotypes are reported on the x axis (AB: wild-type AB; *del4/del4*: *ap1s1*^{del4/del4}), while total number of the observed larvae is reported above each bar. Represented data were obtained by two cumulated experiments and statistical analysis has been performed using chi-square test (ns: not significant; $p > 0.05$).

7.6 PRELIMINARY ANALYSIS OF *ap1s1* EXPRESSION THROUGH REAL TIME PCR

The effect of the Cas9-induced mutation (del4) on the expression level of the *ap1s1* gene was analysed through quantitative Real Time PCR technique on the cDNA obtained from pool of wild-type AB and *ap1s1*^{del4/del4} embryos at 3 dpf stage. Each pool was processed according to the RNA extraction protocol described in “Materials and methods”, followed by a cDNA reverse transcription reaction. The specific pair of qRT-PCR primers for the investigated gene is reported in “Materials and methods” (quantification was performed using *rpl13a* as internal control). Preliminary data generated by a first Real Time PCR experiment showed a decrease of 77.5% in the level of *ap1s1* expression in *ap1s1*^{del4/del4} individuals when compared to wild-type controls at 3 dpf, (Fig. 52). This result suggests that the transcript of the del4 allele is degraded by nonsense-mediated decay (NMD). Further analyses should be performed to confirm and to analyse the statistical significance of these data.

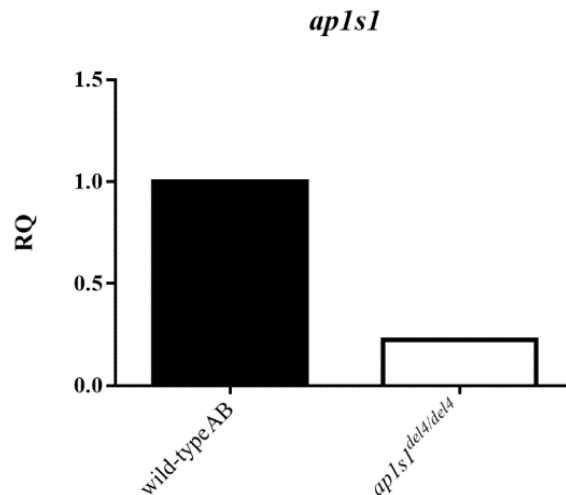


Figure 52. Preliminary analysis of qPCR on *ap1s1* mutant embryos at 3 dpf.

7.7 SPATIAL-TEMPORAL EXPRESSION OF NEUROGENESIS MARKERS

The spatial-temporal expression of relevant genes during the neurogenesis process was qualitatively and preliminary investigated to detect any phenotypic abnormalities in the development of the central nervous system (CNS). Preliminary RNA *in situ* hybridizations experiments were performed using specific antisense RNA probes for *pax2a* (30 hpf), *neurog1* (30 hpf), *neurod1* (24 hpf and 48 hpf) and *dlx2a* (48 hpf) neurogenesis markers (specific information for these genes are reported in “Materials and Methods”). In general, the expression of markers of the neurogenesis is maintained in the *ap1s1*^{del4/del4} mutant line, with subtle alterations not always defined.

Expression of *pax2a* at 30 hpf

Analysis of *pax2a* at 30 hpf (prim-15) revealed the expected expression pattern in the CNS of mutant individuals, although lower and more disorganized probe signal seems to characterize the *ap1s1*^{del4/del4} embryos when compared to wild-type AB at the level of the otic vesicle and the midbrain hindbrain boundary (Fig. 53).

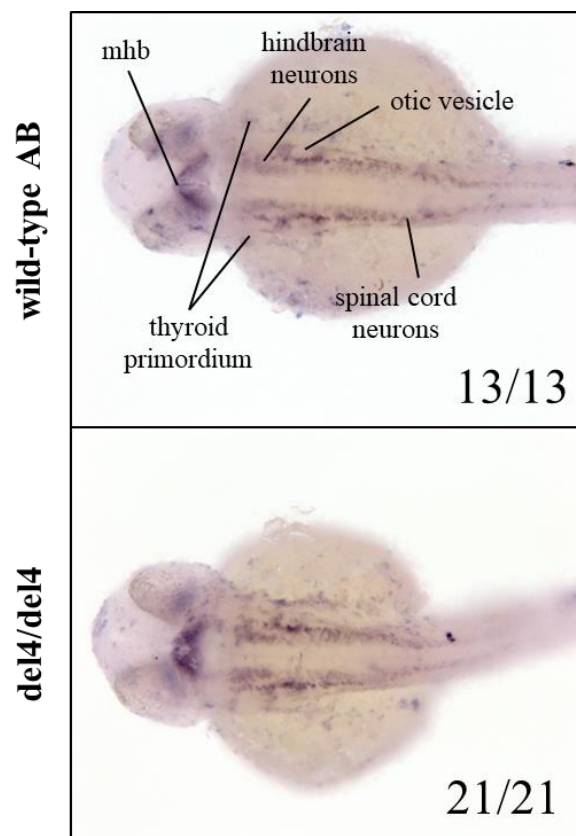


Figure 53. *In situ* hybridizations for *pax2a* at 30 hpf. Representative embryos are reported for the investigated mutant line (del4) and the wild-type AB line. Anatomical references are reported in wild-type AB control for comparison, while genotype reference is indicated on the left. The numbers of the analysed embryos are shown in figure.

Expression of *neurog1* at 30 hpf

Transcription factor *neurog1* has been investigated since it starts to be expressed from the very early neurogenesis, in particular at the gastrula embryonal stage; subsequently, its expression continues until the hatching/long-pec developmental stage.

Preliminary analyses at 30 hpf showed, in general, no difference of expression between wild-type and mutants in the brain regions of interest; in particular, these regions investigations were performed: from the lateral view (Fig. 54, left side), in the region corresponding to the anterior part of the brain, telencephalon, diencephalon, pretegmentum and anterior tegmentum, in the posterior hindbrain region and in the spinal cord neurons. In the dorsal views (Fig. 54, right side), slight differences at the level of the cranial ganglia can be appreciated between mutants and relative controls.

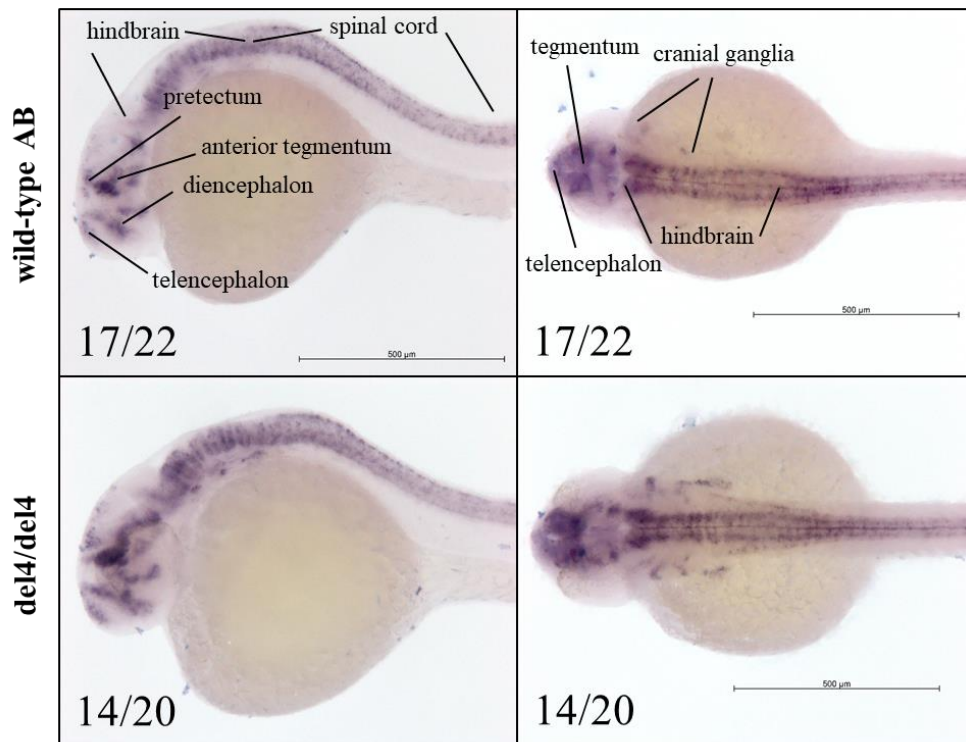


Figure 54. *In situ* hybridizations for *neurog1* at 30 hpf. Representative embryos are reported for the investigated mutant line (*del4*) and the wild-type AB line. Anatomical references are reported in wild-type AB controls for comparison, while genotype reference is indicated on the left. The numbers of the analysed embryos are shown in figure.

Expression of *neurod1* at 24 hpf and 48 hpf

neurod1 is a transcription factor that is activated after the expression of *neurogenin*, the gene product of *neurog1*, which represents its upstream regulator during neurogenesis process (Thomas, Ochocinska, Hitchcock, & Thummel, 2012). Preliminary analysis of *neurod1* expression at 24 hpf revealed no evident differences between mutants and the relative wild-type AB controls in the expected regions of the nervous system. Nevertheless, a slight weaker probe signal was observed in *ap1s1*^{del4/del4} individuals when compared to controls in the region of the anterodorsal/anteroventral lateral line/facial placodes ganglia (ad/av/f), optic placode and the posterior line ganglia, along with the anterior part of the brain, corresponding to the telencephalic (t) area (Fig. 55, up). *neurod1* was investigated also at later embryonal stages (48 hpf), revealing maintained expression in mutants in all the interested anatomic regions, ranging from the anterior part of the brain to the hindbrain organization (Fig. 55, down). However, the probe signal at the level of the rhombomeres was less evident in these hybridizations. Additional experiments should be performed to clarify the expression pattern of *neurod1* in this area.

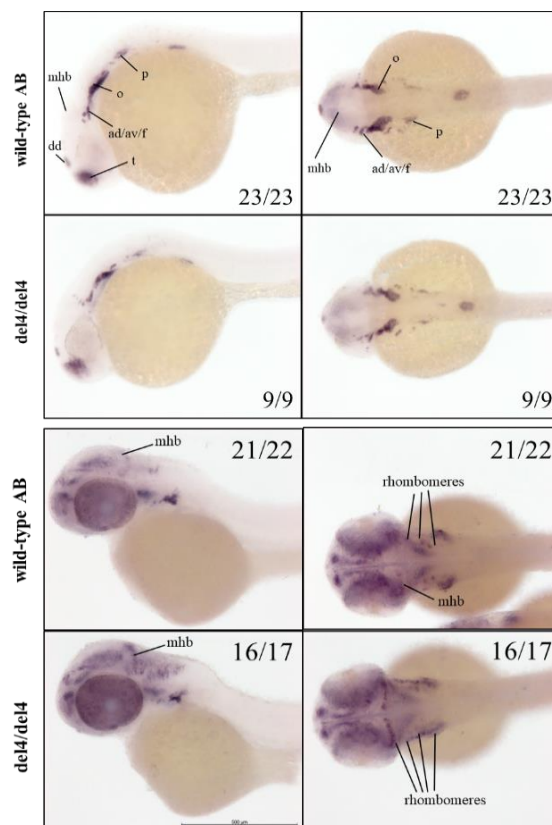


Figure 55. *In situ* hybridizations for *neurod1* at 24 hpf (upper panel) and 48 hpf (lower panel). Representative embryos are reported for *ap1s1* mutant line (del4) and wild-type AB. Anatomical references are reported for comparison, while genotype is indicated on the left. Abbreviations: ad/av/f: anterodorsal/anteroventral lateral line/facial placodes ganglia dd: dorsal diencephalon; mhb: midbrain hindbrain boundary; o: octaval/statoacoustic ganglia; p: posterior lateral line ganglia; t: telencephalon. The numbers of the analysed embryos are shown in figure.

Expression of *dlx2a* at 48 hpf

To highlight any alterations in the anterior portion of the brain further analyses have been performed by hybridizing both the mutants and the wild-type individuals with the antisense RNA probe for *dlx2a*, a transcription factor involved in several processes, including neurogenesis of the frontal brain. Preliminary experiments showed no expression pattern differences between *ap1s1*^{del4/del4} embryos and wild-type AB: the expression of *dlx2a* resulted maintained and correctly organized in the telencephalon, the dorsal prethalamus and in the hypothalamus (Fig. 56, dorsal view). In Fig. 56. (lateral view) *dlx2a* expression in the region of the pharyngeal arches can be appreciated.

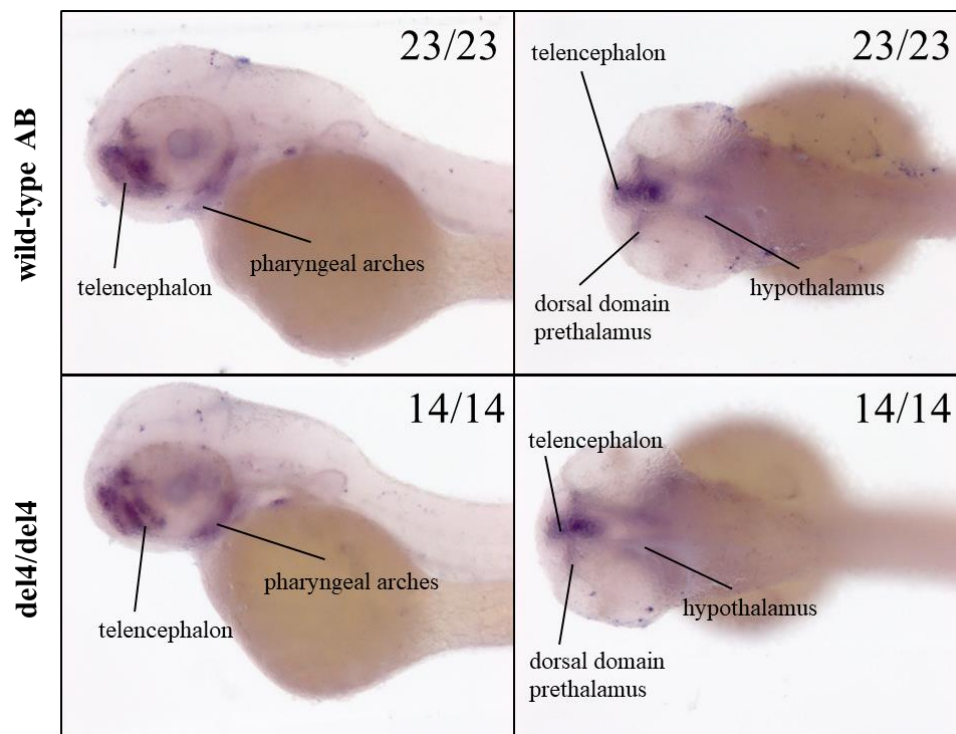


Figure 56. *In situ* hybridizations for *dlx2a* at 48 hpf. Representative embryos are reported for the investigated mutant line (del4) and the wild-type AB line. Anatomical references are reported for comparison, while genotype reference is indicated on the left. The numbers of the analysed embryos are shown in figure.

7.7.1 SPATIAL-TEMPORAL EXPRESSION OF *myod* DURING EMBRYONAL DEVELOPMENT

Patients with MEDNIK syndrome present a wide clinical picture with systemic involvement. Although there is no direct evidence of correlation between the pathogenic mutation and a muscular phenotype, it was decided to investigate the possible impact that *ap1s1* loss of function mutations may have on muscle development. In particular, *ap1s1*^{del4/del4} embryos at 16.5 hpf stage (20 somites) were subjected to *in situ* hybridization using an antisense RNA probe for *myod*, a relevant transcriptional factor involved in muscle development. A total of 43 wild-type AB embryos and 25 *ap1s1*^{del4/del4} mutants have been analysed. No significant differences have been observed by the comparison between the two experimental groups, indicating that *myod* is regularly expressed in the early developmental stages even in the mutant line (Fig. 57).

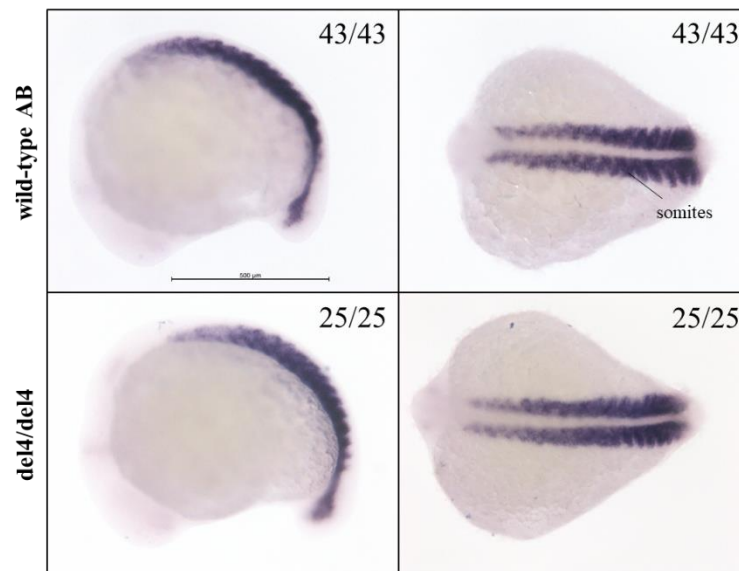


Figure 57. *In situ* hybridizations for *myod* at 16.5 hpf. Representative embryos are reported for wild-type AB and the investigated mutant line (del4). Anatomical references are reported in wild-type AB controls for comparison, while genotype reference is indicated on the left. The numbers of the analysed embryos are shown in figure.

Further analyses should be performed to investigate muscle development and organization at later developmental stage, *e.g.* by using phalloidin staining protocol at 48 hpf. Nevertheless, preliminary qualitative observations in birefringence of *ap1s1*^{del4/del4} embryos after 48 hours of development showed no obvious muscle phenotypes when compared to wild-type controls (data not reported). Birefringence is a method based on the use of lenses able to polarize the light. This analysis permits the observation of the macroscopic organization of the muscle in embryos at 48 hpf, since the polarized light is absorbed by the fibres depending on their macroscopic arrangement; disorganization of fibres will result in a lower absorbed light as it is not able to pass through the sample.

7.8 BEHAVIOUR ANALYSIS ON LARVAE

Preliminary behavioural analysis on 5 dpf larvae was performed using a standard protocol to evaluate the animal response to the “dark-light” visual stimulus (described in “Materials and Methods”). Individuals subjected to this test have been obtained from crossing between *ap1s1*^{del4/del4} adult fish (n = 96) and compared to wild-type AB larvae (n = 96), respectively. Data generated through EthoVision XT (Noldus) have been analysed and reported in graph (Fig. 58). Locomotor activity of each experimental group was evaluated calculating the total distance moved from central point at regular intervals of time (10 min; Fig. 58A). The same parameter was also evaluated as average distance travelled for each experimental group in the corresponding phases (Fig. 58B). Results showed a significant higher predisposition of *ap1s1* mutants to travel longer distances when compared to controls ($p < 0.0001$); thus, mutants were characterized by faster movements in the different phases.

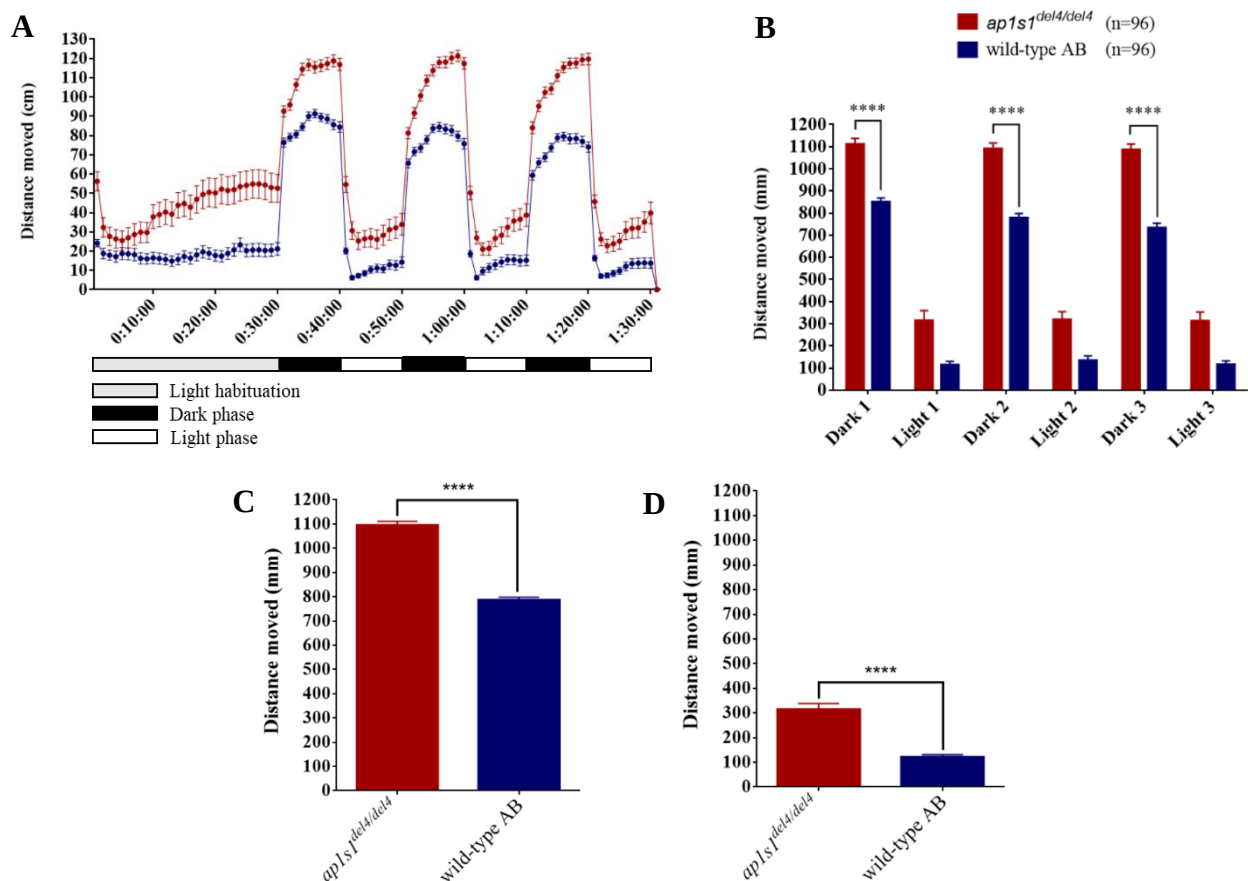


Figure 58. (A) Distance moved by fish at 5 dpf in the different experimental phases at intervals of 10 minutes each. Dark phases were characterized by higher distance travelled; moreover, *ap1s1*^{del4/del4} seemed to respond to dark stimulus by travelling longer distances at higher velocity when compared to wild-type AB. **(B) Total distance moved by fish at 5 dpf in the different experimental phases.** Data reported are the result of a single experiment with larvae generated from crossing between *ap1s1*^{del4/del4} individuals (n = 96) compared to wild-type AB (n = 96). Statistical analysis has been performed using ordinary Two-way ANOVA test (****: $p < 0.0001$). **(C) Total distance moved by fish at 5 dpf in cumulated three dark phases** (****: $p < 0.0001$, unpaired t- test). **(D) Total distance moved by fish at 5 dpf in cumulates three light phases** (****: $p < 0.0001$, unpaired t- test).

The different behaviour observed between mutants and controls does not appear to be characteristic of a particular phase and the cumulative analysis of the average distance covered during the three dark phases (Fig. 58C) and the three light phases (Fig. 58D), respectively, showed a statistically significant difference ($p < 0.0001$), according to parametric unpaired t-test. The described experiment has been performed by disposing the two different groups of larvae (wild-type and mutants) in two different 96-well plates, which were inserted one after the other inside the tracking instrument. For accuracy, the experiment should be repeated in more formal conditions, inserting inside the same plate controls and mutants. Up to now, replicates have been performed by comparing this experiment to behavioural tests performed during treatments with copper solutions (see below), confirming the phenotypical behaviour of wild-type and *ap1s1* mutants. Further investigations have been performed at earlier developmental stages (30 hpf) and data are being processed to assess whether *del4* mutants of *ap1s1* show visible differences at the level of the primary motor behaviour, which consists of spontaneous tail coiling movements involving side-to-side contractions of the trunk.

7.9 EFFECTS OF TREATMENTS WITH COPPER SOLUTIONS ON *ap1s1* MUTANTS

It is known from literature that deleterious mutations of *AP1S1* in humans lead to defective copper metabolism; thus, very preliminary experiments have been performed by treating *ap1s1*^{*del4/del4*} individuals and wild-type controls with high doses of copper solutions (500-1100 µg/L of CuSO₄ · 5 H₂O dissolved in common fish water solution) from 0 hpf to 5 hpf, replicating the treatment conditions published in the literature for copper salts (Johnson, Carew, & Sloman, 2007). Treated embryos have been observed and characterization has been performed by analysing the mortality and the hatching ability of the embryos from 48 hpf to 72 hpf. Moreover, preliminary studies on cell death mechanisms have been performed through acridine orange staining at 72 hpf, while evident alteration of the locomotor behaviour has been investigated at 5 dpf.

The experiments described below were carried out in the final part of the PhD period and should therefore be repeated in order to validate or not the results obtained. In addition to that, further experimental approaches will be necessary, considering the recent guidelines for the validation of chemicals in zebrafish (OECD, 2013). The guidelines provide that the percentages of embryos for which at least one of the endpoints is registered should be linked to the exposure concentrations, so that the results can be processed and the value calculated of the LC₅₀. However, the tests described in this thesis did not generate sufficient data to perform a statistical evaluation of the danger of the treatment solution. For this reason, general observations have been reported on the effects observed in embryos and larvae, in particular regarding the exposure solutions at higher concentrations (as reported in (Johnson, Carew, & Sloman, 2007)).

Mortality and Hatching

During the 72 h test period, mortality and hatching of both wild-type controls and *ap1s1* mutants for each concentration was recorded at 2, 3, 5, 24, 26, 48 and 72 hpf, as performed by Johnson et al., 2007. Death in early embryos was classified as those exhibiting a marked loss in translucency, change in colouration or degree of fungal infection (Ozoh, 1979) (Scudder, Carter, & Leland, 1988). A cessation in heartbeat was the criteria for death in more developed embryos and larvae (Shazili & Pascoe, 1986). Wild-type AB seemed to be unaffected by the treatments (Fig. 59, left), unlike what is described in literature at the same concentrations of exposure, where increasing doses of copper solutions are accompanied by progressively higher mortality rates. This type of trend occurs, as expected, in *ap1s1*^{del4/del4} treated embryos, at the higher dose (1100 µg/L) showing higher mortality rates when compared to the lower dose of copper solution (500 µg/L) and the non-treated individuals (Fig. 59, right.). Despite this, levels of mortality after the exposure to copper solutions are extremely low and not comparable with those reported in literature. It will then be necessary to repeat the experiments using higher copper concentrations in order to obtain more relevant percentages of cell death in the different genotypic classes.

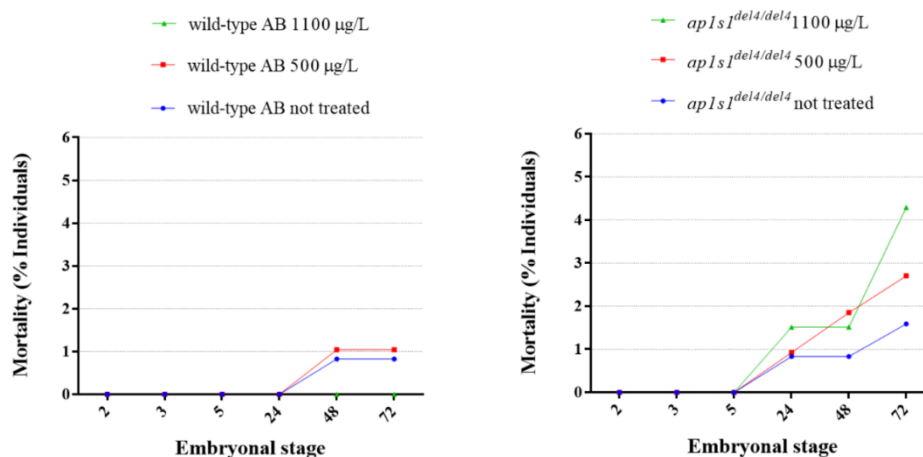


Figure 59. Analysis of mortality in copper treated embryos. Mortality was evaluated in wild-type AB (left) and *ap1s1*^{del4/del4} embryos (right) and reported for each concentration of copper solution. Data were generated analysing at least 6 replicates with 9-13 embryos for each dose.

Increasing copper concentrations had an inhibitory effect on hatching capacity (Fig. 60), with a general tendency of *ap1s1* mutant embryos and wild-type to remain in the chorion for longer time at higher doses of solution (comparison between wild-type and mutants was performed by One-way ANOVA statistic test, without showing a significant difference between the groups; $p > 0.05$). In particular, treatments with the lower dose of copper solution (500 µg/L) did not show significant differences when compared to controls within each treated line.

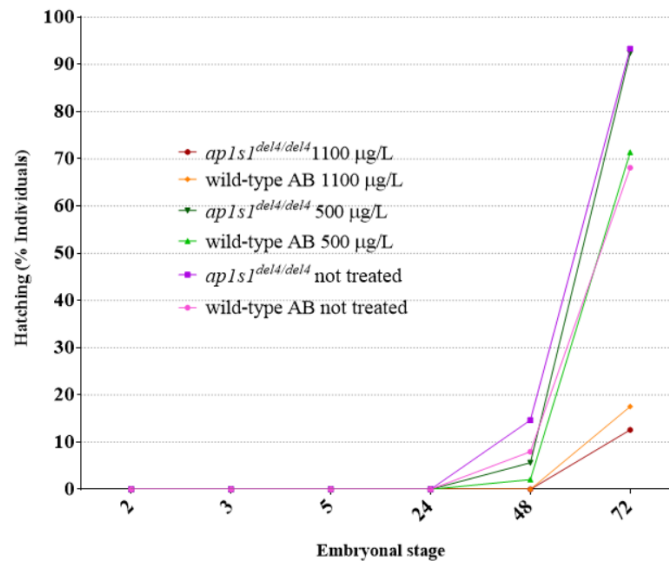


Figure 60. Hatching of zebrafish embryos exposed to each copper treatment. Data were generated analysing at least 6 replicates with 9-13 embryos for each dose.

Acridine orange staining

Both *ap1s1*^{del4/del4} and wt were subjected to acridine orange staining to evaluate the amount of apoptotic cells (Fig. 61); replicates of 5 embryos for each conditions were evaluated, for a total number of 20 individuals for each dose and control groups, without revealing significant differences within the experimental groups and conditions (ANOVA, $p > 0.05$). In particular, the number of apoptotic cells has been evaluated along the entire anterior-posterior development of the larvae, starting from the first somite, and calculated on the left side of each individual. In Fig. 61 is reported the average number of dead cells that were counted in $n = 5$ embryos for each experimental condition when comparing the untreated embryos and the highest doses of copper solution (1100 µg/L) for both wild-type AB and *ap1s1* mutants. Representative images of stained embryos are reported on the left.

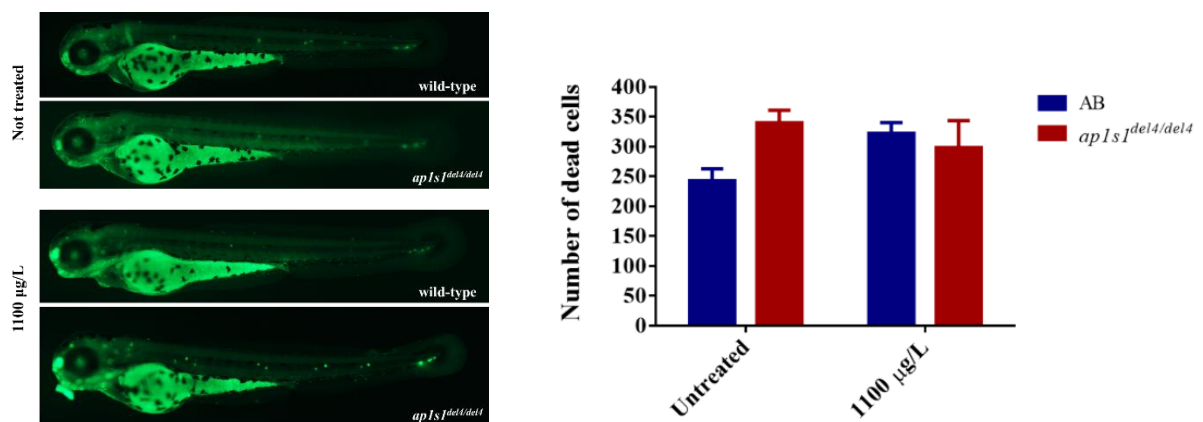


Figure 61. Acridine orange staining in copper treated embryos. Representative images of the staining are reported on the left (a total number of 20 embryos for each condition has been evaluated). Representative quantification of dead cells on $n = 5$ embryos for each experimental condition is reported on the left.

Behaviour analysis on larvae treated with copper solutions

Behavioural analyses have been performed, confirming the previously obtained results (described above) with respect to the untreated control larvae, respectively wild-type and *ap1s1^{del4/del4}*. Nevertheless, it was possible to appreciate how at the highest dose of copper solution (1100 µg/L) a specific effect on the locomotor activity of the mutants could be observed, characterized by significantly shorter distances travelled when compared to wild-type at the same experimental conditions, as well as their littermates treated with the lower copper dose (Fig. 62). On the contrary, locomotor activity of wild-type AB larvae revealed no significant differences within the different doses of treatment with copper solutions (ANOVA, $p > 0.05$).

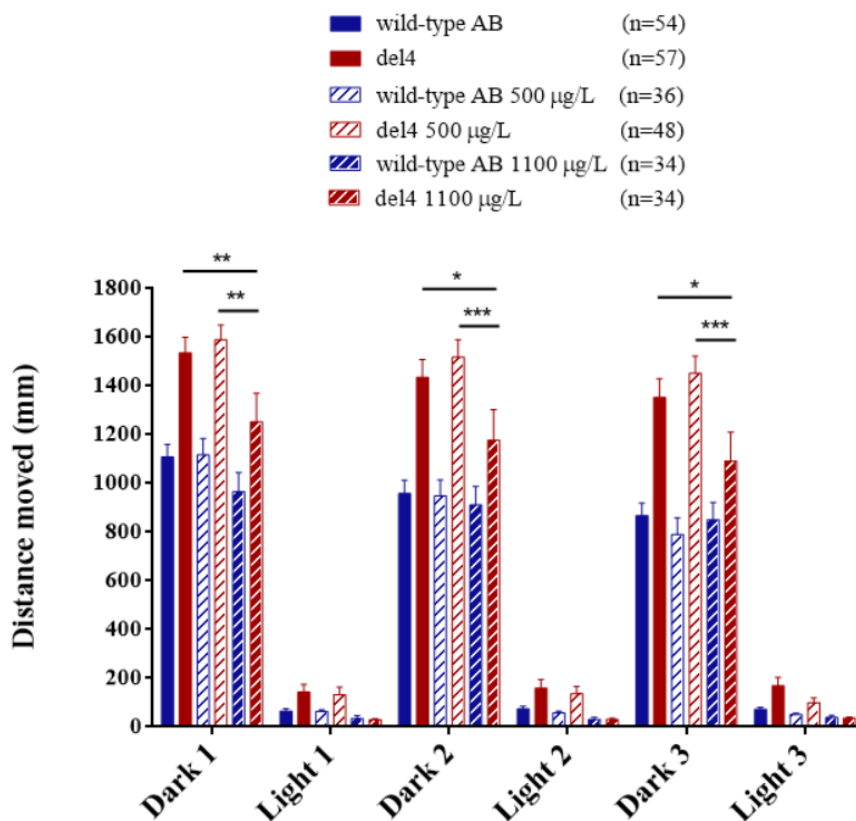


Figure 62. Locomotor activity of wild-type AB and *ap1s1* mutant larvae (5 dpf) treated with copper solutions. Total distance moved (5 dpf) in the different experimental phases are reported. Total analysed individuals are reported next to each genotype reference. Statistical analysis has been performed using Ordinary Two-way ANOVA test (***: $p < 0.001$).

8 RESULTS FOR *ap1s2* KNOCKOUT LINES

8.1 *IN SILICO* ANALYSIS OF *ap1s2* GENE

One of the aims of this thesis is the integration of previous knowledge on AP-1 complex, deepening the role of σ 1B-coding gene in zebrafish. Since mutations in human *AP1S2* identified in Pettigrew syndrome patients mostly are loss of function variations, we anticipated that knockout of *ap1s2* in zebrafish could lead to the generation of a suitable vertebrate model of the disease.

The nature of the σ 1B-coding gene was investigated using several bioinformatics tools and genome sequence databases. A query of the ZFIN database was performed using the *AP1S2* gene name, revealing the presence of a single zebrafish gene, *ap1s2*, mapping on chromosome 11 (<http://zfin.org/ZDB-GENE-030131-5448>).

A sequence homology search using as a query the human *AP1S2* protein (NP_001259000) performed on *D. rerio* genome assembly (Genome Reference Consortium Zebrafish build 11 - GRCz11) using BLAT (Basic Local Alignment Search Tool-Like Alignment Tool) from the UCSC Genome Browser (<https://genome.ucsc.edu/cgi-bin/hgBlat>) led the identification of two identical entries corresponding to the *ap1s2* gene indicating that, based on the current genome sequencing data, the region harbouring the gene could be located in either one or in another locus on chr 11. Other entries have been identified by the homology search one corresponding the *ap1s1* gene on chromosome 7, and two for *ap1s3a* and *ap1s3b* gene paralogs, on chromosome 15 and 2, respectively. Zebrafish *ap1s2* encodes for a 157 aa protein with 91.7 % identity to the human counterpart (160 aa) in a global alignment. Based on these data we started our experimental work aimed at generating inactivating mutations in the zebrafish *ap1s2* gene. Only several months after our initial *in silico* analysis we decide to revise and update the bioinformatic data. This led to the identification of an additional putative *ap1s* paralog in zebrafish that had initially been overlooked, probably because its genomic sequence is partial. The gene is named *zgc:162858* (<http://zfin.org/ZDB-GENE-030131-7909>) and is located on chr 23. Although its genomic sequence is not complete in the GRCz11 assembly, a NCBI Reference Sequence exists (NP_001082809.1) obtained from the sequencing of a full-length zebrafish cDNA clone. *zgc:162858* encodes for a 181 aa protein with 89,8% identity to the human counterpart and 82.8% to zebrafish *Ap1s2* based on a Clustal Omega alignment at EMBL-EBI (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>).

A phylogenetic analysis was performed using NGPhylogeny.fr server (<https://ngphylogeny.fr>) including the five zebrafish proteins obtained from BLAT at UCSC (Ap1s1, Ap1s2, Zgc:162858, Ap1s3a and Ap1s3b) together with the sequence of human AP1S1, AP1S2 and AP1S3 polypeptides. Fig. 63 shows a phylogram tree layout that indicates that Ap1s2 appears to be the zebrafish polypeptide closest to human AP1S2.

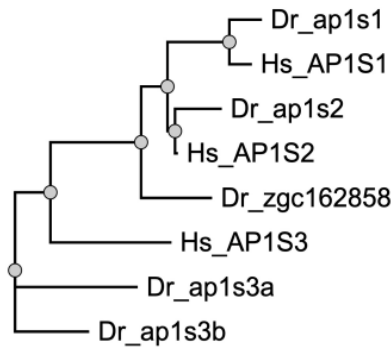


Figure 63. Phylogram tree generated by the NGPhylogeny.fr phylogenetic analysis tool starting from the same AP1S proteins used for multiple sequence alignment. Abbreviation: Dre: *Danio rerio*; HS: *Homo sapiens*.

Genomic organization of *ap1s2* in *D. rerio* was investigated using the University of California, Santa Cruz (UCSC) – Genome Browser, showing the localization of the gene on chromosome 11 and the length in base pairs (Fig. 64). In *D. rerio* the gene is composed of 5 exons and 4 introns, with a RefSeq transcript of ~1700 bp (NM_205558.1; ENSDART00000054837.8) encoding for a protein of 157 amino acids (ENSDART00000054837.8; NP_991121).

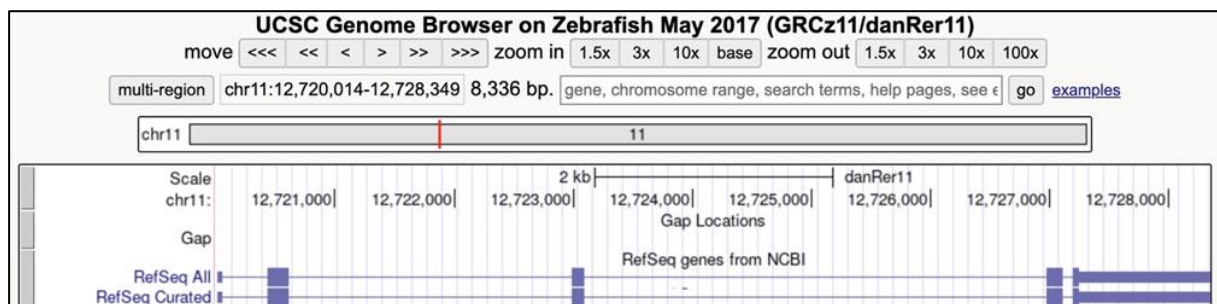


Figure 64. Genomic organization of *ap1s2* gene within zebrafish chromosome 11 (UCSC Genome Browser).

The exon-intron organization of *zgc:162858* on chromosome 23 is incomplete in the GRCz11 genome assembly from the UCSC Genome Browser, with the region that include the putative second exon missing (data not shown). Besides the RefSeqs, additional isoforms have been annotated and reported both for *ap1s2* and *zgc:162858* transcripts, all protein-coding and likely originated either from events

of alternative splicing or the usage of alternative promoter, as emerged mainly from RNA-Seq studies. With reference to the recently identified paralogous gene, the organization of the different isoforms is not yet clear, as conflicting data are present in various databases.

A synteny analysis of the *AP1S2* locus performed using Genomicus web server (<https://www.genomicus.bio.ens.psl.eu>) identified conserved limited synteny blocks for both *ap1s2* (chromosome 11) and *zgc:162858* (chromosome 23) and the genomic region of the X-chromosome harbouring human, mouse and rat *AP1S2* genes (Fig. 65). Sequence homology and synteny data both indicate that the two *AP1S2*-like genes exist in zebrafish, likely as the result of the genome-wide duplication event occurred in teleost fish during evolution, resulting in a gene on chromosome 11 (*ap1s2*) and one on chromosome 23 (*zgc:162858*), with a subsequent selective loss of some of the flanking genes on both chromosomes.

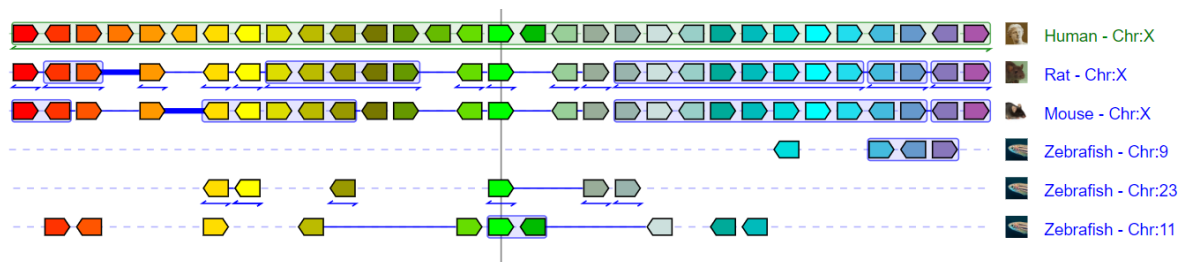


Figure 65. Synteny analysis for *ap1s2* in *H. sapiens*, *R. norvegicus*, *M. musculus* and *D. rerio* performed using Genomicus web server.

Sequence identity between *ap1s2* and *zgc:162858* was analysed using Clustal Omega multiple alignment tool at EMBL-EBI (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), revealing a high level of sequence identity both for cDNA and protein sequences. In particular, alignment between Ap1s2 (NP_991121) and Zgc:162858 (NP_001082809) showed a 82.80% of sequence identity, excluding the 24 amino acids at the 5' of the *ap1s2* paralogue gene (Fig. 66). Fig. 65 also highlights the presence of a single mismatch between the sgRNA-EX2 target sequence on *ap1s2* and the corresponding sequence on *zgc:162858*.

ap1s2_NM_205558.1	tatgccagctgttactttgtctgtgtctgtggaggaccaggaaaatgagttgataactctg	240
zgc_001089340.1	tacgtcagctgtatttctgtctgccatcaggaccaggacaacgagctgatcacgctg	240
	*** ** ***** ** ***** ** * ***** ** ** ** *	
ap1s2_NM_205558.1	gagatcatccaccgatactgtggagctctggacaagtattttggcagtggtgtgagctg	300
zgc_001089340.1	gagatcatccaccgatactgtggagctctggacaatacttcggcagcgtgtgtgggctg	300
	***** ** ***** ** ***** ** * ** *	
ap1s2_NM_205558.1	gacattatcttcaactttgagaaagcctattacattctggatgagtttatactggcggt	360
zgc_001089340.1	gacataatcttcaacttcgagaaggcctacttcatctggacgagttctctgctggcgga	360
	***** ***** ** ** *	
ap1s2_NM_205558.1	gaagcccaagagacatcgaagaagaacgttctaaaggccatagagcaagcggacatgtg	420
zgc_001089340.1	gaagctcaggagacgtccaagaagaacgtgctgaaggccatcgagcagcgccactactg	420
	***** ** ***** ** ***** ** ***** ** ** *	

CLUSTAL O(1.2.4) multiple sequence alignment

Hsa_AP152	MQFMLLFSRQGLKRLQKWVPLSDKEKKKITRELVTQTLARKPKMCSFLEWRDLKIVYKR	60
Dre_Ap1s2	MQFMLLFSRQGLKRLQKWVPLSDTKKKISREVIQMLARKPKMCSFLEWRDLKIVYKR	60
Dre_Zgc:162858	MQFMLLFSRQGLKRLQKWVPLCDKEKKKITRELVTQTLARKPKMCSFLEWRDLKIVYKR	60
	***** ** ***** ** ***** ** ***** ** *****	
Hsa_AP152	YASLYFCCAIEDQDNELITLLEIIHRYVELLDKYGVSVCLEIDIINFKEKAYILDEFLLGG	120
Dre_Ap1s2	YASLYFCCAIVEDQENELITLLEIIHRYVELLDKYGVSVCLEIDIINFKEKAYILDEFILGG	120
Dre_Zgc:162858	YASLYFCCAIEDQDNELITLLEIIHRYVELLDKYGVSVCLEIDIINFKEKAYILDEFLLGG	120
	***** ** ***** ** ***** ** ***** ** *****	
Hsa_AP152	EVQETSKKNVLKATIEQADLLQEEAETPRSVLEEIGLT	157
Dre_Ap1s2	EAQETSKKNVLKATIEQADMLQEEAETPRSVLEEIGLT	157
Dre_Zgc:162858	EAQETSKKNVLKATIEQADLLQESQHEWGGLANDETL	157
	* ***** ** ***** ** ***** ** *****	

Figure 66. Upper Panel: sequence alignment between a portion of corresponding regions of *ap1s2* (NM_205558.1) and *zgc:162858* (NM_001089340.1) RefSeq transcript sequences. The sgRNA-EX2 target sequence is indicated in red and the single nucleotide mismatch (A>C) is labelled in orange. **Lower panel: sequence alignment between Ap1s2 (NP_991121.1) and Zgc:162858 (NP_001082809, excluding the 24 amino acids at the 5' of the protein sequence).** Amino acids are labelled in different colours according to their different chemical properties. Sequence alignments were performed with Clustal Omega at EMBL-EBI.

8.2 EXPRESSION ANALYSES AT DIFFERENT DEVELOPMENTAL STAGES THROUGH WHOLE MOUNT *IN SITU* HYBRIDIZATION

According to a RNA-Seq analysis of a wide pattern of zebrafish genes (up to 23,642 genes) (White, et al., 2017), *ap1s2* (ENSDARG00000058504) expression is higher in all embryonal stages when compared to *zgc:162858* (ENSDARG00000098990). Data are reported in Fig. 67 and expressed in TPM (transcript per million). TPM are calculated from the number of reads that mapped to each particular gene sequence taking into account the gene length and the sequencing depth.

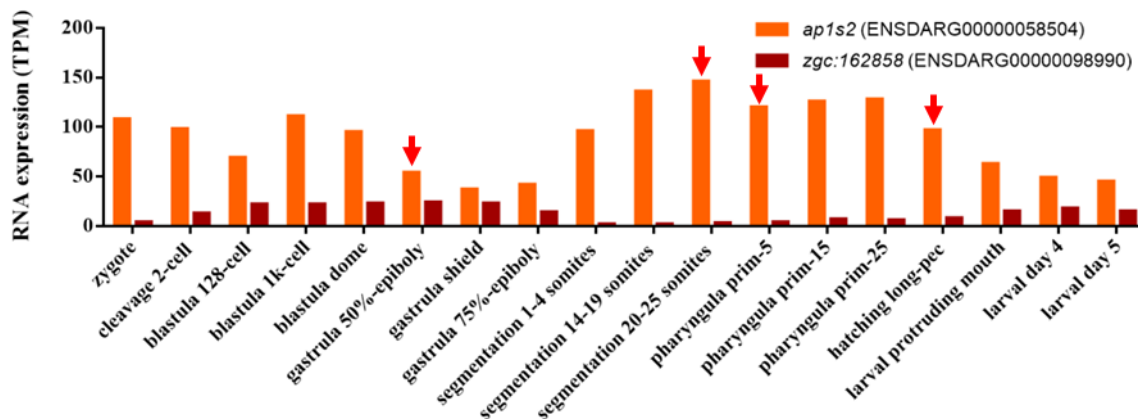


Figure 67. Expression levels of *ap1s2* (ENSDARG00000098990) and *zgc:162858* (ENSDARG00000058504) transcripts, expressed at different zebrafish embryonal stages (White, et al., 2017). Red arrows indicate the different developmental stages that were analysed through whole mount *in situ* hybridizations.

In situ hybridizations were performed on wild-type AB embryos at different embryonal stages (indicated in Fig. 66) to investigate the spatial temporal expression of *ap1s2* using an antisense RNA probe that specifically binds *ap1s2* mRNA (see “Materials and Methods”).

8.2.1 SPATIAL-TEMPORAL ANALYSIS OF *ap1s2* THROUGH *IN SITU* HYBRIDIZATION

The *ap1s2* antisense and sense probes were synthesized starting from a 550 bp fragment of *ap1s2*, including the coding portion of the third exon of *ap1s2* which is rather divergent from *zgc:162858* and does not include repetitive sequences. The expression of *ap1s2* transcript was analysed by performing *in situ* hybridization on wild-type AB embryos at different timing points: two earlier developmental stages, corresponding to 5.25 hpf (50% epiboly) and 16.50 hpf (15 somites), and two later developmental stages, corresponding to 24 hpf (prim 5) and 48 hpf (long pec). Hybridization experiments showed a more diffuse and ubiquitous *ap1s2* expression at the early stages, which became more related to the central nervous system (CNS) at 24 hpf and 48 hpf, with particular expression in the midbrain hindbrain boundary (mhb) region. At 24 hpf (prim 5) *ap1s2* expression was localized also in the tectum, in the cerebellum and at the level of the rhombomeres, while a weaker and more diffuse signal was observed in the telencephalon (anatomical references of the zebrafish brain are reported in Fig. 68)³.

In situ hybridization using the specific synthesized *ap1s2* antisense RNA probe were performed also on mutant embryos (as described below) to assess the effect on *ap1s2* transcript stability in CRISPR/Cas9 generated lines.

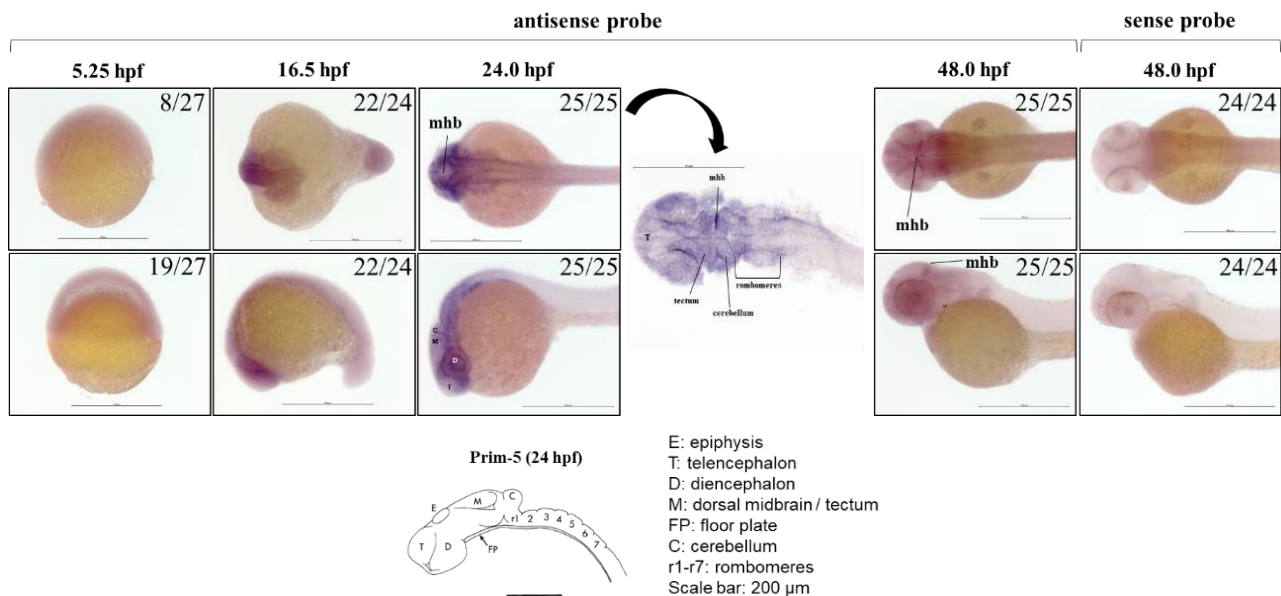


Figure 68. *In situ* hybridizations on AB wild-type embryos using *ap1s2* antisense and sense RNA probes. Hybridization experiments were performed on *wild* AB line at different timing points: from the left, embryos at 5.25 hpf (50% epiboly), 16.5 hpf (15 somites), 14.0 hpf (prim 5) and 48 hpf (long pec) are reported. The ubiquitous expression characterizing the earlier developmental stages is probably due to the maternal expression of the transcript that is still present. At later developmental stages, compartmentalization of the signal is more related to the developing central nervous system. Lateral and flat view at 24 hpf demonstrate the remarkable *ap1s2* expression in defined brain regions (anatomical references 24 hpf are reported for comparison). Representative hybridization at 48 hpf using *ap1s2* sense probe is reported on the right for comparison with the same experiments using antisense probe.

³ Sense probe was tested both at 24 hpf and 48 hpf, showing weaker or barely absent signal when compared to antisense hybridizations.

8.3 GENERATION OF *ap1s2* KNOCKOUT LINES IN ZEBRAFISH USING CRISPR/Cas9

We generated loss-of-function (LOF) mutations in the coding region of *ap1s2* gene using the CRISPR/Cas9 technology (see “Materials and Methods”). After microinjection of a synthetic guide RNA provided by SYNTHGO (targeting the second coding exon of *ap1s2* and thus referred to as *ap1s2*-EX2 sgRNA) and Cas9 protein (EnGen™ Cas9 NLS; *S. pyogenes* – New England Biolabs), zebrafish embryos have been incubated at 28 °C and allowed to grow to the 5 dpf developmental stage. Subsequently, genotyping on non-denaturing polyacrylamide (12%) gel has been performed on DNA extracted from the embryos. This was followed by Sanger sequencing to validate the presence of *ap1s2* mutations in selected embryos. Relevant information about microinjection are reported in Table 3.

DATE	sgRNA	MICROINJECTED EMBRYOS	MORTALITY 24 hpf	GENOTYPING
18/04/2019	<i>ap1s2</i> -EX2 (500 pg/embryo)	148	58/148 (39.19% ⁴)	16 larvae + 10 adults

Table 3. Scheme of *ap1s2*-EX2 injection in wild-type AB embryos at one-cell stage.

Electrophoretic analysis of genotyping PCR products from 5 dpf embryos (16 samples) on native polyacrylamide (12%) gel revealed the presence of several DNA molecules of higher molecular weight compared to the expected 112 bp-amplification product (not shown). This suggests the presence of possible heteroduplex between complementary DNA molecules whose sequence are not 100% identical (*e.g.* wt and mutated sequence). These data indicate that the sgRNA and the experimental procedure used for the *ap1s2* genome editing approach were effective. Based on this observation, the remaining embryos have been left to grow until they reached the adult stage. DNA extraction from adult tails has been performed for 10 fish, followed by a second electrophoretic analysis under non-denaturing condition. Also in this case, several bands of lower intensity were detected migrating above the expected amplification product in all the investigated samples but not in the non-injected control (Fig. 69). None of these adult fish showed an altered visible phenotype.

⁴ Non-injected (NI) embryos usually show a mortality rate around 20%.

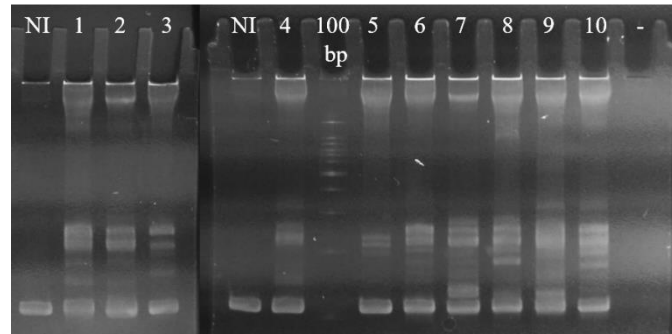


Figure 69. Electrophoresis of genotyping PCR products from DNA extracted from tails of injected AB adult fish on non-denaturing polyacrylamide (12%) gel. DNA molecules migrating above the expected amplification product suggest the presence of heteroduplex. NI: non-injected control (PCR on DNA extracted from AB wild-type fish).

Some of the PCR samples (n. 4, 6, 7, 8, 9, 10 and a non-injected embryo) have been sequenced using the *ap1s2* forward primer (see “Materials and Methods”; Fig. 70). The DNA sequences were analysed using the bioinformatic tool Poly Peak Parser; besides this, a sequencing chromatogram that presents double or indecipherable peaks starting from a region surrounding the PAM is a good indication of a samples composed by a mix of different DNA molecules, hopefully wild-type and mutated ones.

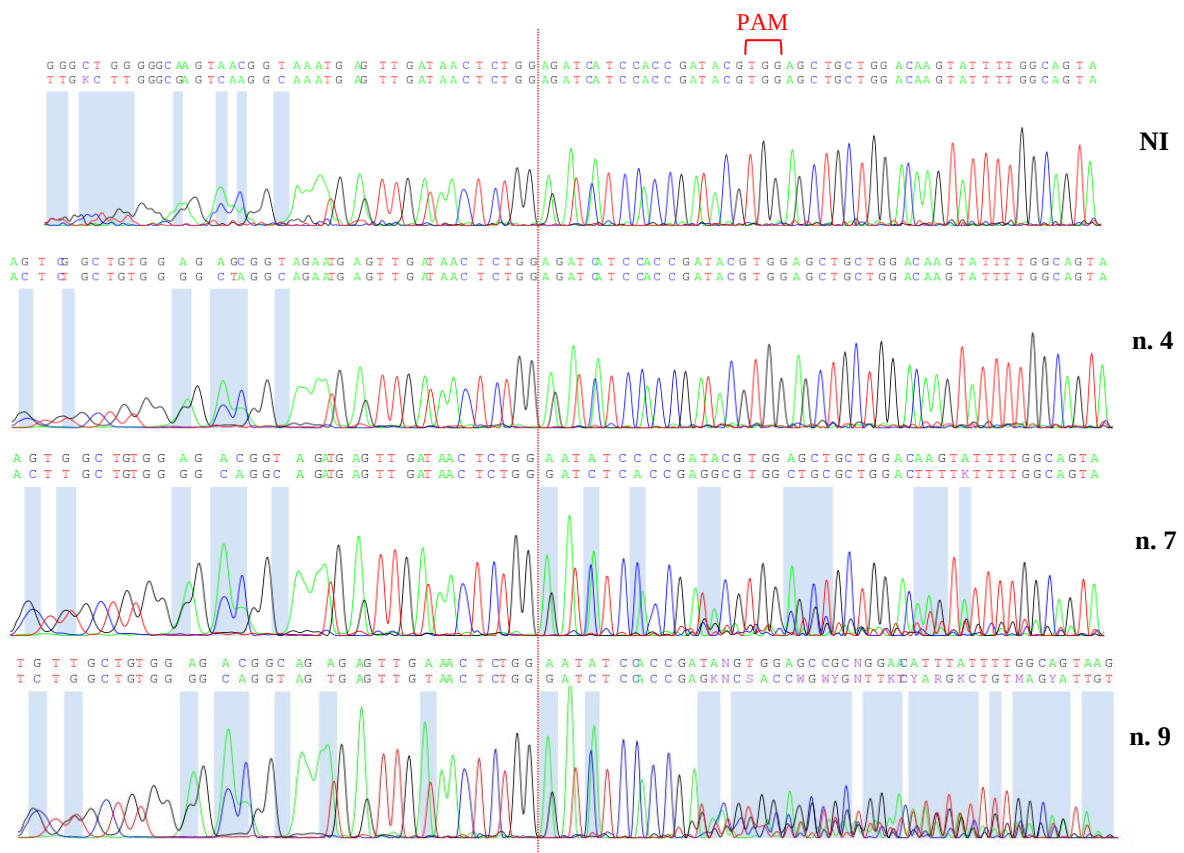


Figure 70. DNA sequencing chromatograms of genotyping PCR products from adult fish derived from embryos injected with the SYNTHOGO-EX2 sgRNA (4, 7, and 9) and from a non-injected AB wild-type (NI). The PAM sequence adjacent the sgRNA target site is shown in red (TGG).

Except for sample n. 4 and the non-injected control (NI), all the other samples show these characteristics, with n. 9 representing the most promising founder. These data suggest the presence of mutational events in *ap1s2* induced by our genome editing approach.

Once reached the sexual maturity (~3 months), it was possible to identify the gender of the fish selected for sequence analysis, which resulted to be all males. Founder n. 9 has been crossed with two wild-type AB females to obtain heterozygotes (F1 generation) for the CRISPR/Cas9-induced mutations. Fertilized eggs have been collected and observed until 5 dpf; at 24 hpf mortality rate (n = 20/112, 17.86%) was not considerably different with respect to NI embryos (~20%). 16 larvae at 5 dpf have been sacrificed and their DNA subjected to PCR genotyping (7 of those showed altered morphology), while the remaining ones have been transferred to a proper tank in the aquarium system. Electrophoresis on non-denaturing polyacrylamide (12%) gel showed the presence of 6 putative heterozygote embryos out of 16 (37.5%; not shown).

An important consideration is that founder fish are often a genetic “mosaic”, as a result of the delayed action of the genome editing mechanism, the persistent activity of the CRISPR/Cas9 system after the one-cell stage, or the variety of DNA-repair mechanisms. Cells and tissues derived from different/independent genome editing events may have different genotypes, thus yielding mosaicism in founders not only in somatic tissues but also in the germline. One advantage of mosaicism is that it can lead to the generation of various mutant strains with different nucleotide changes that can differently affect the gene product. Thus it has not been surprising that Sanger sequencing revealed the presence of different *ap1s2* mutations in fish of F1 generation from a single founder (Fig. 71).

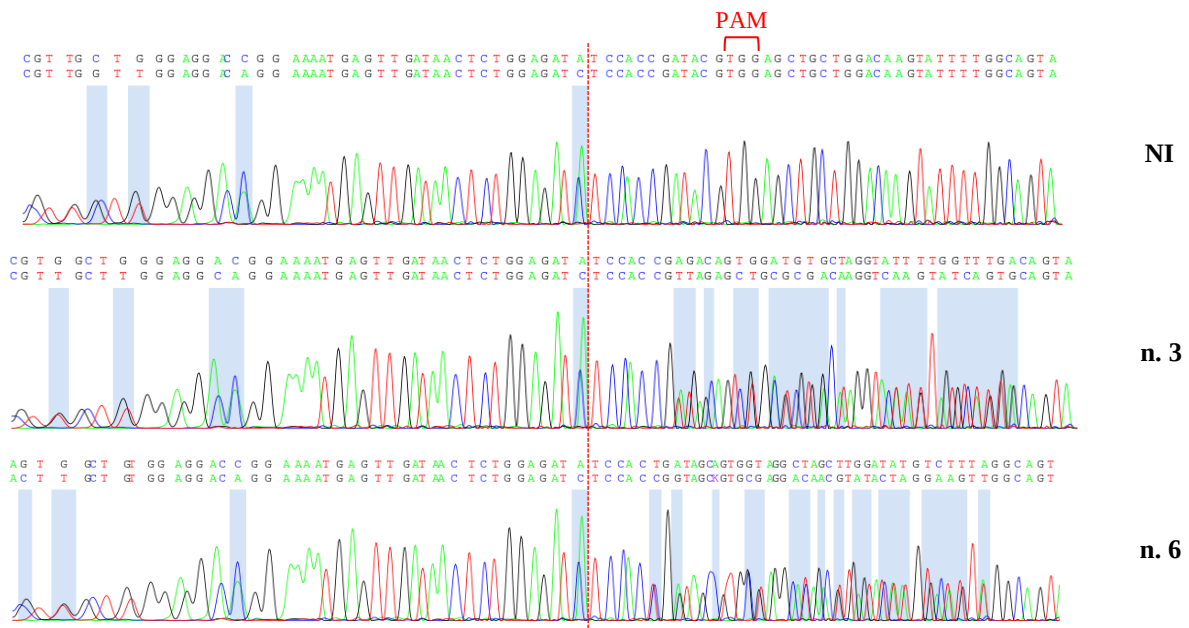


Figure 71. DNA sequencing chromatograms of genotyping PCR products from F1 larvae (5 dpf). From the top: NI (non-injected, AB/wild-type adult); two putative heterozygote larvae for a mutation in the *ap1s2* gene. The PAM sequence adjacent the sgRNA target site is shown in red.

The DNA sequencing chromatograms have been both visually interpreted and analysed with Poly Peak Parser, leading to the identification of two different mutations, a deletion of five and one of seven nucleotides (data not shown). These data indicate that founder n. 9 is able to transmit indel mutations to the F1 progeny. Among the individuals that have been grown to the adult stage, 32 have been genotyped by PCR using the DNA extracted from fish tails. The electrophoretic analysis on polyacrylamide (12%) gel under non-denaturing conditions revealed that 17 F1 fish out of 32 (53.12%) were likely heterozygotes for mutations in *ap1s2*. Interestingly, the gel presents different electrophoretic patterns, one corresponding to the homozygosis for the wt allele, the other representing three putative different *ap1s2* mutations in heterozygosis (Fig. 72).

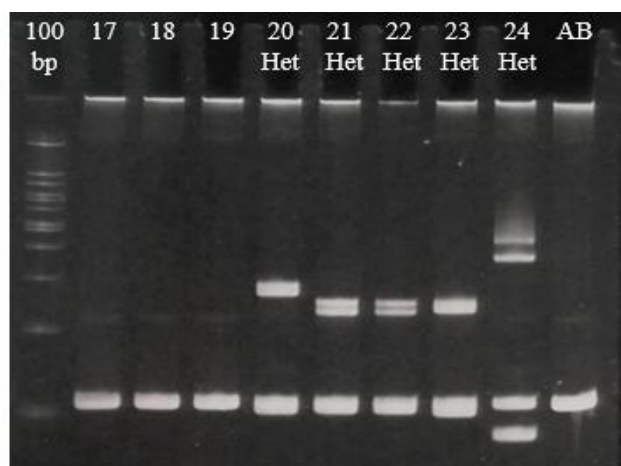


Figure 72. Electrophoresis on non-denaturing polyacrylamide (12%) gel of genotyping PCR products from F1 generation adult fish. Electrophoresis shows different migration patterns, which can be easily discriminated by eye. Putative heterozygotes are indicated as Het.

Validation followed by Sanger sequencing. Double peaks in the chromatograms are clearly visible in samples 1, 3 and 14 starting a few nucleotides before the PAM (Fig. 73). The analysis of the DNA sequences was performed using both Poly Peak Parser and visual inspection to identify the mutation present in the different *ap1s2* alleles generated. A definitive confirmation of the sequence of mutated alleles was only possible following the analysis of the sequence of F2 individuals homozygous for the mutation who present an easily interpretable chromatogram (with no double peaks).

The prediction of the protein sequence encoded by the different *ap1s2* alleles has been performed using both DNA Strider and ORF Finder, As expected del5, del7 resulted to be frameshift mutations, leading to the formation of truncated (and chimeric) proteins (Fig. 74). The del6 and del27 alleles should allow the production of Ap1s2 proteins with 2 and 9 amino acid less, respectively, than the wild-type one.

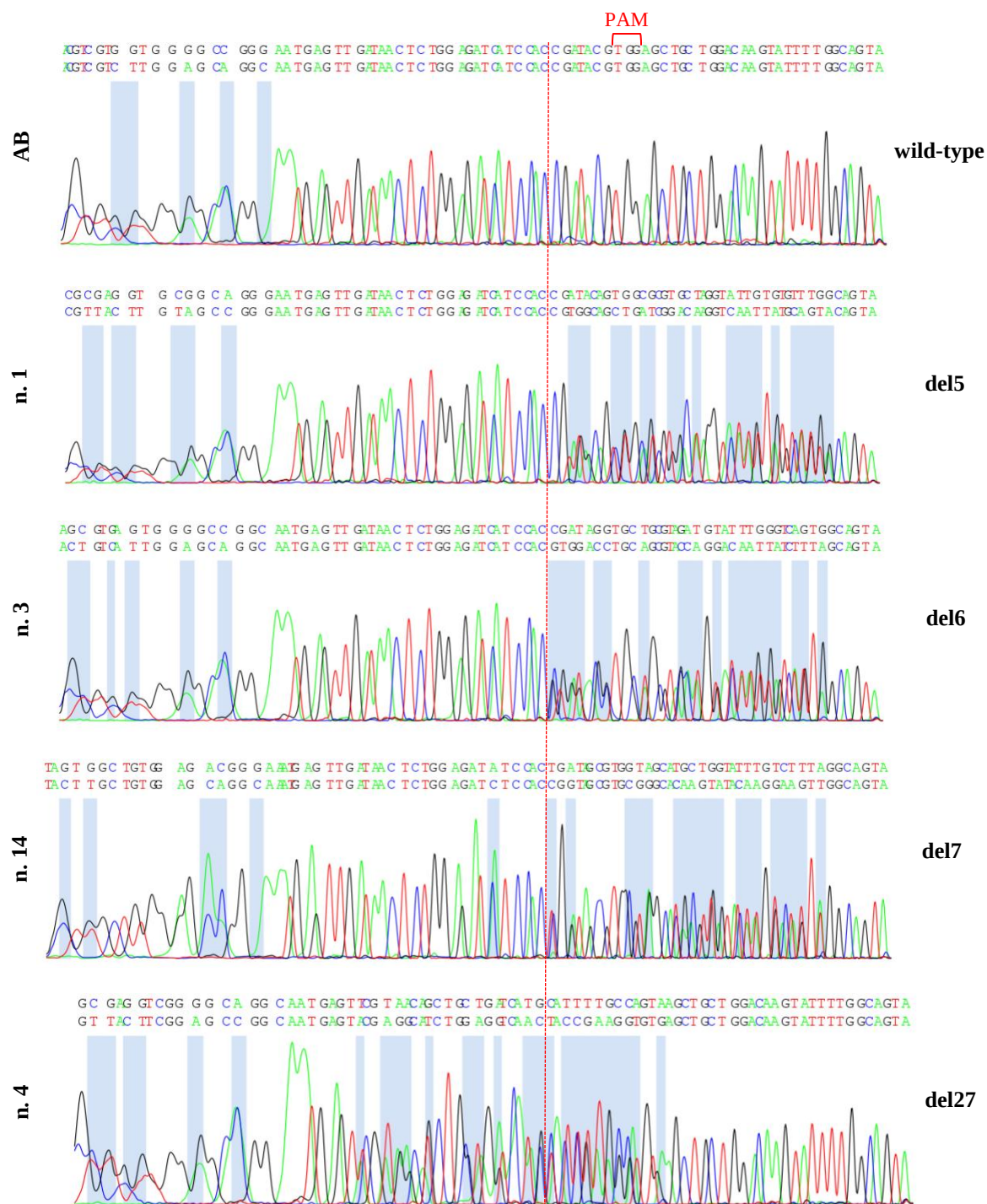


Figure 73. DNA sequencing chromatograms of genotyping PCR products from F1 adults. From the top: AB (wild-type AB adult); four illustrative heterozygotes for each identified mutation are shown. The number of F1 individuals analysed is indicated on the left. Interpretation of the effect of the Cas9-induced mutation is reported on the right. PAM sequence adjacent the sgRNA target site is shown in red.

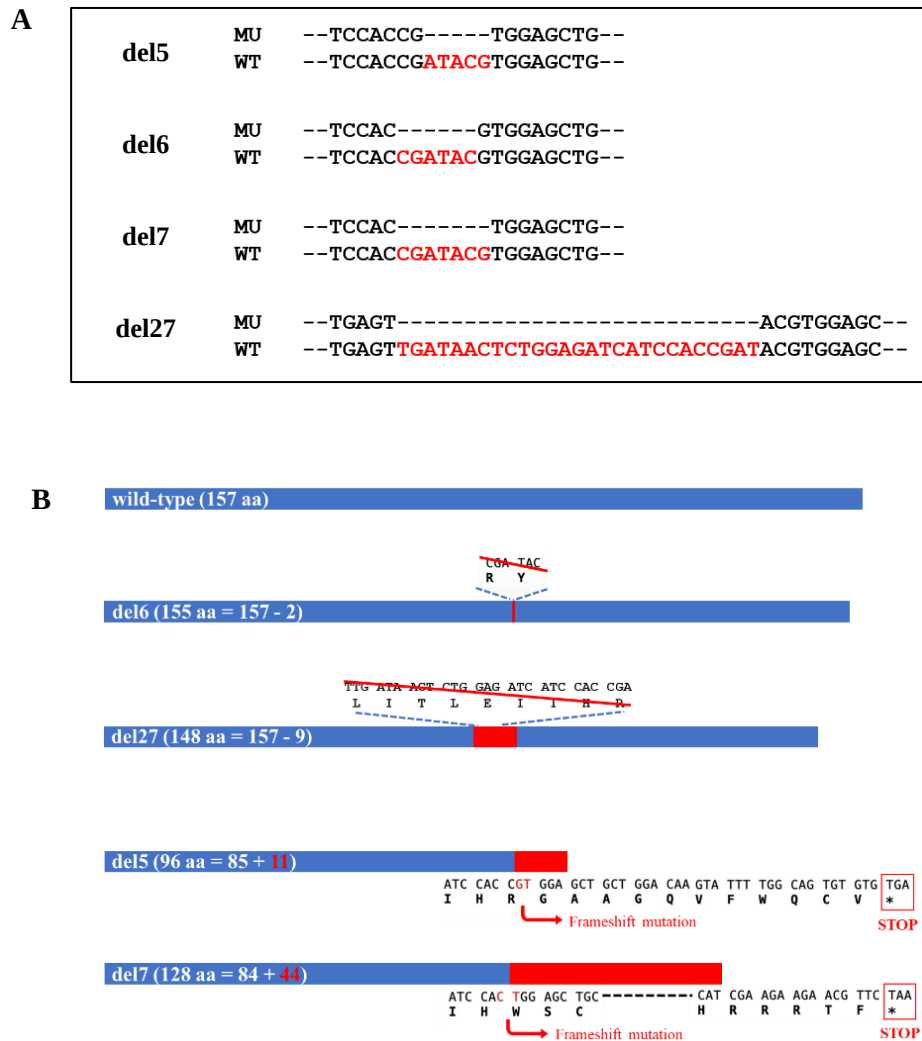


Figure 74. (A) Ap1s2 protein isoforms encoded by the four different mutated alleles identified. (B) ORF prediction for the 4 different *ap1s2* mutant alleles isolated. The protein encoded by each allele is shown. In blue is indicated the portion corresponding to the wild-type Ap1s2 protein, in red the polypeptides originating by frameshift mutations in del5 and del7 alleles.

Once reached the sexual maturity, crossbreeding between F1 heterozygotes had been arranged to obtain the F2 generations, including the three expected genotypic classes, *ap1s2*^{+/+}, *ap1s2*^{+/-}, *ap1s2*^{-/-} for each type of mutation. We decided to characterize two mutated lines for *ap1s2* gene, the del5 and the del27, the first harbouring a frameshift causing mutation and the second with an in frame deletion generating an Ap1s2 polypeptide lacking 9 amino acids.

⁵ Symbols + and - are used when referring to wild-type or mutated alleles, respectively, within *ap1s2* zebrafish gene.

Previous studies of the AP-1 structure highlighted the involvement of the larger subunits in the direct assembly of the adaptor protein complex, while the role of smaller subunits is still unclear. It has been described that $\sigma 1$ and $\mu 1N$ have essentially no direct interaction, with each contacting the N-terminal part of the large chain that primarily embraces the other. Thus, all four chains may be required for a fully stable assembly, and the two halves (γ - $\sigma 1$ and $\beta 1$ - $\mu 1$) may be only marginally stable on their own (Heldwein, et al., 2004). From literature, no specific functional domain for the $\sigma 1$ subunit, in the context of the AP-1 assembly, has been reported, showing that smaller subunits could be involved in the stabilization of the adaptor complex itself. It is likely that both the identified mutations could have a deleterious impact on the adaptor complex, leading to the possible appearance of a phenotype that should be visible in the homozygous individuals. In the first case (*ap1s2^{del5/del5}*), qPCR data strongly suggest that the transcript of the mutated allele is degraded through a NMD mechanism, preventing the production of a strongly altered protein; in the second case (*ap1s2^{del27/del27}*), the in frame mutation should lead to the generation of a protein with a consistent loss of amino acids (9 out of 157 aminoacids, which are highly conserved in the protein during evolution and 100% identical between man and zebrafish), thus likely compromising the secondary/tertiary structure of the polypeptide and therefore its biological activity. However, it is possible that the mutants we generated show milder phenotypes than expected due to the activation of compensatory mechanisms of other genes, first of all the putative paralogue of *ap1s2*, *zgc:162858*. In this case, the injection in mutants of a morpholino specific for *zgc:162858* into mutant embryos could help clarify this aspect, facilitating the identification of a specific phenotype. Preliminary injection experiments were conducted on *del5* line and the results obtained are described and discussed later in the text.

8.4 GENOTYPE SEGREGATION ANALYSIS

Segregation of the three genotypic classes for both del5 and del27 mutations was analysed on 5 dpf larvae and on adult tails (~ 3-6 mpf) by screening PCR products on native acrylamide (12%) gel. Mendelian ratios were generally conserved from larval stage until adulthood (Fig. 75) and no significant differences were found between the distributions of the three genotypes, with respect to each *ap1s2* line, and the expected distribution, according to chi-square statistic test ($p > 0.05$), with except to the del27 line ($p = 0.0362$), for which the percentage of homozygous individuals reaching adulthood is however not reduced for del27 mutation.

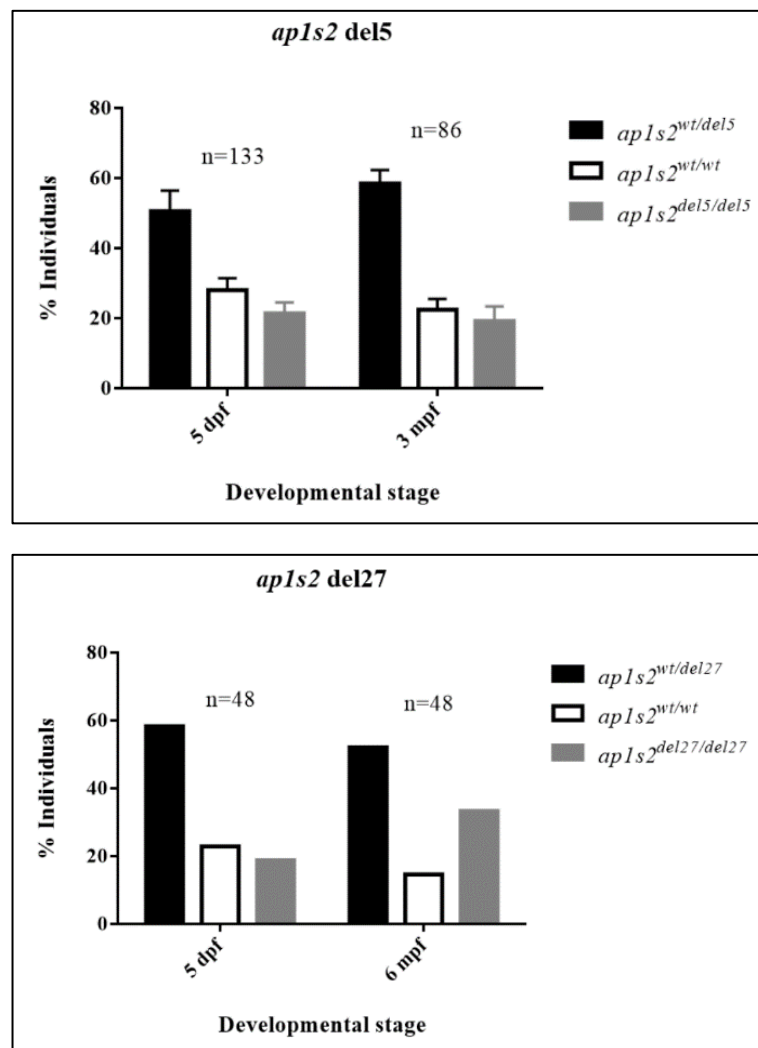


Figure 75. Analysis of the genotypic segregation on populations derived from crosses of heterozygous individuals for del5 and del27 mutations. For each developmental stage, results were obtained from three different (del5 line, up) and a single (del27 line, down) experiment, respectively, for a total number of individuals indicated above each group. Statistical analyses have been performed using chi-square test ($p > 0.05$), comparing the mean distributions of *ap1s2* F2 genotypes, respectively for del5 and del27 lines at the considered developmental stage, and the expected Mendelian ratios. Abbreviations: wt: wild-type; del5: deletion of 5 nucleotides; del27: deletion of 27 nucleotides; dpf: days post fertilization; mpf: months post fertilization.

8.5 MORPHOLOGICAL ANALYSES ON LARVAE

Development of zebrafish larvae has been investigated by analysing the morphological presentation of *ap1s2*^{del5/del5} and *ap1s2*^{del27/del27} individuals at 5 dpf, along with relative wild-type controls. Observed phenotypes have been divided into three different classes, indicated as “wild-type like”, “deflated swim bladder” and “severe phenotype”. In Fig. 76 are reported representative individuals for each observed morphological phenotype (left side), along with their distribution in the different mutant lines and controls (right side). In particular, similar distribution within the different morphological classes of phenotype were observed between *ap1s2*^{del5/del5} and relative wild-type AB controls; analysed *ap1s2*^{del27/del27} mutants revealed the presence of a higher percentage of 5 dpf individuals with correctly developed swim bladder. Taken together, these data suggest that Cas9-induced mutations do not affect morphology of the individuals, which are able to correctly develop until the larval stage.

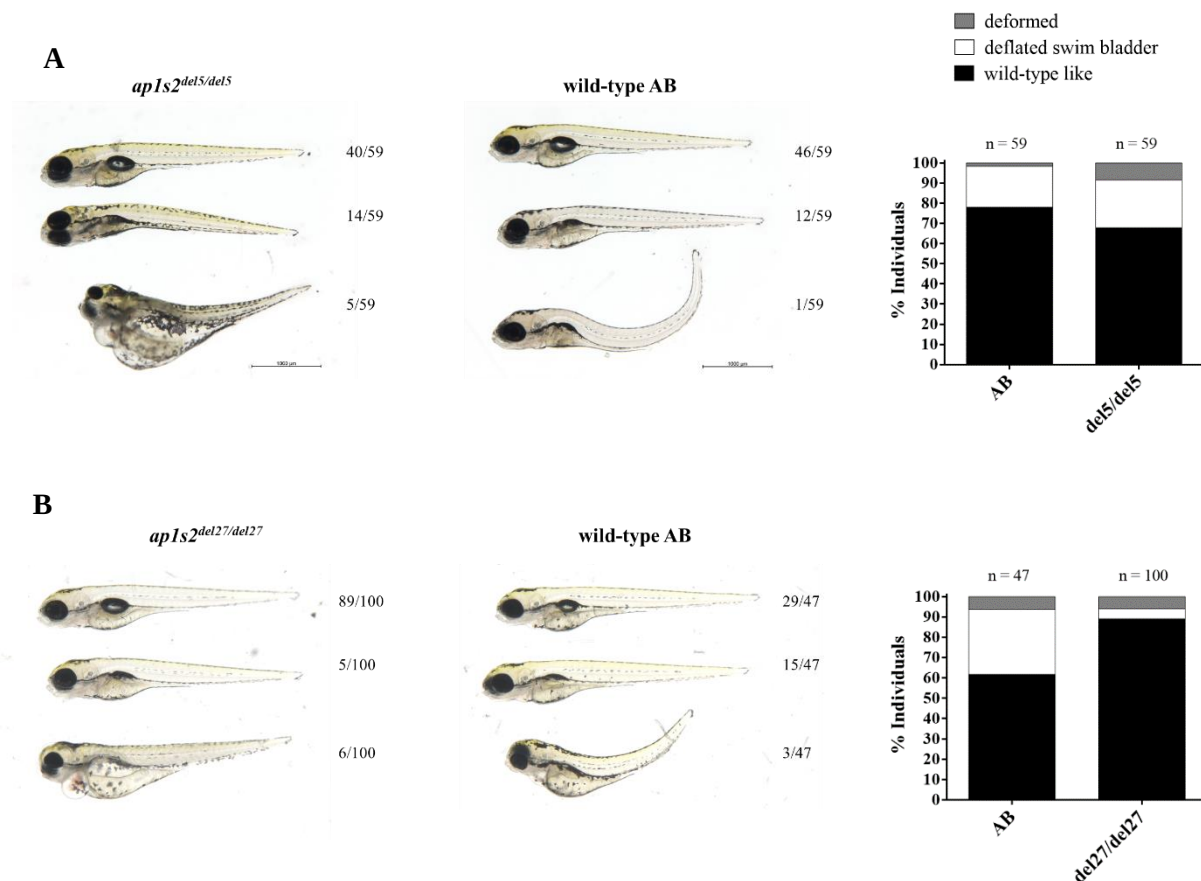


Figure 76. Morphological classes observed for *ap1s2* mutants and wild-type individuals (A-B, left side). Represented mutant larvae were generated by crossings between *ap1s2*^{del5/del5} (A) or *ap1s2*^{del27/del27} (B) individuals. **Distribution of the observed morphological phenotypes in 5 dpf larvae (A-B, right side).** Results were obtained from a single and preliminary investigation. References to the fish line and genotypes are reported on the x axis (AB: wild-type AB; del5/del5: *ap1s2*^{del5/del5}; del27/del27: *ap1s2*^{del27/del27}), while total number of the observed larvae is reported above each bar.

8.6 MORPHOLOGICAL ANALYSIS ON ADULTS

Homozygous individuals for the *del5* and *del27* mutations have been bred until adulthood and regularly mated. No major alterations have been observed between the two mutant lines and wild-type individuals. Patients affected by Pettigrew syndrome show rather evident clinical phenotypes, with malformations mainly involving the central nervous system. To assess whether the *del5* frameshift mutation, similarly to the loss-of-function causative mutations of the described mental retardation, could lead to the generation of morphological malformations of this type, preliminary targeted histological investigations have been carried out for *ap1s2*^{*del5/del5*} adult brains. In Fig. 77 are reported the representative images corresponding to the brain coronal sections of *ap1s2*^{*del5/del5*} and *ap1s2*^{*wt/wt*} individuals, respectively, at 10 mpf of development.

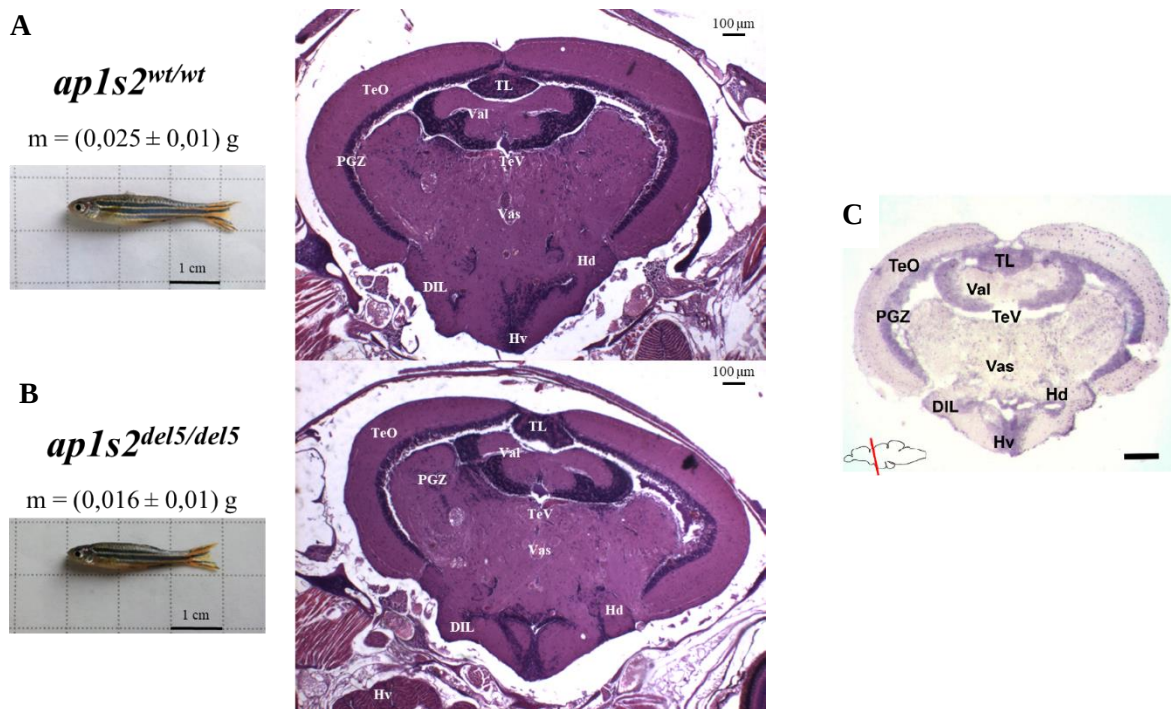


Figure 77. Histological coronal sections of 10 mpf zebrafish on brain. (A) *ap1s2*^{*wt/wt*} (4X magnification); (B) *ap1s2*^{*del5/del5*} (4X magnification). Genotype references are reported on the left, along with the representative image of the fish (respective masses are indicated in figure). **(C) Coronal section of zebrafish brain (Wong, et al., 2020).** Abbreviations: DIL: diffuse nucleus of the inferior lobe; Hd: dorsal zone of periventricular hypothalamus; Hv: ventral zone of periventricular hypothalamus; TeO: tectum opticum; TL: torus longitudinalis; TeV: tectal ventricle; Val: lateral division of valvular cerebelli; Vas: vascular lacuna of area postrema.

Comparative analysis revealed properly organized anatomical regions and no significant differences were observed between the mutant and the control fish (references are reported for comparison). Additional analyses on different anatomical compartments and at different stages will be necessary to complete the morphological presentation of *ap1s2* adult mutants.

8.7 THE EFFECT OF del5 MUTATION ON *ap1s2* SPLICING THROUGH RT-PCR

CRISPR/Cas9 allowed the introduction of different mutations in *ap1s2* gene of zebrafish at the level of the second coding exon. We decided to investigate whether the deletions induced by CRISPR/Cas9 (del5 and del27) are also able to alter the correct mRNA splicing as a result of the activation of cryptic splice sites. The effect of del5 and del27 alteration on the correct splicing of the transcript has been investigated by analysing the mRNA from adult tails of different genotypes (*ap1s2*^{wt/wt}, *ap1s2*^{wt/del5}, *ap1s2*^{del5/del5}, *ap1s2*^{wt/del27} and *ap1s2*^{del27/del27}), followed by RT-PCR reaction. Subsequently, Sanger sequencing was performed on the cDNA of the samples characterized by the frameshift mutation (del5). RNA extraction protocol was carried out following the guidelines described in “Materials and Methods”.

After extraction, 700 ng of total RNA from each sample was reverse transcribed. Later, RT-PCR was performed on the total cDNA using specific primers (**for primer:** CAGATATGCCAGCCTGTACTTTT; **rev primer:** GCTGAACACTTAGACTTTTAAGGGA) which should allow to obtain amplification product of 799 bp (wt), 794 bp (del5) and 772 bp (del27) when correct splicing occurs (Fig. 78).

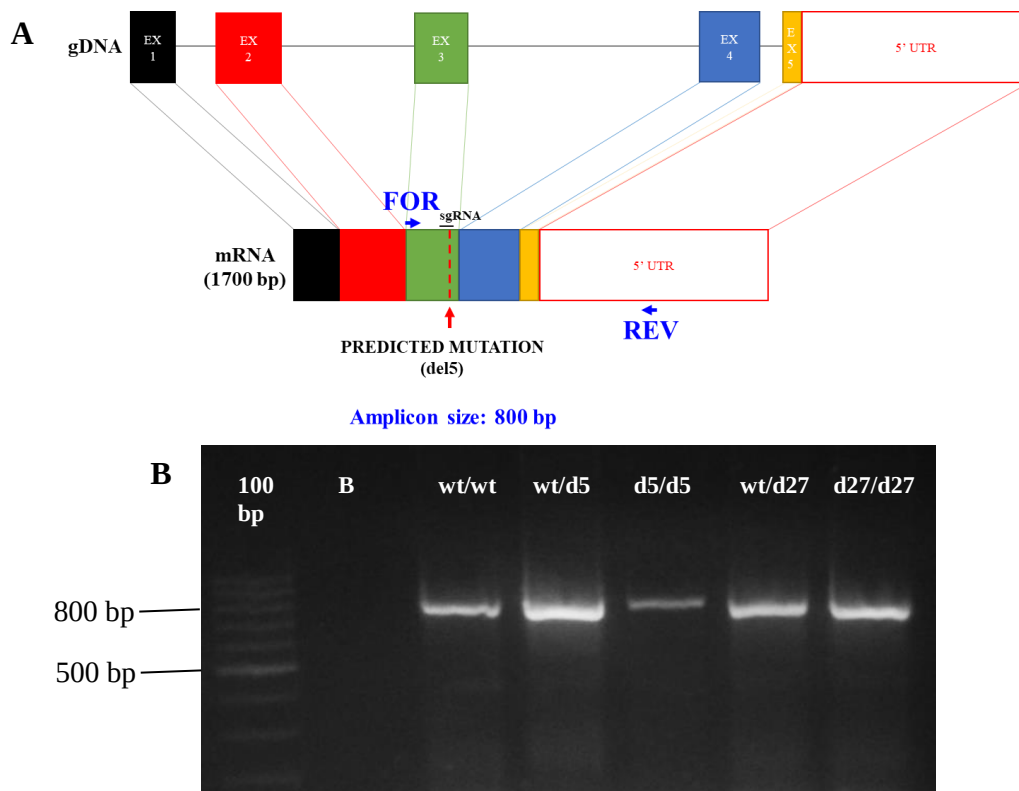


Figure 78. (A) RT-PCR amplification scheme of *ap1s2* cDNA. Localization of forward and reverse primers is reported (the figure includes both coding and non-coding exon of the gene). An amplification product of 799 bp is expected as a result of the correct splicing for the wt allele. Relative position of the sgRNA is also reported. **(B) Electrophoresis analysis on agarose gel of the amplification products obtained from *ap1s2* cDNA.** Abbreviations: B: blank of the PCR; wt/wt: *ap1s2*^{wt/wt}; wt/d5: *ap1s2*^{wt/del5}; d5/d5: *ap1s2*^{del5/del5}; wt/d27: *ap1s2*^{wt/del27}; d27/d27: *ap1s2*^{del27/del27}.

The analysis of the electrophoresis on agarose gel showed that a single amplification product of the expected size (from 772 to 799 bp, indistinguishable by this type of electrophoresis) was obtained from all the cDNA samples and no additional amplification products at different molecular weight were present in samples *ap1s2*^{wt/del5}, *ap1s2*^{del5/del5}, *ap1s2*^{wt/del27} and *ap1s2*^{del27/del27}, indicating that del5 and del27 have no obvious effect on gene transcript splicing.

Results were confirmed for del5 mutation by investigating the three PCR products (*ap1s2*^{wt/wt}, *ap1s2*^{wt/del5}, *ap1s2*^{del5/del5}) through Sanger sequencing. The analysis of the sequences evidenced that, as expected; *ap1s2* cDNA derived from the homozygous individual for the mutation (*ap1s2*^{del5/del5}) presents a deletion of five nucleotides, similarly to the deletion on the genomic DNA. The chromatogram corresponding to the heterozygote individual (*ap1s2*^{wt/del5}) does not show the presence of the amplification product from the deleted allele, indicating that the mutated allele is much less expressed in wt/del5 compared to wild-type fish, suggesting selective degradation by nonsense-mediated decay mechanism. Although the RT-PCR experiment is not quantitative, in Fig. 79 it is noted that the amount of amplified individual *ap1s2*^{del5/del5} is lower than the other PCR product and, even if not particularly robust from an experimental point of view, this data supports a lower stability of the mutated allele. Further experiments, including qualitative *in situ* hybridization with the specific *ap1s2* synthesized probe and quantitative methods (Real Time PCR) have been performed to corroborate this hypothesis.

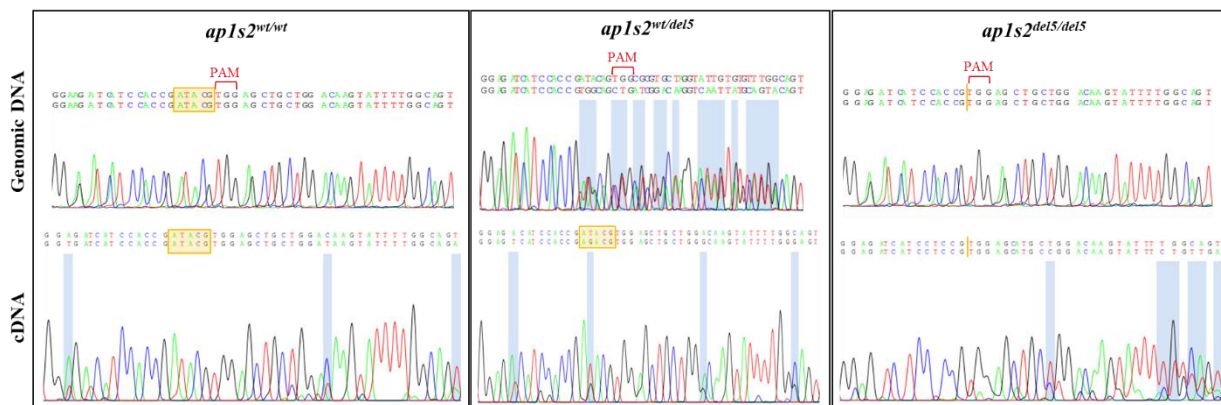


Figure 79. Chromatograms of the genomic and cDNA obtained from adult tails of different *ap1s2* genotypes. DNA sequence electropherograms from individuals with different genotypes are reported. Alignment between the genomic and the corresponding cDNA sequence is shown for comparison. The genotypes corresponding to each analyses sample are reported in the upper part of the figure. The five base pairs that are deleted in the *ap1s2*^{del5/del5} are reported in squares and the PAM sequence is reported as a reference point for the mutation site.

8.8 SPATIAL-TEMPORAL ANALYSIS OF *ap1s2* THROUGH *IN SITU* HYBRIDIZATION

After defining the localization of *ap1s2* expression in wild-type AB line, ISH on mutants have been performed to analyse the impact of the Cas9-induced mutations on the stability of the gene transcripts expression. Hybridizations have been conducted on *ap1s2*^{del5/del5} and *ap1s2*^{del27/del27} embryos at 48 hpf, along with wild-type AB as controls. Results showed a strong reduction of the *ap1s2* signal at the level of the central nervous system (Fig. 80) and, more in detail, in the portion corresponding to the midbrain hindbrain boundary (mhb) in *ap1s2*^{del5/del5} individuals, when compared to wild-type AB controls, suggesting that events of mRNA degradation by NMD take place in mutants for the frameshift mutation. No obvious differences were observed when comparing *ap1s2* expression of *ap1s2*^{del27/del27} individuals and the relative wild-type AB controls, indicating that this “in frame” mutation (del27) has no consequences on the stability of the encoded mRNA. The encoded protein, lacking 9 amino acids is very likely non-functional. This does not necessarily mean that the generated protein could maintain its original and correct biological function; furthermore, it is possible that other mechanisms of degradation occur at the level of the protein itself.

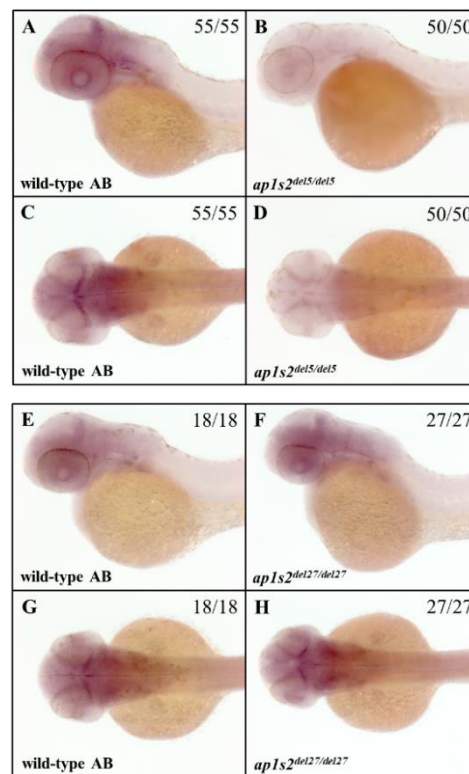


Figure 80. *In situ* hybridizations for *ap1s2* on mutant embryos at 48 hpf. The absence of hybridization signal in *ap1s2*^{del5/del5} embryos (B, D) suggests a possible mRNA degradation. By contrast, *ap1s2* transcript in *ap1s2*^{del27/del27} individuals seems to be unaffected by the presence of the mutation (F, H). Hybridization on wild-type AB embryos are reported for comparison (A, C, E, G). The totality of the individuals analysed and the relative genotype are indicated in figure.

8.9 ANALYSIS OF *ap1s2* EXPRESSION THROUGH REAL TIME PCR

Qualitative results of the RT-PCR investigation and whole mount *in situ* hybridizations on mutant embryos have been confirmed by performing quantitative analyses of Real Time PCR on the cDNA obtained from pool of embryos at different embryonal stage (48 hpf and 5 dpf). Each pool was processed according to the RNA extraction protocol described in “Materials and Methods”, followed by a cDNA reverse transcription reaction. In addition to analyse the *ap1s2* transcript at the selected stages of development, the expression of *zgc:162858*, the paralogue of *ap1s2* located on chromosome 23, was also evaluated. More in detail, expression analyses were performed on different experimental groups, corresponding to these classes of genotypes:

- wild-type AB
- *ap1s2*^{del5/del5}
- *ap1s2*^{del27/del27}

The specific pairs of primers for each of the two genes are reported in “Materials and Methods”.

Results showed a significant reduction of *ap1s2* expression (~ 88.5%) in *ap1s2*^{del5/del5} when compared to AB wild-type at 48 hpf, suggesting that most likely degradation by NMD takes place in this mutant line. Conversely, *ap1s2* expression seems to be unaffected by the presence of the del27 mutation (Fig. 81, left side). No significant differences in *zgc:162858* expression levels were observed in all the three experimental groups (Fig. 81, right side), indicating that the probable absence of a functional Ap1s2 protein does not activate compensation mechanisms involving the *zgc:162858* paralogue gene.

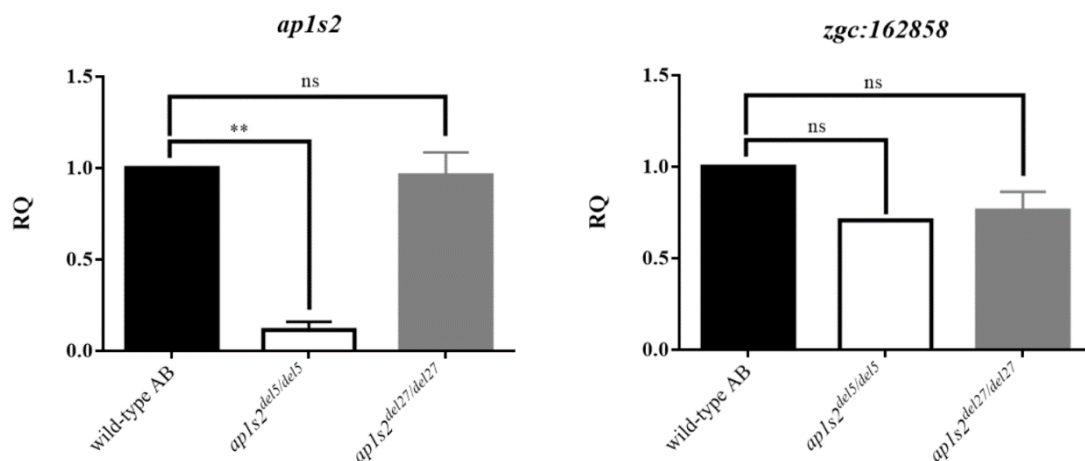


Figure 81. qPCR analyses on *ap1s2* mutant embryos at 48 hpf. On the left: analysis of *ap1s2* expression at 48 hpf. On the right: analysis of *zgc:162858* expression at 48 hpf. Data are represented as the mean $\pm$ SEM of the expression, normalized to wild-type AB expression and as described in “Materials and Methods”. Results were obtained for at least two replicates of each experimental group and statistical analysis was performed according to One-way ANOVA test (**: $p < 0.01$; ns: not significant).

Preliminary data at a later developmental stage (5 dpf) confirmed the results obtained for earlier stages, with a significant reduction of *ap1s2* expression ($\sim 89.6\%$) in *ap1s2*^{del5/del5} mutants when compared to wild-type AB and no significant differences in *ap1s2* expression between *ap1s2*^{del27/del27} and wild-type controls (Fig. 82, left side). The quantitative analysis of *zgc:162858* transcript showed no significant differences of expression between the three experimental groups, although an increase of expression seems to be characteristic of the *ap1s2*^{del27/del27} individuals when compared to the controls (Fig. 82, right side). The absence of statistical significance could be related to variability errors in this single experimental session; therefore, further analyses should be carried out in order to clarify this aspect.

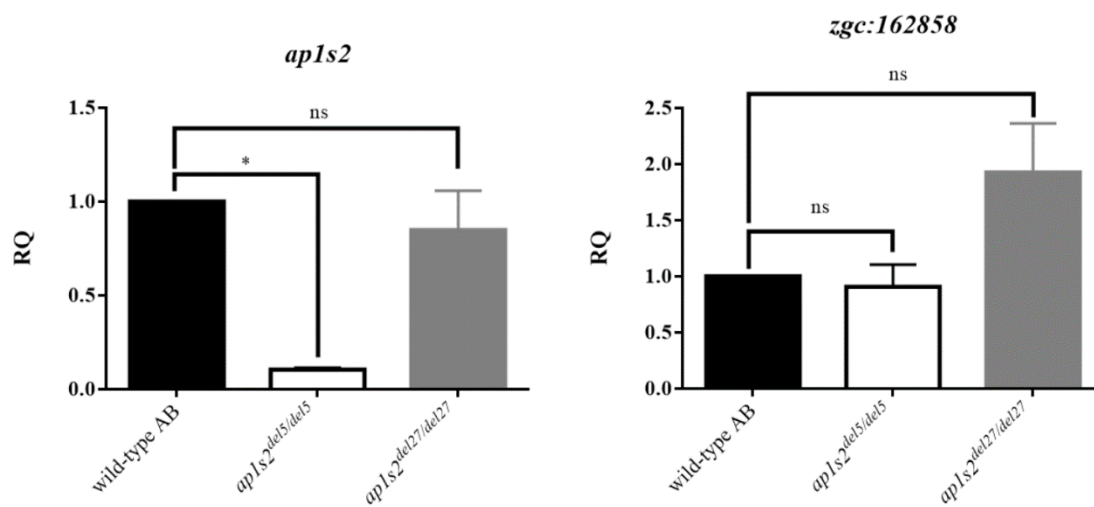


Figure 82. qPCR analyses on *ap1s2* mutant larvae at 5 dpf. On the left: analysis of *ap1s2* expression at 5 dpf. On the right: analysis of *zgc:162858* expression at 5 dpf. Data are represented as the mean $\pm$ SEM of the expression, normalized to AB wild-type expression and as described in “Materials and methods”. Results were obtained for at least two replicates of each experimental group and statistical analysis was performed according to One-way ANOVA test (*: $p < 0.05$; ns: not significant, $p > 0.05$).

8.10 SPATIAL-TEMPORAL EXPRESSION OF KEY NEUROGENESIS MARKERS IN MUTANTS

The spatial-temporal expression of relevant genes during the neurogenesis process was qualitatively investigated to detect any phenotypic abnormalities in the development of the central nervous system, attributable to a possible correlation between the inactivation of *ap1s2* and the expression of the genes examined. *In situ* hybridizations were performed using specific antisense RNA probes for *pax2a* (16 hpf and 24 hpf), *neurog1* (30 hpf) *neurod1* (24 hpf and 48 hpf) and *dlx2a* (48 hpf) neurogenesis markers (specific information for these genes are reported in “Materials and Methods”). In general, the expression of markers of the neurogenesis is maintained in both the mutant types (*ap1s2*^{del5/del5} and *ap1s2*^{del27/del27}), with subtle alterations not always well defined. The numbers of the analysed embryos are shown in the corresponding images.

Expression of *pax2a* at 16 hpf

Analysis of *pax2a* expression at ~ 16 hpf (10-14 somites) showed no differences between mutants and the relative wild-type AB controls in all the interested anatomical regions: a defined signal of expression was observed in the optic stalk and the anterior retina; a marked signal was present at the level of the midbrain hindbrain boundary and *pax2a* expression in the spinal cord neurons seemed unaffected (Fig. 83).

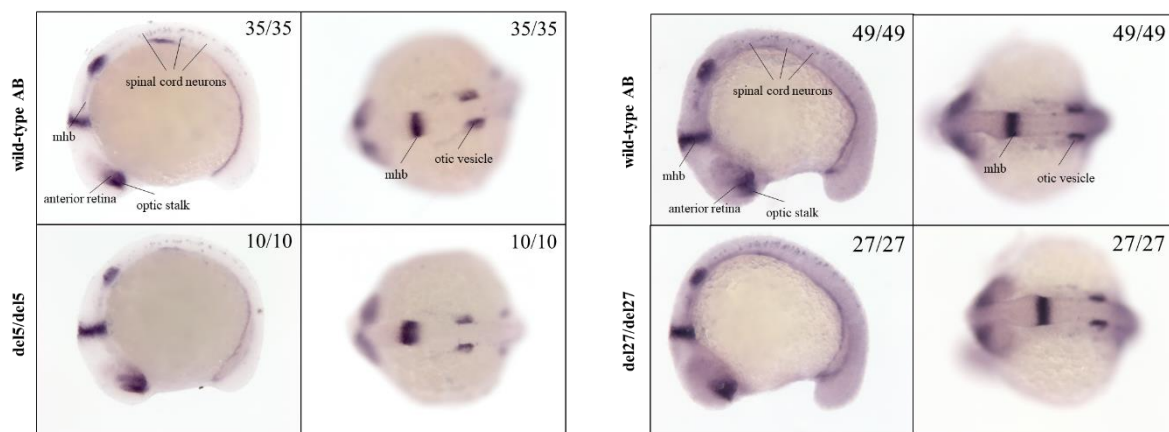


Figure 83. *In situ* hybridizations for *pax2a* at 16 hpf. Representative embryos are reported for each investigated mutant line (del5, on the left; del27, on the right). Anatomical references are reported in wild-type AB controls for comparison, while genotype reference is indicated on the left.

Expression of *pax2a* at 24 hpf

Analysis of *pax2a* expression at 24 hpf (prim-5) revealed expression of the neural marker in the expected anatomical regions, although weaker hybridization signals were observed at the level of the midbrain hindbrain boundary and the hindbrain neurons in *ap1s2^{del5/del5}* when compared to wild-type AB (Fig. 84), while the same analyses revealed no differences between *ap1s2^{del27/del27}* and their controls. Despite this, the expression of *pax2a* in the region of the optic stalk, the spinal cord neurons and in the pronephric duct is not altered.

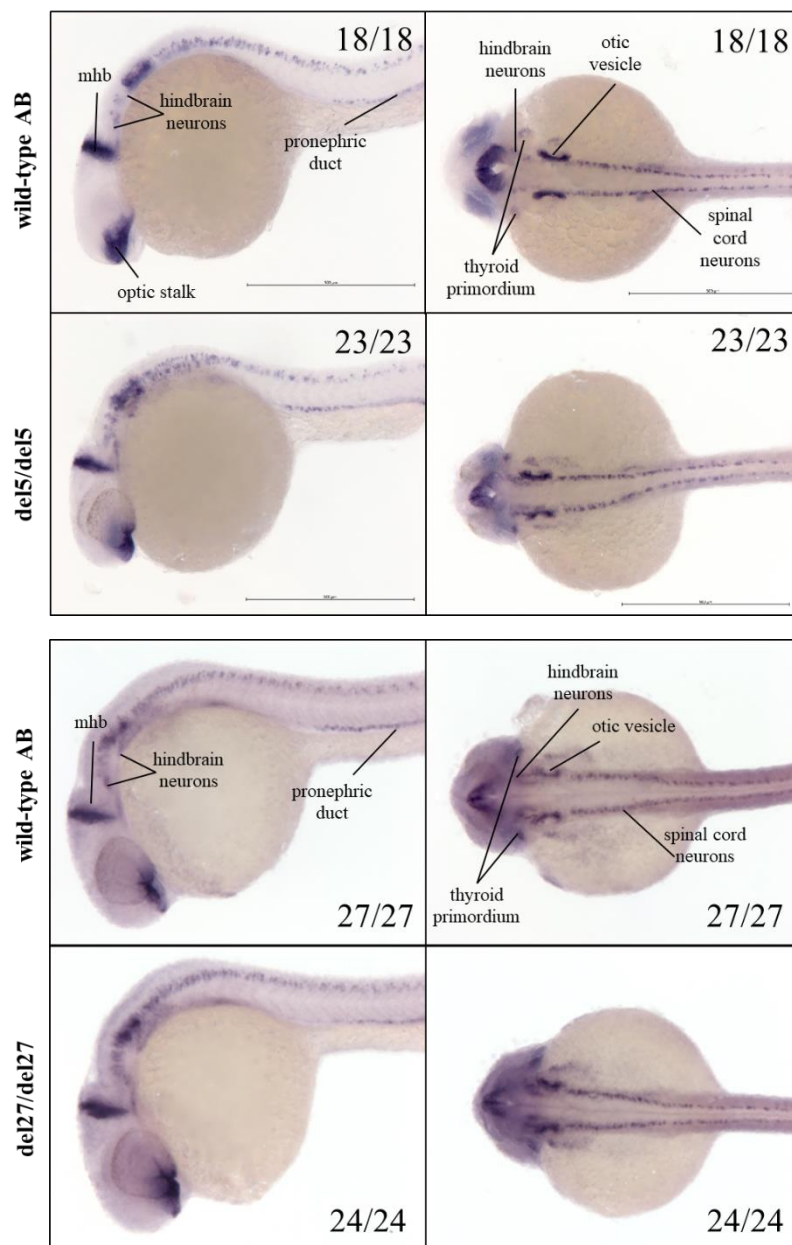


Figure 84. *In situ* hybridizations for *pax2a* at 24 hpf. Representative embryos are reported for each investigated mutant line (*del5* and *del27*). Anatomical references are reported in wild-type AB controls for comparison, while genotype reference is indicated on the left.

Expression of *neurog1* at 30 hpf

The expression of the transcription factor *neurog1* has been investigated and preliminary analyses at 30 hpf revealed a lower intensity hybridization signal at the level of the cranial ganglia in *ap1s2*^{del5/del5} individuals but not in *ap1s2*^{del27/del27} ones, when compared to wild-type AB embryos. In Fig. 85, from the dorsal view, a slight weaker hybridization signal in the anterior part of the brain can be appreciated, in the regions corresponding to the diencephalon and the telencephalon, which is more evident between *ap1s2*^{del5/del5} individuals and wild-type AB.

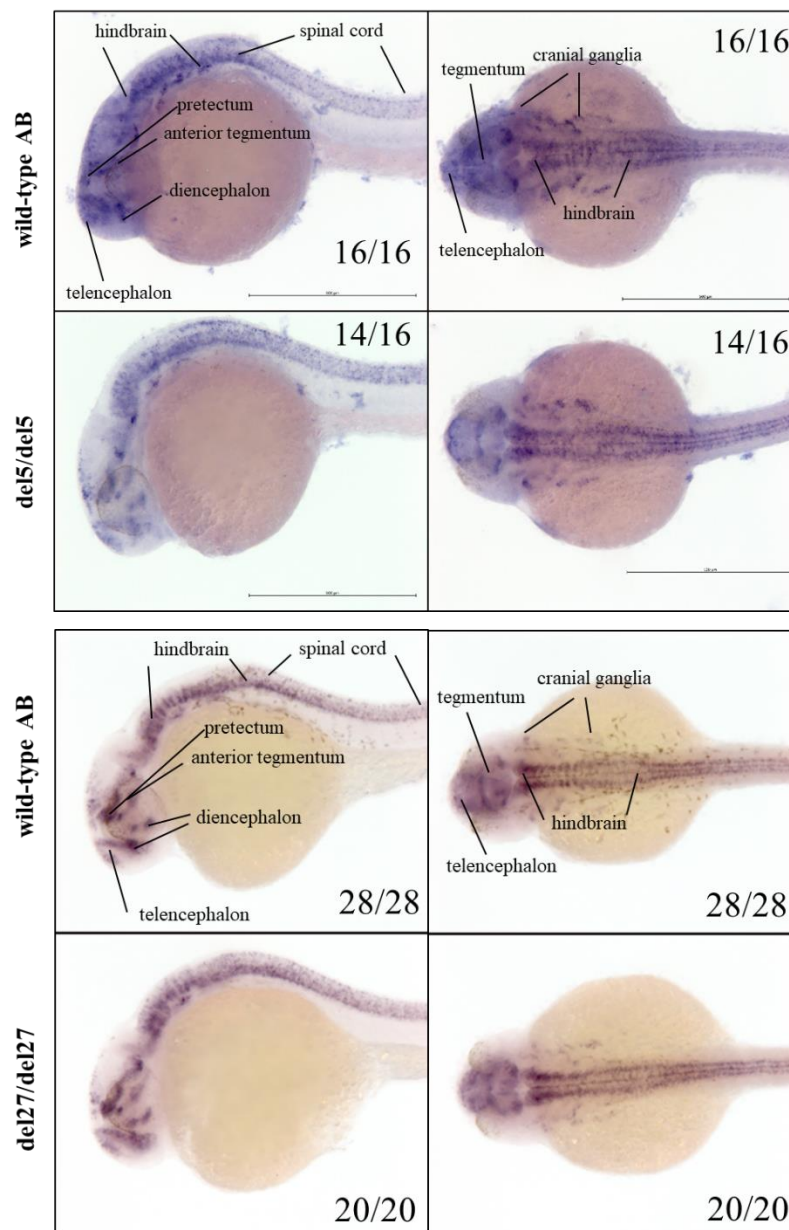


Figure 85. *In situ* hybridizations for *neurog1* at 30 hpf. Representative embryos are reported for each investigated mutant line (del5 and del27). Anatomical references are reported in wild-type AB controls for comparison, while the genotype is indicated on the left. Hybridization experiments were performed also at early developmental stages (16 hpf and 24 hpf) with particular attention to *ap1s2*^{del5/del5} individuals, but no significant differences were observed (data not reported).

Expression of *neurod1* at 24 hpf

Later transcription factor of the neurogenesis was investigated using the antisense probe for *neurod1*, whose upstream regulator is represented by *neurogenin*, the gene product of *neurog1* (Thomas, Ochocinska, Hitchcock, & Thummel, 2012). Analysis of *neurod1* expression at 24 hpf revealed no strong differences between mutants and the relative wild-type AB controls in the expected neuronal regions, although a slight weaker hybridization signal was observed in the region corresponding to the anterodorsal/anteroventral lateral line/facial placodes ganglia (ad/av/f) in *ap1s2^{del5/del5}* individuals but not in *ap1s2^{del27/del27}* ones when compared to controls. Moreover, wild-type embryos were characterized by a slight hybridization stronger signal in the telencephalon (t) and the octaval statoacoustic ganglia (o) when compared to *ap1s2^{del5/del5}* mutants (Fig. 86).

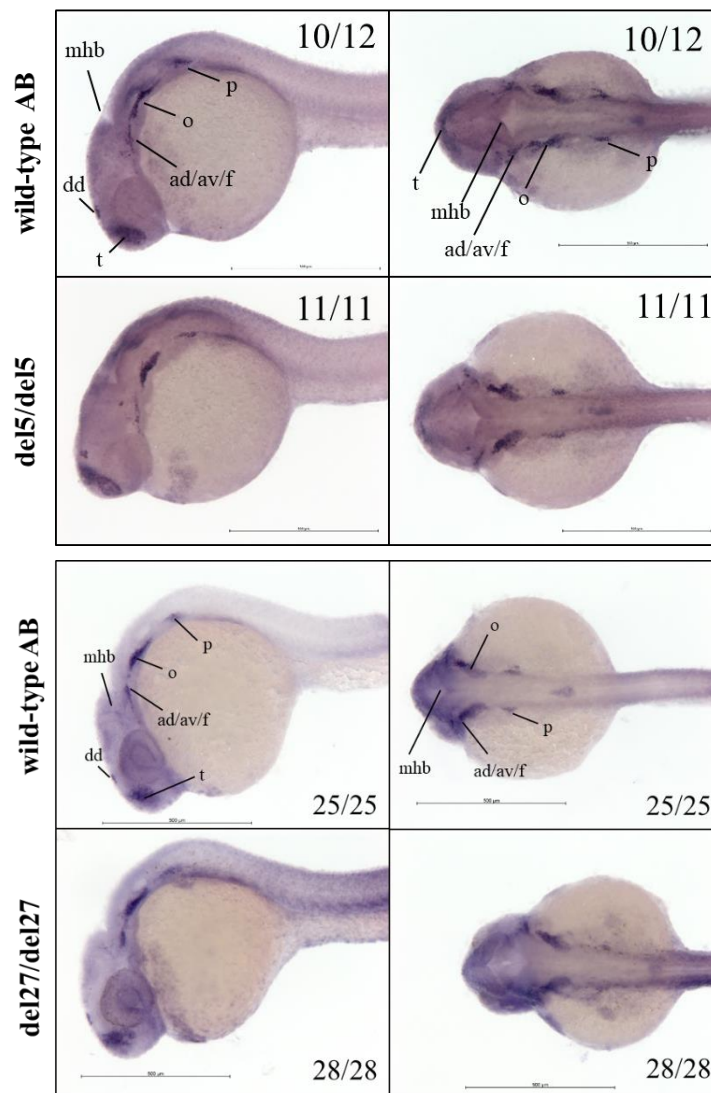


Figure 86. *In situ* hybridizations for *neurod1* at 24 hpf. Representative embryos are reported for each investigated mutant line (del5 and del27). Anatomical references are reported in wild-type AB controls for comparison, while the genotype is indicated on the left. Abbreviations: ad/av/f: anterodorsal/anteroventral lateral line/facial placodes ganglia; dd: dorsal diencephalon; mhb: midbrain hindbrain boundary; o: octaval/statoacoustic ganglia; p: posterior lateral line ganglia; t: telencephalon.

Expression of *neurod1* at 48 hpf

neurod1 was investigated even at later embryonic stages; Fig. 87 highlights the expression of the neural marker at 48 hpf, showing differences in *ap1s2*^{del5/del5} mutants when compared to wild-type AB in specific brain regions: a weaker expression was observed starting from the midbrain hindbrain boundary to the region of the hindbrain. Interestingly, a less evident and organized hybridization signal was visible at the level of the rhombomeres of the homozygous individuals for the del5 mutation but not for the homozygous for the del27 mutation, whose pattern of expression is more similar to that of the controls.

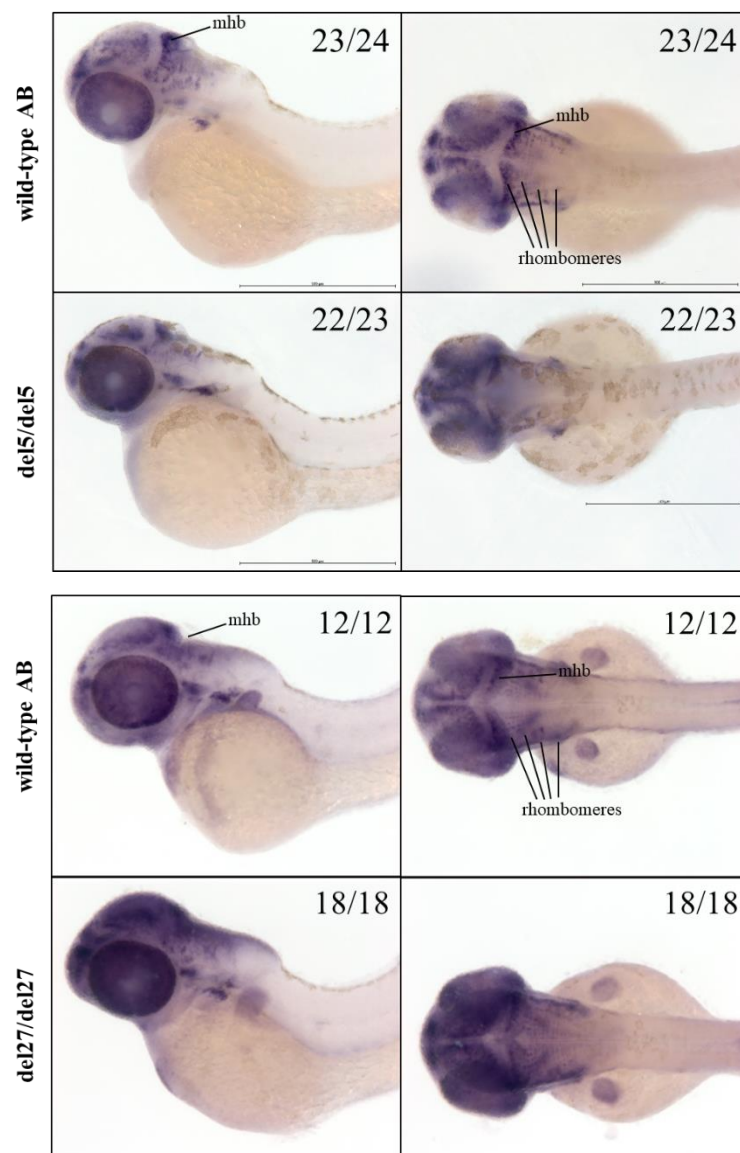


Figure 87. In situ hybridizations for *neurod1* at 48 hpf. Representative embryos are reported for each investigated mutant line (del5 and del27). Anatomical references are reported in wild-type AB controls for comparison, while the genotype is indicated on the left. Abbreviations: mhb: midbrain hindbrain boundary.

Expression of *dlx2a* at 48 hpf

Considering the subtle differences observed concerning the expression of neural markers in the anterior portion of the brain, neurogenesis was analysed using *dlx2a* antisense RNA probe. Hybridizations showed slight differences in *ap1s2*^{del5/del5} mutant embryos when compared to wild-type ones, revealing weaker and less organized signal of expression in the telencephalic region and in the dorsal domain prethalamus (Fig. 88, left side). Conversely, no significant differences were seen between del27 mutants and AB controls (Fig. 88, right side).

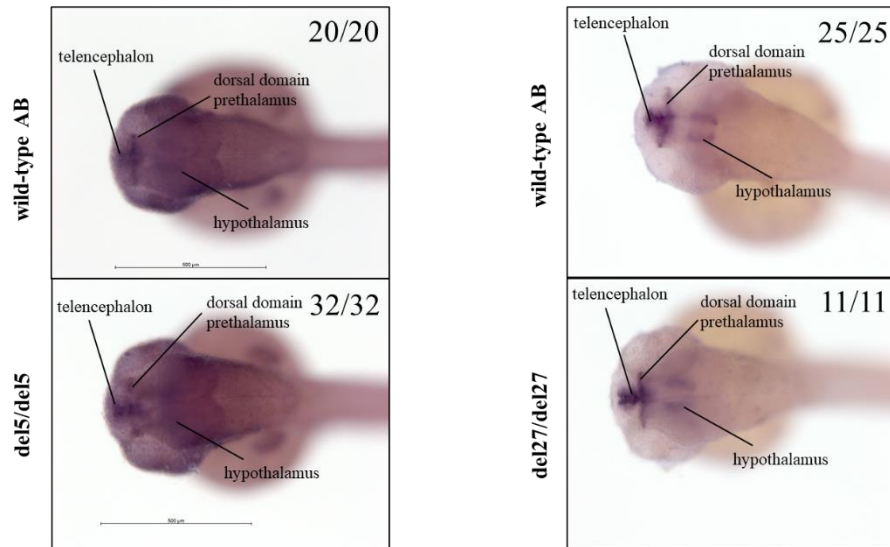


Figure 88. *In situ* hybridizations for *dlx2a* at 48 hpf. Representative embryos are reported for each investigated mutant line (del5 and del27). Anatomical references are reported for comparison, while genotype reference is indicated on the left.

The images of representative dorsal flats of *ap1s2*^{del5/del5} individuals and relative controls reveal disorganization of the hybridization signal in the most anterior part of the brain. Moreover, a weaker expression is observed in the region corresponding to the hypothalamus in *ap1s2*^{del5/del5} (Fig. 89).

The apparent different size of the two embryos reported is due solely to a procedural artefact during the realization of the flats.

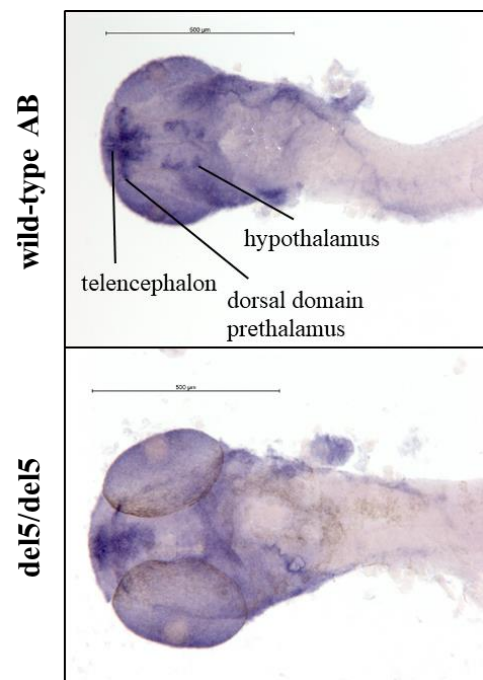


Figure 89. Dorsal flats of *in situ* hybridizations for *dlx2a* at 48 hpf.

8.11 ANALYSIS OF THE EFFECT OF *ap1s2*-INACTIVATING MUTATIONS ON LIPID DISTRIBUTION

Studies on a murine knockout model for the $\sigma 1B$ subunit evidenced that altered sortilin sorting in adipocytes deficient for the $\sigma 1B$ -containing AP-1 complex leads to the inhibition of adipogenesis (Baltes, et al., 2014). To investigate whether Cas9-induced mutations could have an impact on lipid metabolism, some preliminary analyses have been performed on 5 dpf *ap1s2*^{del5/del5} and *ap1s2*^{del27/del27} larvae, compared to wild-type AB controls, through Oil Red O staining (ORO). The lipophilic dye employed for this technique allows to stain neutral lipids, cholesteryl esters, and lipoproteins. ORO has been widely used for intracellular lipid staining and tissue staining due to its cellular permeability, neutral fats, mainly triglycerides, can be stained with an orange-red tint. Larvae have been processed according to published guidelines (Wang, et al., 2023) and deposition of lipids could be observed. Images of the larvae were obtained using the Axio Zoom.V16 microscope; later, quantification of the staining has been performed as described in “Materials and Methods”. More in detail, quantification was performed on three different regions, defined as “head”, “body” and “tail”. Representative larvae and relative OD measurements are reported below. Preliminary measurements revealed a lower OD for both the mutant lines when compared to wild-type individuals in the region of the head, suggesting a lower deposition of adipose tissue in this part; in addition to that significant decrease in OD are observed in the central part of the body when comparing *ap1s2*^{del5/del5} and wild-type AB (Fig. 90). Additional investigations should be performed to support these data and future inspections should clarify the role of *ap1s2* in the lipid metabolism in adulthood in the fish; for these reasons, histological sections and specific staining should be performed.

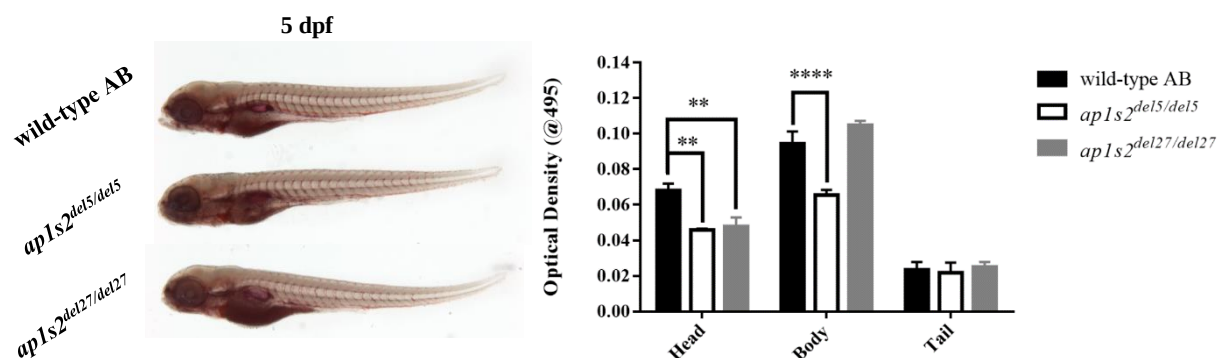


Figure 90. Staining for neutral triglycerides and lipids in wild-type AB and *ap1s2* mutant larvae (left side) and quantification of ORO staining (right side). Optical Density (OD) analysis of ORO stained larvae using a microplate spectrophotometer is reported; quantification took into account the intensity and amount of ORO staining present within the three different parts of the larvae (head, body, tail). Data is represented as optical density measurements analysed at a wavelength of 495 nm. Data is represented as the mean $\pm$ SEM of the staining for 60 larvae at 5 dpf (5 replicates with 12 larvae each). Statistical analysis was performed according to Two-way ANOVA test (**: $p < 0.01$; ****: $p < 0.0001$). @ = measured at wavelength 495nm.

8.12 SPATIAL-TEMPORAL EXPRESSION OF *myod* DURING EMBRYONAL DEVELOPMENT

Patients with Pettigrew syndrome are characterized by several symptoms, including hypotonia and motor delay. To assess whether Cas9-induced mutations could affect the process of muscular development and organizations, investigations have been performed through *in situ* hybridizations at 16.5 hpf (20 somites) using antisense RNA probe for *myod*, a transcription factor acting as an upstream of or within pharyngeal muscle development, skeletal muscle tissue development and striated muscle development.

A total of 13 wild-type AB and 12 *ap1s2*^{del5/del5} were observed in a first experiment, while 43 wild-type controls and 44 *ap1s2*^{del27/del27} individuals were analysed in a second experiment.

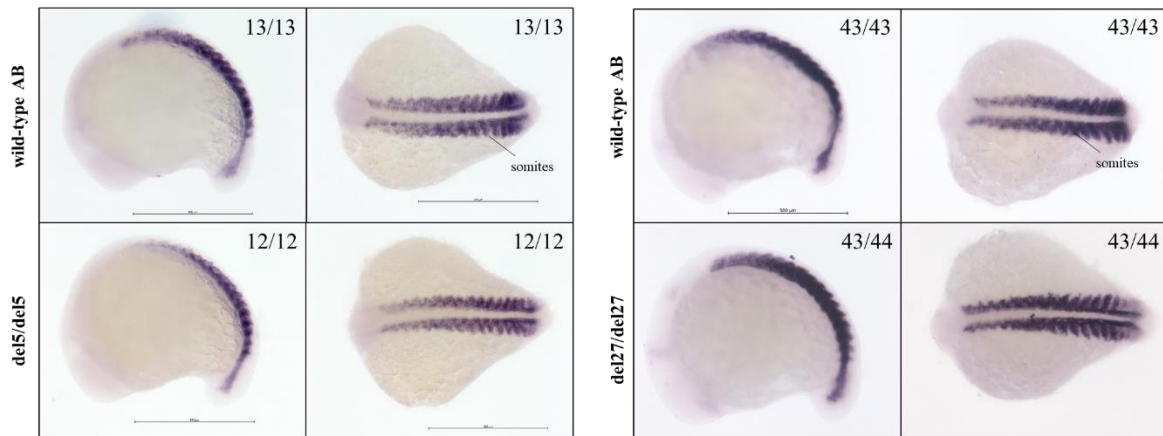


Figure 91. *In situ* hybridizations for *myod* at 16.5 hpf. Representative embryos are reported for each investigated mutant line (del5 and del27). Anatomical references are reported in wild-type AB controls for comparison, while the genotype is indicated on the left.

Preliminary experiments have shown that *myod* expression is maintained in the various anatomical regions of the mutants. From the signal detected at the level of the somites, it was also possible to evaluate the organization and length of the same, which is not significantly different in the two mutant groups compared to the controls (Fig. 91). Further investigations on larger groups of embryos should be performed to confirm this data, evaluating in particular the statistical significance concerning the length of somites.

8.13 ANALYSIS OF THE ORGANIZATION OF MUSCLE FIBRES THROUGH PHALLOIDIN STAINING

To further investigate the possible correlation between the Cas9-induced mutations and the muscle development, embryos at 48 hpf have been subjected to phalloidin staining assay. More in detail, phalloidin is a highly selective bicyclic peptide used for staining actin filaments (also defined as F-actin). Its conjugation with fluorescent molecules allows the observation of the actin fibres using a classic fluorescence microscope. Qualitative observation of muscle fibres could be analysed by using ZEISS Axio Zoom.V16, which was also employed to realize the images reported below at different magnifications (Fig. 92).

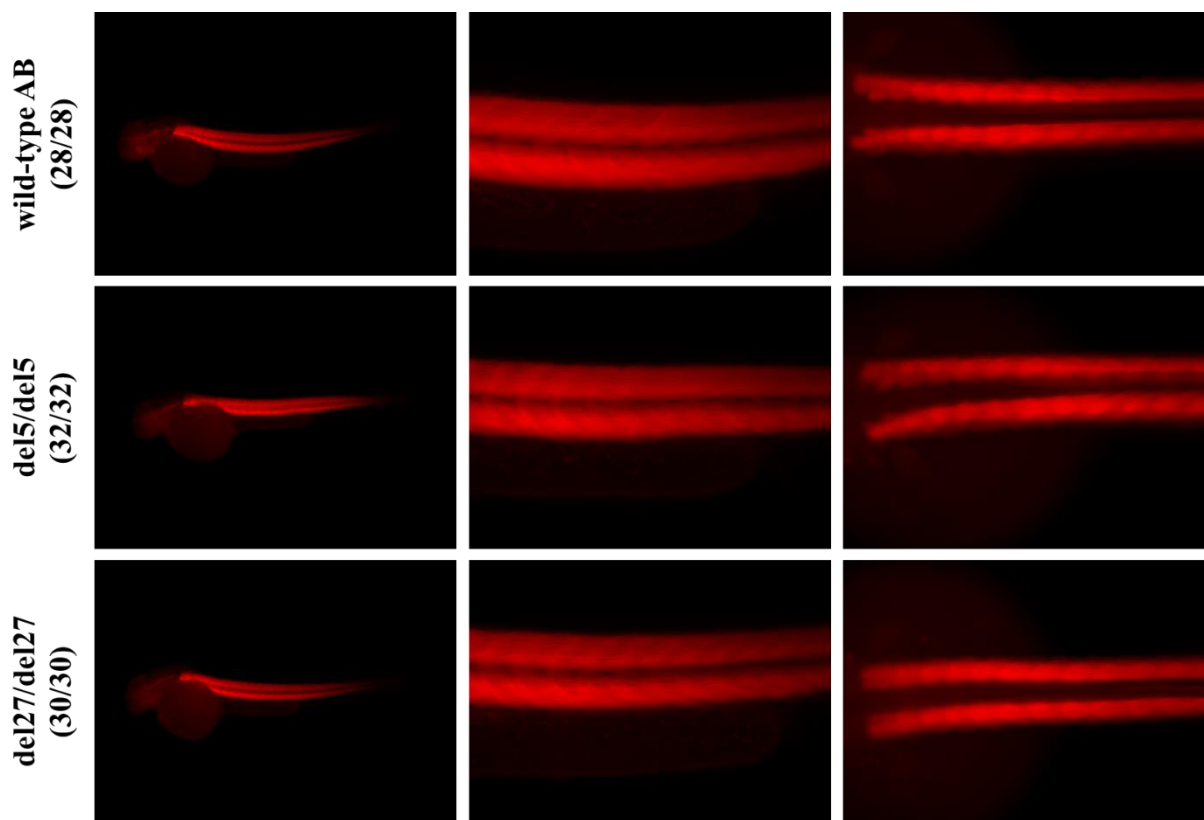


Figure 92. Phalloidin staining on wild-type AB embryos and *ap1s2* mutants at 48 hpf. Representative embryos are reported for wild-type controls and for each investigated mutant line (del5 and del27). Different magnifications are reported (40X for the whole mount embryo, lateral view, and 80X for the higher magnifications, lateral and dorsal view). By comparison with wild-type AB staining, F-actin filaments are correctly organized in all the individuals of the mutant lines, clearly visible from the lateral and the dorsal view. Genotypes and numbers of analysed embryos are indicated on the left.

8.14 BEHAVIOURAL ANALYSIS ON ZEBRAFISH LARVAE

The capability of the animals to respond to visual stimulus was evaluated by behavioural analysis at 5 dpf developmental stage. In particular, *ap1s2^{del5/del5}*, *ap1s2^{del27/del27}* and relative wild-type individuals have been subjected to the standard “dark/light” routine described in “Materials and Methods” using DanioVision (Noldus) instrument. Locomotor activity of each experimental group was evaluated by comparison of different parameters calculated with EthoVision XT (Noldus) software, which included the distance travelled and the velocity in the different experimental phases. Fig. 93 shows the distance covered by the groups of larvae, expressed in different ways: on the left side is reported as the average distance covered (in cm) in the different experimental phases (habituation, dark phases and light phases); on the right side the graph shows the distance at regular intervals (10 min each) from the start of the experiment to its conclusion.

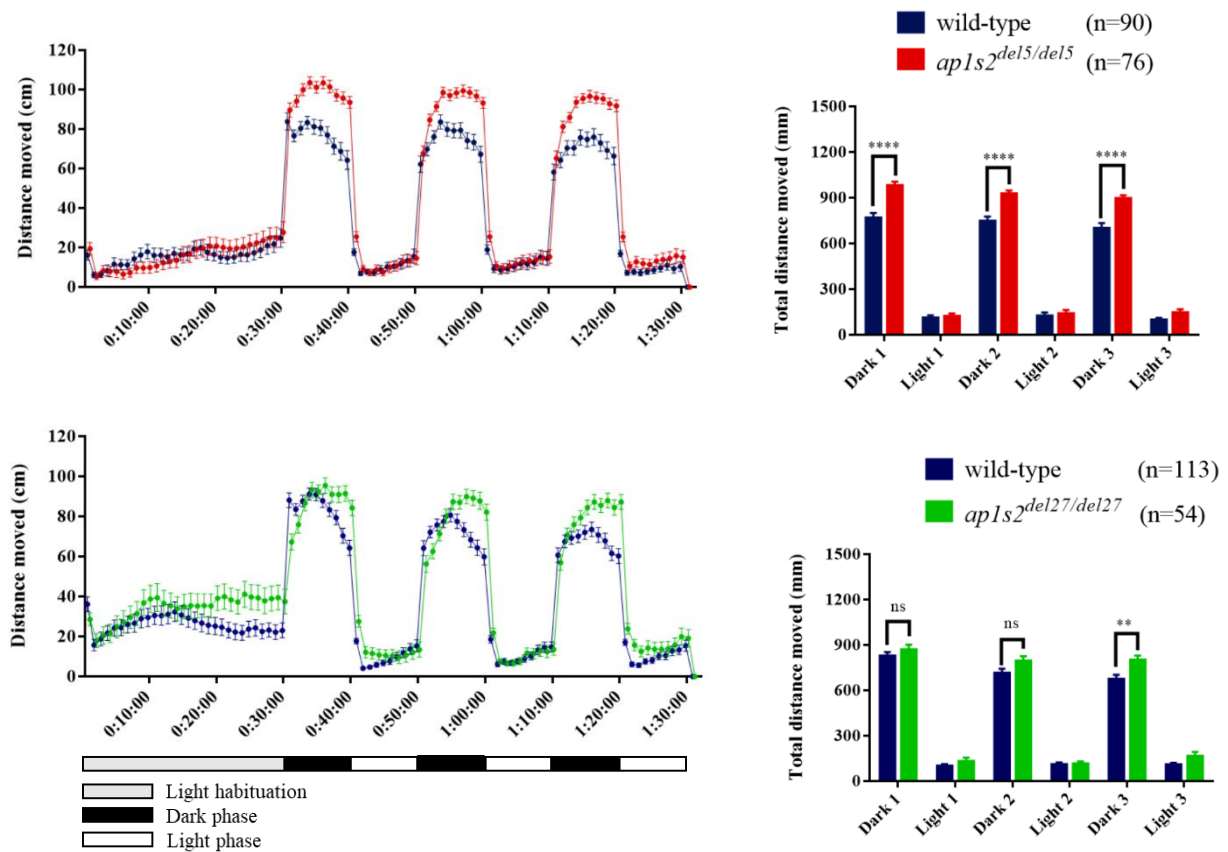


Figure 93. Left side: distance moved by *ap1s2* mutants and the relative control larvae (5 dpf) in the different experimental phases at intervals of 10 minutes each. Dark phases are characterized by higher distance travelled; moreover, homozygous for the frameshift *del5* mutation respond to dark stimulus by travelling long distances when compared to controls; conversely, *ap1s2^{del27/del27}* do not show differences with wild-type individuals. **Right side: total distance moved by *ap1s2* mutants and relative control larvae (5 dpf) in the different experimental phases.** Travelled distance between *ap1s2^{del5/del5}* individuals and the corresponding controls (upper graph) and between *ap1s2^{del27/del27}* mutants and their relative wild-type (lower graph) are reported. Data were generated upon n = 2 experiments (for each experimental pair) and are presented as the mean $\pm$ SEM of the distance. Statistical significance has been evaluated by Two-way ANOVA test (ns: not significant; ****: $p < 0.0001$).

It is well known from literature that “dark” phases promote movement of the zebrafish larvae, which cover a greater distance and move with a higher speed when compared to the “light” phases (Basnet, Zizioli, Taweedet, Finazzi, & Memo, 2019). Results of behavioural tests on the *ap1s2* mutant larvae confirm this aspect and therefore suggest a significant predisposition of *ap1s2*^{del5/del5} mutants to move faster and travel larger distances when compared to controls, while no significant differences were found when analysing *ap1s2*^{del27/del27} individuals and the relative wild-type larvae. Further investigations have been performed at earlier developmental stages (30 hpf) and data are currently being processed to assess whether del5 mutants of *ap1s2* show specific behavioural phenotypes already at the level of the primary motor behaviour, which consists of spontaneous tail coiling movements involving side-to-side contractions of the trunk.

8.15 *zgc:162858* MORPHOLINO INJECTION IN *ap1s2*^{del5/del5} EMBRYOS

The recently described *zgc:162858* gene located on chromosome 23 of zebrafish genome shares over 80% of sequence identity with *ap1s2* within their coding regions. In particular the 20 bp *ap1s2* target sequence of the *ap1s2*-EX2 sgRNA presents only a single mismatch with a homologous sequence within *zgc:162858*. A group of 16 *ap1s2*^{+/-} individuals from the F1 generation have been genotyped for the *zgc:162858* locus with partial complementarity to the *ap1s2*-EX2 sgRNA and no evidence of heteroduplex DNA molecules was observed by native electrophoresis on acrylamide gel of the PCR products. This suggests that *ap1s2*-EX2 sgRNA is specific in targeting the *ap1s2* gene (chr 23) and that the F1 we generated with mutations in *ap1s2* likely possess a wild-type *zgc:162858* sequence. On the other hand the presence of a functional paralog could explain the very subtle phenotypes in *ap1s2* zebrafish compared to KO mice and Pettigrew syndrome patients. Thus, the presence of a functional Ap1s2-like protein encoded by *zgc:162858* could potentially have a role within the compensation mechanisms activated by the cell in response to the *ap1s2* gene knockout. To assess whether *zgc:162858* could play a role in this type of mechanism, preliminary experiment of transient knockdown have been performed through morpholino injections into one cell-stage embryos derived from crosses between *ap1s2*^{del5/del5} mutants. The specific morpholino (indicated as “*zgc162858*-EX3ACC”, also abbreviated in “*zgc*-MO”) was designed on the acceptor splice site of the third exon of *zgc:162858* gene, as described in “Materials and Methods”, leading to the skipping of the exon 3. The sequence of this MO has been selected by the company Gene Tools who also indicated that is non optimal, being only 19 nt long. Different amounts of the morpholino were initially injected into wild-type embryos, allowing to select the appropriate concentrations for the experiments of injection (0.04 pmol/embryo, 0.4 pmol/embryo), which revealed the lowest toxic effect on zebrafish embryos.

A standard control morpholino oligonucleotide (STD-MO) was used as negative control. During the analysis, the observed phenotypes have been classified and annotated as “wild-type like”, “mild”, “moderate”, “severe”. In Fig. 94 are reported the representative images for each class. Notably, mild phenotype was discriminated by the presence of small head and eyes, but a general maintained development of the anterior-posterior axis; moderate phenotype was characterized by smaller size of the head and the eye in addition to at least one of other phenotypical appearances, including alteration of the yolk extension and/or of the somite organization; severe phenotype was discriminated by the presence the combination of seriously compromised anterior-posterior development with small head, absence of the eyes (or severe defects of their development), along with pronounced edema. In particular, the first reported (0.04 pmol/embryo) should be considered as the best dose of injection, characterized by the appearance of mild (14.17%) and moderate phenotypes (8.97%), but which maintain a fair share of wild-type-like individuals (60.75%); on the contrary, 0.8 pmol/embryo would represent the most deleterious injection dose, with the significant reduction of wild-type-like individuals (6.69%) and the appearance of mostly severe phenotypes (69.84%), which are more easily attributable to off-target effects of the morpholino.

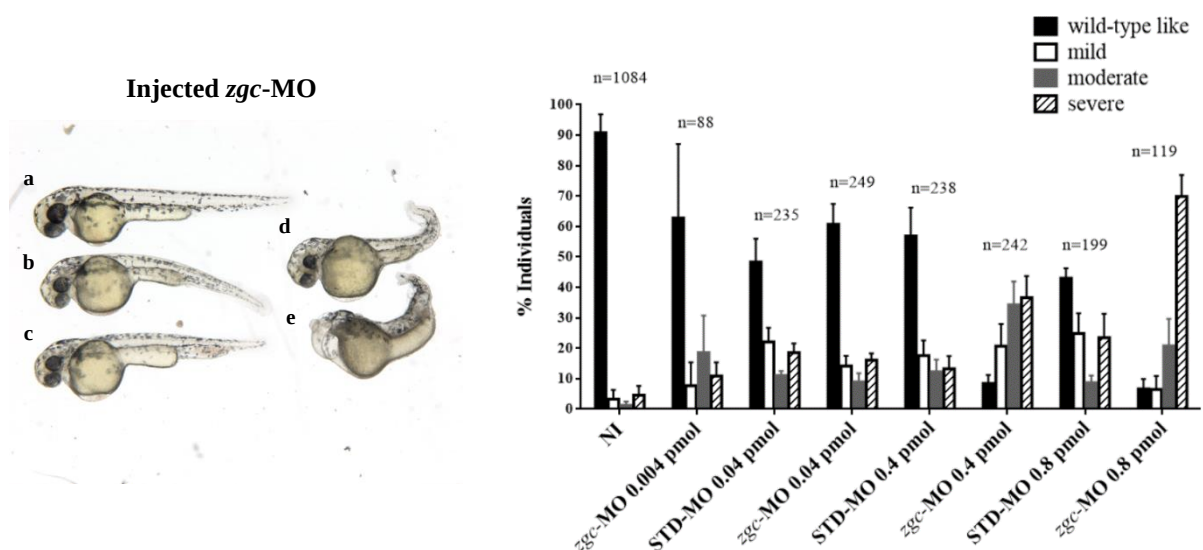


Figure 94. Left side: classes of morphological phenotypes observed after injection of *zgc162858*-EX3ACC morpholino, abbreviated in “*zgc*-MO”. Wild-type like (a), mild (b), moderate (c), severe (d, e). Criteria adopted to distinguish phenotypes are described in the text. Right side: percentage of individuals observed within each morphological class after injection of morpholino in wild-type lines (right side). Total number of injected individuals observed at 48 hpf is reported above each experimental condition. Concentrations of injections are reported on the x axis as pmol/embryo (abbreviated in “pmol”).

To assess the effect of morpholino on the CRISPR/Cas9 lines of *ap1s2*, all the three doses were injected into *ap1s2^{del5/del5}* embryos (Fig. 95). The objective was to evaluate a significant variation in the observed phenotypes within the morphological classes between injections in wild-type and those in *ap1s2^{del5/del5}* embryos. We expected to observe a decrease in the population of wild-type-like or mild individuals and a consequent increase in more severe phenotypes in response to the absence of both the products of *ap1s2* and *zgc:162858*.

As expected, the number of compromised embryos is progressively higher as the injection dose increases. In particular, 0.04 pmol/embryo injection dose was characterized by about 1.6 times fewer wild-type like individuals and about 2.7 times more severe individuals than injected control individuals. Similarly, injection with 0.4 pmol/embryo dose showed a higher number of severely deformed embryos (about 1.8 times higher) than wt individuals injected under the same conditions.

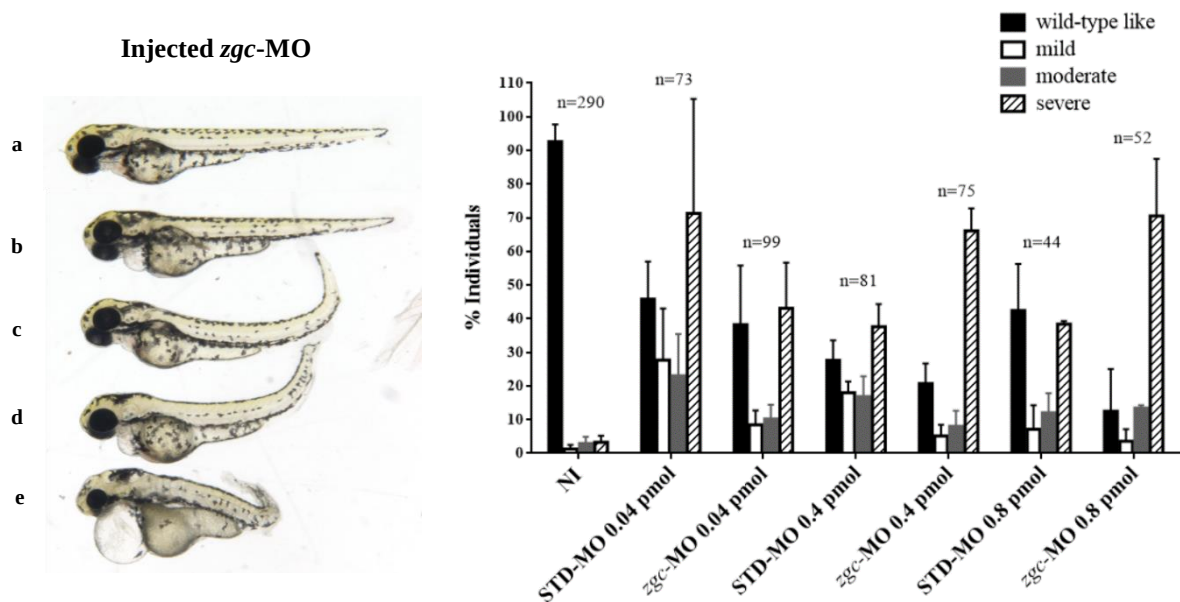


Figure 95. Left side: classes of morphological phenotypes observed after injection of *zgc162858*-EX3ACC morpholino, abbreviated in “*zgc*-MO”. Wild-type like (a), mild (b), moderate (c), severe (d, e). Criteria adopted to distinguish phenotypes are described in the text. Right side: percentage of individuals observed within each morphological class after injection of morpholino in *ap1s2^{del5/del5}* mutant line (right side). Total number of injected individuals observed at 48 hpf is reported above each experimental condition. Concentrations of injections are reported on the x axis as pmol/embryo (abbreviated in “pmol”).

The consequences of the injections were evaluated at the molecular level, through agarose gel electrophoresis of PCR products obtained from cDNA amplification of injected embryos, at 48 hpf. Since the number of remaining embryos after the injections was relatively low, it was decided to proceed by pooling all the individuals injected with a given dose of *zgc:162858*-EX3ACC morpholino. If the splicing morpholino had acted correctly, the reaction would have led to a 261 bp

amplification product, 138 bp shorter than that from correctly spliced transcript (399 bp). Fig. 96 shows representative electrophoresis of some RT-PCR samples corresponding to 0.4 and 0.8 pmol/embryo doses of MO injection. The electrophoresis relative to RT-PCR on the pool of embryos injected with 0.8 pmol/embryo of the splice morpholino for *zgc:162858* (*zgc*-MO) shows a product corresponding to the amplicon expected after the skipping of exon 3 (261 bp). A product corresponding to the size of the transcript that retains the third exon is also present in the sample, indicating that the MO is only partially effective in interfering with correct splicing. In general, the results of the RT-PCR are difficult to interpret: the amplification product of the correctly spliced *zgc:162858* transcript is not present with its correct size (399 bp) both in the non-injected and in the pool of individual injected with the STD-MO. RT-PCR on the pool of embryos injected with 0.4 pmol/embryo of splice MO again suggests a partial efficacy of the MO in inhibiting the correct splicing. This analysis will need to be repeated after optimization of the RT-PCR conditions, probably requiring the identification of a new pair of oligonucleotide primers.

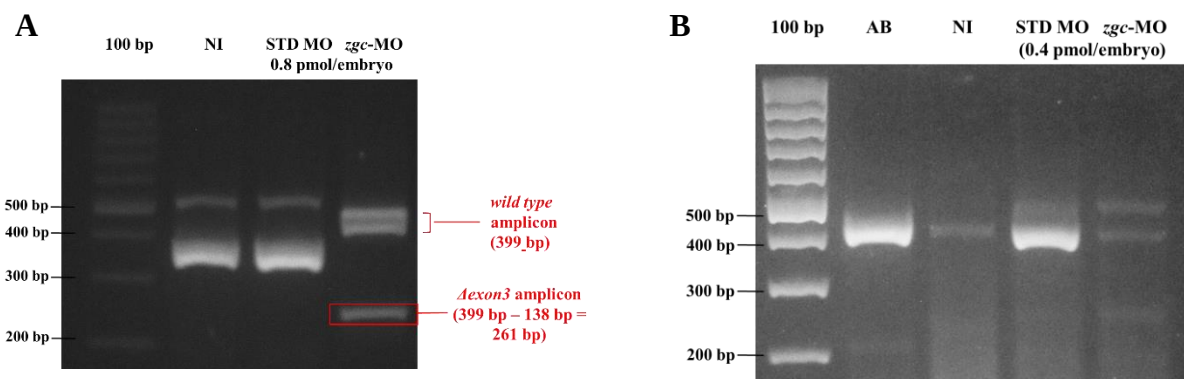


Figure 96. Electrophoresis on agarose gel of products obtained from cDNA amplification of injected embryos, pooled at 48 hpf. Doses of injections are reported on the images: 0.8 pmol/embryo (A) and 0.4 pmol/embryo (B). Description of the wild-type and deleted amplicon are reported in red. Abbreviations: NI (not injected), STD MO (standard morpholino), *zgc:162858*-EX3ACC morpholino (*zgc*-MO).

The UniProt database (<https://www.uniprot.org/uniprotkb/Q5U3I2/entry>) shows a likely localization of zebrafish Slc7a6os protein in cytoplasm and nucleus likewise the human counterpart (Subcellular localization databases of Jensenlab, <https://compartments.jensenlab.org>, Fig. 98).

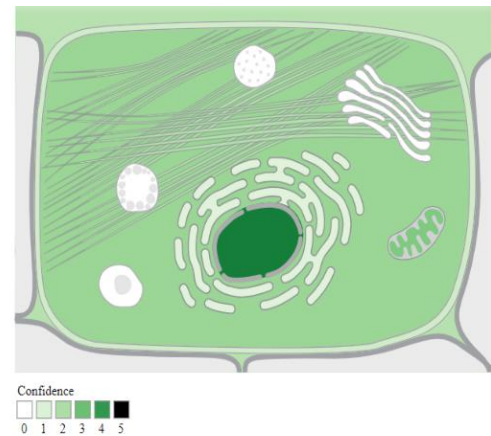


Figure 98. Model of the localization of SLC7A6OS protein in human cell (<https://compartments.jensenlab.org>).

The genomic organization of *slc7a6os* in *D. rerio* was investigated using the University of California, Santa Cruz (UCSC) – Genome Browser, showing the localization of the gene on chromosome 11 and the length in base pairs. In zebrafish the gene is organized in 5 exons and 6 introns (Fig. 99). Besides the former RefSeq (NM_001007344.2, ENSDART00000019991.9), an additional isoform has been annotated and reported according to Ensembl database (<https://www.ensembl.org/index.html>), leading to a protein product of 302 amino acids (ENSDART000000159339.3).

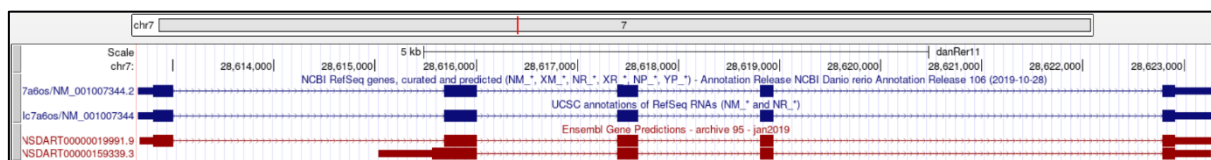


Figure 99. Genomic organization of *slc7a6os* gene within zebrafish chromosome 7 (UCSC Genome Browser).

The analysis of the genomic regions flanking the *slc7a6os* gene were already performed in the study of Benini *et al.*, 2015, using Synteny Database and Genomicus. Synteny is conserved between the regions in human chromosome 16 and *Danio rerio* chromosome 7. It should be noted that zebrafish *slc7a6os* gene, like its human counterpart, is flanked in position 3' by the *slc7a6* gene, although, unlike humans, there is currently no evidence of overlap of the two gene transcripts. The sequence of the first exon belonging to the *SLC7A6OS* human gene, where the mutation c.191A>G responsible for a form of PME present in six patients of two unrelated family is located, has been analysed in detail. This region was compared to that of the first exon belonging to the zebrafish orthologue. The mutation generates a change in the consensus splice site from AGgt to GGgt, thus making the removal of the first intron sequence from the primary transcript of the human gene less efficient. Intron 1 (121 bp) retention in a significant percentage of mature mutate allele transcripts leads in patients either to the production of a truncated protein, characterized by only 86 aa in length (in contrast to the 309 aa

of the wt protein). This is caused by the generation of a premature stop codon within the intron 1 sequence (Fig. 100). Alternatively, the aberrant transcript is degraded by the nonsense-mediated decay (NMD) mechanism. It is interesting to note that the donor splicing sequence (AG) of zebrafish *slc7a6os* exon 1 is identical to that of human wild-type allele (Fig. 101)

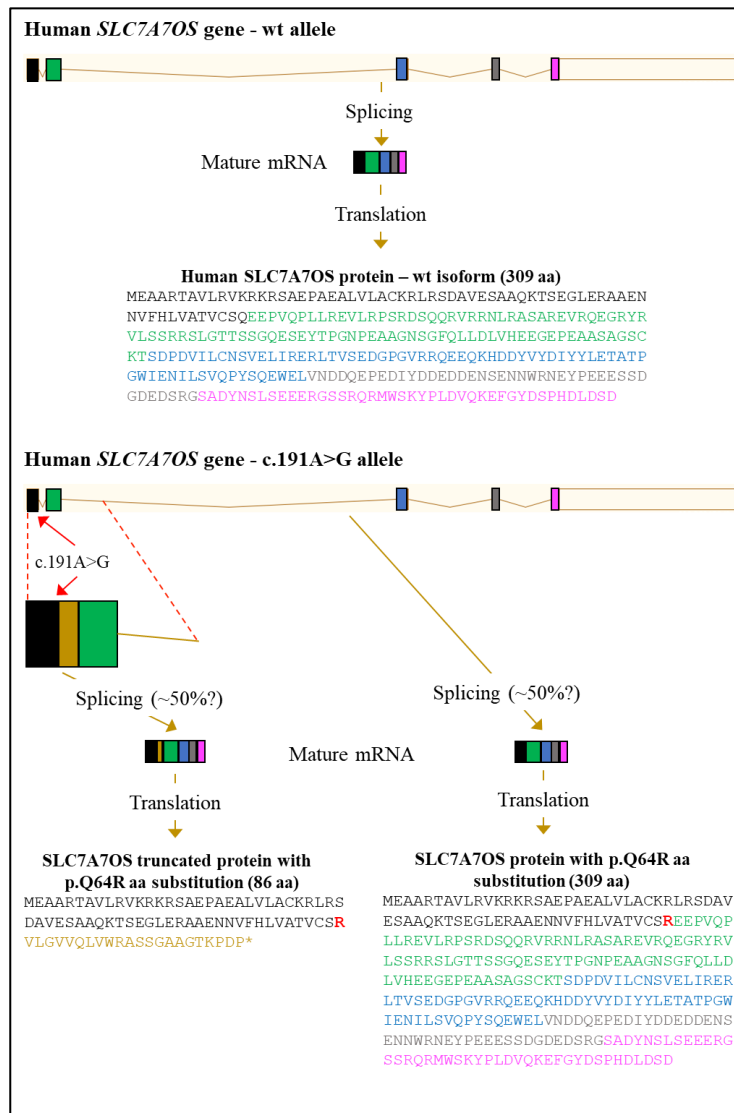


Figure 100. Effect of human c.191A>G (p.Q64R) mutation in PME patients. The reported mutation reduces the splicing effect responsible for the removal of intron 1, generating a significant percentage of mature transcripts which retain that sequence and lead to the synthesis of a truncated protein of only 86 aa (down, left side) or are degraded through a nonsense mediated decay mechanism. The same variation represents also a missense mutation, so even those transcripts that undergo proper splicing give rise to a protein (down, right side) that presents an arginine (instead of a glutamine) in a position (residue 64) which has a high level of conservation during evolution. The five exons of the gene are reported in different colours, as well as their encoded portion of protein.

The c.191A>G sequence variation, besides being a splicing mutation, is also a missense mutation, leading to the substitution of the amino acid in position 64 (glutamine to arginine). Thus, although in PME patients is still present a 309 aa SLC7A6OS protein generated by the translation of properly processed mRNA, this polypeptide presents the p.Q64R mutation that could be detrimental to the functionality of the gene product. Data obtained by western blot experiments indicate a protein amount of about 50% in lymphoblast cells of a subject with PME (with homozygous mutation c.191A>G) when compared to control individuals. Analysis using bioinformatics tools to predict the potential effect of a missense sequence change classifies the p.Q64R mutation as probably damaging (Polyphen, <http://genetics.bwh.harvard.edu/pph2/>) and deleterious (SIFT, <https://sift.bii.a-star.edu.sg/>). In contrast, the recent AlphaMissense predictions classify this as a likely benign substitution with a 0.117 pathogenicity score. It is interesting to note that the c.191A>G sequence variation therefore represents an uncommon case in which a mutation is able both to interfere with the correct splicing of the transcript and to behave as a missense mutation.

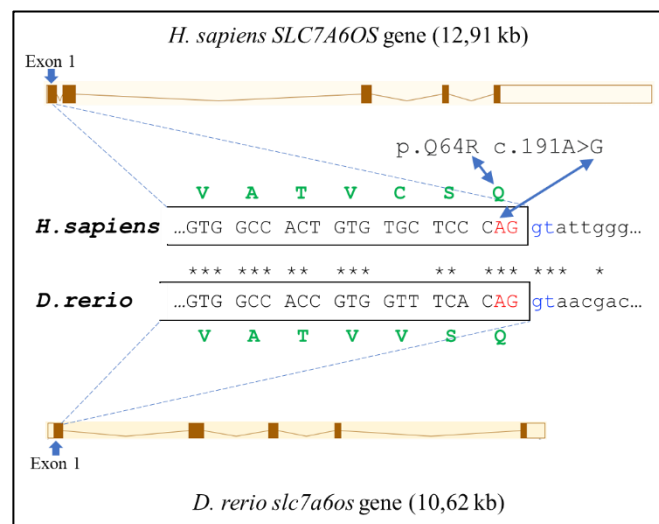


Figure 101. Donor splicing sequence in exon 1 of human *SLC7A6OS* gene and in zebrafish orthologue. Consensus sequence (donor splicing) AGgt is labelled in red (AG) and blue (gt). Relative position of the c.191A>G mutation (responsible for a form of PME) is reported above the human sequence, thus leading to the alteration of the consensus splicing sequence, along with the last codon of the exon 1. Amino acid sequence of the two polypeptides is labelled in green.

9.2 EXPRESSION ANALYSES AT DIFFERENT DEVELOPMENTAL STAGES

RNA-Seq analyses provided by White *et al.* mRNA expression time course of zebrafish development across 18 time points from 1 cell to 5 dpf (White, et al., 2017), allowed to analyse the expression of the investigated gene, revealing that *slc7a6os* (ENSDARG00000010596) expression is relatively low in the embryonal stages; higher expression from 2-cell to blastula stage suggests that *slc7a6os* gene is a “maternal gene”, with accumulation of maternal derived mRNA in the early stages of development. Results are reported in Fig. 102 and expressed in TPM (Transcripts Per Million), a normalised measure of the proportion of transcripts in RNA samples. TPM are calculated from the number of reads that mapped to each particular gene sequence taking into account the gene length and the sequencing depth.

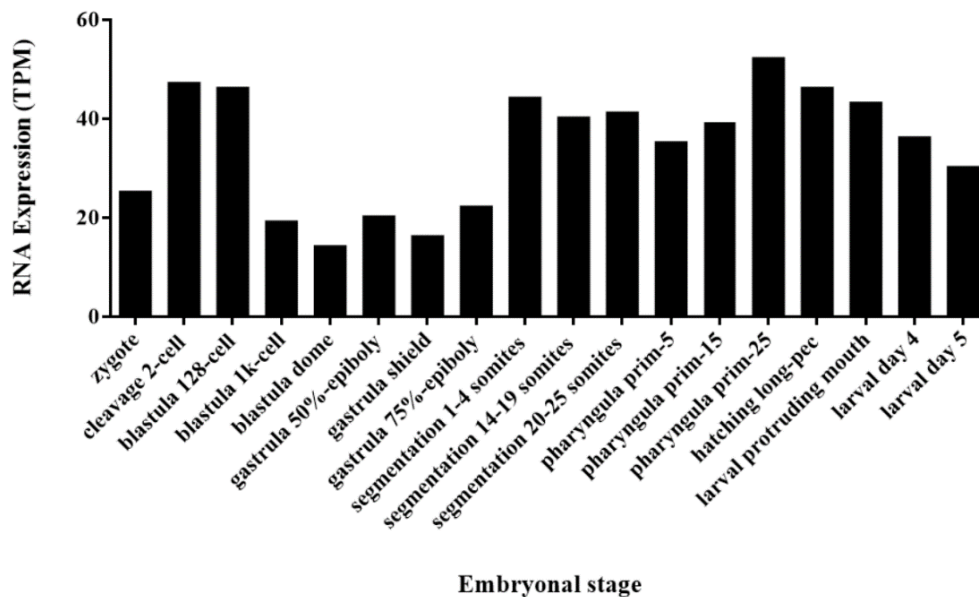


Figure 102. Expression levels of *slc7a6os* expressed in TPM at different zebrafish embryonic stages (White, et al., 2017).

9.3 GENERATION OF *slc7a6os* KNOCKOUT LINES IN ZEBRAFISH USING CRISPR/CAS9

The recent development of CRISPR/Cas9 technology and its relative simplicity of application in *D. rerio* enabled to generate mutant lines carrying loss-of-function (LOF) mutations in the coding region of *slc7a6os* gene (see “Materials and Methods”). Compared to preliminary studies of morpholino injections (Benini, et al., 2015), the generation of stable mutants for *slc7a6os* allows to characterize mutant phenotype also in adulthood.

Sequence of the single guide RNA (sgRNA) was defined using the “CHOP-CHOP” bioinformatics tool, which allowed to predict a specific sgRNA to minimize the possibility to have off-target effects. Among the possible sequences of sgRNA proposed by the tool, we selected one targeting the third exon of *slc7a6os* without relevant sequence identities to other regions of the zebrafish genome. This would minimize any mutations in loci other than the selected one. Validation of the sgRNA sequence was performed using “Verify Guide Design”, provided by SYNTHIGO. Details are reported in Fig. 103.

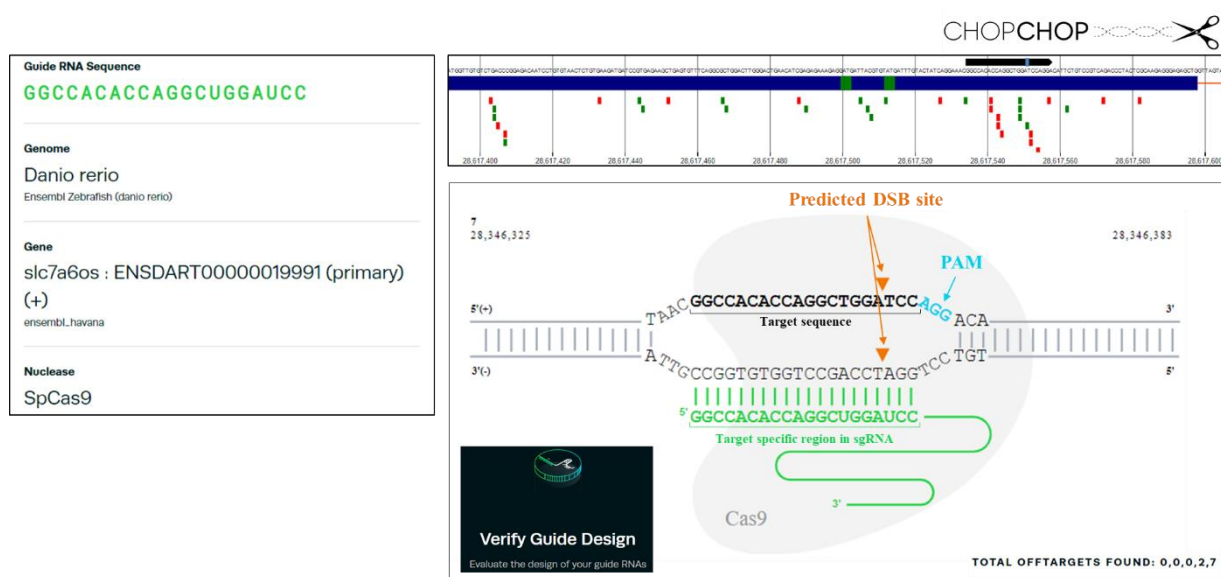


Figure 103. Verification of the *slc7a6os* sgRNA using “Verify Guide Design” (SYNTHIGO): the upper panel show the position of the selected sgRNA using “CHOP-CHOP” bioinformatic tool, while the lower panel shows the target sequence and the predicted site of double strand breaks verified by the tool provided by SYNTHIGO (<https://design.synthego.com/#/validate>).

Embryos at one cell stage were microinjected with the synthetic guide RNA (~ 680 pg/embryo), in addition to the Cas9 endonuclease (EnGen™ Cas9 NLS; *S. pyogenes* – New England Biolabs), to obtain the individuals defined as founders, which represent the F0 generation. These are characterized

by the presence of only some mutated *slc7a6os* cells within the organism, while others may not be mutated or have different mutations (mosaic individuals).

After microinjection, zebrafish embryos were placed at 28 °C and allowed to grow to the 5 dpf developmental stage. Subsequently, genotyping on non-denaturing polyacrylamide (12%) gel has been performed on some individuals of this F0 generation. PCR was performed using specific oligonucleotides that amplify the portion of the third exon of the gene. Analysis of the electrophoretic migration pattern of F0 investigated samples, showed that most of the PCR products are characterized by the presence of multiple bands that suggest the presence of heteroduplex between wild-type and mutated DNA (not shown). Conversely, PCR product on wild-type AB non-injected larvae was characterized by the presence of a single band corresponding to the 110 bp expected amplicon. Part of the PCR products have been subjected to Sanger sequencing and analysed using Poly Peak Parser bioinformatic software. Besides this a sequencing chromatogram that presents double or indecipherable peaks starting from a region surrounding the PAM is a good indication of a samples composed by a mix of different DNA molecules, hopefully wt and mutated ones. In Fig. 104 are reported two exemplificative chromatograms, respectively of a wild-type AB and a putative potential founder. These data suggest the presence of mutational events in *slc7a6os* induced by our genome editing approach.

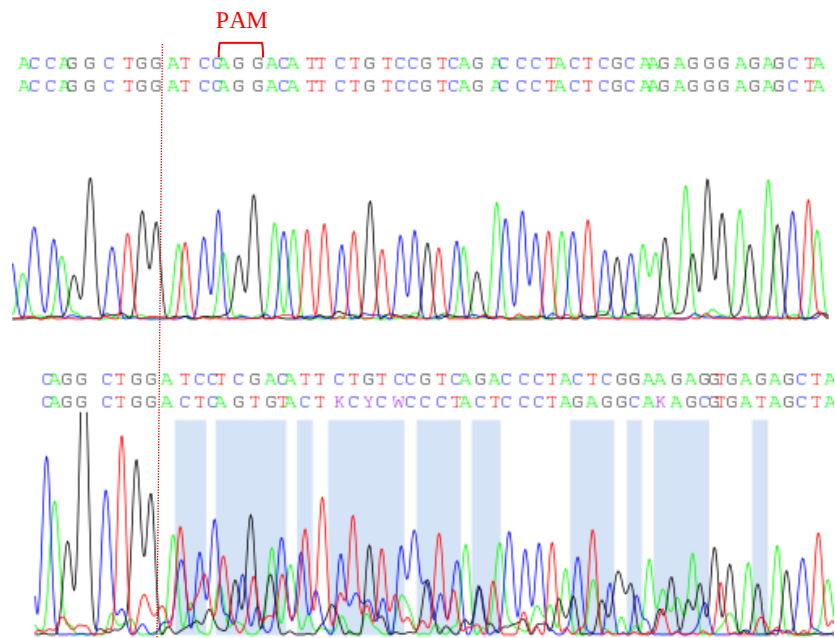


Figure 104. DNA sequencing chromatograms of genotyping PCR products from 5 dpf larvae. Exemplificative chromatograms are shown for a non-injected wild-type AB (NI, upper panel) and an injected larva with *slc7a6os*-EX3 sgRNA, which represents a putative potential founder. The PAM sequence adjacent the sgRNA target site is shown in red (AGG).

The larvae that were not sacrificed for genotyping were placed in a proper tank of the cabinet-aquarium system and allowed to grow until adulthood was reached (~ 3 mpf), when they were subjected to genotyping using the genomic DNA extracted from adult tails. A total number of eight individuals were screened using PCR. The analysis showed results that were more difficult to interpret, with electrophoretic bands at higher molecular weight, attributable to the presence of heteroduplex, less numerous and evident (data not shown). Sanger DNA sequencing revealed that individuals 2, 3, 4 and 5 show evidence of mosaicism; conversely, individuals n. 1, 6, 7 and 8 are characterized by wild-type chromatograms (Fig. 105).

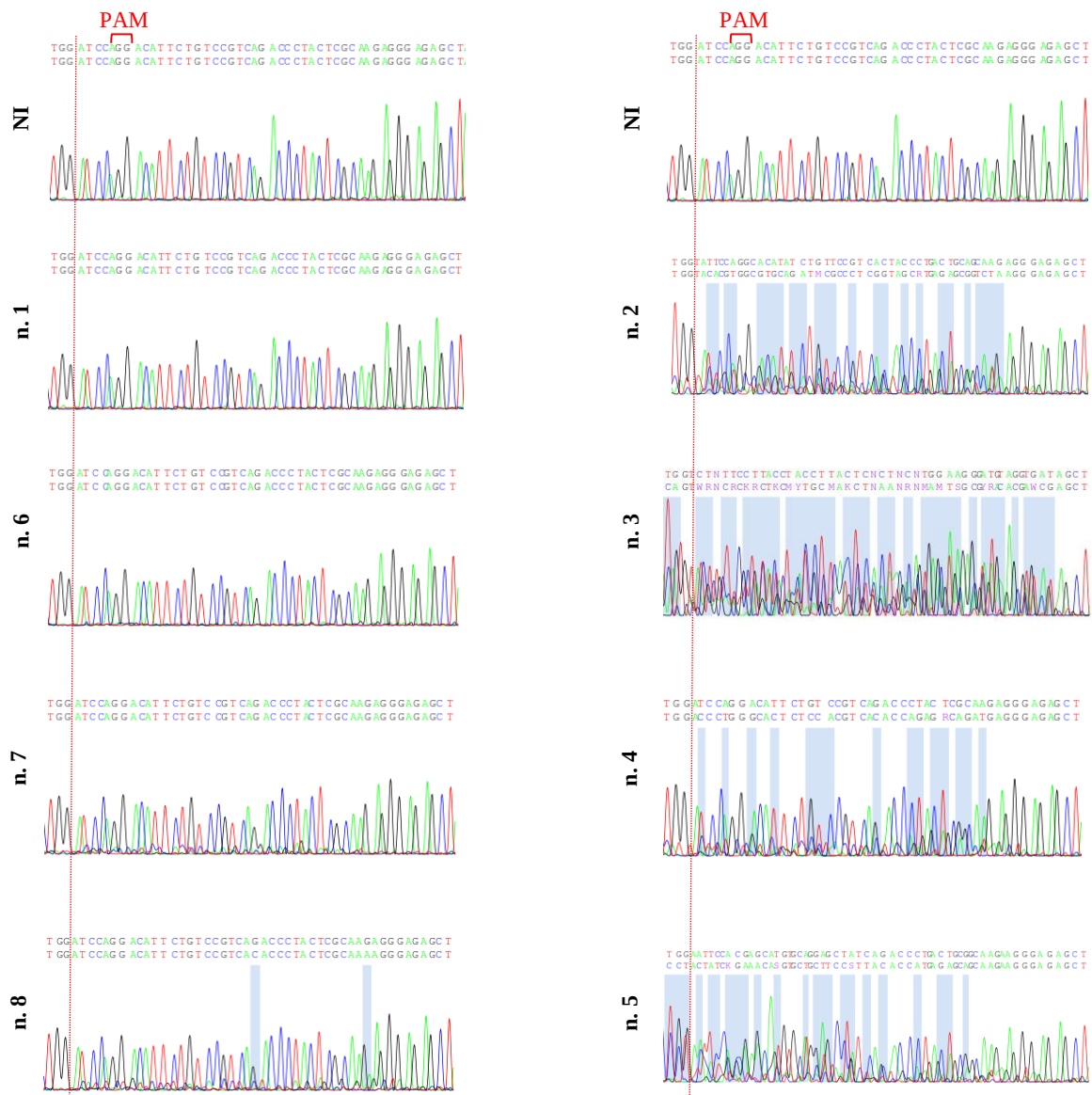


Figure 105. DNA sequencing chromatograms of PCR genotyping products from 3 months old fish. Individuals characterized by wild-type chromatograms are reported on the left side, while those fish characterized by evidence of mosaicism are reported on the right side, along with an exemplificative chromatogram of a non-injected wild-type AB fish (NI). The PAM sequence adjacent the sgRNA target site is shown in red (AGG).

Although experimental evidence was obtained on genomic DNA extracted from adult tails, it is possible to assume that mosaicism is present in other somatic and germinal tissues. The presence of germ cells containing mutations for *slc7a6os* gene would allow the founders to produce gametes able to transmit sequence variations to the F1 generation. Once reached sexual maturity, those fish identified as mosaic were crossed with wild-type AB to obtain individuals (F1 generation) that could be heterozygous in all the cells of the body for the CRISPR/Cas9-induced mutations.

Male founder n. 2, when crossed with wild-type AB, originated a population of embryos with no evident abnormal phenotypes at 5 dpf. Genotyping analyses revealed the presence of two different mutations in F1 generation heterozygotes (Fig. 106): a deletion of two nucleotides (*slc7a6os*^{wt/del2}) and a deletion of eleven nucleotides (*slc7a6os*^{wt/del11}), respectively.

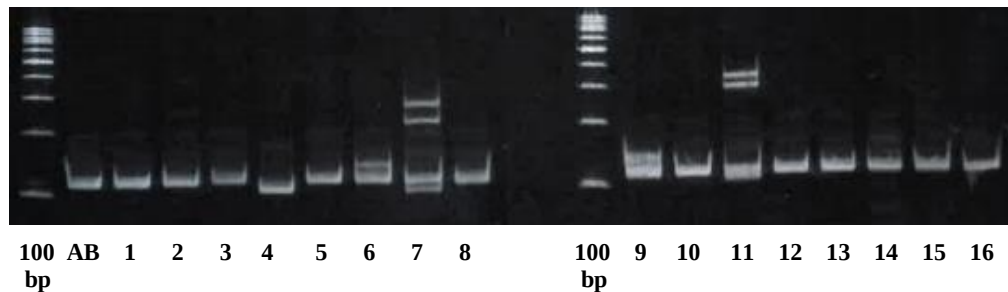


Figure 106. Electrophoresis of genotyping PCR products from DNA of 5 dpf F1 larvae on non-denaturing polyacrylamide (12%) gel.

The described alterations represent frameshift mutations that could lead to the production of truncated proteins (lacking the last NLS) for the presence of premature stop codons in the transcript. More frequently, the transcript of such alleles does not even lead to the production of an abnormal polypeptide due to its degradation by nonsense-mediate decay (NMD) mechanism. While initially the analyses were conducted on both mutant lines, in the absence of evident phenotypic differences between the two strains it was subsequently decided to use mainly the del11 line which allows for easier genotyping of the individuals.

Crossings between heterozygous for the same mutation of the F1 led to the creation of the F2 generation, giving raise to those three genotypic classes that were subjected to characterization experiments (*slc7a6os*^{wt/wt}, *slc7a6os*^{wt/del11}, *slc7a6os*^{del11/del11}).

In Fig. 107 are summarized the characteristic chromatograms of homozygous F2 individuals for del2 or del11 mutations, respectively, along with a representation of the consequences on the protein products.

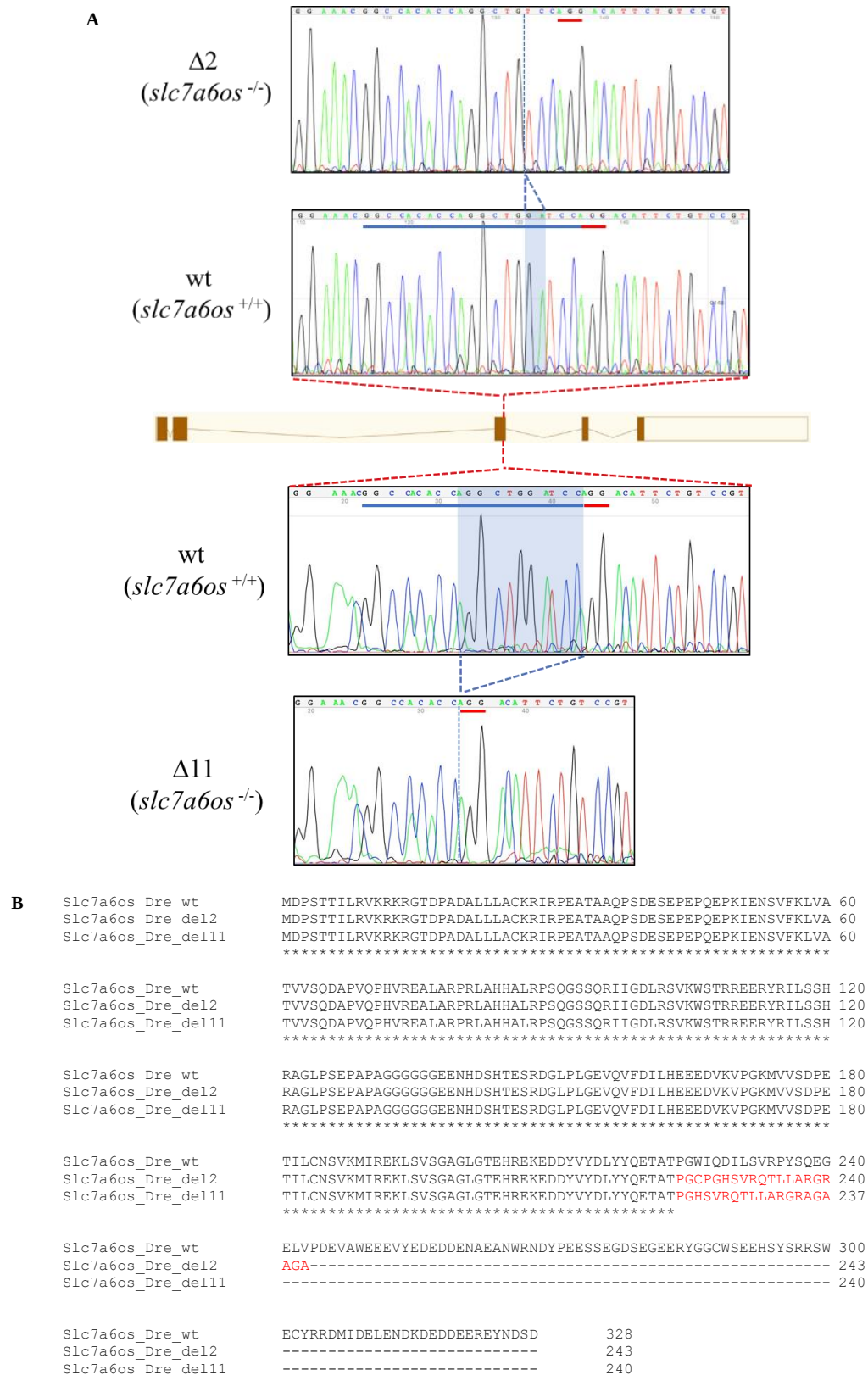


Figure 107. DNA Sequencing chromatograms around the sgRNA target sequence on the exon 3 of *slc7a6os* gene. (A) Representative chromatograms of F2 homozygous individual for the del2 (upper side) and del11 (lower side) are reported, along with the chromatogram of a wild-type individual for comparison. The specific sgRNA target sequence is labelled in blue, while PAM is reported in red; deleted nucleotides are highlighted in light blue. Exon-intron organisation of the *Danio rerio* *slc7a6os* gene is reported in brown. (B) Predicted effect of del2 and del11 frameshift mutations on the protein product: the presence of premature stop codons would lead to the production of truncated proteins (243 aa and 240 aa in length, respectively).

9.4 GENOTYPE SEGREGATION ANALYSIS

Segregation of the three genotypic classes (*slc7a6os*^{wt/wt}, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}) was investigated from 5 dpf to 3 mpf developmental stages on different population of individuals, generated from crosses between del11 heterozygotes. The expected Mendelian ratios of the three genotypic classes was conserved until 1.5 months post fertilization, when a considerable loss of *slc7a6os*^{del11/del11} individuals was detected (Fig. 108). Interestingly, among those fish who reached the adult stage, no homozygous mutant females were present, suggesting that del11 mutation may be in some way impair female sexual development.

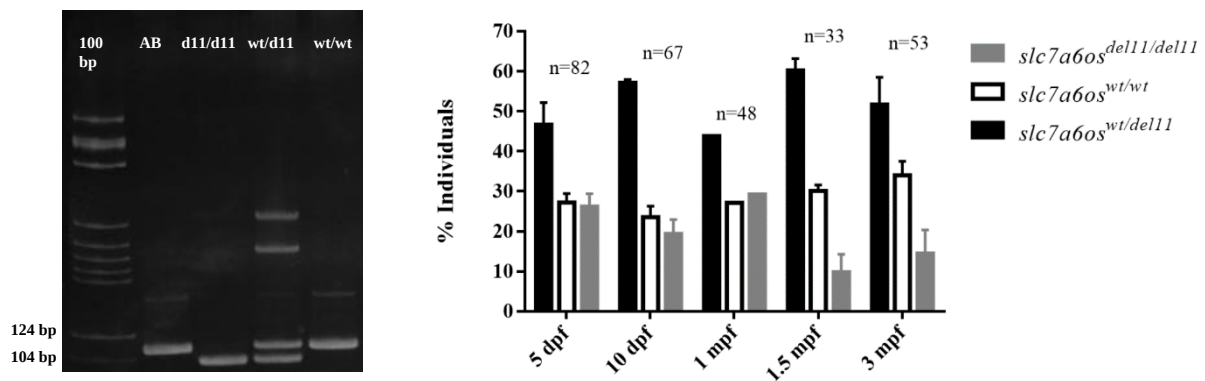


Figure 108. Analysis of the genotypic segregation on populations derived from crosses of individuals heterozygous for the del11 mutation. For each developmental stage, results were obtained from experimental analyses carried out at least twice (excluding the 1 mpf stage, whose data relate to a single experiment), for a total number of individuals indicated above each experimental group. Analysis has been performed using chi-square statistic test, comparing the distributions of the individuals within the three genotypic classes and the expected Mendelian ratios. In particular, significant differences were observed for 1.5 mpf ($p = 0.0025$) and 3 mpf ($p = 0.0169$) developmental stages. Next to the histogram a representative figure is reported, showing the polyacrylamide gel electrophoretic migration (12%) of PCR products from individuals with different genotypes, together with wild-type control sample (AB). Abbreviations: wt: wild-type; d11: deletion of 11 nucleotides; dpf: days post fertilization; mpf: months post fertilization.

In order to analyse in detail the mortality of each genotypic class, two survival experiments have been set up according to the following experimental protocol: larvae generated from crosses between *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11} individuals have been genotyped and left to grow in separated tanks within the aquarium system, annotating the mortality every day. Preliminary data on the two cumulated experiments revealed that fewer homozygotes reached the adulthood, showing a slight higher mortality rate (80.77%) when compared to *slc7a6os*^{wt/del11} (69.77%). Those individuals who reach sexual maturity were only males; conversely, heterozygotes for the same mutation developed balanced populations of males and females. Additional experiments will be necessary to assess the statistical significance of this difference and specific analyses should be performed to thoroughly investigate the process of sexual maturity in *slc7a6os* zebrafish mutants.

9.5 THE EFFECT OF del11 MUTATION ON *slc7a6os* TRANSCRIPT

Although the del11, located in the third exon of the *slc7a6os* gene, represents a frameshift mutation, we cannot exclude that it may induce aberrant splicing of the primary transcript by the creation of cryptic splice sites. To test this hypothesis, we performed RT-PCR experiments with primers located in the second (forward) and the fifth (reverse) exon of the gene. The analysis was carried out on total RNA obtained from the tails of the following individuals:

- Tg(*neurod1:EGFP*) with *slc7a6os*^{del11/del11} genotype
- Tg(*neurod1:EGFP*) *slc7a6os*^{wt/del11} genotype
- wild-type fish with *slc7a6os*^{wt/wt} genotype

The three samples all show the presence of a single amplification product of the expected size (463 bp, Fig. 109) indicating that the del11 mutation has no obvious effect on *slc7a6os* transcript splicing.

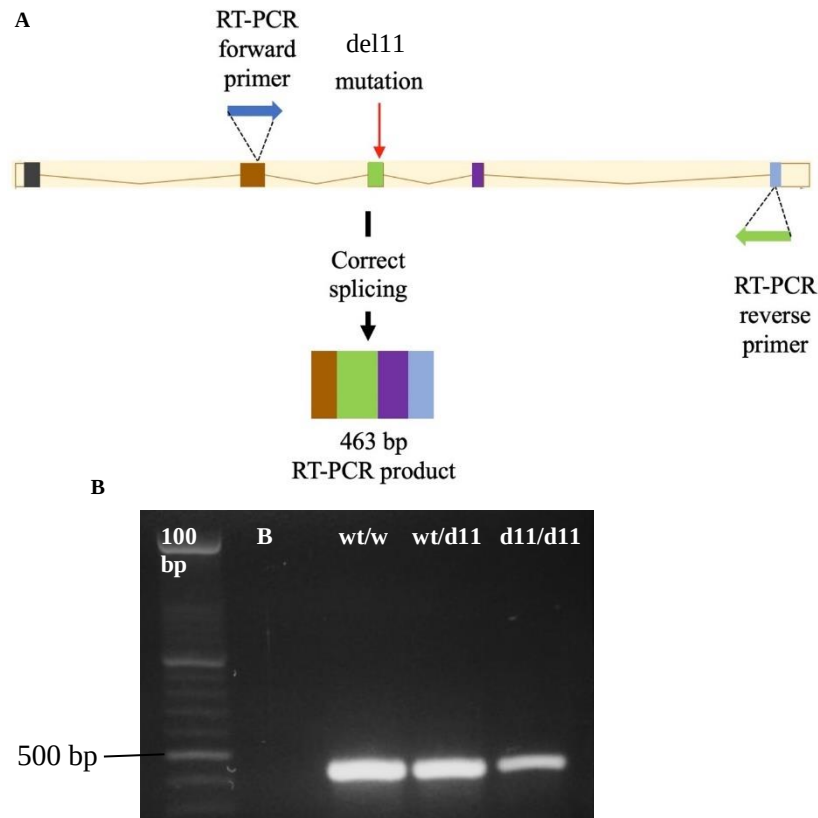


Figure 109. (A) RT-PCR amplification scheme on *slc7a6os* cDNA. Forward and reverse primers are reported as complementary to the second and the fifth coding exon, respectively. Following proper splicing of the primary transcript, an amplification product of 463 bp is expected, consisting of a portion of the exon 2 (in brown), the complete exon 3 (in green) and 4 (purple) and a portion of exon 5 (in blue). **(B) Electrophoretic analysis on agarose gel of the amplification products obtained from *slc7a6os* cDNA.** Abbreviations: B: blank of the PCR; wt/wt: *slc7a6os*^{wt/wt}; wt/d11: *slc7a6os*^{wt/del11}, d11/d11: *slc7a6os*^{del11/del11}.

Results were confirmed by investigating the three PCR products with Sanger DNA sequencing analysis (Fig. 110): as expected, *slc7a6os* cDNA derived from the homozygous individual for the mutation (*slc7a6os*^{del11/del11}) show the 11 nucleotides deletion also present in the gDNA. It is interesting to note that in the cDNA of the heterozygous individual (*slc7a6os*^{wt/del11}), the chromatogram corresponding to the wild-type allele is decidedly prevalent compared to that of the mutated allele, which is present as a sort of “background noise”. This result suggests that a higher amount of cDNA molecules belonging to the wild-type allele is present in the heterozygote RT-PCR sample in contrast to the product of the del11 allele. This suggests that the allele transcript characterized by frameshift mutation undergoes nonsense-mediated RNA decay. Although the RT-PCR experiment is not quantitative, in Fig. 109 the amount of amplified individual *slc7a6os*^{del11/del11} is lower than that of *slc7a6os*^{wt/wt} and *slc7a6os*^{wt/del11}, further suggesting a lower stability of the mutated allele. Further experiments with quantitative methods (*e.g.*, Digital Droplet PCR) will be needed to reinforce the hypothesis.



Figure 110. DNA sequencing chromatograms of the cDNA obtained from adult tails of different *slc7a6os* genotypes. The genotypes corresponding to each analysed sample are reported on the left. The eleven bp that are deleted in the *slc7a6os*^{del11/del11} are reported in red square.

9.6 MORPHOLOGICAL ANALYSIS ON LARVAE

To assess the presence of morphological changes that could be related to inactivating mutations of *slc7a6os* gene in zebrafish, larvae resulting from cross between *slc7a6os*^{del11/del11} and *slc7a6os*^{wt/del11} individuals (later genotyped) were observed at 5 dpf developmental stages (n = 82) and compared with wild-type AB controls (n = 59). Morphological analysis was conducted using the ZEISS AXIO Zoom.V16 microscope, leading to the identification of three morphological classes: “wild-type like”, “deflated swim bladder”, “deformed” and “severely deformed” (Fig. 111).

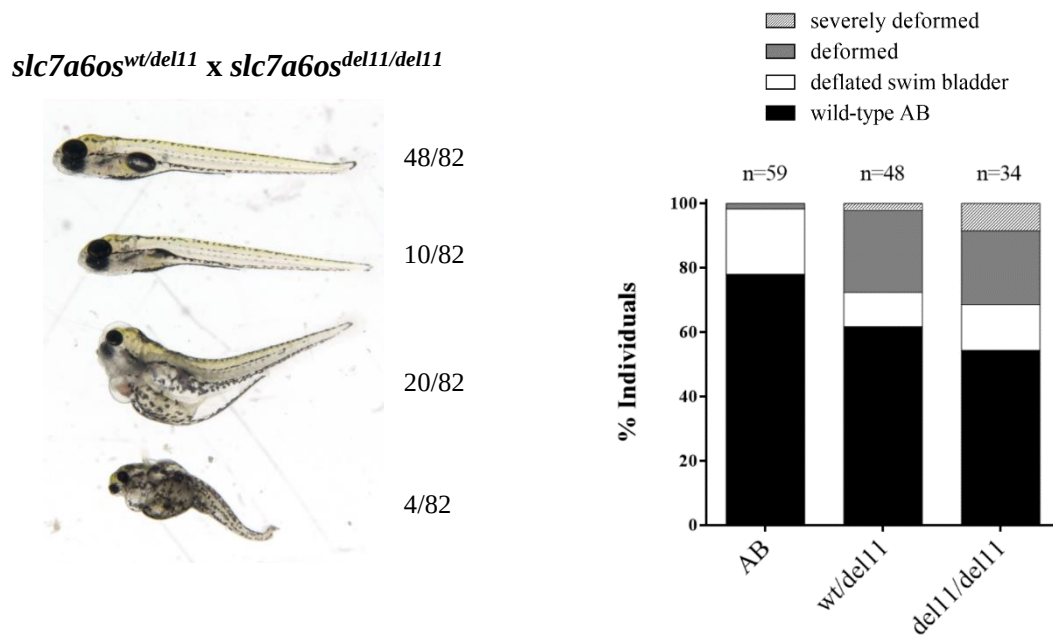


Figure 111. Morphological classes observed for *slc7a6os* mutant individuals (left side). Represented larvae were generated by crossings between *slc7a6os*^{del11/del11} and *slc7a6os*^{wt/del11} individuals. **Distribution of the observed morphological phenotypes in 5 dpf larvae (right side).** Results were obtained from a single preliminary observation. References to the fish line and genotypes are reported on the x axis (AB: wild-type AB; wt/del11: *slc7a6os*^{wt/del11}; del11/del11: *slc7a6os*^{del11/del11}), while total number of the observed embryos is reported above each bar.

“Deformed” larvae were discriminated by the presence of smaller head and eyes, edema and moderate altered antero/dorsal development, while “severely deformed” showed compromised antero/dorsal development, serious edema, small head and eyes and development of necrotic areas. Preliminary data suggested that a higher percentage of individuals with altered morphology resulted from *slc7a6os*^{del11/del11} and *slc7a6os*^{wt/del11} coupling than wild-type AB controls. More in detail, comparisons between heterozygotes and homozygotes for del11 mutation showed a tendency of *slc7a6os*^{del11/del11} larvae to develop abnormal phenotypes when compared to *slc7a6os*^{wt/del11} individuals. Further morphological analyses on a larger number of fish at different stages of development will be necessary to investigate more thoroughly the presence of phenotypic alterations that explain the high mortality of homozygous individuals starting from one month of age.

9.7 SPATIAL-TEMPORAL ANALYSIS OF KEY NEUROGENESIS GENES IN MUTANTS

The spatial-temporal expression of relevant genes during the neurogenesis process was investigated to detect phenotypic abnormalities in the development of the central nervous system in *slc7a6os* mutants. Moreover, these experiment should confirm previously generated data on the morpholino knockdown model using the same probes. *In situ* hybridizations were performed using specific antisense RNA probes for *pax2a* (16 hpf and 30 hpf) and *neurod1* (24 hpf and 72 hpf) neurogenesis markers (specific information for these genes are reported in “Materials and methods” paragraph). For each experiment, groups of embryos from crosses between *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11} were used and about 30 wild-type AB embryos as controls. The observation of the embryos was carried out using the ZEISS Axio Zoom.v16 microscope in bright field and at different magnifications, allowing to obtain high-resolution photographs of the examined individuals. After hybridizations, the embryos were divided according to the differences observed in the expression of the analysed marker. Subsequently, genotyping was performed on selected and representative individuals for each phenotypic class of expression, in order to verify a possible correlation between expression patterns and the genotype.

***pax2a* expression at 16 hpf**

At 16 hpf 20 wild-type AB and 18 embryos generated from cross between *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11} individuals have been analysed after hybridization with *pax2a* antisense RNA probe (Fig. 112). By this preliminary experiment no strongly evident differences in *pax2a* expression have been observed in the pool of individuals obtained from *slc7a6os*^{wt/del11} X *slc7a6os*^{del11/del11} when compared to wild-type controls, with respect to the interested anatomical regions. Thus, five embryos of the pool were selected and genotyped, revealing the presence of the two expected genotypic classes: *slc7a6os*^{wt/del11} (2/5) and *slc7a6os*^{del11/del11} (3/5), respectively. It is interesting to note that *neurod1* expression signal suggested a possible downregulation in the region of the midbrain hindbrain boundary of *slc7a6os*^{del11/del11} individuals, which therefore was shorter when compared to those of *slc7a6os*^{wt/del11} and the relative controls.

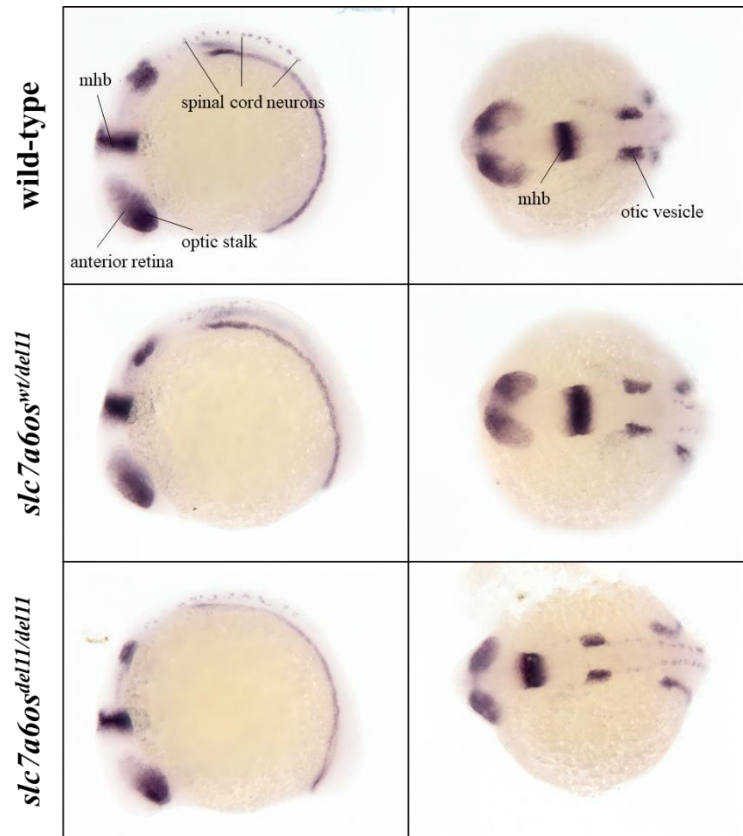


Figure 112 *In situ* hybridizations for *pax2a* at 16 hpf. From the top, representative embryo of: wild-type AB, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}, respectively. On the left: lateral view. On the right: dorsal view. Anatomical references are reported for comparison, while genotype reference is indicated on the left. Abbreviations: mhb: midbrain hindbrain boundary.

***pax2a* expression at 30 hpf**

At 30 hpf, 24 wild-type AB and 43 embryos generated from cross between *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11} individuals have been analysed after the hybridization with *pax2a* antisense RNA probe (Fig. 113). In preliminary experiments it was possible to observe slight differences within the pool of *slc7a6os*^{wt/del11} X *slc7a6os*^{del11/del11} embryos, in the region corresponding to the midbrain-hindbrain boundary and the hindbrain neurons. It was therefore possible to divide the 43 embryos into two subclasses: embryos with wild-type *pax2a* expression patterns (26/43) and embryos with slightly altered spatial-temporal expression (17/43). Subsequently, 4 representative embryos were selected and genotyped for each group. The analysis showed that all embryos with wild-type expression patterns resulted to be *slc7a6os*^{del11/del11}, while all embryos with slightly altered expression phenotype *slc7a6os*^{wt/del11}, leading to a difficult interpretable data. This is a very preliminary data that will have to be confirmed by further investigations on a larger number of embryos in order to establish an effective genotype/phenotype correlation. However, it cannot be excluded that compensation mechanisms may be activated by mutations induced by Cas9, resulting in mild or no phenotypes.

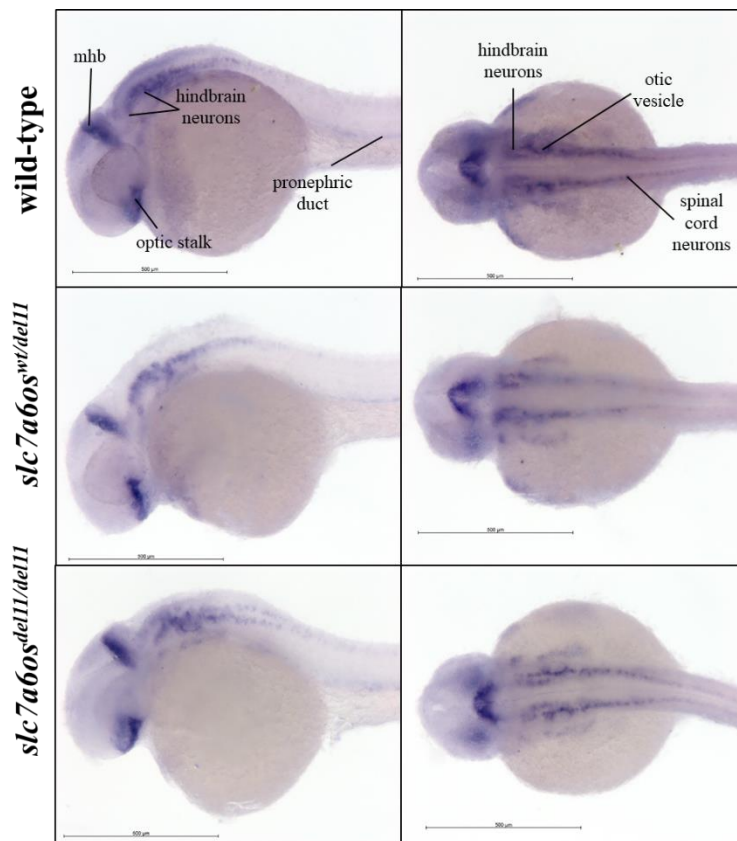


Figure 113. *In situ* hybridizations for *pax2a* at 30 hpf. From the top, representative embryo of: wild-type AB, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}, respectively. On the left: lateral view. On the right: dorsal view. Anatomical references are reported for comparison, while genotype reference is indicated on the left. Abbreviations: mhb: midbrain hindbrain boundary.

***neurod1* expression at 24 hpf**

At 24 hpf 20 wild-type AB embryos and 50 embryos generated from cross between *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11} individuals have been analysed after hybridization with *neurod1* antisense RNA probe (Fig. 114). Slight differences of the expression signal were observed when comparing *slc7a6os*^{wt/del11} X *slc7a6os*^{del11/del11} embryos and the relative wild-type controls at the level of the telencephalon (t); in addition to that, a small alteration was visible at the level of the octaval statoacoustic ganglia (o) compared to controls. It was therefore possible to divide the 50 embryos into two subclasses: a first group, including those embryos with wild-type-like pattern of expression (31/50) and a second group, including those individuals with altered pattern of expression (19/50). The embryos were subsequently subjected to genotyping, which revealed the presence of three homozygous individuals for the del11 mutation characterized by the presence of an altered phenotype (Fig. 32 summarizes the *neurod1* spatial-temporal expression of two representative individuals with *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11} genotypes, respectively). On the contrary, no homozygous individuals for the del11 mutation were found in the pool of wild-type-like embryos. These are preliminary data that will have to be confirmed by further investigations on a larger number of embryos in order to establish an effective genotype/phenotype correlation.

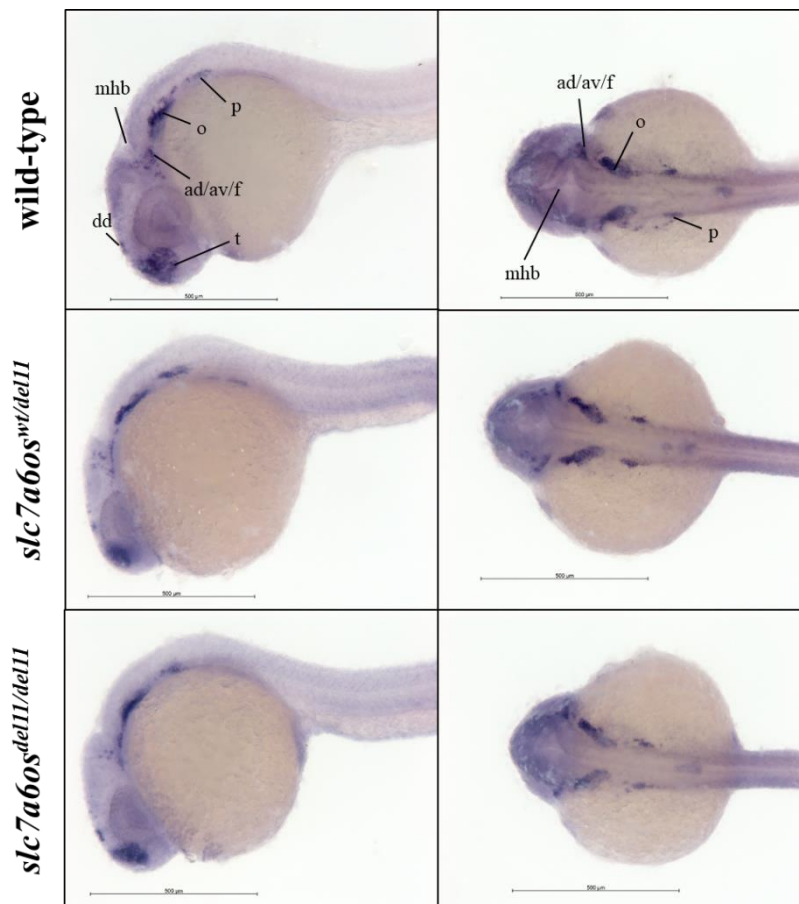


Figure 114. *In situ* hybridizations for *neurod1* at 24 hpf From the top, representative embryo of: wild-type AB, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}, respectively. On the left: lateral view. On the right: dorsal view. Anatomical references are reported for comparison, while genotype reference is indicated on the left. Abbreviations: ad/av/f: anterodorsal/anteroventral lateral line/facial placodes ganglia dd: dorsal diencephalon; mhb: midbrain hindbrain boundary; o: octaval/statoacoustic ganglia; p: posterior lateral line ganglia; t: telencephalon.

***neurod1* expression at 72 hpf**

At 72 hpf, 31 wild-type AB and 32 embryos generated from cross between *slc7a6os*^{wt/del11} and *slc7a6os*^{wt/del11} individuals have been analysed after hybridization with *neurod1* antisense RNA probe⁶ (Fig. 115). Slight differences in *neurod1* expression were observed at the level of midbrain-hindbrain boundary (mhb) with respect to wild-type controls in this pool of embryos. Eight representative individuals were then selected and genotyped. From this analysis it was possible to verify the presence of individuals belonging to all three expected genotypic classes. In Fig. 114 are reported the images of one representative embryo for each genotype, respectively *slc7a6os*^{wt/wt}, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}.

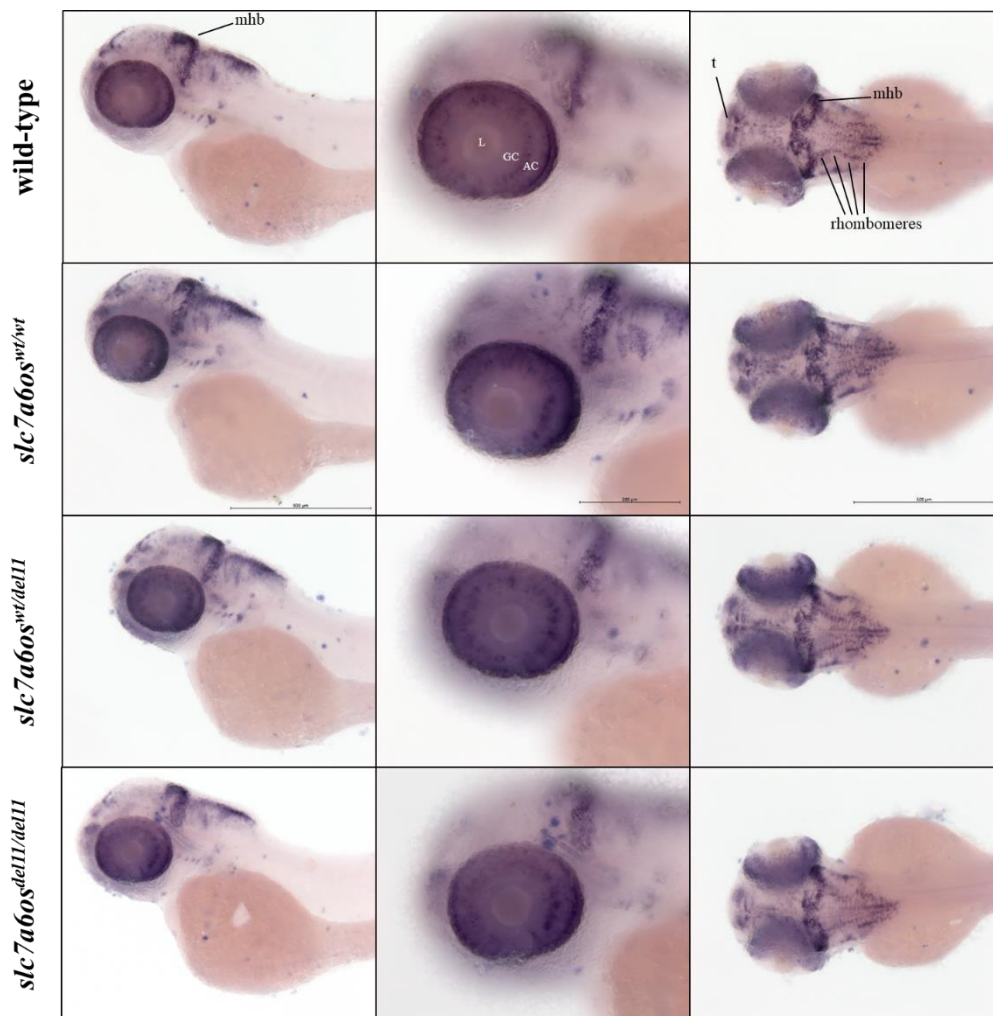


Figure 115. *In situ* hybridizations for *neurod1* at 24 hpf From the top, representative embryo of: wild-type AB, *slc7a6os*^{wt/wt}, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}, respectively. On the left: lateral view. On the right: dorsal view. Anatomical references are reported for comparison, while genotype reference is indicated on the left. Abbreviations: mhb: midbrain hindbrain boundary; t: telencephalon; L: lens; GC: ganglion cells; AC: amacrine cells.

⁶ During the period in which the couplings were set up in order to obtain the embryos to be fixed for the hybridization experiments, *slc7a6os*^{del11/del11} males started to show obvious difficulties in reproduction, without exhibiting the typical behaviour during the mating, not stimulating the females, and tended to remain isolated on the bottom of the tank. It was therefore decided to proceed by setting up crossings between heterozygous individuals for the mutation.

Through a more accurate analysis of the eye, it has been possible to observe some differences in *neurod1* expression in some neuronal populations of the central portion of the eye, which appeared to be characterized by a weaker and disorganized signal in *slc7a6os^{del11/del11}* individuals when compared to *slc7a6os^{wt/wt}*, *slc7a6os^{wt/del11}* and wild-type AB controls. Further analyses should be performed based on these preliminary data and, in particular, histological sections on hybridized embryos of known genotype may be useful to investigate more in detail the effect of *del11* mutation on *neurod1* expression in this type of neuronal cell population.

It is important to underline that *in situ* hybridization is a technique that allows to verify the presence of alterations in the spatial-temporal expression of genes of interest from a qualitative point of view. For this reason, further quantitative analysis should be performed, including quantitative PCR (qPCR) or Digital PCR.

9.8 GENERATION OF THE TRANSGENIC/KNOCKOUT LINE Tg(*neurod1*:EGFP) *slc7a6os del11*

The Zebrafish Facility at the University of Brescia (Department of Molecular and Translational Medicine) allows the housing and breeding of several transgenic reporter lines for genes involved in neurogenesis. In particular, the Tg(*neurod1*:EGFP) transgenic line is available (Thomas, Ochocinska, Hitchcock, & Thummel, 2012), expressing EGFP under the control of *neurod1* promoter, a transcription factor involved in the process of neurogenesis in diencephalon, telencephalon and ganglia. To investigate more thoroughly the impact of the *slc7a6os* induced mutation during the neurogenesis, a transgenic/knockout line have been generated by crossing the CRISPR/Cas9 mutant line characterized by the *del11* mutation with individuals of the Tg(*neurod1*:EGFP) transgenic line. Males individual of the Tg(*neurod1*:EGFP) transgenic line have been crossed with heterozygotes females with *slc7a6os^{wt/del11}* genotype to obtain the F1 generation. Screening for fluorescence has been performed at 48 hpf and positive individuals have been placed at 28 °C until 5 dpf, when they have been transferred to the aquarium system and allowed to grow until adulthood. Once reached sexual maturity they have been genotyped to identify heterozygotes for *del11* mutation. Subsequent crosses between *slc7a6os^{wt/del11}* fish allowed to obtain the F2 transgenic/knockout line that was employed to perform the characterization analyses.

9.8.1 ANALYSIS OF *neurod1* EXPRESSION IN Tg(*neurod1*:EGFP) *slc7a6osdel11* THROUGH LIGHT SHEET MICROSCOPY

To investigate the expression of *neurod1* transcription factor in the generated double transgenic/knockout line “Tg(*neurod1*:EGFP) *slc7a6osdel11*”, pools of embryos at 3 dpf have been subjected to screening for fluorescence using the ZEISS Axio Zoom.V16 microscope to select those individuals who presented a comparable level of fluorescence. Subsequently, selected larvae have been genotyped by cutting the tail fin and extracting the DNA and then fixed in PFA 4%. Later, two Tg(*neurod1*:EGFP) *slc7a6os^{del11/del11}* individuals were analysed through using ZEISS LightSheet microscope V1 and compared with relative Tg(*neurod1*:EGFP) controls characterized by wild-type *slc7a6os* sequence. From an initial comparative investigation of the obtained three-dimensional reconstruction, it was possible to observe how the brain structures in which *neurod1* is expressed were maintained, both in the control subjects and in the mutants, in the dorsal portion (Fig. 116, A-B) and in the ventral portion (Fig. 116, C-D). These data confirm previously investigation using *in situ* hybridization technique at the same developmental stage.

Interestingly, a more detailed analysis of the transversal view (single plane) allowed to observe some differences in the cellular organization of the eye, in the regions corresponding to the marginal zone and the inner nuclear layer (indicated by red arrows in Fig. 116, F). It is well known from literature that *neurod1* plays a key role in the proliferation of photoreceptor progenitor cells in zebrafish retina; its expression during the development of the teleost is dynamic and present both during the early retinogenesis and the maturation of the photoreceptors of the cones. In early retinogenesis, expression of *neurod1* expands from a small region of the ventro-nasal retina through the remaining retinal neuroepithelium. As retinogenesis progresses, the expression of *neurod1* is limited to amacrine cells, immature cones, and cells of rod and cone lineages. This expression reaches an adult pattern by 96 hpf, after which the temporal expression pattern of *neurod1* in the central retina is spatially summarized at the germinative margin (Ochocinska & Hitchcock, 2007).

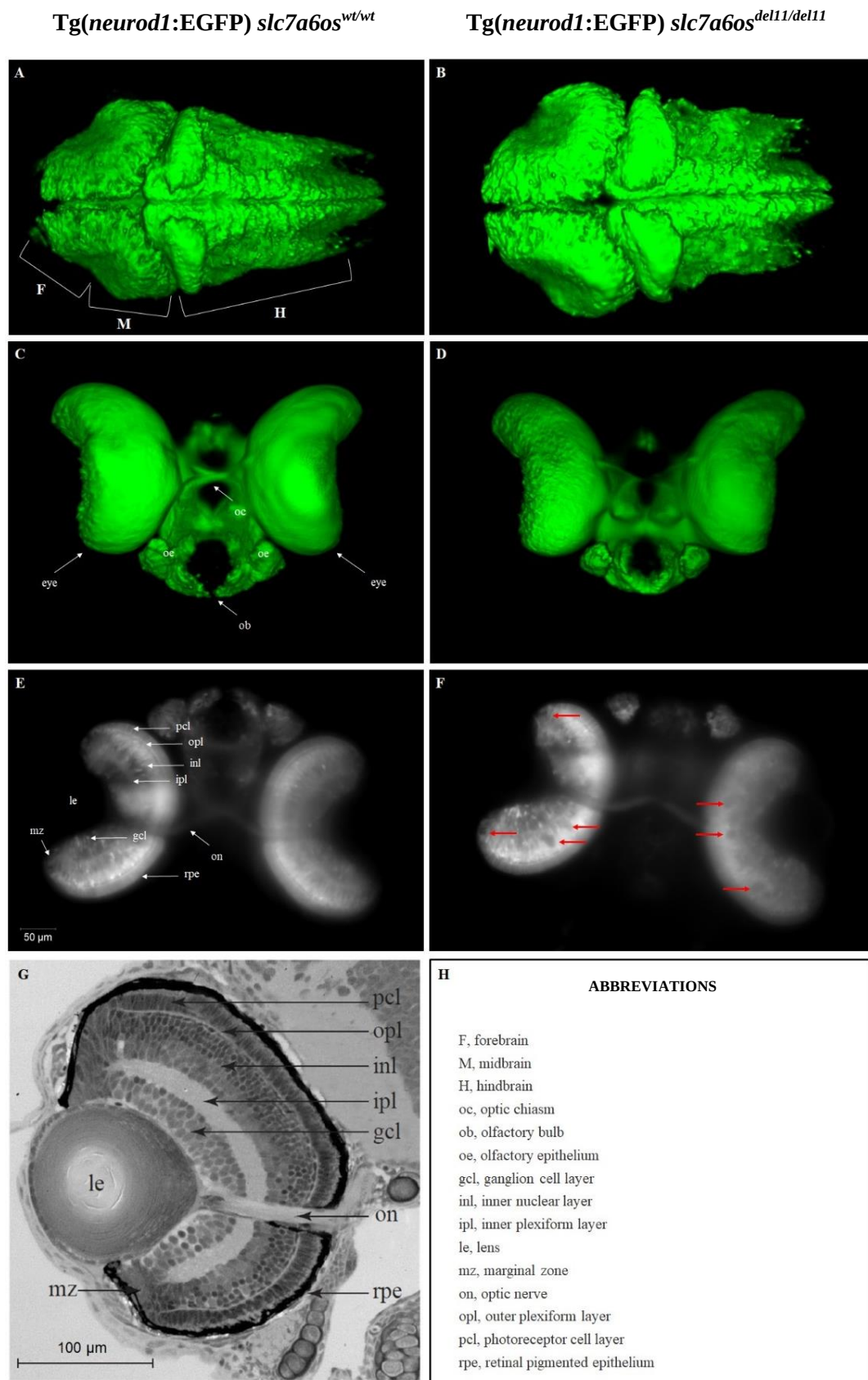


Figure 116. Analysis of *neurod1* expression at 72 hpf through light sheet fluorescence microscopy. (A, C, B, D) Three-dimensional dorsal and ventral reconstruction of embryos Tg(*neurod1*:EGFP) *slc7a6os*^{wt/wt} and Tg(*neurod1*:EGFP) *slc7a6os*^{del11/del11}, respectively. (E, F) Single ventral plane of MIP of Tg(*neurod1*:EGFP) *slc7a6os*^{wt/wt} and Tg(*neurod1*:EGFP) *slc7a6os*^{del11/del11}, respectively. Anomalies of the cellular organization are indicated by red arrows. (G) Histological section of zebrafish eye at 72 hpf for interpretation of E and F images (Malicki, et al., 1996).

The hypothesis formulated was that there may be a disorganization at the level of the retinal cells of *slc7a6os^{del11/del11}* individuals. To exclude the possibility that the observed differences were the result of experimental artefacts, new-born larvae, derived from the crossing of heterozygous individuals for the *del11 slc7a6os* mutation, have been genotyped and fixed in PFA 4%. Subsequently, individuals, respectively *slc7a6os^{del11/del11}* and *slc7a6os^{wt/wt}*, were processed for histological sections and subsequent staining in haematoxylin and eosin.

The main objective of the analysis was the possibility to correctly distinguish all the layers of the retina and the respective cellular populations. Consequently, before being processed for histology, the embryos were left to grow to a stage closer to 4 dpf, to reduce the variability generated by the possible asynchrony of development of the various individuals. Preliminary sections were made from *slc7a6os^{wt/wt}* and *slc7a6os^{del11/del11}* embryos. The figure below (Fig. 117) shows representative images of a single individual for each genotype, respectively. It is possible to observe a correct organization of the retinal layers in the mutant, where no noticeable changes are observed in the different cell populations that can be recognized as aberrant phenotypes known from the literature (Malicki, et al., 1996). Unlike the expected, the experiment showed that wt individual presents a less defined organization of the retina, with a thin and almost absent inner plexiform layer (IPL) and not correctly developed retinal layers. Although this may be an experimental artefact, further analysis will be carried out to clarify this aspect. This represent a single and preliminary experiment that should be repeated in order to assess the presence of a specific phenotype. More accurate histological sections will be performed, selecting those characterized by a greater correspondence between the plans.

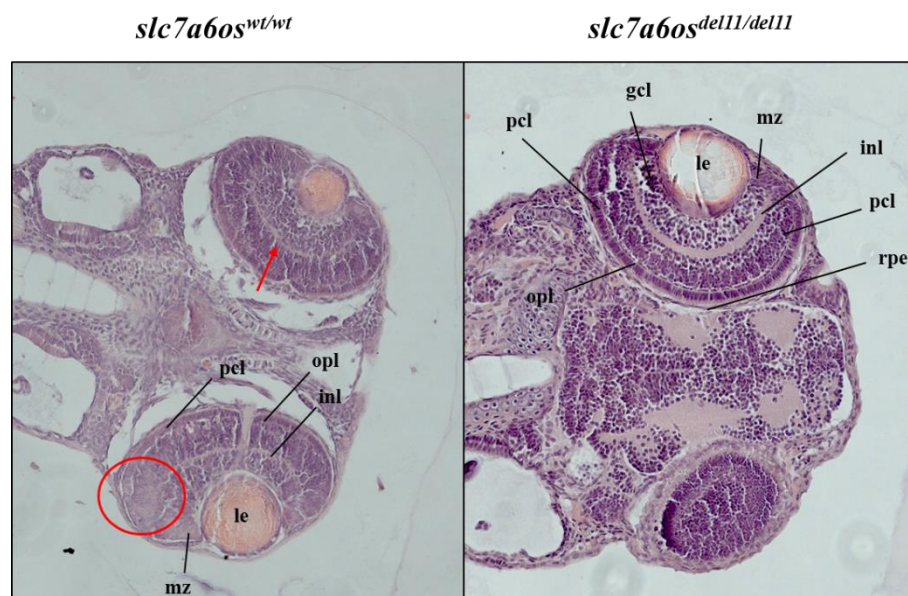


Figure 117. Histological sections on 4 dpf zebrafish. Genotype reference is reported above each figure. Abbreviations are reported in Fig. 115.

9.9 BEHAVIOUR ANALYSIS ON LARVAE

Behavioural analysis on 5 dpf larvae was performed using a standard protocol to evaluate the animal response to the “dark-light” visual stimulus (described in “Materials and Methods”). Individuals subjected to this test have been obtained from crossing *slc7a6os*^{wt/del11}. Subsequent genotyping has been performed on larvae at 5 dpf. Data generated through EthoVision XT (Noldus) have been analysed and reported in graph. Locomotor activity of each experimental group was evaluated calculating the total distance moved from central point at regular intervals of time (10 minutes, Fig. 118, left side). The same parameter was also evaluated as average distance travelled for each experimental group in the corresponding phase (Fig. 118, right side).

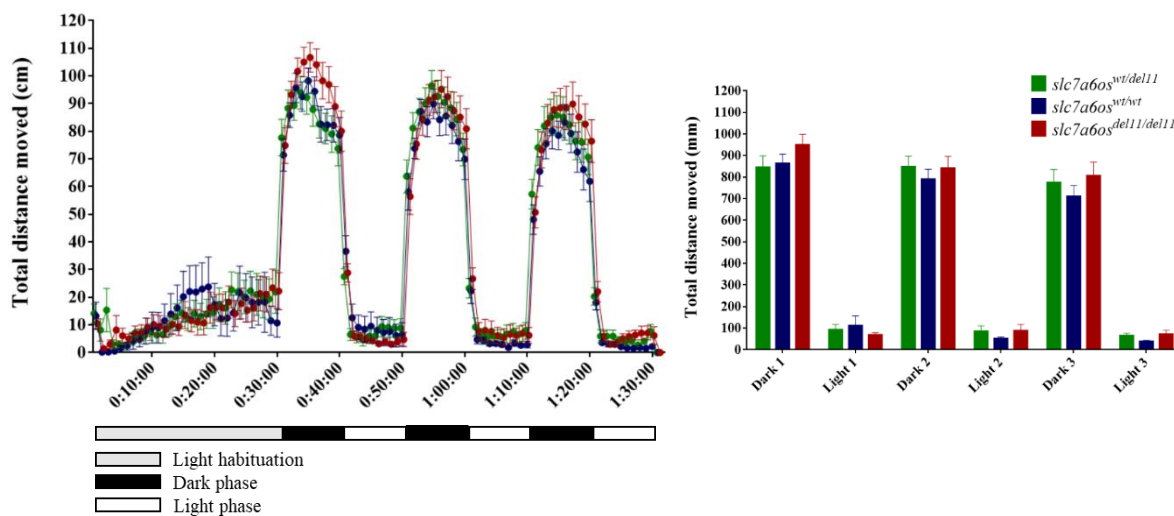


Figure 118. Left side: distance moved (5 dpf) in the different experimental phases at intervals of 10 minutes each. Dark phases are characterized by higher distance travelled; moreover, *slc7a6os*^{del11/del11} seems to respond to dark stimulus by travelling longer distances at higher velocity when compared to wild-type littermates; conversely heterozygotes are characterized by variable behaviour during dark phases when compared to controls. **Right side: total distance moved (5 dpf) in the different experimental phases.** Data reported are the result of a single experiment with larvae generated from a single cross between heterozygotes for del11 fish. Multiple comparison between *slc7a6os*^{del11/del11} and *slc7a6os*^{wt/del11}, respectively, with the littermates wild-type for *slc7a6os* was not significant according to an ordinary Two-way ANOVA test ($p > 0.05$). The described represents the duplicate of a previous experiment (not reported), which confirms the behavioural trend within the various experimental classes, but not the reproducibility of the measurement (in this second experiment the individuals show a significantly greater distance travelled).

Data showed a predisposition of *slc7a6os*^{del11/del11} individuals to travel longer distances when compared to *slc7a6os*^{wt/wt}, although multiple comparison using ordinary Two-way ANOVA was not significant. Conversely, distance covered by heterozygotes was characterized by higher variability when compared to controls. Although these represent extremely preliminary data, they are suggestive of a slight difference of response to light stimuli between mutants and wild-type controls: more in general, both homozygotes and heterozygotes for del11 mutation show a tendency to move faster

when compared to wild-type controls, both in dark and in light phases. Reproducibility of these results should be evaluated with a subsequent test following the same experimental conditions. The obtained data were consistent with our expectations: the tendency of the homozygous mutants to move at higher speed and to travel longer distances could be an indication of an anomalous behavioural phenotype, likewise suggesting the possibility to be characterized by an epileptic-like behaviour, as described in literature (Kalueff, Stewart, & Gerlai, 2014). Further behavioural analyses for the study of a model of epilepsy should be performed, including specific tests, in order to analyse more thoroughly the observed behavioural outcome.

9.10 BEHAVIOUR ANALYSIS ON ADULTS AND MORPHOLOGICAL STUDIES

Preliminary investigations were carried out using a basic test called “novel tank test” on adult zebrafish (description of the procedure is reported in “Materials and Methods”). Generated data and the interpretation of the tracked movements were performed using the EthoVision XT (Noldus). The analysis was performed on 12 adult individuals at the age of 6 months, divided as follows: 4 *slc7a6os*^{wt/del11} individuals (2 females and 2 males) and 4 *slc7a6os*^{del11/del11} male individuals, along with 4 wild-type AB male individuals as controls (Fig. 119). The intention was to evaluate the capability of adult zebrafish to respond to a specific stimulus (new environment). For this reason, the observational tank (indicated as “arena”) has been divided into three different zones: the upper (S1), the central (S2) and the lower (S3) zone, respectively.

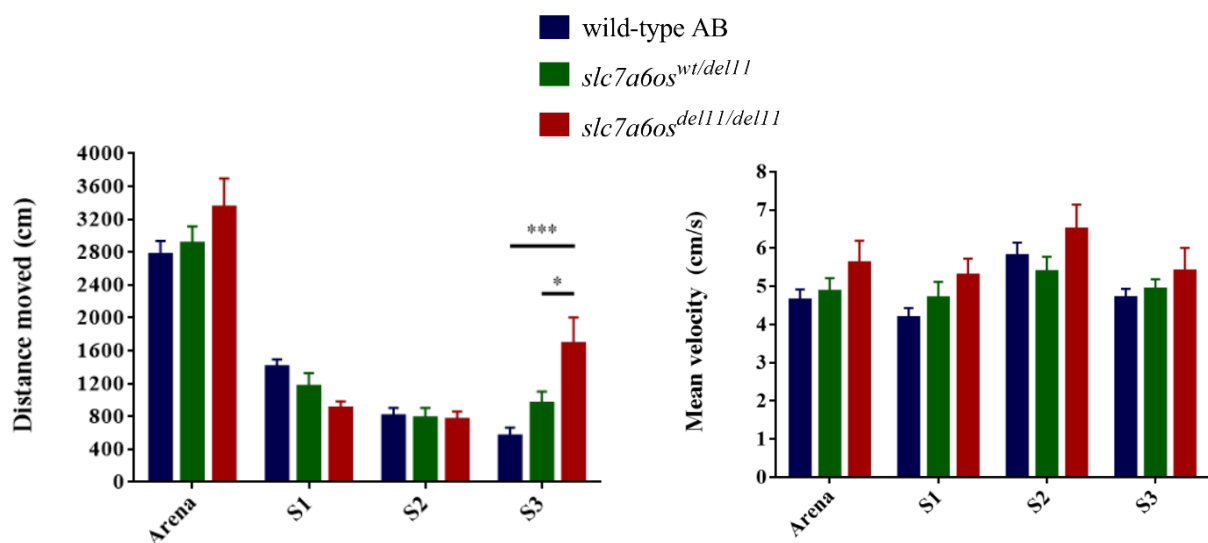


Figure 119. (A) Observational tank to analyse the adult behaviour. Observational tank has been divided into three different zones: S1 (uppero zone), S2 (central zone), S3 (lower zone). **(B) Distance moved in cm (left side) and mean velocity in cm/s (right side).** Statistical analysis has been performed using ordinary Two-way ANOVA test, resulting in significant differences between *slc7a6os*^{del11/del11} distance moved and both heterozygotes (*: $p < 0.05$, ***: $p < 0.001$).

Locomotor activity parameters, including distance travelled and velocity, were calculated by the software, revealing that *slc7a6os*^{del11/del11} individuals were characterized by longer distance travelled when compared to heterozygotes for the same mutation and the relative wild-type AB controls. Otherwise, multiple comparison of the velocity showed no significant differences between the different experimental groups in the total arena during the experimental session and, moreover, within the different zones of the tank during the test. By providing this specific information, Noldus tool allows to calculate latency in each zone, generating qualitative heat maps for each experimental group, with the aim to facilitate visual inspection of the animal movements (Fig. 120). When introduced into a new environment, zebrafish naturally lay on the bottom of the tank and later starts to gradually explore the new environment: a long stay at the bottom of the tank is an indication of a state of anxiety and fear (as well as the latency in entering the upper area); on the contrary, a long stay in the central and upper zone indicates that the fish is not showing an anxious-like behaviour (Kalueff, Stewart, & Gerlai, 2014). The analysis was obtained from two replicates under the same experimental conditions. Comparisons between the generated heat maps (Fig. 120) and the graph of the travelled distance (Fig. 119) confirmed previously described results, showing that *slc7a6os*^{del11/del11} individuals travel shorter distances in the upper zone (S1) of the tank when compared to heterozygotes and wild-type controls; on the contrary, they spend more time at the bottom of the tank (S3), covering higher distances when compared to the other individuals. Heterozygotes show an intermediate phenotype, moving indistinctly in all the three zones (S1, S2, S3).

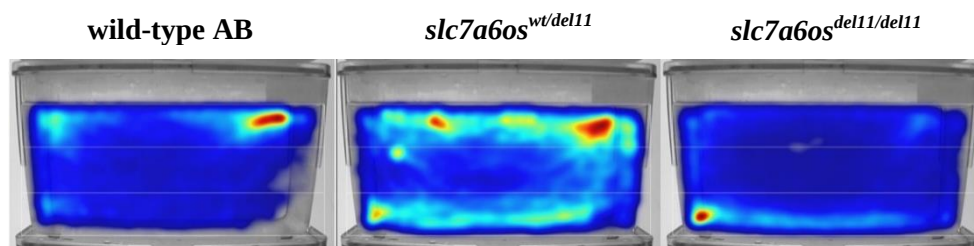


Figure 120. Heat maps for latency generated using EthoVision XT (Noldus) software *slc7a6os* mutants and wild-type control. Representative images of the generated heat maps for latency zone of a single experimental session are reported. References of the different genotypes are reported above each figure.

The described behaviour shown by homozygotes of del11 mutation (reduction of exploration) is suggestive of an anxiety-like and fear behaviour, as described in literature (Kalueff, Stewart, & Gerlai, 2014), that should be clarified with specific tests.

Morphological observations on the adults excluded the presence of major abnormalities that could affect the quality of swimming of the tested individuals, as reported in Fig. 121.



Figure 121. Morphological analyses on adult zebrafish. Representative images are reported for each class of genotype (*slc7a6os*^{wt/wt}, *slc7a6os*^{wt/del11} and *slc7a6os*^{del11/del11}); heterozygotes and homozygotes for del11 mutation, which were subjected to “novel tank test”, were observed and compared to wild-type individuals which were not subjected to behavioural tests.

Despite this, observation of the remaining part of individuals housed in the aquarium system showed that *slc7a6os*^{del11/del11} often develop important abnormalities after the sexual maturity (significant slimming, difficulty in maintaining balance, abnormalities of the anterior-posterior development), which compromise swimming of the adults, leading the individuals to lay at the bottom of the tank, often failing to reach the upper part and showing difficulties in feeding.

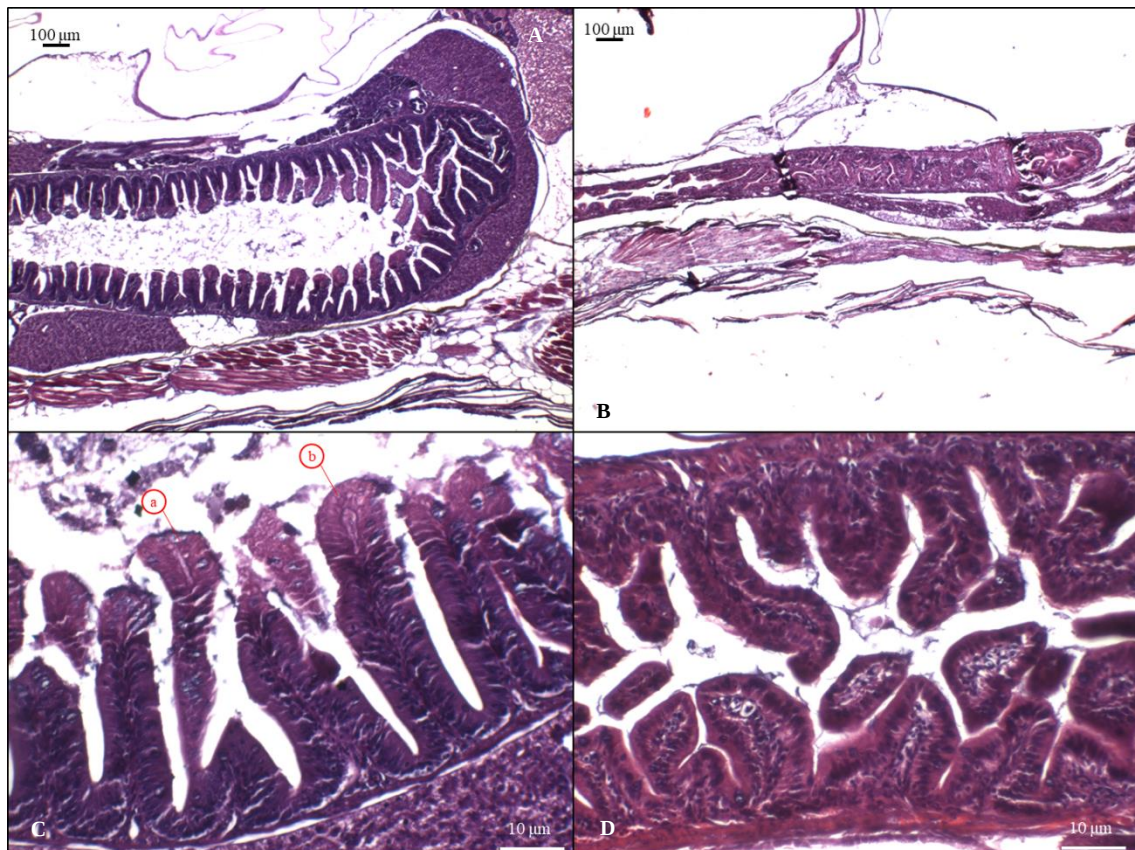


Figure 122. Histological sections on 3 mpf zebrafish of the gastrointestinal tract. (A) *slc7a6os*^{wt/wt} (4X magnification); (B) *slc7a6os*^{del11/del11} (4X magnification); (C) *slc7a6os*^{wt/wt} (20X magnification); (D) *slc7a6os*^{del11/del11} (20X magnification). References: (a) goblet cell; (b) columnar-shaped absorptive enterocyte (Menke, Spitsbergen, Wolterbeek, & Woutersen, 2011).

In addition to that, homozygous males often showed obvious difficulties in reproduction, without exhibiting the typical behaviour during the mating, not stimulating the females, and tending to remain isolated on the bottom of the mating tank.

Histological sections on adults have been performed and, interestingly, evident alterations were observed in different anatomic compartments. In particular, the gastrointestinal tract of *slc7a6os^{del11/del11}* showed shorter intestinal villi when compared to those of the control, with a lack of organization of the cellular components and the presence of a narrow intestinal lumen (Fig. 122). Below are reported different images at greater magnification, in which defects observed at the level of the intestinal villi is more appreciable, respectively in control individual and mutant (Fig. 123).

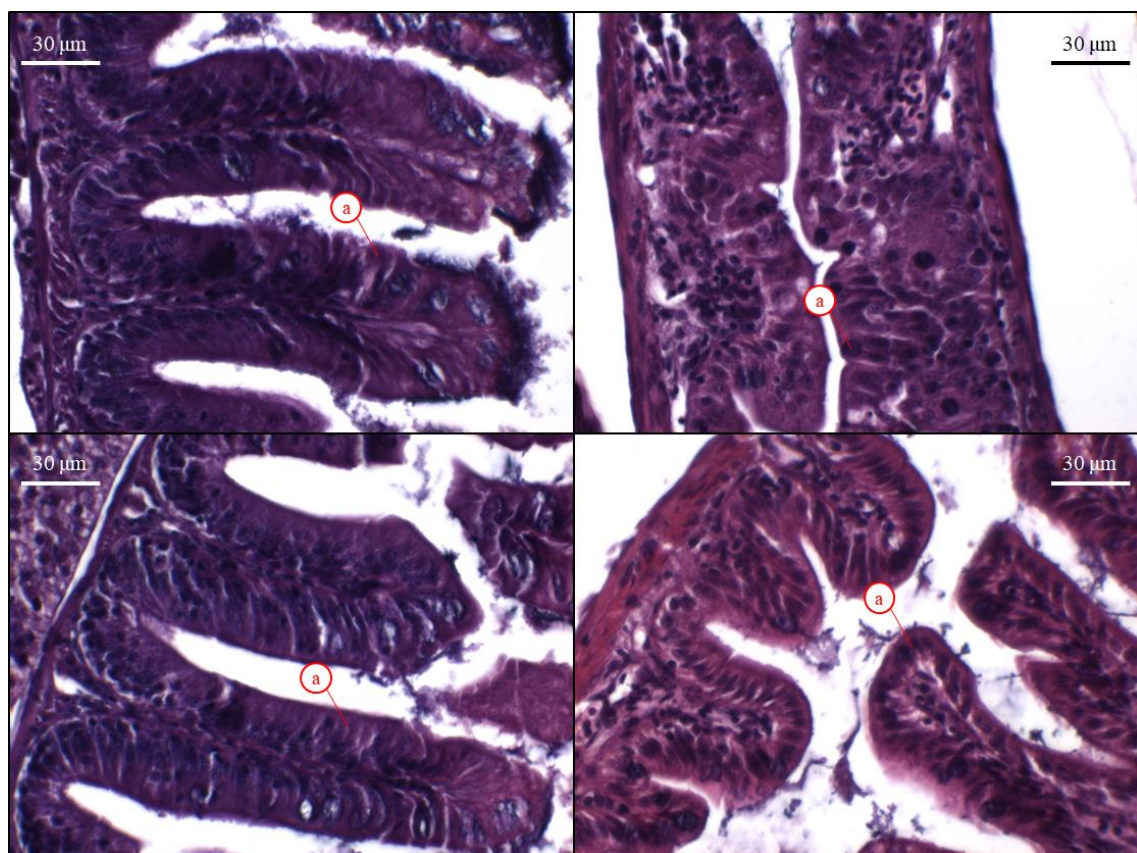


Figure 123. Histological sections on 3 mpf zebrafish of the gastrointestinal tract. (A, C) *slc7a6os^{wt/wt}* (40X magnification); (B, D) *slc7a6os^{del11/del11}* (40X magnification); References: (a) columnar-shaped absorptive enterocyte (Menke, Spitsbergen, Wolterbeek, & Woutersen, 2011).

Visual inspection of histological sections on testis (Fig. 124) showed a lack of germinal tissue organization, with a reduced number of mature spermatids (g) and a more prominent number of earlier germ cells (d, e, f).

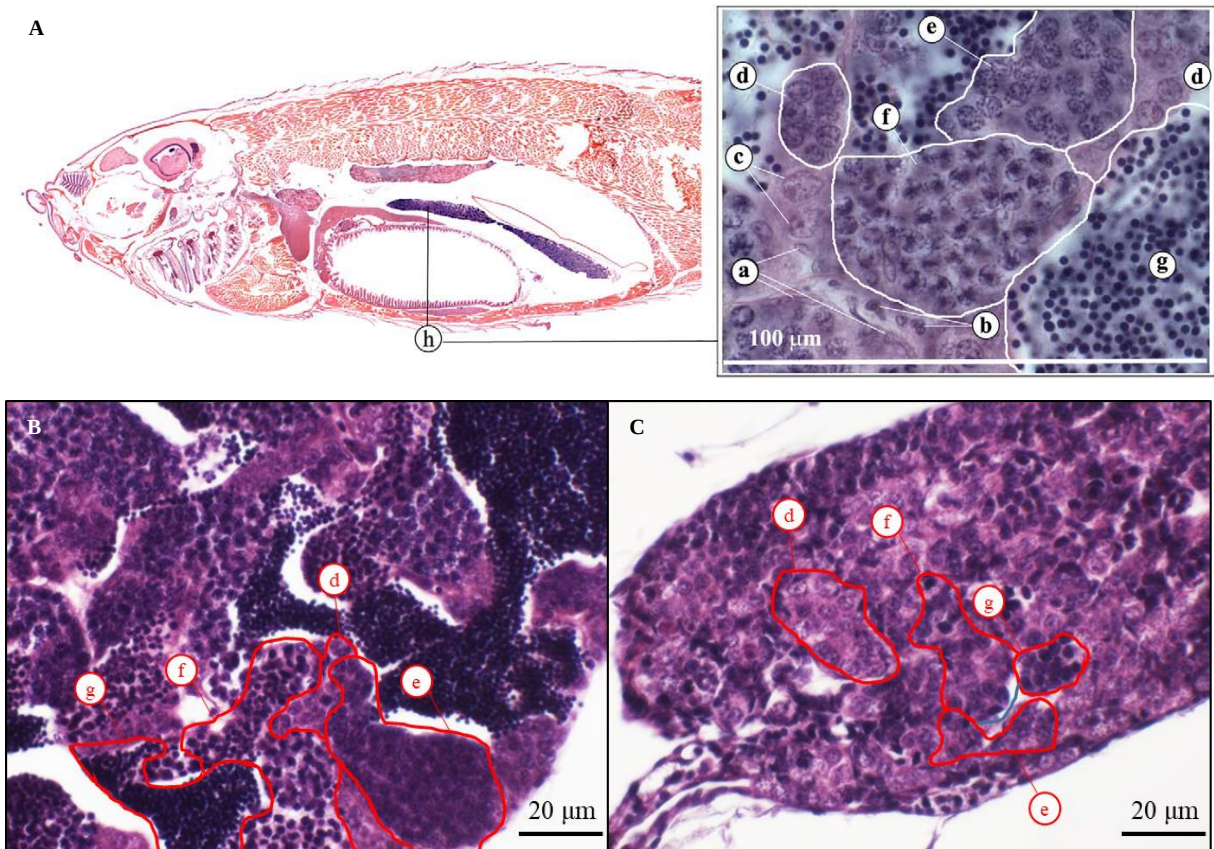


Figure 124. Histological sections on 3 mpf *slc7a6os*^{del11/del11} fish. (A) Longitudinal section of an adult zebrafish male (left side) and representative section of the testis (right side) (Menke, Spitsbergen, Wolterbeek, & Woutersen, 2011). (B) Testis of control *slc7a6os*^{wt/wt} 3 mpf male (40X magnification). (C) Testis of *slc7a6os*^{del11/del11} 3 mpf male (40X magnification). References: (a) Sertoli cells; (b) Leydig cells; (c) spermatogonia type A; (d) spermatogonia type B; (e) pachytenic spermatocytes; (f) dyplotenic spermatocytes and first meiotic division; (g) mature spermatids; (h) testis.

On the other hand, sections of the brain did not present obvious abnormalities between *slc7a6os^{del11/del11}* and the relative wild-type controls, with mutant individuals showing well developed and maintained brain compartments (Fig. 125).

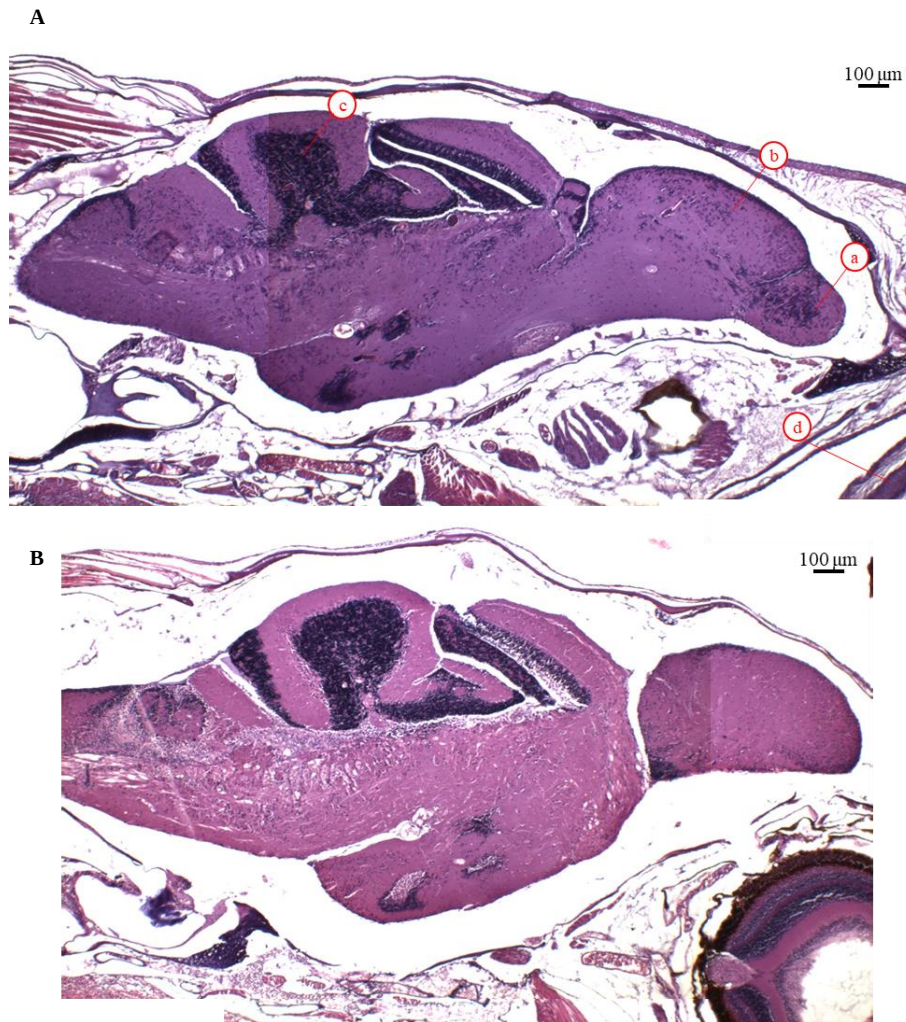


Figure 125. Histological sections of 3 mpf zebrafish on brain. (A) *slc7a6os^{wt/wt}* (4X magnification); (B) *slc7a6os^{del11/del11}* (20X magnification). References: (a) olfactory bulb; (b) telencephalon; (c) cerebellum (Menke, Spitsbergen, Wolterbeek, & Woutersen, 2011).

Additional and systematic investigation at different times of development and adult life will be necessary to both validate the morphological abnormalities observed and define the timing of their onset.

10 DISCUSSION

CRISPR/Cas9 represents an innovative genome editing approach that enables the introduction of targeted mutations in desired genomes, allowing the modelling for functional studies on the genes and the corresponding protein products. In these sense, CRISPR/Cas9 technology has been exploited to obtain knockout lines for the *in vivo* characterization in *Danio rerio* of *ap1s1*, *ap1s2* and *slc7a6os*, three genes of high biomedical relevance whose mutations in the human orthologues are causative of rare forms of neurodevelopmental and neuromuscular disorders: the MEDNIK syndrome (MIM # 609313), the Pettigrew syndrome (MIM # 304340) and a rare form of progressive myoclonic epilepsy (MIM # 619191), respectively. Phylogenetic and synteny analyses indicated that the described genes are highly conserved during evolution. This represents an encouraging premise for studies where the CRISPR/Cas9 genome editing technology may be applied not only to generate knock-outs in zebrafish but also to introduce by knock-in the specific disease-causing mutation of the patients for the study of the pathogenesis and the course of the disease. Moreover, the easy manipulation of zebrafish and its fast reproduction would allow a large-scale screening of chemical compounds aimed at developing therapeutic approaches.

The first line of research was aimed at characterizing *ap1s1* and *ap1s2*, which encode for the small subunit ($\sigma 1$ or $\sigma 2$, respectively) of the adaptor protein 1 (AP-1), a complex involved in vesicular trafficking between the *trans*-Golgi and the early or recycling endosomes. This project, aimed at creating zebrafish genetic models of MEDNIK and Pettigrew syndrome and at a better understanding of the function of the AP-1 adaptor complex (Robinson, 2015), is part of a line of research started years ago in our laboratory (Zizioli, et al., 2010) (Zizioli, et al., 2017) (Mignani, et al., 2023). Conversely, the second line of research was focused on the characterization of *slc7a6os*, a gene conserved during evolution, although few information on its biological function is available. Studies on the putative orthologue in *S. cerevisiae* (named *IWR1*) highlighted its role in the nuclear import of RNA polymerase II (Czeko, Seizl, Augsberger, Mielke, & Cramer, 2011).

For all three genes that have been mutated the absence of protein product in the homozygous lines for the interested mutations could not be assessed; however, the effect of the induced deletions has been evaluated through alternative strategies, such as *in situ* hybridizations, quantitative PCR (qPCR) and cDNA sequencing, which showed high instability of the transcript encoded by the frameshift alleles.

Generation and characterization of *ap1s1* mutants

In human, MEDNIK syndrome is caused by the presence of mutated forms of the *AP1S1* gene and it is characterized by a multifactorial picture, which mainly affects the central nervous system and the skin; in addition to that, patients often show severe perturbation of copper homeostasis with hypocupremia, hypoceruloplasminemia, liver copper accumulation and intrahepatic cholestasis (Martinelli & Dionisi-Vici, 2014).

Our CRISPR/Cas9 approach led to the generation of a single loss-of-function frameshift mutation, corresponding to a deletion of four nucleotides, in *ap1s1* coding sequence of zebrafish. Segregation analyses allowed to exclude that these are lethal mutations both in embryogenesis and at later developmental stages in zebrafish: homozygous individuals for the investigated mutation, without evident altered morphologies, are present in a slight lower percentage, at the adult stage, when compared to the expected Mendelian ratio. In addition to that, individuals who reach sexual maturity maintain functional reproductive capabilities, generating fertile progenies.

Characterization was performed at different stages of development, showing mild phenotypes which therefore do not recapitulate the majority of clinical features presented by patients affected by MEDNIK syndrome (Martinelli, et al., 2013) and even those that characterize the zebrafish knockdown model (Montpetit, et al., 2008). The lack of a severe phenotype in our *ap1s1* mutant line could be due to genetic compensation mechanisms as discussed in the last part of this section.

Preliminary investigations on the transcript of the deleted allele by qPCR showed a significant decrease of its level of expression (77.5%) when compared to the wild-type transcript, suggesting the probable degradation by the activation of nonsense-mediated mRNA decay mechanisms.

The spatial-temporal expression of relevant genes during the neurogenesis process was analysed by *in situ* hybridization, revealing the presence of subtle alterations in homozygotes when compared to controls in the anterior portion of the brain: in particular, a slight weaker *neurod1* signal was visible at 24 hpf in the regions of the anterodorsal/anteroventral lateral line/facial placodes ganglia, optic placode and the posterior line ganglia, along with the telencephalic area; at 30 hpf a lower and disorganized *pax2a* expression characterizes the midbrain hindbrain boundary (mhb) and the otic vesicles of *ap1s1^{del4/del4}* mutants. On the contrary, no differences were found between *ap1s1^{del4/del4}* mutants and wild-type by preliminary investigations of the muscular development both with *in situ* hybridization for *myod* marker at the early stages (16.5 hpf) and with birefringence analyses at later stages (48 hpf, data not reported).

Interestingly, significant differences were observed when analysing the capacity of the zebrafish larvae to respond to light stimuli. The specific test highlighted the tendency of the homozygotes to move faster and cover longer distances during dark periods when compared to wild-type controls.

Since it is known from the literature that *AP1S1* mutations result in defective copper metabolism in MEDNIK syndrome, *ap1s1* mutants have been treated with high doses of copper solutions (500-1100 µg/L of CuSO₄ · 5 H₂O dissolved in fish water solution), along with wild-type controls, from 0 hpf to 5 dpf. Within each of the two tested groups, the trend of hatching confirmed the published data in literature (Johnson, Carew, & Sloman, 2007), with progressively increasing difficulty of the embryos to leave the chorion at higher doses; conversely, mortality was not comparable to previously obtained data, with levels of mortality after the exposure to copper solutions extremely low. Both *ap1s1*^{del4/del4} and wt were subjected to acridine orange staining, without revealing significant differences within the experimental groups and conditions. Although acridine orange is often used to assess the level of cellular apoptosis in zebrafish animal model (Beckman, 2021), it represents a cell-permeable dye that interacts with DNA by intercalation, thus not allowing a specific discrimination of the cell death mechanisms. At the moment it is not to exclude the possible activation of cellular death mechanisms directly related to the presence of copper (cuproptosis); thus, it would be interesting to investigate these mechanisms through specific tests and at different developmental stages (Wang, et al., 2023). Behavioural analyses have been performed, confirming the previously obtained results (described above) with respect to the untreated control larvae, respectively wild-type and *ap1s1*^{del4/del4}. Nevertheless, it was possible to appreciate how the highest dose of treatment (1100 µg/L) suggested a specific effect on the locomotor activity of the mutants, characterized by significantly shorter distances travelled when compared to wild-type at the same experimental conditions and their littermates treated with the lower copper dose. This is in line with current knowledge from literature that correlates copper and zebrafish behaviour. Although copper is an essential element in many biological processes, it may exert toxic effects at levels in excess to metabolic requirements. As an example, 120 hpf embryos exposed to low doses of copper solutions possessed significantly fewer functional neuromasts, an effect which was associated with a reduced ability to orientate in a current (Johnson, Carew, & Sloman, 2007). Additionally, a relatively recent work described the effect of copper on adult zebrafish: repeated novel tank diving assays on wild-type individuals were performed and results demonstrated how copper exposure had no effect on the latency of entry into the upper zones of the tank, nor on the total time spent therein, but did cause a greater number of freezing bouts in comparison to the control group (Pilehvar, Town, & Blust, 2020). It would be therefore interesting to deepen the role of copper in *ap1s1* mutants by reproducing similar investigations at larval and adult stages; the analyses should be accompanied by biochemical studies and quantitative investigations in the different anatomical compartments, to assess the effect of copper surplus in individual organs and to clarify the metabolic impact that is exacerbated in patients affected by MEDNIK syndrome.

Generation and characterization of *ap1s2* mutants

Pettigrew syndrome is defined as a rare form of X-linked mental retardation, characterized by choreoathetosis, hydrocephalus, Dandy-Walker malformation, seizures and iron or calcium deposition in the brain (Cacciagli, et al., 2014). The generation of *ap1s2* knockout lines in *D. rerio* had as main objective to integrate previous knowledge obtained from human cells (Borck, et al., 2008) and a targeted knockout model (Glyvuk, et al., 2010), where $\sigma 1B$ -deficient mice are viable but show reduced motor coordination and severely impaired long-term spatial memory, caused by altered synaptic vesicle recycling. More recent studies on the same murine model evidence that altered sortilin sorting in adipocytes deficient for the $\sigma 1B$ -containing AP-1 complex leads to the inhibition of adipogenesis (Baltes, et al., 2014).

In silico analysis revealed two putative co-orthologues of the human *AP1S2* gene in zebrafish: on the chromosome 11, encoding for the *ap1s2* gene, and 23, named *zgc:162858*. The injection of CRISPR/Cas9 components allowed to specifically introduce mutations in zebrafish *ap1s2* coding sequence, without affecting the paralogous gene on chr 23. We decided to analyse the two mutated lines for *ap1s2*, characterized respectively by a deletion of 5 and of 27 nucleotides. The first is a loss-of-function frameshift mutation leading to a transcript that undergoes to NMD. The second is an in-frame deletion, likely causing a loss-of-function is a gene with an extremely high level of sequence conservation in vertebrates. The transcript of the del27 allele is not expected to undergo NMD. Genotype segregation confirmed Mendelian ratios of the three genotypic classes for both del5 and del27 lines at larval (5 dpf) and adult stage (3-6 mpf) of development, indicating that mutations are not lethal in zebrafish. Moreover, morphological analyses have shown that both the categories of mutants develop correctly, reaching adulthood and giving rise to fertile progenies. Preliminary histological investigations have been performed on adult brains from *ap1s2^{del5/del5}* and *ap1s2^{wt/wt}* individuals, revealing proper organization of the nervous tissue and the distribution of the cellular components. Further studies will be necessary to complete the representative picture of the adult mutants, investigating other anatomical districts. Mild phenotypes are characteristics of the *ap1s2* zebrafish mutants, which are therefore not comparable to the mouse knockout model and the clinical presentation of Pettigrew syndrome.

RT-PCR experiments allowed to exclude that del5 and del27 deletions could also have effects on the regular transcript splicing. Interestingly sequencing data of the RT-PCR product corresponding to the heterozygote individual (*ap1s2^{wt/del5}*) did not show in the chromatogram the presence of sequence of the amplification product from the deleted allele, indicating that the frameshift transcript is much less expressed compared to wild-type one, suggesting selective degradation by NMD. The same experiment should be performed in future also on cDNAs from individuals heterozygous for the del27

mutation to assess whether, as expected, the transcript of the mutated allele is present at a level comparable to that of the wild-type one.

In situ hybridization experiments using a specific antisense probe for *ap1s2* at 48 hpf allowed to qualitatively investigate the spatial-temporal expression of the gene; the probe signal in the central nervous system, localized in particular at the level of the midbrain hindbrain boundary (mhb), is present in both *ap1s2*^{del27/del27} individuals and wild-type controls but not in *ap1s2*^{del5/del5} mutants, suggesting a significant decrease in transcript levels in del5 mutants. These data were supported by qPCR analyses at the same developmental stage, which also evidenced no significant differences in the expression levels of the paralogue, *zgc:162858*, in both the *ap1s2*^{del5/del5} mutants and the wt controls at 48 hpf and 5 dpf stages. Taken together, these results indicate that gene compensation mechanism, most likely do not occur through the overexpression of *zgc:162858* paralogous gene. Nevertheless, the presence of a *zgc:162858* protein product expressed at its physiological level could be enough to allow the development of *ap1s2*^{del5/del5} individual without a striking phenotype. Alternatively, the presence of functional proteins generated by the other *ap1s* genes, particularly *ap1s1*, could compensate the absence of the specific product of *ap1s2*, allowing the correct biological role of the sigma subunit of AP-1. It would be interesting to generate both *ap1s2/zgc:162858* and *ap1s2/ap1s1* double knockouts to evaluate the effect of the absence of two related *ap1s* gene products on zebrafish phenotype.

The spatial-temporal expression of relevant genes during the neurogenesis process was analysed by *in situ* hybridization, revealing the presence of subtle alterations in homozygotes for del5 when compared to controls and *ap1s2*^{del27/del27} individuals, particularly involving the anterior part of the zebrafish brain: a slight weaker signal was observed at 24 hpf for *pax2a*, at the level of the midbrain hindbrain boundary and the hindbrain neurons, and for *neurod1* transcription factor, in the region corresponding to the anterodorsal/anteroventral lateral line/facial placodes ganglia, the telencephalon and the octaval statoacoustic ganglia; lower intensity *neurog1* hybridization signal was observed at 30 hpf at the level of the cranial ganglia and the anterior part of the brain, in the regions corresponding to the diencephalon and the telencephalon; later stages (48 hpf) were characterized by a weaker expression of *neurod1* marker from the midbrain hindbrain boundary to the region of the hindbrain and a less evident and organized hybridization signal was visible at the level of the rhombomeres. Disorganization of the hybridization signal in the telencephalic region and in the dorsal domain prethalamus were observed also by hybridization experiments for *dlx2a* neurogenesis marker. Conversely, no differences were found between *ap1s2* mutants and wild-type controls by preliminary investigations of the muscular development, through *in situ* hybridization for *myod* marker at the early stages (16.5 hpf), and of muscle fibres organization, through phalloidin red staining.

The effect of *ap1s2* inactivating mutations on the lipid component was investigated by preliminary Oil Red O (ORO) staining on 5 dpf larvae, resulting in a significant decrease of the measured ORO optical density in the mutants, when compared to wild-type, in particular in the brain. More detailed experiments should concern the study of the adipose component in adult zebrafish, both through histological investigations and biochemical strategies, to verify and replicate the studies the mouse model of Baltes *et al.*, 2010.

The capability of the animals to respond to visual stimulus was evaluated by behavioural analysis at 5 dpf developmental stage. Interestingly, the obtained data suggest a significant predisposition of *ap1s2*^{del5/del5} mutants to move faster and travel larger distances when compared to controls, while no significant differences were found when analysing *ap1s2*^{del27/del27} individuals and the relative wild-type larvae. At the moment it is difficult to find an explanation for the behavioural differences observed between *ap1s2*^{del5/del5} and *ap1s2*^{del27/del27} mutants. A more detailed study of the type of movements performed by the larvae could be useful to verify the presence of behavioural phenotypes typically related to pathological conditions (Kalueff, Stewart, & Gerlai, 2014). In addition to that, the implementation of adult-specific behavioural experiments could help in testing whether zebrafish mutants show the same impaired reduced motor coordination and impaired long-term spatial memory observed in mice (Glyvuk, et al., 2010).

As previously mentioned, the presence of a functional paralogue of the *ap1s2* gene could explain the very subtle phenotypes in *ap1s2* zebrafish compared to KO mice and Pettigrew syndrome patients. To assess whether *zgc:162858* could play a role in this type of mechanism, preliminary experiment of transient knockdown have been performed through morpholino injections (0.04, 0.4 and 0.8 pmol/embryo) into one cell-stage embryos derived from crosses between *ap1s2*^{del5/del5} mutants. In relation to the first two doses indicated, differences between *ap1s2*^{del5/del5} injected mutants and wild-type in the same conditions were observed. In particular, 0.04 pmol/embryo injection dose was characterized by about 1.6 times fewer wild-type like individuals and about 2.7 times more severe individuals than injected control individuals. Similarly, injection with 0.4 pmol/embryo dose showed a higher number of severely deformed embryos (about 1.8 times higher) than wt individuals injected under the same conditions. The consequences of the injections were evaluated at the molecular level but, unfortunately, the results of the RT-PCR are difficult to interpret, with RT-PCR on the pool of embryos injected with 0.4 pmol/embryo of splice MO suggesting a partial efficacy of the MO in inhibiting the correct splicing. The analysis will need to be repeated after optimization of the RT-PCR conditions, probably requiring the identification of a new pair of oligonucleotide primers.

Generation and characterization of *slc7a6os* mutants

SLC7A6OS is a gene conserved during evolution (from unicellular eukaryotes such as the yeast *Saccharomyces cerevisiae* to vertebrates), although few information on its biological function are available in animal kingdom. *In silico* analyses indicated that is a “singleton” gene, not belonging to any multi-member gene family and, therefore, not showing paralogues within the same species. Studies on the putative orthologue in *S. cerevisiae* (named *IWR1*) highlighted its role in the nuclear import of RNA polymerase II (Czeko, Seizl, Augsberger, Mielke, & Cramer, 2011), transcriptional elongation and genome stability (Gómez-Navarro, Peiró-Chova, & Estruch, 2017). Interestingly, mutations in human *SLC7A6OS* gene were identified as causative of a rare form of an autosomal recessive and progressive myoclonic epilepsy.

Our decision to implement CRISPR/Cas9 technique for *slc7a6os* in zebrafish is due to the possibility of generating permanently stable mutant lines, opposite to the transient gene silencing induced by the use of morpholino oligonucleotides, objective of previous studies on the same gene in our laboratory (Benini, et al., 2015). Contrary to what has been observed in this work, zebrafish *slc7a6os* mutants more frequently show mild phenotypes, which however do not fully summarize the clinical picture presented by patients affected by PME.

When we initially planned the genome editing strategy, we were aware that the mutants generated, characterized mostly by loss-of-function mutations, would not be models of human pathology (PME). The splicing mutation identified in PME patients in fact allows the production of a quota of correctly spliced mRNA. Since this same mutations is also a missense variation as an end result patients present a reduction of the mRNA characterized by correct splicing (likely less than 50%) which encodes for a protein with a p.Q64R substitution that could be detrimental to the functionality of the polypeptide. In the presence of an obvious embryonic phenotype in *slc7a6os*^{-/-} individuals, it would have been possible to test a transient phenotypic rescue by injecting the mutant embryos at one cell stage of development with the wild-type sequence of the human *SLC7A6OS* transcript and, in parallel, with the mRNA corresponding to the sequence of the correctly spliced transcript of the PME patients with the p.Q64R mutation. Unfortunately, this is not currently feasible in the absence of a clear embryonal phenotype in *slc7a6os*^{-/-} individuals.

Injection of Cas9 with the selected sgRNA in wild-type AB line led to the generation of two mutant lines with frameshift loss-of-function mutations in *slc7a6os*, corresponding to a deletion of two and eleven nucleotides, respectively. After an initial characterization of both lines, we decided to perform subsequent studies only on the del11 line, due to the simpler genotyping.

The absence of antibodies specific for the Slc7a6os zebrafish protein prompted us to investigate the fate of the transcript from the mutated allele. Relevant information was obtained through RT-PCR

analysis from individuals belonging to each of the three genotypic classes followed by Sanger sequencing, suggesting that the allele transcript characterized by frameshift mutation undergoes nonsense-mediated RNA decay. Additional experiments with quantitative methods (*e.g.*, Digital Droplet PCR) will be needed to validate this hypothesis.

Crosses between heterozygous mutant individuals produce viable progeny in the expected Mendelian genotypic ratios until 1.5 mpf, when we assist to a decrease in *slc7a6os*^{del11/del11} individuals, which are represented only by the male sex. Two alternative hypotheses could explain the lack of homozygous *-/-* mutant females: female-specific lethality or female-to-male sex reversal. From the analysis of the genotype segregation between 5 dpf and 3 mpf of development, in which homozygote mutant males are not far from the attended 12.5%, the evidence leans more towards the first hypothesis. Otherwise, female-to-male sex reversal phenotype would have had as result the presence of a 25% of *-/-* individuals, even if all males, as it happens in a similar case for *fancl*^{-/-} mutants (Rodríguez-Marí, et al., 2005). Domesticated zebrafish do not have a fixed sex chromosome and its sex is determined by a complex polygenic system and the environment, such as social interactions or temperature. Zebrafish gonads start out as a dual-potential organ with germ cells and immature oocytes. When the sex differentiation occurs at 20–25 dpf, the immature oocytes in the potential females continue to mature through oogenesis, resulting in adult females. On the other hand, the potential males lose their immature oocytes through apoptosis, and their gonads turn into testis. Thus, the number of oocytes and their molecular signals are crucial for sexual development and are necessary for ovary formation (Kossack & Draper, 2019). Besides *fancl*, other mutants have been described that disrupt oocyte meiosis and/or cause early germ cell loss, and like *fancl*, result in an all-male phenotype (*e.g.* *zili*, *tdrd1*, *vasa*, *mps1*) (Kossack & Draper, 2019) due to female-to-male sex reversal, while little could be found in the scientific literature on female-specific lethality after sex determination. Additional studies will be necessary first to confirm the female-specific lethality hypothesis and subsequently to investigate on the molecular basis of this peculiar phenotype.

Interestingly mutations in the putative ortholog of *slc7a6os* in *Drosophila* (*fs(2)ltoPP43*) are responsible for sterility in females (Butler, et al., 2001). Unfortunately, these data are from an old study describing collectively 38 *Drosophila* genes with limited information on each one. The FlyBase *Drosophila* database indicate that *fs(2)ltoPP43* is involved in chorion-containing eggshell formation, further supporting its role in oogenesis.

Preliminary morphological investigations were performed through observational studies: comparisons between heterozygotes and homozygotes for *del11* mutation showed a tendency of *slc7a6os*^{del11/del11} larvae to develop abnormal phenotypes when compared to *slc7a6os*^{wt/del11} individuals and wild-type AB.

The spatial-temporal expression of relevant genes during the neurogenesis process was preliminarily analysed, showing subtle alterations in *slc7a6os*^{del11/del11} mutants, which are not comparable to the results obtained in the previous study on the morphants with the same probes. Results suggest a possible downregulation of *pax2a* at the earlier developmental stages (16 hpf) at the level of the midbrain hindbrain boundary (mhb); slight differences and lower hybridization signals were observed at 24 hpf for *neurod1* in the telencephalon and at the level of the octaval statoacoustic ganglia, while slight *neurod1* downregulation is suggested by hybridizations at 48 hpf, where mutant individuals therefore present weak differences in some neuronal populations of the central portion of the eye, which appeared to be characterized by a weaker and disorganized signal when compared to wild-type.

The generation of a double transgenic/knockout line, named Tg(*neurod1*:EGFP), allowed to investigate *neurod1* expression in *slc7a6os*^{del11/del11} mutants at 72 hpf. Light sheet fluorescence microscopy suggested an altered organization of retinal cells localized in the marginal zone and the inner nuclear layer, which, however, has not yet been confirmed by histological studies on larvae.

The capability of the animals to respond to visual stimulus was evaluated by behavioural analysis at 5 dpf. Data showed a predisposition of *slc7a6os*^{del11/del11} individuals to travel longer distances when compared to *slc7a6os*^{wt/wt}, while distance covered by heterozygotes was characterized by higher variability when compared to controls. Taken together, these results are indicative of a possible anomalous behavioural phenotype showed by *slc7a6os*^{del11/del11} that should be clarified by specific tests, at different developmental stages (Kalueff, Stewart, & Gerlai, 2014). In particular, behaviour shown by homozygotes for del11 mutation could be indicative of the presence of a similar-epileptic phenotype, characteristic of PME patients, which would should be analysed in the future with more in-depth analysis. In the last decade, zebrafish embryos/larvae have emerged not only as organisms useful for disease modelling but also as alternative preclinical models for drug discovery and screening, since they enable rapid evaluation of the efficiency and potential toxicity of new drug candidates. Thus, it would be interesting in the future to test wild-type individuals and mutants of *slc7a6os* in order to analyse the presence of a different sensitivity to substances that induce epileptic seizures (chemoconvulsants), including primarily pentylenetetrazol (PTZ), kainic acid (KA), and pilocarpine (D'Amora, et al., 2023).

Preliminary survival analyses have been performed on the three different genotypic classes of the F2 generation, showing significant mortality of male *slc7a6os*^{del11/del11} individuals. Only few males survive until adulthood that are characterized by significant defects, including weight loss, problems in maintaining balance and swimming, along with difficulties in reaching the upper zone of the tank. Thus, homozygotes tend to lay and feed directly from the bottom of the tank; moreover, preliminary

histological examinations on the adults revealed visible alterations in the germinal tissue and in the intestinal tract.

It is not surprising that morphological alterations are reflected in abnormal behaviour in adults: individuals subjected to the “novel tank test” showed that *slc7a6os*^{del11/del11} spend more time at the bottom of the tank, in contrast to *slc7a6os*^{wt/del11} fish, which explore all the three different zones of the tank. This kind of behaviour, even if could be indicative of an anxiety-like status specifically shown by homozygous mutants, cannot ignore the morphological context of the mutants, thus additional specific tests will be necessary to clarify the presence of a specific pathological behavioural phenotype. In addition to that, it would be also interesting to investigate adult behaviour in a social context, to verify the possible development of aggressive behaviour within the same population of individuals, which could be indicative of a subsequent suffering status and therefore an increase in mortality of *slc7a6os*^{del11/del11}.

Considerations on genetic compensation mechanisms and conclusion

The application of CRISPR/Cas9 technology in zebrafish animal model allowed to generate different stable mutant lines of the three genes of high biomedical relevance, *ap1s1*, *ap1s2* and *slc7a6os*. The presence of phenotypes in *ap1s1* and *ap1s2* mutant fish which are much milder than those observed in morphants, mouse knockout and patients, makes them not suitable as a model for the study of the pathogenesis of MEDNIK and Pettigrew syndrome disease. *slc7a6os* mutant lines we generated also show very limited alteration during CNS development in larvae compared to the morphants for the same gene. This could be due to activation of compensation mechanism by factors other than Slc7a6os that could mediate the RNA Pol II nuclear import (their existence has already been demonstrated in yeast). However, inactivating mutations in *slc7a6os* lead to an adult fish phenotype (female lethality and morphological and behavioural alterations in males) that is different and more severe than the human PME disease. This could be explained by the fact that in PME patients is likely present a residual SLC7A6OS activity that is completely absent in our mutant zebrafish line.

In all three genes frameshift loss-of-function mutations have been generated and expression studies suggest, as expected, a low stability of the mutant transcript probably due to NMD.

The absence of an obvious phenotype can be explained by the possible activation of gene compensation mechanisms to counterweigh for the lack of the gene product. Genetic compensation, also called “genetic buffering”, is a phenomenon in which an organism with a pathogenic mutation does not develop the expected adverse phenotype due to compensatory actions of another gene or genes, which functionally compensate for the loss-of-function genotypes, restoring more normal

physiological function. Genetic compensation may result from several mechanisms, including upregulation of genes with redundant functions, or from the activation of more complex responses within metabolic, signalling or transcriptional networks. This phenomenon may be result from an upregulation of a paralogue gene, an action of a genetic modifier or the generation of a plethora of genetic changes. Interestingly, many reports have been published showing discrepancies between knockdown and mutant (knockout) phenotypes in genetic models, especially in zebrafish organism. Recent studies reveal that in some cases the absence of the phenotype is due to genetic compensation, which involves the upregulation of either gene paralogs or genes with sequence homology, triggered by nonsense-mediated-decay (NMD) of the mutant mRNA, also referred to as transcriptional adaption response (Buglo, et al., 2020).

ap1s1 and *ap1s2* genes belong to a family with different paralogs which could be the first candidate as compensatory genes. Expression studies should be performed on these family members to provide evidence of such a mechanism in our mutant lines. In addition, a double *ap1s1/ap1s2* could be created to evidence if the lack of a severe phenotype in *ap1s2* somehow involve the more ubiquitously expressed *ap1s1* gene.

Moreover, a number of studies have been published reporting discrepancies between the phenotype of CRISPR/Cas9-generated stable homozygous mutant lines at F0 and the phenotype of F0 mosaic models (the so called crispants) for the same investigated gene (Buglo, et al., 2020). By comparing F0 crispants and multigenerational mutant larvae, phenotypic compensation in the stable mutants occur, in contrast to mosaic F0 crispants that do not exhibit genetic compensation and replicate robust and rescuable phenotypes which were previously reported in morpholino knockdowns. Moreover, RNA sequencing showed that the absence of a phenotype in the mutants of the investigated gene could be associated with significant changes in expression of candidate compensatory genes. The analysis of the transcriptome of the mutant zebrafish lines by RNA-Seq could be informative about the activation of compensatory mechanisms by other genes, facilitating the identification of the molecular pathways involved and directing future characterization studies. Since the generated *ap1s1* and *ap1s2* mutants do not constitute suitable models of the corresponding human disease, in future studies it will be appropriate to evaluate parallel or alternative approaches including for example the generation of crispants for studying the impact of mutations in the earliest stages of embryonal development. For studies on genetic mutants it will be necessary to develop new skills necessary for adequate phenotypic analyses on adult fish; however, this will require a longer period of time to ensure that the animal would be able to develop correctly upon reaching sexual maturity or, in any case, an appropriate age for performing such tests.

Elena Massard

11 SCIENTIFIC PRODUCTION

As a PhD student, I've been collaborating to other studies that are described in the following scientific publications:

1. Int J Mol Sci. 2023 Sep 1;24(17):13578. doi: 10.3390/ijms241713578.

Downregulation of Zebrafish Cytosolic Sialidase Neu3.2 Affects Skeletal Muscle Development.

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Sialidases remove terminal sialic acids residues from the non-reducing ends of glycoconjugates. They have been recognized as catabolic enzymes that work within different subcellular compartments and can ensure the proper turn-over of glycoconjugates. Four mammalian sialidases (NEU1-4) exist, with different subcellular localization, pH optimum and substrate specificity. In zebrafish, seven different sialidases, with high homology to mammalian counterparts, have been identified. Zebrafish Neu3.2 is similar to the human cytosolic sialidase NEU2, which is involved in skeletal muscle differentiation and exhibits a broad substrate specificity toward gangliosides and glycoproteins. In zebrafish neu3.2, mRNA is expressed during somite development, and its enzymatic activity has been detected in the skeletal muscle and heart of adult animals. In this paper, 1-4-cell-stage embryos injected with neu3.2 splice-blocking morpholino showed severe embryonic defects, mainly in somites, heart and anterior-posterior axis formation. Myog and myod1 expressions were altered in morphants, and impaired musculature formation was associated with a defective locomotor behavior. Finally, the co-injection of Neu2 mouse mRNA in morphants rescued the phenotype. These data are consistent with the involvement of cytosolic sialidase in pathologies related to muscle formation and support the validity of the model to investigate the pathogenesis of the diseases.

DOI: 10.3390/ijms241713578

PMCID: PMC10487903

PMID: 37686385

2. Int J Mol Sci. 2023 Apr 12;24(8):7108. doi: 10.3390/ijms24087108.

Deficiency of AP1 Complex Ap1g1 in Zebrafish Model Led to Perturbation of Neurodevelopment, Female and Male Fertility; New Insight to Understand Adaptinopathies.

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In vertebrates, two homologous heterotetrameric AP1 complexes regulate the intracellular protein sorting via vesicles. AP-1 complexes are ubiquitously expressed and are composed of four different subunits: γ , $\beta 1$, $\mu 1$ and $\sigma 1$. Two different complexes are present in eukaryotic cells, AP1G1 (contains $\gamma 1$ subunit) and AP1G2 (contains $\gamma 2$ subunit); both are indispensable for development. One additional tissue-specific isoform exists for $\mu 1A$, the polarized epithelial cells specific to $\mu 1B$; two additional tissue-specific isoforms exist for $\sigma 1A$: $\sigma 1B$ and $\sigma 1C$. Both AP1 complexes fulfil specific functions at the trans-Golgi network and endosomes. The use of different animal models demonstrated their crucial role in the development of multicellular organisms and the specification of neuronal and epithelial cells. Ap1g1 ($\gamma 1$) knockout mice cease development at the blastocyst stage, while Ap1m1 ($\mu 1A$) knockouts cease during mid-organogenesis. A growing number of human diseases have been associated with mutations in genes encoding for the subunits of adaptor protein complexes. Recently, a new class of neurocutaneous and neurometabolic disorders affecting intracellular vesicular traffic have been referred to as adaptinopathies. To better understand the functional role of AP1G1 in adaptinopathies, we generated a zebrafish ap1g1 knockout using CRISPR/Cas9 genome editing. Zebrafish ap1g1 knockout embryos cease their development at the blastula stage. Interestingly, heterozygous females and males have reduced fertility and showed morphological alterations in the brain, gonads and intestinal epithelium. An analysis of mRNA profiles of different marker proteins and altered tissue morphologies revealed dysregulated cadherin-mediated cell adhesion. These data demonstrate that the zebrafish model organism enables us to study the molecular details of adaptinopathies and thus also develop treatment strategies.

DOI: 10.3390/ijms24087108

PMCID: PMC10138411

PMID: 37108275

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13 ACKNOWLEDGMENTS

Eccomi giunta al termine della corsa, un periodo intenso e non privo di difficoltà, che mi ha sicuramente arricchita di insegnamenti professionali e per la vita. Vorrei ringraziare innanzitutto il Prof. Presta, che in questi anni mi ha permesso di formarmi presso la Facility di Zebrafish dell'Università degli Studi di Brescia. Ringrazio inoltre il Prof. Giuseppe Borsani e la Dott.ssa Daniela Zizioli, che mi hanno affidato i progetti diventati oggetto di questa tesi, la Prof.ssa Elisa Borsani, per la collaborazione nella realizzazione e nelle analisi delle sezioni istologiche, il Prof. Dario Finazzi, per il contributo scientifico nel corso dello svolgimento dell'attività sperimentale e i componenti della Sezione di Biologia e Genetica del Dipartimento di Medicina Molecolare e Traslazionale. Grazie a tutti i membri della Facility di Zebrafish di Brescia, con i quali ho vissuto un pezzo importante della mia vita, in particolare a Chiara, per i preziosi insegnamenti e i consigli di cui farò sempre tesoro; a Barbara, per tutto l'aiuto e il sostegno; a Elena, per la sua grinta; a Silvia, senza di lei le giornate sarebbero sicuramente state meno colorate; a Jessica, per il suo contributo e la sua pazienza; a Elisa, che non smette mai di sorprendermi; a Imane, che mi è sempre stata accanto, con la sua dolcezza e determinazione; al Dott. Davide Barbetta, per la sua comprensione e la sua disponibilità ogni giorno; al Prof. Marco Schiavone, per la sua vicinanza durante questo periodo.

Vorrei ringraziare Roberta, Cristina, Francesca e Stefania, che mi hanno sostenuta in tutti questi anni. Un ringraziamento speciale va anche a Silvia, Germano e Monica, per il loro supporto e la loro comprensione. A Ilaria, Alessia, Alex, Marco, Fede, Sabrina, Anna e Giacomo, per la loro vicinanza e tutti gli incoraggiamenti che hanno saputo darmi.

Ringrazio anche chi mi ha lasciato o ha preferito intraprendere una strada differente, perché mi ha ricordato che non bisogna mai arrendersi. Ringrazio tutti i miei amici, coloro che ho ritrovato e che non se ne sono mai andati, e tutti coloro che sono entrati a far parte della mia vita: Giulia, che nonostante la distanza e gli impegni, non scorda mai l'importanza di passare del tempo assieme; Andrea O., che non si è perso nemmeno un passo di questo lungo cammino; Davide, in cui ho trovato un vero amico e una spalla; Arianna e Federico, Chiara e Paolo, che mi hanno ricordato quanto l'amicizia e il sostegno reciproco siano essenziali; Marco T. e Max, Mara e Daniele, Cloh e Marco M., per tutti i bei momenti che abbiamo vissuto assieme e per avermi supportato, sempre, soprattutto in questo ultimo anno.

Un grande grazie va a Stefen, per il suo appoggio e la sua forza, non cambiare mai!

Un grazie va anche a mio cugino Nicola, senza il quale non ce l'avrei certamente fatta in certe situazioni.

Voglio dire grazie alla mia famiglia: ai miei genitori, che mi hanno ricordato quanto sia essenziale cogliere tutti gli insegnamenti che la vita ci pone, anche nelle situazioni peggiori; alle mie sorelle: a Paola, che ha decisamente più forza e tenacia di quanto possa averla una montagna, da lei non posso fare altro che imparare; ad Arianna, che non voglio mai vedere smettere di sognare e pensare “in grande”; ad Alberto, l'esatto esempio di chi è in grado di donare tutto se stesso per aiutare l'altro, il suo conforto è stato di estrema importanza per me; ad Andrea, che è entrato nella mia vita proprio quando oramai non credevo più a nulla; sono bastate tre sue parole per risvegliare quella parte di me che avevo oramai scordato da tempo: “Credo in te”.

MODULO DI EMBARGO DELLA TESI
(da compilare solo se si richiede un periodo di segretazione della tesi)

Il/La sottoscritto/a..... Elena Massardi Nato/a il..... 24/07/1996
a (indicare anche l'eventuale paese estero)..... Brescia
provincia di (ovvero sigla del paese estero)..... Brescia
Dottorato di Ricerca in GENETICA MOLECOLARE, BIOTECNOLOGIE E MEDICINA SPERIMENTALE
.....

DICHIARA

- che il contenuto della tesi **non può essere immediatamente consultabile per il seguente motivo**

I risultati riportati nella tesi di dottorato saranno oggetto di pubblicazioni scientifiche peer-reviewed e non possono quindi essere resi immediatamente disponibili a causa della competitività a livello internazionale della ricerca scientifica svolta.

La motivazione deve essere dettagliata e controfirmata obbligatoriamente dal Primo Supervisore di tesi
(Brevetto, segreto industriale, motivi di priorità nella ricerca, motivi editoriali, altro)

- che il testo completo della tesi potrà essere reso consultabile dopo:

☐ 6 mesi dalla data di conseguimento titolo

☒ 12 mesi dalla data di conseguimento titolo

- che sarà comunque consultabile immediatamente l'abstract della tesi, che viene caricato in Esse3, profilo studente.

Luogo e Data

Milano 20-05-2024

Brescia 20-05-2024

Firma del Dichiarante



Controfirma del Primo Supervisore di tesi
per la motivazione di embargo e il periodo.

