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**Looking beyond the *NF1* and *NF2* genes:
identification of additional players contributing
to phenotypic severity and variability in
NF1 and *NF2* microdeletion patients**

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Abstract

Neurofibromatosis type 1 (NF1) and Neurofibromatosis type 2 (NF2) are rare autosomal dominant disorders primarily caused by point mutations or small insertions/deletions in the *NF1* and *NF2* genes, respectively. Both syndromes are multisystemic and characterized by a predisposition to tumor development. In rare cases, patients present with microdeletions encompassing not only the causative gene but also flanking genes, often resulting in a more severe and variable phenotype. These observations indicate that the pathogenesis of both syndromes extends beyond the mere *NF1* or *NF2* gene haploinsufficiency.

NF1 microdeletions, classified as typical or atypical based on breakpoint location, have been extensively characterized in terms of gene content and possible genotype-phenotype correlations. However, these correlations alone do not fully explain the clinical complexity observed in affected individuals. *NF2* microdeletions are even rarer, highly heterogeneous, and poorly described due to the absence of recurrent breakpoints; therefore, their underlying pathogenic mechanisms remain largely unexplored.

The aim of this research project was to investigate multiple pathogenic mechanisms contributing to the complex phenotypes observed in patients with *NF1* and *NF2* microdeletions.

Regarding NF1, a potential dysfunction of the Polycomb Repressive Complex 2 (PRC2), which includes SUZ12, a protein encoded by one of the genes commonly deleted in *NF1* microdeletions, was investigated. Altered expression of PRC2 core components and target genes was detected by qPCR assays in patients with both *NF1* microdeletions and intragenic mutations. This dysregulation is likely driven by Ras pathway hyperactivation and may contribute to the tumorigenic phenotype characteristic of these individuals. To provide a platform for future molecular and functional analyses, induced pluripotent stem cell (iPSC) lines were generated from two patients carrying the typical type-1 *NF1* deletion and an atypical *NF1* deletion. Regarding NF2, the extent of two novel 22q12.1q12.2 microdeletions was characterized by array-CGH. To define the deletion breakpoints at the single-base resolution, the Long-Read Sequencing (LRS) was carried out. The deletions, encompassing 3.1 and 2.4 Mb, include 44 and 47 protein-coding genes,

respectively, and display breakpoints located within repetitive sequences of low mutual homology. These findings suggest that both rearrangements were likely generated by non-homologous end joining (NHEJ) or alternative microhomology-based mechanisms, including microhomology-mediated end joining (MMEJ) and replication-associated processes such as fork stalling and template switching and microhomology-mediated break-induced replication (FoSTeS/MMBIR). Analysis of the topological and regulatory architecture of the 22q12 region revealed predicted alterations in the 3D chromatin organization and regulatory interactions following *NF2* deletions. These changes were consistent with a position effect impacting the expression of several flanking genes, a finding further confirmed by qPCR analyses. LRS also enabled the detection of additional variants and CNVs, enhancing the molecular characterization of both patients. We proposed a potential correlation between the loss of one allele of *APIB1* and cognitive impairment, as well as between a pathogenic variant in the modifier gene *EVC* and the cardiac defects observed in the corresponding patient.

Overall, the findings suggest that disease phenotypes may result not only from the loss of deleted genes but also from the dysregulation of flanking genes and the influence of variants in modifier genes. This study represents a pilot investigation that opens new perspectives in the understanding of pathogenic mechanisms underlying microdeletion syndromes. The results presented here have potential implications for the diagnosis, prognosis, and clinical management of patients carrying *NF1* and *NF2* microdeletions and may guide the identification of novel therapeutic targets for the development of personalized pharmacological treatments.

Riassunto

La Neurofibromatosi di tipo 1 (NF1) e la Neurofibromatosi di tipo 2 (NF2) sono malattie rare a trasmissione autosomica dominante, causate principalmente da mutazioni puntiformi o da piccole inserzioni/delezioni, rispettivamente nei geni *NF1* e *NF2*. Entrambe le sindromi sono multisistemiche e caratterizzate da una predisposizione allo sviluppo di tumori. Più raramente, i pazienti presentano microdelezioni che coinvolgono non solo il gene causativo, ma anche geni fiancheggianti, con conseguente fenotipo più severo e variabile. Queste osservazioni indicano che la patogenesi delle Neurofibromatosi va oltre la semplice aploinsufficienza dei geni *NF1* o *NF2*.

Le microdelezioni *NF1*, classificate come tipiche o atipiche in base alla localizzazione dei *breakpoint*, sono state ampiamente caratterizzate in termini di contenuto genico e di possibili correlazioni genotipo-fenotipo. Tuttavia, tali correlazioni non spiegano completamente la complessità clinica osservata nei soggetti affetti. Le microdelezioni *NF2* sono invece ancora più rare, altamente eterogenee e poco descritte a causa dell'assenza di *breakpoint* ricorrenti; di conseguenza, i loro meccanismi patogenetici rimangono in gran parte inesplorati. L'obiettivo del mio progetto di ricerca è stato indagare i molteplici meccanismi patogenetici che contribuiscono ai fenotipi complessi osservati nei pazienti con microdelezioni *NF1* e *NF2*.

Per quanto riguarda la NF1, è stata analizzata una possibile disfunzione del Complesso Repressivo Polycomb 2 (*Polycomb Repressive Complex 2*, PRC2), che include SUZ12, una proteina codificata da uno dei geni comunemente inclusi nelle microdelezioni *NF1*. L'espressione alterata delle subunità principali e di geni *target* di PRC2 è stata rilevata mediante saggi di qPCR in pazienti con microdelezioni e con mutazioni intrageniche del gene *NF1*. Questa disregolazione, probabilmente indotta dall'iperattivazione del *pathway* di Ras, potrebbe contribuire al fenotipo tumorale caratteristico di questi individui. Al fine di fornire un modello per future analisi molecolari e funzionali, sono state generate linee di cellule staminali pluripotenti indotte (*induced Pluripotent Stem Cell*, iPSC) da due pazienti, uno con la tipica delezione *NF1* di tipo 1 e uno con una delezione *NF1* atipica.

Per quanto riguarda la NF2, l'estensione di due nuove microdelezioni 22q12.1q12.2 è stata caratterizzata mediante array-CGH. Per definire i *breakpoint* a livello di singola base, è stato eseguito il Long-Read Sequencing (LRS). Le delezioni, di 3,1 e 2,4 Mb, comprendono rispettivamente 44 e 47 geni codificanti per proteine e presentano *breakpoint* localizzati in sequenze ripetute a bassa omologia reciproca. Questi risultati suggeriscono che entrambi i riarrangiamenti siano stati probabilmente generati tramite il *non-homologous end joining* (NHEJ) o meccanismi alternativi basati sulla micro-omologia, inclusi il *microhomology-mediated end joining* (MMEJ) e processi associati alla replicazione, come il *fork stalling and template switching and microhomology-mediated break-induced replication* (FoSTeS/MMBIR). L'analisi dell'architettura topologica e regolatoria della regione 22q12 ha portato a predire alterazioni nell'organizzazione tridimensionale della cromatina e nelle interazioni regolatorie a seguito delle delezioni NF2. Tali modifiche delle interazioni DNA-DNA risultano coerenti con un effetto posizione che influenza l'espressione di diversi geni adiacenti, dato ulteriormente confermato da saggi di qPCR. Il LRS ha inoltre permesso di rilevare ulteriori varianti e CNV, migliorando la caratterizzazione molecolare di entrambi i pazienti. È stata proposta una potenziale correlazione tra la perdita di un allele di *APIB1* e il deficit cognitivo, nonché tra una variante patogenetica nel gene modificatore *EVC* e le anomalie cardiache osservate nel paziente portatore della variante stessa.

Complessivamente, i risultati suggeriscono che i fenotipi patologici possano derivare non solo dalla perdita dei geni deleti, ma anche dalla disregolazione dei geni fiancheggianti la delezione e dal contributo di varianti in geni modificatori. Questo rappresenta uno studio pilota che apre nuove prospettive nella comprensione dei meccanismi patogenetici alla base delle sindromi da microdelezione. I risultati presentati hanno potenziali implicazioni per la diagnosi, la prognosi e la gestione clinica dei pazienti portatori di microdelezioni NF1 e NF2 e potrebbero guidare l'identificazione di nuovi bersagli terapeutici per lo sviluppo di trattamenti farmacologici personalizzati.

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List of Abbreviations

3C	Chromosome Conformation Capture
3D	Three-dimensional
4C	Circular Chromosome Conformation Capture
5C	Chromosome Conformation Capture Carbon Copy
aCGH	Array-based Comparative Genomic Hybridization
ACMG	American College of Medical Genetics and Genomics
ADHD	Attention-Deficit/Hyperactivity Disorder
ADLD	Adult-onset Demyelinating Leukodystrophy
AFP	Alpha-Fetoprotein
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
APP	Amyloid- β (A4) Precursor Protein
array-CGH	Array-based Comparative Genomic Hybridization
ASD	Autism Spectrum Disorder
BAM	Binary Alignment Map
BLAST	Basic Local Alignment Search Tool
BP	Breakpoint
BSA	Bovine Serum Albumin
cDNA	complementary DNA
ChIP	Chromatin Immunoprecipitation
CI	Confidence Interval
CMS	Congenital Myasthenic Syndrome
CNR	Consiglio Nazionale delle Ricerche
CNV	Copy Number Variation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CSRD	Cysteine/Serine-Rich Domain
CTD	C-Terminal Domain

CTRL	Control
CY-3	Cyanine 3
CY-5	Cyanine 5
DAE	Differential Allelic Expression
DIN	DNA Integrity Number
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dUTP	Deoxyuridine Triphosphate
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
ExAC	Exome Aggregation Consortium
ExoI	Exonuclease I
FBS	Fetal Bovine Serum
FERM	Four-point-one, Ezrin, Radixin, Moesin
FGF	Fibroblast Growth Factor
FISH	Fluorescence In Situ Hybridization
FoSTeS	Fork Stalling and Template Switching
GAP	GTPase-Activating Protein
gDNA	genomic DNA
GDP	Guanosine Diphosphate
GnomAD	Genome Aggregation Database
GPCR	G Protein Coupled Receptor
GRCh38	Genome Reference Consortium Human build 38
GRD	GAP-related domain
GTE _x	Genotype-Tissue Expression
GTP	Guanosine Triphosphate
H3K27	Lysine 27 of Histone H3
H3K27me3	trimethylation at Lysine 27 of Histone H3
HD	Healthy Donor

HEMA	Hydroxyethyl Methacrylate
Hi-C	High-throughput Chromosome Conformation Capture
HiFi	High-Fidelity
huES	human Embryonic Stem cell
ID	Identification Code
IF	Immunofluorescence
IgG	Immunoglobulin G
IGV	Integrative Genomics Viewer
iPSC	induced Pluripotent Stem Cell
IRCCS	Istituti di Ricovero e Cura a Carattere Scientifico
ITB	Istituto di Tecnologie Biomediche
JNCL	Juvenile Neuronal Ceroid Lipofuscinosis
LCRs	Low Copy Repeats
LINE	Long Interspersed Nuclear Elements
LOH	Loss Of Heterozygosity
LRS	Long-Read Sequencing
LTR	Long Terminal Repeat elements
MAF	Minor Allele Frequency
Mb	Megabase
MEDNIK	Mental retardation, Enteropathy, Deafness, peripheral Neuropathy, Ichthyosis, and Keratoderma
MERCs	Mitochondria-Endoplasmic Reticulum Contact Sites
MLPA	Multiplex Ligation-dependent Probe Amplification
MMBIR	Microhomology-Mediated Break-Induced Replication
MMEJ	Microhomology-Mediated end-Joining
MOI	Multiplicity Of Infection
MPNST	Malignant Peripheral nerve Sheath Tumor
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
NA	Not Available
NAHR	Non-Allelic Homologous Recombination

NCBI	National Center for Biotechnology Information
NEG	Negative control
NF	Neurofibromatoses
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
NGS	Next-Generation Sequencing
NHEJ	Non-Homologous End-Joining
NIH	National Institutes of Health
NTC	No-Template Control
OMIM	Online Mendelian Inheritance in Man
ONT	Oxford Nanopore Technologies
OPG	Optic Pathway Glioma
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PF	Passing Filter
PFA	Paraformaldehyde
pLI	probability of Loss-of-function Intolerance
PRC2	Polycomb Repressive Complex 2
PRS	Pierre-Robin Sequence
PT	Patient
QFQ	Q-bands by Fluorescence using Quinacrine
qPCR	quantitative Polymerase Chain Reaction
RefSeq	NCBI Reference Sequence Database
RNA	Ribonucleic Acid
RNA-Seq	RNA-Sequencing
ROCKi	ROCK inhibitor
RT	Room Temperature
SAP	Shrimp Alkaline Phosphatase
SBD	Substrate-Binding Domain

SCF	Stem Cell Factor
SD	Standard Deviation
SEM	Standard Error of the Mean
SINE	Short Interspersed Nuclear Elements
SNF	Spinal Neurofibromatosis
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
STR	Short Tandem Repeats
SV	Structural Variant
SWN	Schwannomatosis
TAD	Topologically Associating Domain
TBD	Tubulin-Binding Domain
TBE	Tris/Boric Acid/EDTA
TDB	Topological Domain Boundary
TE	Tris-EDTA
UPD	Uniparental Disomy
UTR	Untranslated Region
VS	Vestibular Schwannoma
VUS	Variant of Uncertain Significance
WES	Whole Exome Sequencing
WT	Wild Type

1. INTRODUCTION

1.1 Neurofibromatoses

Neurofibromatoses (NF) are neurocutaneous syndromes encompassing a spectrum of distinct genetic disorders that share the common feature of nerve sheath tumor development. Tumors can arise in both the central and peripheral nervous system, affecting the brain, spinal cord, skin, and skeletal structures. Three subtypes of NF have been identified: Neurofibromatosis type 1 (NF1), which accounts for approximately 96% of all cases, Neurofibromatosis type 2 (NF2), representing about 3%, and Schwannomatosis (SWN), observed in fewer than 1% of patients. Each form is caused by different dominantly inherited gene mutations, leading to the development of specific tumor types and a broad spectrum of additional clinical manifestations with variable expressivity (Korf, 2013; Tamura, 2021).

This thesis will focus on the two most prevalent forms of NF: NF1 and NF2. In addition to their shared association with nerve sheath tumor development, both conditions are characterized by alterations in molecular signaling pathways that, although classically considered distinct, are increasingly recognized to interact at a functional level (Zinatizadeh *et al.*, 2019).

1.1.1 Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1, OMIM #162200), also known as Von Recklinghausen's disease, is an autosomal dominant genetic disorder with an estimated incidence of 1 in 2,500 live births and a prevalence of 1 in 3,000 individuals worldwide (Lee *et al.*, 2023). NF1 is mainly caused by heterozygous loss-of-function mutations in the tumor suppressor gene *NF1* (OMIM #613113) (Shofty *et al.*, 2015; Stewart *et al.*, 2018), which encodes neurofibromin, a protein essential for embryonic development (Abramowicz and Gos, 2014) and involved in multiple cellular signaling pathways (Bergoug *et al.*, 2020). Neurofibromin functions as a negative regulator of the Ras/MAPK (Ras/mitogen-activated protein kinase) pathway, a signaling cascade critical for cellular differentiation,

proliferation, and survival (Larizza *et al.*, 2009). Consequently, NF1 is classified among the RASopathies, a group of genetic syndromes caused by germline mutations in genes encoding proteins that regulate the Ras/MAPK pathway, resulting in its hyperactivation through increased phosphorylation of its downstream effector, ERK (Hebron *et al.*, 2022).

The *NF1* gene, mapped to chromosome 17q11.2, was definitively identified and characterized in 1990 (Cawthon *et al.*, 1990). It spans approximately 280 kilobases (kb) and contains 60 exons, of which 57 are constitutive and three (exons 9a, 23a, and 48a) are involved in alternative splicing (Biayna *et al.*, 2021). The *NF1* gene is driven by a weak promoter, resulting in generally low expression levels across most cell types (Zou *et al.*, 2004). Although ubiquitously expressed during both embryonic and adult stages, its expression is enriched in certain cell populations of the central and peripheral nervous system, including neurons, oligodendrocytes, astrocytes, and Schwann cells (Nordlund *et al.*, 1993). Transcriptomic analyses have revealed extensive alternative splicing, with 39 transcript variants reported in the Ensembl database, differing in length (11-13 kb) and tissue-specific expression patterns.

The principal transcript (Ensembl ID: ENST00000356175.7) comprises 57 exons, with an open reading frame (ORF) of 8,457 nucleotides, a 5' untranslated region (UTR) of approximately 350 bp, and a 3' UTR of ~3.5 kb. It encodes neurofibromin isoform 1, a 2,818 amino acid (aa) protein predominantly expressed in central nervous system neurons. Isoform 2, which is widely expressed across human tissues, differs from isoform 1 by the inclusion of exon 23a, resulting in an additional region of 21 aa. Isoforms 1 and 2 represent the biologically most relevant neurofibromin variants (Naschberger *et al.*, 2021). Two further isoforms are produced by the inclusion of exon 48a encoding 18 aa (isoform 3) and exon 9a encoding 10 aa (isoform 4). Isoform 3 is preferentially expressed in cardiac, skeletal, and smooth muscle, whereas isoform 4 expression is restricted to the central nervous system (Gutmann *et al.*, 1993; Danglot *et al.*, 1995).

The neurofibromin protein has a molecular weight of approximately 287 kDa (DeClue *et al.*, 1991) and can assemble into a high-affinity homodimer (Sherekar

et al., 2020). It is primarily localized in the cytoplasm, where it associates with microtubules, actin filaments, mitochondria, and the endoplasmic reticulum. However, nuclear localization has also been reported, facilitated by a nuclear localization signal encoded by *NF1* exon 43 (Vandenbroucke *et al.*, 2004). Neurofibromin is a multifunctional protein comprising several functional domains (Fig. 1): the cysteine/serine-rich domain (CSRD) located at the N-terminal region (Mangoura *et al.*, 2006); the tubulin-binding domain (TBD), which interacts with tubulin and leucine-rich pentatricopeptide repeat motif-containing protein (LRPPRC), thereby modulating cytoskeletal dynamics (Arun *et al.*, 2013); the GAP-related domain (GRD), which confers GTPase-activating protein (GAP) activity (Martin *et al.*, 1990; DeClue *et al.*, 1991); the SEC14 domain, implicated in the regulation of subcellular protein localization (D'Angelo *et al.*, 2006); the pleckstrin homology (PH) domain, which interacts with SEC14 (Walti *et al.*, 2011); the C-terminal domain (CTD) (Luo *et al.*, 2014); the substrate-binding domain (SBD), through which neurofibromin can interact with syndecans (Abramowicz and Gos, 2015).

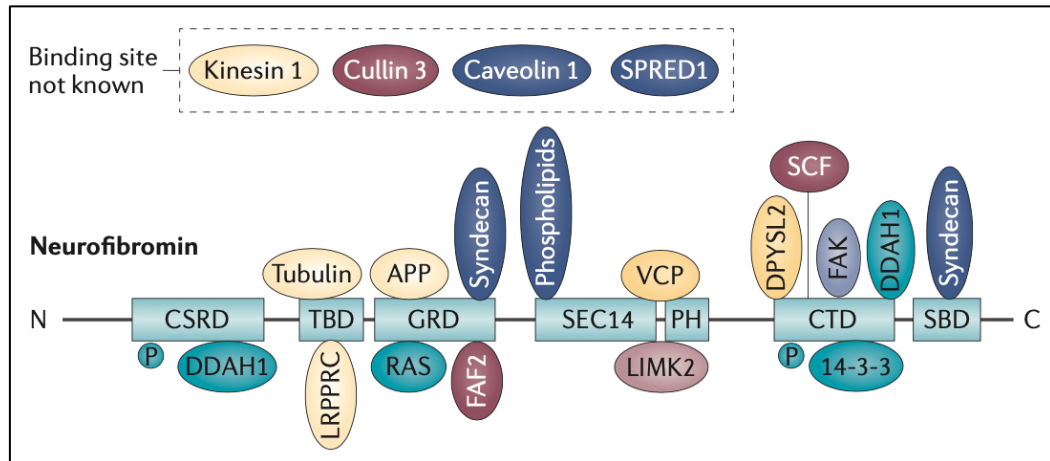


Figure 1. Functional domains and interactors of neurofibromin. Neurofibromin comprises several functional domains, depicted as light blue rectangles. Its interactors are represented as ovals, color-coded according to their respective functions: cellular signaling in turquoise, intracellular trafficking in light yellow, neuronal differentiation in dark yellow, membrane localization in dark blue, actin cytoskeleton remodeling in light pink, ubiquitination in dark pink, and cell adhesion in purple. Interactors enclosed within dashed boxes correspond to proteins for which the specific neurofibromin binding domain has not yet been identified (adapted and modified from Ratner and Miller, 2015).

Due to its multiple domains, neurofibromin participates in several signaling pathways, such as the Ras/MAPK, PI3K/Akt/mTOR, Rho/ROCK/LIMK/cofilin, and cAMP/PKA pathways, and in different cellular processes, including proliferation and migration, cytoskeletal dynamics, and neurite outgrowth (Fig. 2) (Philpott *et al.*, 2017; Bergoug *et al.*, 2020).

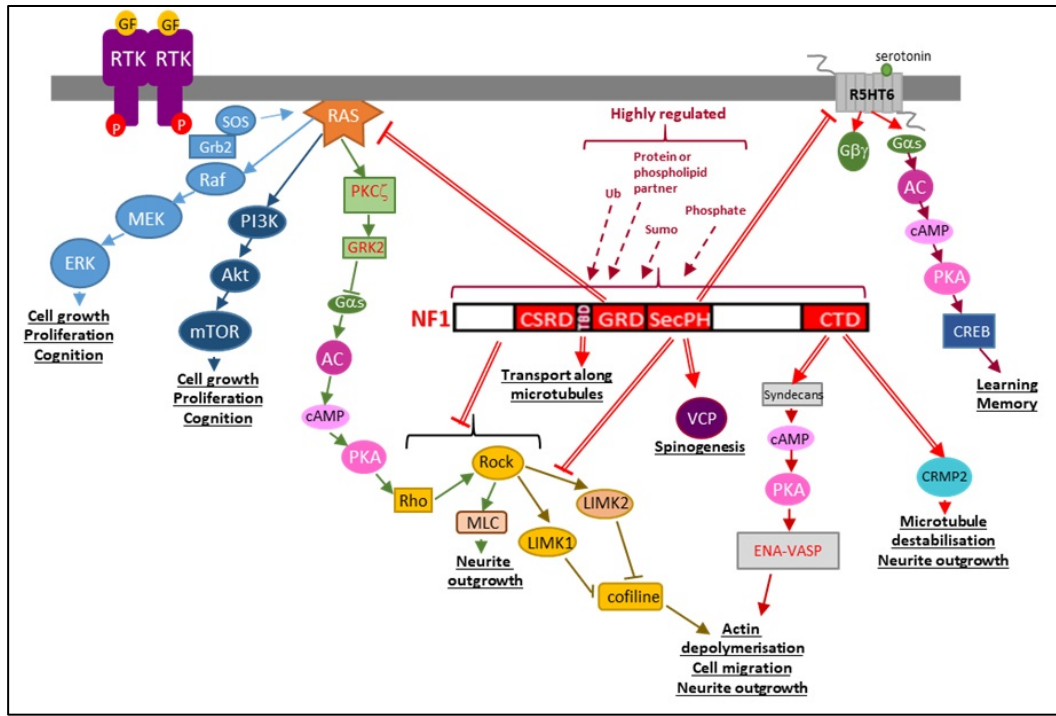


Figure 2. Mechanisms of action of neurofibromin. NF1 inhibits Ras activity in normal cells. Therefore, the NF1 loss-of-function leads to increased Ras activity, which induces the Ras/MAPK and PI3K/Akt/mTOR pathways, resulting in dysregulated cell growth and tumorigenesis. NF1 can also modulate the cAMP-PKA and Rho/ROCK/LIMK/cofilin signaling pathways, affecting cytoskeletal dynamics and neurite outgrowth (adapted from Bergoug *et al.*, 2020).

The active site of neurofibromin, spanning approximately 300-400 aa and encoded by eight *NF1* exons (21-27a), corresponds to the GRD domain (Martin *et al.*, 1990). Neurofibromin is therefore specifically classified as a Ras-GAP, as it enhances the intrinsic GTPase activity of Ras (Upadhyaya *et al.*, 1997), accelerating the hydrolysis of GTP, bound to active Ras-GTP, and thereby promoting its conversion to the inactive GDP-bound state (Ras-GDP), thus preventing prolonged Ras activation (Le and Parada, 2007). Isoform 2 of *NF1*, which incorporates exon 23a within the GRD domain, exhibits a ~10-fold reduction in GAP activity compared with isoform 1. The presence or absence of exon 23a modulates Ras/ERK

signaling and has been shown to influence memory and learning processes (Nguyen *et al.*, 2017). Ras proteins are encoded by three proto-oncogenes – *HRAS*, *KRAS*, and *NRAS* – and play a pivotal role in receptor-mediated signaling pathways that regulate cell growth, proliferation, differentiation, and survival (Nguyen *et al.*, 2017). In its active form (Ras-GTP), Ras triggers the MAPK signaling cascade by interacting with and activating RAF, a kinase that phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. ERK subsequently phosphorylates downstream targets, including kinases and transcription factors such as CREB (cAMP response element-binding protein) (Fig. 2). The ultimate outcome of this signaling cascade is the induction of cell proliferation. Consequently, loss-of-function mutations in *NF1* result in elevated intracellular levels of active Ras-GTP, leading to sustained hyperactivation of the Ras/MAPK pathway and uncontrolled cell proliferation, thereby increasing the risk of neoplastic transformation (Philpott *et al.*, 2017). Thus, *NF1* is recognized as a tumor suppressor gene, with its protein product functioning as an inhibitor of Ras activation. In addition, neurofibromin plays an essential role in embryonic development, as aberrant stimulation of the Ras/MAPK pathway has been implicated in abnormal neural crest cell formation during embryogenesis (Bezniakow *et al.*, 2014).

A further signaling cascade activated by excessive levels of Ras-GTP is the PI3K/Akt/mTOR pathway (Phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin) (Fig. 2), which contributes to protection against apoptosis. Enhanced activity of this pathway results in increased cellular proliferation and survival (Philpott *et al.*, 2017). Furthermore, PI3K activates not only Akt but also Rac proteins, which are involved in cytoskeletal reorganization and cell migration (Ingram *et al.*, 2001).

Neurofibromin also acts as a positive regulator of intracellular cAMP levels (Fig. 2) by mediating the activation of adenylate cyclase downstream of G protein coupled receptors (GPCRs), through both Ras-dependent and Ras-independent mechanisms (Sutton *et al.*, 2019). Increased intracellular cAMP levels inhibit cell proliferation. Therefore, pathogenic *NF1* mutations promote cellular growth also through impaired cAMP signaling (Bergoug *et al.*, 2020).

Through its multiple functional domains, neurofibromin can interact with a wide variety of phospholipids and proteins, including tubulin, amyloid- β (A β) precursor protein (APP), protein kinases A and C, syndecans, and caveolin (Fig. 1). These neurofibromin interactors participate in diverse cellular processes, such as intracellular trafficking, actin cytoskeleton remodeling, ubiquitination, neuronal differentiation, cell adhesion, and cellular signaling (Arun *et al.*, 2013; Hsueh, 2012; Vallée *et al.*, 2012; Tan *et al.*, 2011; Wang *et al.*, 2011; Khanna *et al.*, 2012).

The entire *NF1* locus is considered a mutational hot spot in the human genome, as its spontaneous germline mutation rate – estimated to range between 1×10^{-4} and 3.1×10^{-5} gametes per generation – is approximately tenfold higher than that of most other genes associated with hereditary disorders (Clementi *et al.*, 1990; Upadhyaya *et al.*, 2008). Mutational events include: point mutations, distributed across the entire *NF1* sequence, ~80% of which are nonsense (resulting in truncated proteins) and ~20% missense (causing amino acid substitutions with loss or alteration of protein function); small insertions/deletions (indels), which may induce frameshifts and premature termination; chromosomal translocations; point mutations or indels leading to aberrant mRNA splicing; and intragenic copy number variations (CNVs), such as exon deletions or duplications, identified in ~2% of patients (Philpott *et al.*, 2017; Pros *et al.*, 2008). Consequently, NF1 is characterized by extensive allelic heterogeneity, with more than 4,700 pathogenic variants of *NF1* currently reported in the ClinVar database, ~50% of which are *de novo* (sporadic cases), while the remaining 50% are inherited (familial cases) (Gottfried *et al.*, 2006).

Although *NF1* pathogenic mutations display complete penetrance, NF1 is clinically marked by variable expressivity: individuals carrying the same mutation, even within the same family, may exhibit different phenotypic manifestations; moreover, genotype-phenotype correlations are generally weak, with only a few specific mutations associated with distinctive phenotypes (Carey and Viskochil, 1999). The principal clinical features emerge early in life – 50% of cases within the first year and 97% by the age of eight (Karwacki and Woźniak, 2006) – and include: café-au-lait macules, the most common clinical sign (present in ~90% of patients), which appear at birth and progressively increase in size and number with age (Fig.

3A); axillary and/or inguinal freckling (Fig. 3B); Lisch nodules (iris hamartomas) (Fig. 3C); and neurofibromas, benign peripheral nerve sheath tumors typically appearing after the age of eight, ranging in number from a few to over a thousand per patient, and often increasing in response to hormonal changes. Neurofibromas may be cutaneous (Fig. 3D) or plexiform (Fig. 3E), the latter with potential progression to malignant peripheral nerve sheath tumors (MPNSTs). In addition to these primary manifestations, patients may present with optic pathway gliomas (OPGs) (Fig. 3F), skeletal dysplasias (Fig. 3G-H), cognitive impairments, and an increased predisposition to both benign and malignant neoplasms (Miller *et al.*, 2019; Saleh *et al.*, 2023).

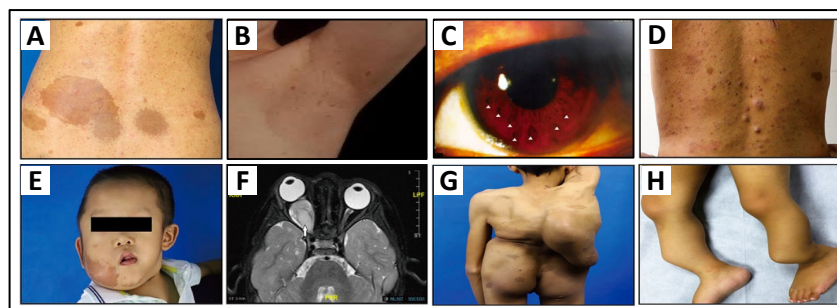


Figure 3. Common clinical features of Neurofibromatosis type 1. (A) Café-au-lait macules. (B) Axillary freckling. (C) Lisch nodules. (D) Cutaneous neurofibromas. (E) Plexiform neurofibromas. (F) OPGs. (G) Scoliosis. (H) Pseudoarthrosis (adapted and modified from Wang *et al.*, 2021).

Patients in whom NF1 arises from postzygotic somatic mutations occurring during embryonic development present with mosaicism, i.e., genetically distinct cell lineages. The clinical impact depends on the embryonic stage at which the somatic mutation occurs. In approximately 0.02% of NF1 patients, mutations arise during early embryogenesis, resulting in generalized mosaicism, which manifests with the classical clinical features of NF1. If the mutation occurs later in development, patients exhibit segmental mosaicism, characterized by clinical signs restricted to specific body regions, a condition reported in ~0.002% of patients. Even more rarely, NF1 patients may present with gonadal mosaicism, arising from mutations in germ cells; in this case, the affected individual does not display clinical manifestations, but the mutation is transmitted to the offspring, who develop the disease (Hall, 1988; Kehrer-Sawatzki and Cooper, 2008).

The diagnosis of NF1 is based on the international diagnostic criteria established at the National Institutes of Health (NIH) Consensus Development Conference in 1987 (NIH, 1988), and revised by an international panel of experts in 2021 (Table 1) (Legius *et al.*, 2021). In individuals without an affected parent, a diagnosis of NF1 requires the presence of at least two diagnostic criteria listed in Table 1. In contrast, if a parent has a confirmed diagnosis of NF1, the presence of only one diagnostic criterion is sufficient to establish the diagnosis.

Table 1. Revised diagnostic criteria for Neurofibromatosis type 1

Clinical and genetic features	Diagnostic criteria
Café-au-lait macules	Six or more café-au-lait macules (greatest diameter > 5 mm in prepubertal individuals and > 15 mm in postpubertal individuals)
Skinfold freckling	Freckling in axillary or inguinal region
Neurofibromas	Two or more neurofibromas of any type or one plexiform neurofibroma
Optic pathway glioma	Presence of optic pathway glioma
Ophthalmologic findings	Two or more iris Lisch nodules or choroidal abnormalities
Osseous lesions	A distinctive osseous lesion (sphenoid dysplasia, anterolateral bowing of the tibia, pseudarthrosis of a long bone)
Genetic testing	A heterozygous pathogenic <i>NF1</i> variant with a variant allele fraction of 50% in apparently normal tissue

Three distinct forms of NF1 can be distinguished: classical NF1, spinal neurofibromatosis (SNF), and NF1 microdeletion syndrome. Classical NF1, clinically defined by the features previously described, is caused by point mutations or small indels within the *NF1* gene, and accounts for more than 90% of all NF1 cases. Patients with SNF also harbor *NF1* point mutations, predominantly missense variants, but are clinically characterized by bilateral neurofibromas affecting all 38

spinal nerve roots (and, in some cases, all major branches of the peripheral nerves). These tumors often occur in the absence of other clinical signs of classical NF1 (Ruggieri *et al.*, 2015; Pattera *et al.*, 2022; Bettinaglio *et al.*, 2023). By contrast, patients with NF1 microdeletion syndrome (~5-10% of NF1 cases) carry a deletion encompassing the entire *NF1* gene along with a variably sized contiguous chromosomal region. These individuals typically present with a more severe phenotype compared with classical NF1 (Tritto *et al.*, 2024). NF1 microdeletion syndrome will be discussed in detail later.

1.1.2 Neurofibromatosis type 2

Neurofibromatosis type 2 (NF2, OMIM #101000), also referred to as NF2-related Schwannomatosis, is an autosomal dominant genetic disorder with an incidence of approximately 1 in 33,000 live births and a prevalence of ~1 in 56,000 individuals worldwide (Evans *et al.*, 2010). The disease is primarily caused by heterozygous loss-of-function mutations in the tumor suppressor gene *NF2* (OMIM #607379) (Kluwe *et al.*, 1996; Baser *et al.*, 2004), which encodes neurofibromin 2, also known as Merlin. This protein regulates cell-cell contact, proliferation, and motility by modulating several intracellular signaling pathways (Kim *et al.*, 2024).

The *NF2* gene, located on chromosome 22q12.2, was identified in 1993 (Rouleau *et al.*, 1993). It spans ~250 kb and contains 17 exons subject to alternative splicing (Biayna *et al.*, 2021). As a result, multiple mRNA transcripts are generated, ranging in size from 1 to 6 kb. Among the 21 alternative transcripts reported in the Ensembl database, 17 are protein-coding, with isoforms 1 and 2 being the predominant forms. Isoform 1 (Ensembl ID: ENST00000338641.10) includes exons 1-15 and 17 and encodes a protein of 595 aa, whereas isoform 2 (Ensembl ID: ENST00000672896.1) contains exons 1-16 and encodes a protein of 590 aa. *NF2* is expressed ubiquitously across adult tissues as well as in various embryonic tissues, with the highest expression levels observed in Schwann cells, meningeal cells, lens, and peripheral nerves (Pečina-Šlaus, 2013).

The protein product of *NF2*, Merlin (also called Schwannomin), has a molecular weight of approximately 67 kDa (Stamenkovic and Yu, 2010) and forms homodimers (Yogesha *et al.*, 2011). Merlin (moezin-ezrin-radixin-like protein) belongs to the protein 4.1 superfamily of cytoskeletal elements involved in cytoskeletal dynamics and ion transport regulation (Ceccarelli *et al.*, 2006). This protein is organized into three main structural domains (Fig. 4A): (i) an N-terminal FERM domain, a highly conserved region essential for mediating interactions between cell membrane glycoproteins and the actin cytoskeleton; (ii) a central flexible coiled-coil domain, which connects the N- and C-terminal ends and facilitates intramolecular conformational changes; and (iii) a C-terminal domain, which differs between isoforms and undergoes post-translational modifications that regulate Merlin's conformation and tumor suppressor activity. Merlin exists in two conformational states: a dephosphorylated active “closed” form and a phosphorylated inactive “open” form (Fig. 4B). Specifically, phosphorylation at Ser518 by p21-activated kinase (PAK1) or cAMP-dependent protein kinase A (PKA) and dephosphorylation by myosin phosphatase-1 protein phosphatase-1 (MYPT1-PP1) control this conformational switch. Isoform 2 lacks the C-terminal residues required for intramolecular binding to the N-terminal FERM domain, resulting in a constitutively open conformation (Nouri *et al.*, 2024).

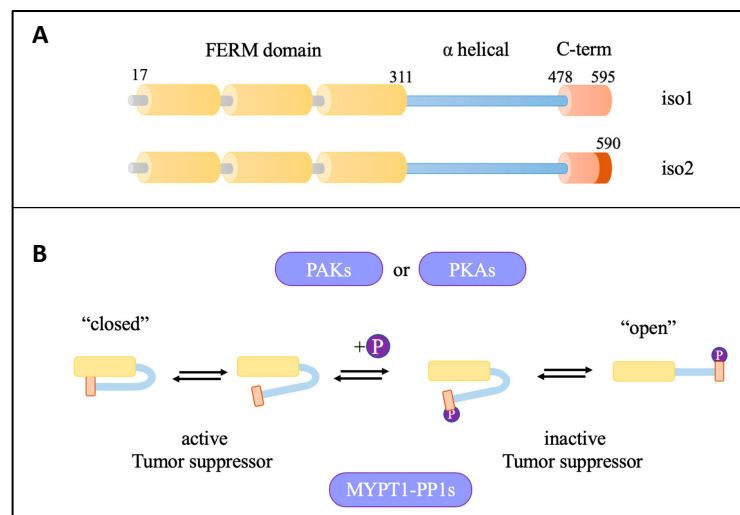


Figure 4. Structure and conformational forms of Merlin protein. (A) The isoforms 1 and 2 of Merlin protein show three main domains, which differ only in the C-terminal ending. (B) Merlin can exist in two conformational forms, with a dephosphorylated active “closed” form and a phosphorylated inactive “open” form (adapted and modified from Nouri *et al.*, 2024).

Merlin exerts its tumor suppressor function by regulating multiple signaling pathways involved in cell growth and division (Fig. 5) (Tamura, 2021).

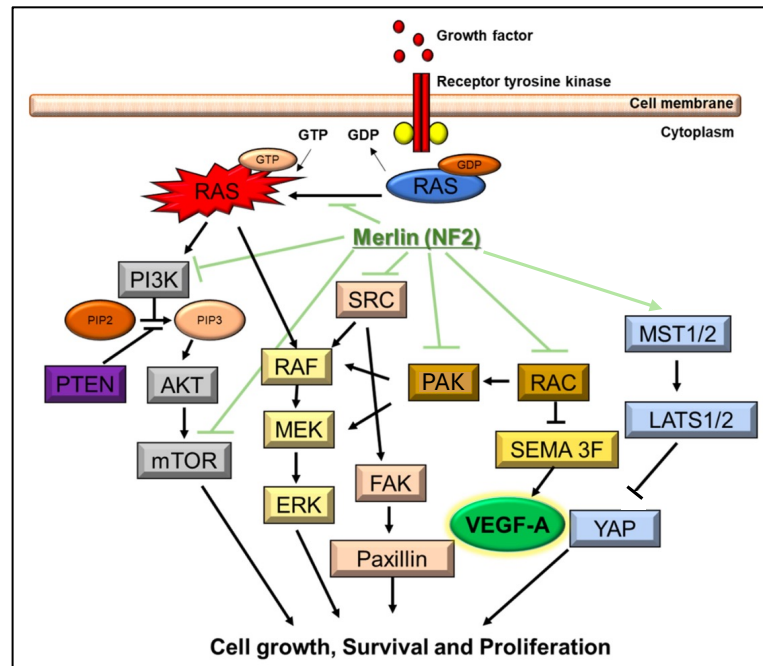


Figure 5. Mechanisms of action of Merlin. NF2 inhibits Ras/MAPK, PI3K/Akt/mTOR, Src/FAK, Rac/JNK, and Hippo pathways in normal cells. Therefore, the NF2 loss-of-function leads to increased activity of these pathways, resulting in dysregulated cell growth and tumorigenesis (adapted and modified from Tamura, 2021).

These pathways include (Fig. 5):

- Hippo. Merlin activates MST1/2 kinases, promoting cell growth inhibition and apoptosis through phosphorylation of downstream targets such as LATS1/2, which in turn inhibit YAP, a key activator of tissue growth (Mota and Shevde, 2020).
- Ras/MAPK. Merlin recruits Ras-GAP, either through an association with receptor tyrosine kinases (RTKs) or via RTK-independent mechanisms, thereby contributing to the inactivation of Ras-GTP and termination of downstream signaling (Cui *et al.*, 2019).
- PI3K/Akt/mTOR. Merlin inhibits the GTPase PIKE-L (phosphatidylinositol 3-kinase enhancer-L), which normally binds to and activates PI3K, thereby suppressing downstream pathway activation (Petrilli and Fernández-Valle, 2016).

- Src/FAK/Paxillin. Merlin inhibits this signaling pathway cascade by binding to ErbB2, thereby preventing ErbB2-mediated Src phosphorylation and the subsequent activation of mitogenic signaling (Houshmandi *et al.*, 2009).
- Rac/JNK. Merlin negatively regulates this pathway both by inhibiting PAK, a downstream effector of Rac, and by participating in a multiprotein complex composed of Angiomotin, Patj, and Pals1, which suppresses the activity of Rich1, a Rac1-activating GAP (Li *et al.*, 2012).

The tumor suppressor function of Merlin implies that loss-of-function mutations in the *NF2* gene lead to the development of NF2, a disease characterized by tumor predisposition. To date, nearly 300 pathogenic variants of the *NF2* gene have been reported in the ClinVar database, approximately 50% of which are inherited, while the remaining 50% occur *de novo*. Among patients with *de novo* mutations, about 25% display post-zygotic mosaicism (Kluwe *et al.*, 2003). Several types of *NF2* gene alterations have been identified as causative of the disease, including nonsense and missense mutations, frameshift deletions and insertions, splicing mutations, deletions of one or more exons, and rare 22q12 microdeletions involving both *NF2* and the flanking chromosomal region. Although genotype-phenotype correlations are not yet fully established, truncating mutations appear to be associated with a more severe phenotype (Evans *et al.*, 1998; Selvanathan *et al.*, 2010).

NF2 is typically considered a disease of young adulthood, as symptoms most often arise in the second or third decade of life, with diagnosis usually occurring between 20 and 30 years of age. Furthermore, age at onset is inversely correlated with disease severity (Arden-Holmes *et al.*, 2016). The condition displays a variable expressivity of clinical phenotypes (Fig. 6) (Parry *et al.*, 1994), although its hallmark feature is the presence, in nearly all patients, of bilateral vestibular schwannomas – benign tumors originating from Schwann cells of the eighth cranial nerve – leading to hearing loss, tinnitus, and gait ataxia. Patients may also develop additional schwannomas affecting other cranial and peripheral nerves. Other tumor types are also observed, including ependymomas (present in ~30% of patients), multiple spinal tumors (~65% of patients), and intracranial or spinal meningiomas (~50% of patients) (Halliday *et al.*, 2019a; Bachir *et al.*, 2021). Additional clinical

manifestations include ophthalmological features, such as decreased visual acuity, retinal hamartomas, and cataracts (Ragge *et al.*, 1997), as well as dermatological features, including dermal plaque-like lesions, subcutaneous nodular tumors, and café-au-lait spots (Ardern-Holmes *et al.*, 2016; Mautner *et al.*, 1997).

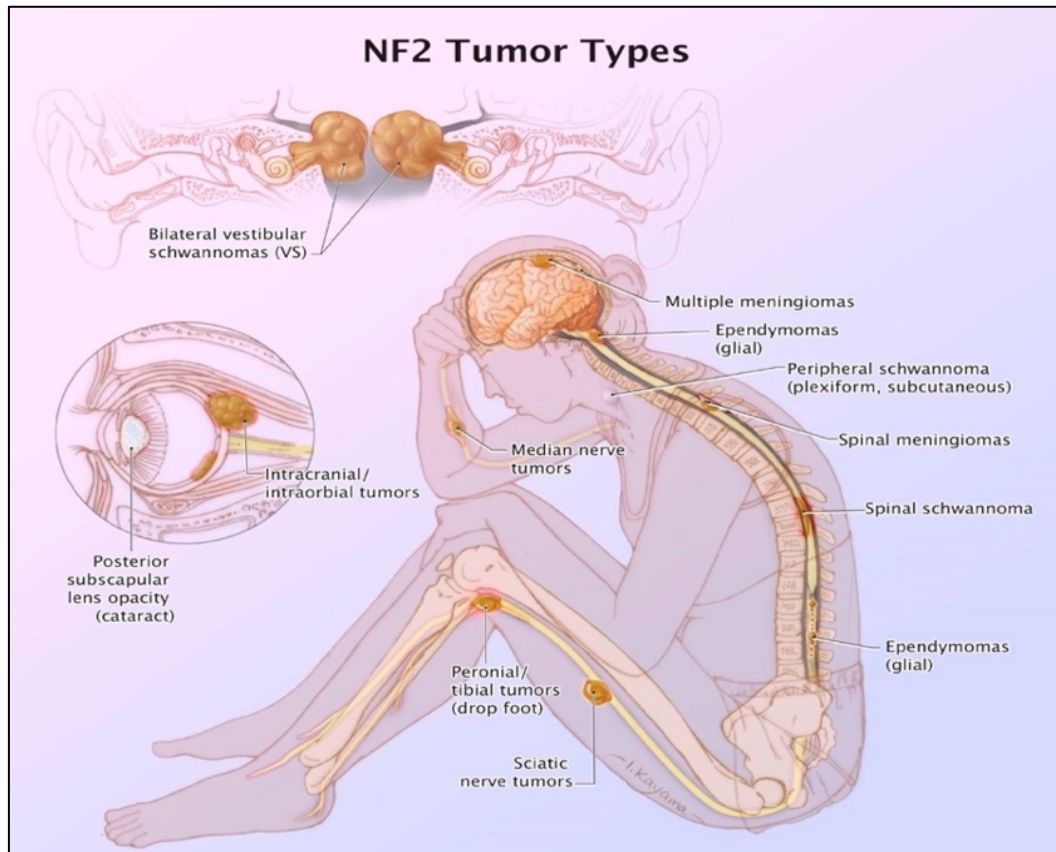


Figure 6. Main clinical signs of Neurofibromatosis type 2. This disorder is associated with the development of bilateral vestibular schwannomas. NF2 patients can also develop other tumors, including schwannomas of the cranial, spinal, peripheral, and cutaneous nerves, meningiomas and ependymomas, along with ophthalmological and dermatological features (adapted from Bachir *et al.*, 2021).

The diagnosis of NF2 is based on international diagnostic criteria, first established by the NIH Consensus Development Conference in 1988 (NIH, 1988). The current diagnostic criteria, resulting from revisions by international expert panels in recent years (Plotkin *et al.*, 2022), state that a diagnosis of NF2 can be established when an individual presents with one of the following: bilateral vestibular schwannomas; the same pathogenic *NF2* variant identified in at least two anatomically distinct NF2-related tumors; or either two major criteria, or one major and two minor criteria, as detailed in Table 2.

Table 2. Revised diagnostic criteria for Neurofibromatosis type 2

A diagnosis of NF2 can be made when an individual has one of the following:	
1. Bilateral vestibular schwannomas 2. An identical <i>NF2</i> pathogenic variant in at least 2 anatomically distinct NF2-related tumors (schwannoma, meningioma, and/or ependymoma) 3. Either 2 major or 1 major and 2 minor criteria	
Major criteria • Unilateral vestibular schwannomas • First-degree relative other than sibling with NF2 • 2 or more meningiomas • <i>NF2</i> pathogenic variant in an unaffected tissue	Minor criteria • Ependymoma, meningioma, schwannoma • Juvenile cataract, retinal hamartoma • Specific pattern of genetic changes in unaffected and tumor tissue

In addition to the classical form of NF2, caused by intragenic *NF2* mutations or by deletions of one or more exons or the entire gene, there are rare patients carrying large deletions in the 22q12 chromosomal region, who exhibit a more severe phenotype (Tritto *et al.*, 2022). These deletions result in the loss of not only the *NF2* gene but also a variable number of flanking genes and will be discussed in detail later.

1.1.3 Functional interplay between NF1 and NF2 signaling pathways

Although NF1 and NF2 are classically associated with distinct signaling pathways, increasing evidence suggests partial functional overlap and crosstalk between their downstream networks. NF1 primarily acts as a negative regulator of Ras signaling, thereby influencing the MAPK and PI3K pathways (Bergoug *et al.*, 2020), while NF2 regulates cell growth and contact inhibition mainly through the Hippo pathway (Ma *et al.*, 2019). However, dysregulation of Ras/MAPK signaling has been shown to impact Hippo pathway components, including YAP/TAZ activity, and conversely, Hippo pathway alterations can modulate MAPK signaling outputs.

Specifically, aberrant Ras-RAF-MEK-ERK signaling can attenuate Hippo pathway kinase activity, leading to decreased phosphorylation and increased nuclear accumulation of YAP/TAZ, thereby promoting their transcriptional co-activator function and altering gene expression patterns linked to cell proliferation and survival (Reddy and Irvine, 2013). Moreover, activated Ras signaling can suppress Hippo pathway activity by promoting the formation of low-activity MST1/MST2 heterodimers, thereby reducing kinase signaling to LATS1/2 and facilitating YAP/TAZ activation and oncogenic transformation (Rawat *et al.*, 2016). In turn, alterations in core Hippo pathway components can feed back on Ras/MAPK signaling, as loss of the Hippo pathway kinases LATS1/2 or increased YAP/TAZ activity has been shown to enhance Ras pathway signaling through upregulation of *EGFR* (Epidermal Growth Factor Receptor) expression, thereby reinforcing mitogenic Ras/MAPK hyperactivation and promoting oncogenic phenotypes (Zinatizadeh *et al.*, 2019).

These functional interactions indicate that NF1- and NF2-related pathways are not entirely independent but instead converge on shared processes such as cell proliferation, survival, and cytoskeletal organization. Such crosstalk may help explain overlapping clinical and tumorigenic features observed in NF1- and NF2-associated neoplasms.

1.2 *NF1* and *NF2* microdeletions

1.2.1 Structure and gene content of 17q11.2 microdeletions

NF1 microdeletion syndrome (OMIM #613675), present in approximately 4.7-11% of NF1 patients, results from a heterozygous deletion encompassing the entire *NF1* gene along with a variable number of flanking genes located within the 17q11.2 genomic region (Riva *et al.*, 2000; Venturin *et al.*, 2004b). This chromosomal region is gene-rich, containing protein-coding genes, pseudogenes, and microRNA-encoding genes, and is also characterized by the presence of paralogous repeated sequences, known as Low Copy Repeats (LCRs).

Based on deletion size, breakpoint (BP) location, and the number of genes included within the deleted region, 17q11.2 deletions underlying NF1 microdeletion syndrome have been classified into four groups (Fig. 7). The first three groups comprise the so-called typical deletions, including type-1 *NF1* deletion, type-2 *NF1* deletion, and type-3 *NF1* deletion. The fourth group consists of atypical *NF1* deletions (Kehrer-Sawatzki and Cooper, 2021; Pacot *et al.*, 2024).

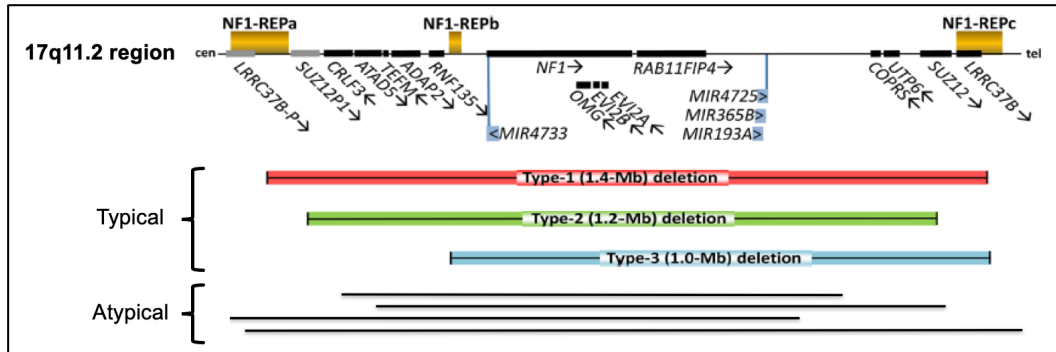


Figure 7. Schematic representation of the 17q11.2 genomic region and *NF1* deletion classes. At the top, the 17q11.2 genomic region is shown, including the genes it contains, among which is *NF1*. Arrows following gene names indicate their transcriptional orientation: cen, centromeric direction; tel, telomeric direction. The bars at the bottom indicate the genomic region encompassed by each deletion class and its size (adapted and modified from Kehrer-Sawatzki *et al.*, 2017).

The type-1 *NF1* deletion (Fig. 7, red bar) is the most recurrent *NF1* microdeletion, present in 70-80% of all patients with NF1 microdeletion syndrome. It spans a chromosomal region of 1.4 megabases (Mb), encompassing 14 protein-coding genes, including *NF1*, five microRNA-coding genes, and one pseudogene. This microdeletion is characterized by BPs located within two LCRs adjacent to the *NF1* gene, designated NF1-REPa (proximal) and NF1-REPC (distal) (Pasmant *et al.*, 2010). NF1-REPa and NF1-REPC serve as hotspots for interchromosomal Non-Allelic Homologous Recombination (NAHR) (Fig. 8A) (De Raedt *et al.*, 2006; Bengesser *et al.*, 2014), an event that can occur during meiosis, resulting in the loss of the genetic material between these sequences. Consequently, the type-1 *NF1* deletion usually arises during meiosis in the germline of one of the affected individual's parents, and it is therefore present constitutionally in all cells of the patient (López-Correa *et al.*, 2000; Hillmer *et al.*, 2016).

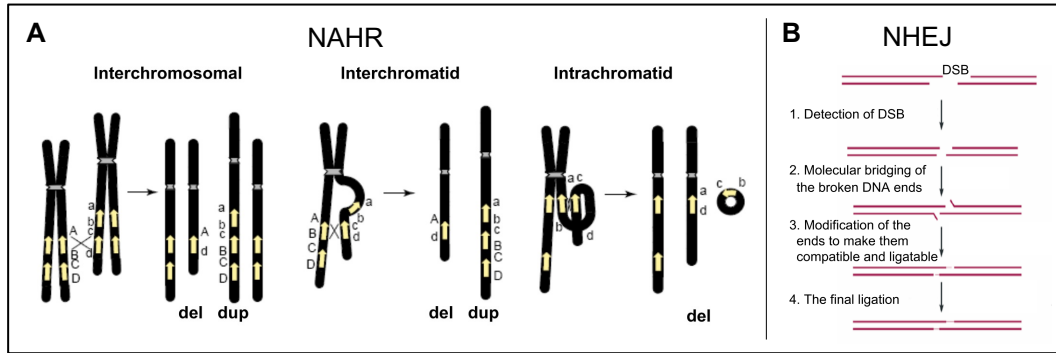


Figure 8. Mutational mechanisms underlying *NF1* microdeletions. (A) Schematic representation of reciprocal duplications and deletions mediated by interchromosomal, interchromatid, and intrachromatid NAHR between pairs of LCRs in direct orientation. Yellow arrows and letters represent LCRs and specific chromosomal sequences, respectively. Interchromosomal and interchromatid NAHR events between direct-orientation LCRs produce reciprocal duplications and deletions, whereas intrachromatid NAHR generates deletions only. (B) Schematic representation of the NHEJ mechanism, which repairs double-strand DNA breaks. The thick lines represent the two DNA strands, while the thin segments in the center depict modifications that the DNA ends undergo before final ligation (adapted and modified from Gu *et al.*, 2008).

Another recurrent deletion, observed in 10-20% of patients with *NF1* microdeletion syndrome, is the type-2 *NF1* deletion (Fig. 7, green bar), which includes the same genes as the type-1 deletion, except for the *LRRC37B* gene, for a total of 1.2 Mb region deleted. This deletion is characterized by BPs within the *SUZ12* gene (distal) and the highly homologous pseudogene *SUZ12P* (proximal). Type-2 *NF1* deletions are generally post-zygotic, as they are predominantly mediated by mitotic NAHR (Fig. 8A) (Vogt *et al.*, 2012; Bengesser *et al.*, 2014). This event gives rise to somatic mosaicism, such that phenotypic effects of the deletion manifest only in the affected regions of the body (Steinmann *et al.*, 2007).

Type-3 *NF1* deletions (Fig. 7, blue bar), observed in only ~1-4% of all *NF1* microdeletion syndrome patients, span 1.0 Mb. These deletions are mediated by NAHR between NF1-REPB and NF1-REPC LCRs (Fig. 8A), resulting in hemizygosity for a total of nine protein-coding genes (Bengesser *et al.*, 2010; Pasmant *et al.*, 2010; Zickler *et al.*, 2012).

Finally, atypical *NF1* deletions (Fig. 7, black bars) account for 8-10% of all *NF1* microdeletions (Pasmant *et al.*, 2010). These deletions are characterized by non-recurrent BPs and substantial heterogeneity, both in terms of size and the

number of genes included, as well as in their underlying mutational mechanisms (Upadhyaya *et al.*, 1996; Riva *et al.*, 2000; Kehrer-Sawatzki *et al.*, 2003; Venturin *et al.*, 2004a; Gervasini *et al.*, 2005; Mantripragada *et al.*, 2006; Kehrer-Sawatzki *et al.*, 2008; Pasmant *et al.*, 2010; Vogt *et al.*, 2014). Atypical deletions may result from different recombination mechanisms, such as NAHR between short highly homologous sequences, located at distinct genomic positions (Fig. 8A), or from DNA repair processes, including Non-Homologous End-Joining (NHEJ), in which DNA double-strand breaks are ligated despite the absence of template homology (Fig. 8B) (Venturin *et al.*, 2004a), or from retrotransposon-mediated mechanisms (Vogt *et al.*, 2014). Atypical deletions can originate either during meiosis or in post-zygotic phase (Taylor Tavares *et al.*, 2013; Vogt *et al.*, 2014).

The 17q11.2 microdeletions encompass, in addition to *NF1*, the genes *EVI2A*, *EVI2B*, and *OMG*, which are located within intron 27b of *NF1* but transcribed in the opposite direction (minus strand) (Li *et al.*, 1995; Zjablovskaja *et al.*, 2017; Venturin *et al.*, 2006). Beyond these genes, 17q11.2 microdeletions may include, upstream and downstream of *NF1*, additional protein-coding genes, one pseudogene, and genes encoding microRNAs (Riva *et al.*, 2000). The gene content of typical *NF1* deletions is specific to each class, as reported in Table 3, whereas the gene content of atypical *NF1* deletions is variable.

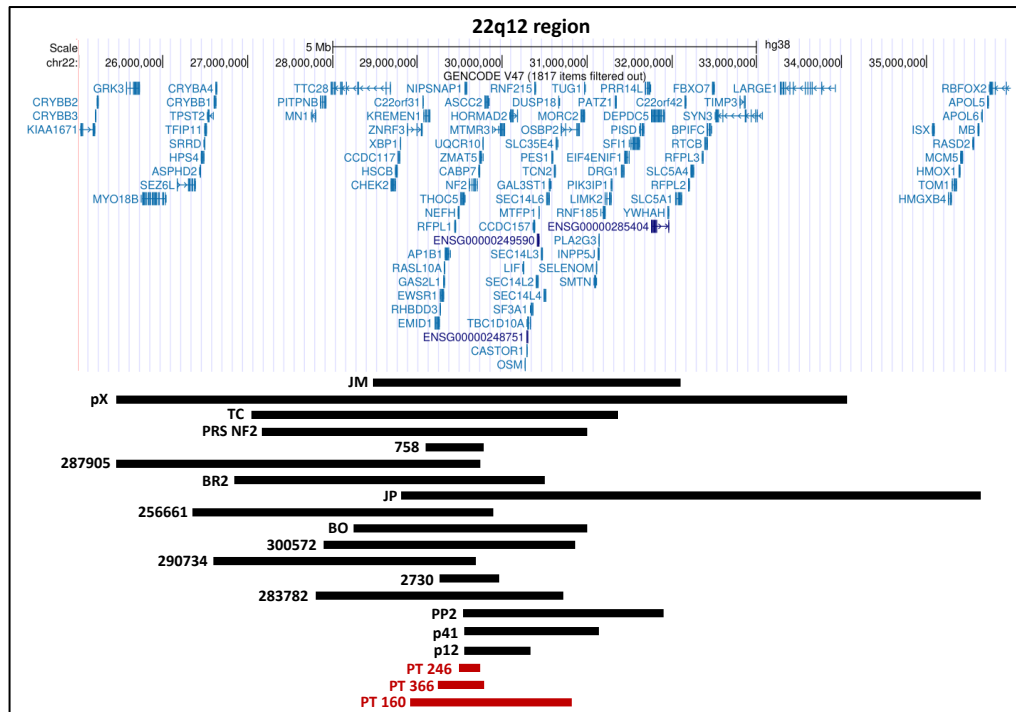
Table 3. Gene content of typical and atypical deletions in the 17q11.2 genomic region

17q11.2 microdeletions	Gene content
Type-1 <i>NF1</i> deletion	<i>SUZ12P, CRLF3, ATAD5, TEFM, ADAP2, RNF135, miR-4733, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, miR-4724, miR-193A, miR-4725, miR-365B, COPRS, UTP6, SUZ12, LRRC37B</i>
Type-2 <i>NF1</i> deletion	<i>SUZ12P, CRLF3, ATAD5, TEFM, ADAP2, RNF135, miR-4733, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, miR-4724, miR-193A, miR-4725, miR-365B, COPRS, UTP6, SUZ12</i>
Type-3 <i>NF1</i> deletion	<i>miR-4733, NF1, OMG, EVI2B, EVI2A, RAB11FIP4, miR-4724, miR-193A, miR-4725, miR-365B, COPRS, UTP6, SUZ12, LRRC37B</i>
Atypical <i>NF1</i> deletions	<i>variable</i>

1.2.2 Structure and gene content of 22q12 microdeletions

NF2 patients may carry a constitutional *NF2* deletion, which occurs in 11-24% of cases. However, in most patients, deletions involve only one or more exons of the *NF2* gene. Rarely, *NF2* microdeletions affecting larger genomic regions and multiple genes across the entire 22q12 band have been described (Barbi *et al.*, 2002; Kluwe *et al.*, 2005; Said *et al.*, 2011; Davidson *et al.*, 2012; Bosson *et al.*, 2016; Breckpot *et al.*, 2016; Bruder *et al.*, 1999; Bruder *et al.*, 2001), and their exact frequency has not yet been determined.

In contrast to typical *NF1* deletions, *NF2* microdeletions are characterized by non-recurrent BPs and highly variable size and gene content. These deletions, ranging from 100 kb to several Mb, can involve from a single gene to nearly hundred genes in addition to *NF2*. The different locations and sizes of 22q12 microdeletions are illustrated in Figure 9, which summarizes 17 cases described in the literature (collected by Trizuljak *et al.*, 2023) along with the three cases reported in our previous study (Tritto *et al.*, 2022).



The lack of recurrent recombination sites is due to the absence of LCRs in the 22q12 region. Instead, this region is enriched in several families of interspersed repetitive sequences that are identical or nearly identical, scattered across the genome through duplication, transposition, or retrotransposition events. These repetitive elements render the 22q12 region prone to chromosomal rearrangements, resulting in a wide variety of genetic lesions (Tritto *et al.*, 2022).

The 22q12 deletions reported in the literature (Fig. 9, black bars) have been identified, in most cases, using the Multiplex Ligation-dependent Probe Amplification (MLPA) or Array-based Comparative Genomic Hybridization (array-CGH) (Bruder *et al.*, 2001), both techniques that do not provide the exact deletions' BPs. However, to establish the molecular mechanism underlying the rearrangement, the DNA sequences involved should be characterized. Therefore, in a previous study, we amplified and sequenced the junction fragments of three *NF2* deletions (Fig. 9, red bars). The high homology of BP regions for each *NF2* deletion, involving repetitive elements all showing a reciprocal homology greater than 80%, prompted us to hypothesize that the events at the basis of these chromosomal rearrangements could be NAHR (Fig. 8A) (Tritto *et al.*, 2022).

1.2.3 Variable expressivity in patients carrying *NF1* and *NF2* microdeletion

Patients harboring *NF1* or *NF2* microdeletions exhibit a marked variable expressivity of clinical signs, often presenting with more severe phenotypes compared to individuals affected by the classical forms of NF1 and NF2, which are typically caused by intragenic mutations in the respective genes (*NF1* and *NF2*) (Tritto *et al.*, 2024; Tritto *et al.*, 2022).

Individuals with NF1 microdeletion syndrome commonly display distinctive craniofacial dysmorphisms, childhood overgrowth, more pronounced cognitive impairment, and various skeletal and cardiovascular malformations. Furthermore, these patients tend to develop benign neurofibromas at an earlier age and show an

increased lifetime risk of MPNSTs (Tritto *et al.*, 2024; Venturin *et al.*, 2005; De Raedt *et al.*, 2003).

Similarly, patients with large-scale *NF2* microdeletions may experience an early onset of classical NF2 manifestations, frequently accompanied by additional features, such as congenital heart defects and intellectual disability (Bruder *et al.*, 1999, Tritto *et al.*, 2022; Halliday *et al.*, 2019b). However, unlike NF1 microdeletion syndrome, clear genotype-phenotype correlations have not yet been established for *NF2* deletion carriers. This lack of correlation is primarily attributable to the limited number of reported cases, their incomplete clinical characterization, and the heterogeneity of the deletion BPs, which are often insufficiently defined at the molecular level.

Comparative analyses have been conducted to evaluate clinical outcomes and overall survival among individuals with *NF2* mutations predicted to produce truncated proteins (nonsense or frameshift variants) and those with alterations resulting in complete loss of protein expression (e.g., large deletions). These studies suggest that nonsense and frameshift mutations are associated with more severe clinical manifestations of NF2. Nevertheless, it is important to note that such evidence derives exclusively from data collected in the population-based United Kingdom NF2 registry, where patients with microdeletions were grouped together with other large-deletion cases for analytical purposes (Selvanathan *et al.*, 2010; Hexter *et al.*, 2015).

1.3 Pathogenic mechanisms in NF1 and NF2 microdeletion syndromes

Patients affected by microdeletion syndromes are often characterized by a broad spectrum of clinical manifestations and a high degree of phenotypic variability (Veltman and Brunner, 2010). Such variability and severity of the phenotype may result not only from the loss of gene content but also from epistatic interactions (Brezo *et al.*, 2014; Viskochil, 2002) and epigenetic mechanisms (Milot *et al.*, 1996; Giorgio *et al.*, 2015), which remain poorly investigated in

patients carrying *NF1* and *NF2* microdeletions (Ferrari *et al.*, 2017; Tritto *et al.*, 2022; Tritto *et al.*, 2024). Indeed, a deletion event may lead to the unmasking of a recessive allele on the non-deleted homologous chromosome or to alterations in chromatin structure, which in turn may result in position effect, a deregulation of the expression of genes flanking the deleted region. Finally, variants in modifier genes may also contribute to the phenotypic variability.

1.3.1 Haploinsufficiency of genes included in the deletion interval

In patients affected by microdeletion syndromes, the most extensively studied and best-known pathogenic mechanism underlying the onset of disease phenotypes is the haploinsufficiency of one or more genes included within the deleted region. Haploinsufficiency refers to a gene's intolerance to the loss of one allele in a diploid organism. Consequently, a deletion or a loss-of-function mutation in a haploinsufficient gene results in altered phenotypes, as a single normal allele is "insufficient" to maintain the wild type state. Haploinsufficiency is therefore recognized as a major cause of several dominantly inherited disorders (Johnson *et al.*, 2019; Collins *et al.*, 2022).

Haploinsufficient, dosage-sensitive genes mainly fall into two functional categories: genes encoding proteins that interact with DNA or receptors, whose activity depends on a quantitative threshold (e.g., transcription factors) (Veitia, 2003; Bhatia *et al.*, 2013); genes encoding proteins that participate in multi-subunit complexes or compete with other proteins, where a precise stoichiometric balance between components is required for proper function (Topalian and Sharpe, 2014).

The concept that haploinsufficiency of genes within deletions could cause disease became evident following the advent of human karyotype cytogenetic analysis, which enabled the association of several developmental syndromes with partial chromosomal deletions. Subsequently, through studies first in *Saccharomyces cerevisiae* and later in model organisms such as *Drosophila*, zebrafish, and mouse, and more recently in human induced Pluripotent Stem Cells

(iPSCs), it has been possible to establish genotype-phenotype correlations and to recapitulate human disorders caused by deletions (Iyer and Girirajan, 2015; De Masi *et al.*, 2020).

The identification of potentially haploinsufficient genes has been further facilitated by the development of computational prediction systems. Based on the large-scale analysis of exome sequencing data from 60,706 individuals of diverse ethnic backgrounds generated by the “Exome Aggregation Consortium” (ExAC), Lek and colleagues developed an algorithm to identify genes under strong selection against loss-of-function variants. This method considers the ratio between the expected and observed number of truncating variants within each gene and assigns a score termed pLI (probability of Loss-of-function Intolerance), ranging from 0 to 1. Genes with a $pLI \geq 0.9$ are predicted to be haploinsufficient (Lek *et al.*, 2016).

Haploinsufficiency of genes besides *NF1*, located within the deletion interval, represents the most extensively studied pathogenic mechanism in NF1 microdeletion syndrome. Efforts have long been directed toward correlating the clinical and phenotypic features of this disorder with the number and type of genes located in the 17q11.2 chromosomal region, which are lost together with *NF1* due to heterozygous deletions (Kehrer-Sawatzki and Cooper, 2021; Pacot *et al.*, 2024). Several studies have identified genotype-phenotype correlations between deleted genes and specific clinical manifestations.

An increased risk of developing MPNSTs in NF1 microdeletion patients has been associated with the loss of function of one allele of *ATAD5* (ATPase family, AAA domain containing 5) and *SUZ12* (SUZ12 polycomb repressive complex 2 subunit), as well as with the loss of two tumor suppressor microRNAs, *miR-193A* and *miR-365B* (Pasmant *et al.*, 2010; Kehrer-Sawatzki *et al.*, 2017). Specifically, reduced levels of ATAD5 protein, a positive regulator of DNA replication and gene expression, lead to slower transcription and replication, increased replicative stress, and genomic instability, as demonstrated in HeLa and yeast cells (Zanotti *et al.*, 2015; Kim *et al.*, 2020). This promotes tumorigenesis, highlighting *ATAD5*’s role as a tumor suppressor gene. The *SUZ12* gene also acts as a tumor suppressor. It encodes a stabilizing subunit of the Polycomb Repressive Complex 2 (PRC2),

which catalyzes the trimethylation of histone H3 at lysine 27 (H3K27me3), thereby mediating transcriptional repression of target genes (Holoch and Margueron, 2017). Loss-of-function mutations in *SUZ12* have been identified at the somatic level in peripheral nerve sheath tumors, high-grade gliomas, and melanomas, where deregulation of PRC2 activity promotes tumorigenesis (De Raedt *et al.*, 2014; Conway *et al.*, 2015). Furthermore, pathogenic *SUZ12* mutations in non-NF1 patients have been associated with overgrowth, facial dysmorphisms, musculoskeletal anomalies, and intellectual disabilities, suggesting that *SUZ12* hemizygosity may contribute to these phenotypic features in NF1 microdeletion patients (Imagawa *et al.*, 2018; Cyrus *et al.*, 2019).

Overgrowth, facial dysmorphisms, increased tumor predisposition, and intellectual disabilities have also been linked to deletions or mutations in *RNF135* (ring finger protein 135) (Ferrari *et al.*, 2017; Douglas *et al.*, 2007; Tastet *et al.*, 2015), which encodes an E3 ubiquitin ligase identified as a biomarker and potential therapeutic target in glioblastoma (Liu *et al.*, 2016).

A further frequent clinical feature in these patients is the occurrence of cardiac defects, correlated with the loss of one copy of *ADAP2* (ArfGAP with dual PH domains 2), which encodes a protein that increases microtubule stability by binding to β -tubulin (Zuccotti *et al.*, 2012). This correlation has been validated by functional studies in zebrafish and further supported by the observation that patients with microdeletions including *ADAP2* gene show an increased incidence of cardiac malformations (Venturin *et al.*, 2014). Additionally, the *SUZ12* and *UTP6* (UTP6 Small Subunit Processome Component) genes, both expressed during human heart development, may contribute additively to cardiomyopathy in patients with deletions encompassing these genes (Serra *et al.*, 2019).

Finally, *CRLF3* (cytokine receptor like factor 3) has been identified as a critical regulator of hematopoiesis (Taznin *et al.*, 2022) and neurogenesis (Wegscheid *et al.*, 2021), as demonstrated in zebrafish and human brain organoids derived from iPSCs, respectively.

Moreover, *ATAD5*, *OMG*, *RAB11FIP4*, and *SUZ12*, in addition to *NF1*, have been identified as dosage-sensitive genes based on their pLI scores ≥ 0.9 . Therefore,

haploinsufficiency of these genes could have pathological consequences in NF1 microdeletion patients, worsening their phenotype compared with NF1 patients without deletions (Tritto *et al.*, 2024).

Unlike NF1 microdeletion syndrome, only a few studies have attempted to establish genotype-phenotype correlations between haploinsufficient genes included in NF2 deletions and specific clinical manifestations observed in patients. Davidson described an NF2 patient carrying a deletion encompassing multiple genes, including NF2 and MNI (meningioma 1), who exhibited classical NF2 symptoms along with Pierre-Robin sequence (PRS). The author attributed the cleft palate and craniofacial abnormalities to MNI haploinsufficiency. *Mn1* acts as an oncogene in murine hematopoiesis and participates as a fusion gene (*TEL/MNI*) in human myeloid leukemias. Interestingly, *Mn1*-haploinsufficient mice display abnormal skull development and secondary cleft palate. Moreover, *Mn1* regulates the maturation and function of calvarial osteoblasts and acts as an upstream regulator of *Tbx22*, a gene associated with cleft palate in both mice and humans (Davidson *et al.*, 2012).

Further genotype-phenotype correlations have been explored starting from the evaluation of the deleted genes' pLI score. In two NF2 unrelated patients with 22q12 deletions of different sizes, both affected by severe symptoms including intellectual disability – a feature uncommon in the NF2 phenotypic spectrum – *AP1B1* emerged among the genes with $pLI \geq 0.9$. This gene has previously been associated with MEDNIK-like (mental retardation, enteropathy, deafness, peripheral neuropathy, ichthyosis, and keratoderma) syndrome, a disorder with autosomal recessive inheritance (Alsaif *et al.*, 2019). It has been hypothesized that partial expression of the MEDNIK-like phenotype, limited to intellectual disability, could result from heterozygous deletion of the *AP1B1* gene (Tritto *et al.*, 2022).

Genotype-phenotype correlations based on haploinsufficiency of genes included in NF1 and NF2 microdeletions do not fully explain the severity and variability of patients' phenotypes. Therefore, in recent years, the research group led by Prof. Riva has focused on investigating additional pathogenic mechanisms (Ferrari *et al.*, 2017; Tritto *et al.*, 2024; Tritto *et al.*, 2022).

1.3.2 Position effect on deletion flanking genes

In microdeletion syndromes, the clinical manifestations observed in patients are not always directly attributable to the loss of one allele of a specific gene. Indeed, gene expression regulation itself can also be disrupted, since the correct spatial, temporal, and quantitative expression of a gene requires not only an intact coding sequence but also the proper function of its regulatory elements. In particular, the main factors determining appropriate gene expression include: the promoter region, which represents the site where the transcriptional machinery assembles on DNA to initiate transcription; enhancer and silencer elements, which are short DNA regions containing binding sites for transcription factors that facilitate the assembly of the transcriptional machinery at the promoter and are often tissue-specific; and the chromatin context in which the gene locus resides, as enhancers and promoters can function correctly only when the chromatin adopts a state that renders these regulatory elements accessible to protein interactions. Consequently, proper gene expression requires chromosomes to fold into complex three-dimensional (3D) structures (Gibcus and Dekker, 2013).

Eukaryotic genomes exhibit a hierarchical organization into multiscale compartments that control chromatin dynamics, thereby promoting or constraining intra- and inter-chromosomal contacts. This organization spans from the nucleosomal scale (nucleosomes) to the nuclear scale (chromosomal territories and nuclear compartments), and includes an intermediate supranucleosomal level comprising chromatin loops, Topologically Associating Domains (TADs), and chromosomal compartments A/B (Fig. 10) (Gibcus and Dekker, 2013; Ea *et al.*, 2015).

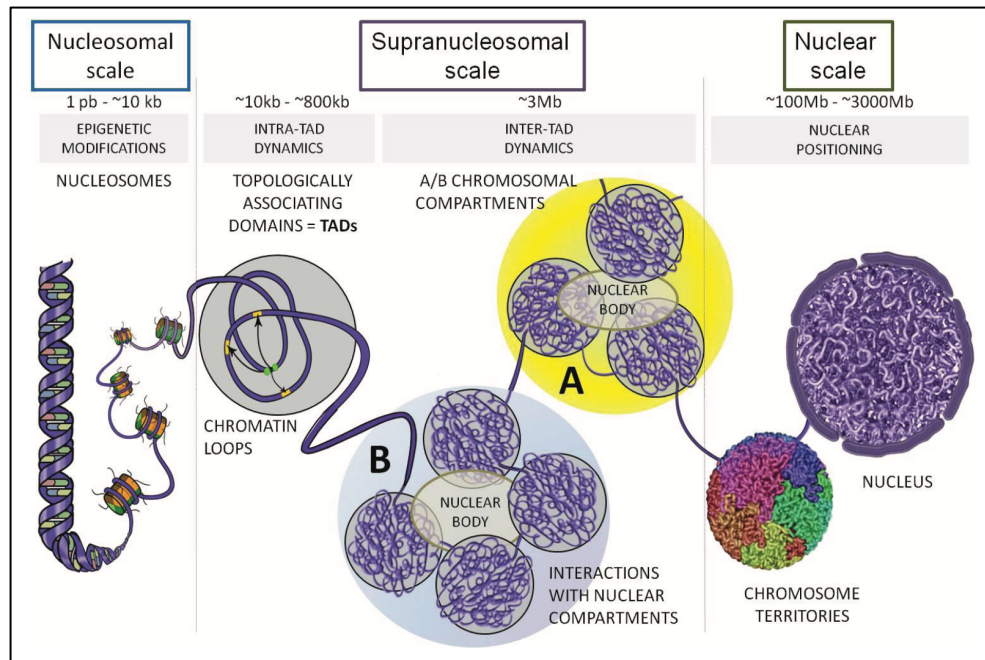


Figure 10. Schematic representation of genome organization in mammals. Between the nucleosomal scale and the nuclear scale, the 3D organization of the genome at the supranucleosomal level includes TADs, chromatin loops, and transcriptionally active (A) or inactive (B) chromosomal compartments (adapted from Ea *et al.*, 2015).

TADs provide a structural and functional framework for the regulatory landscape of the genome. TADs are chromosomal regions, ranging from approximately 40 kb to 3 Mb in mammals, within which DNA sequences frequently establish long-range physical contacts (Nora *et al.*, 2013). Between adjacent TADs lie narrow segments known as Topological Domain Boundaries (TDBs), where chromatin interactions sharply terminate. TDBs restrict interactions within specific genomic domains, thereby preventing inappropriate cross-talk between neighboring chromatin regions – for instance, between enhancers and promoters belonging to different TADs (Gaszner and Felsenfeld, 2006; Ea *et al.*, 2015). Consequently, TADs confine the regulatory activities of the genome within defined but extensive domains, forming a structural backbone that supports tissue-specific regulatory interactions and defines gene expression profiles (Gibcus and Dekker, 2013; Nora *et al.*, 2013; Symmons *et al.*, 2014). This spatial organization of chromatin into TADs is widespread across the genome, stably maintained across different cell types, and highly conserved between mouse and human. Such conservation suggests an evolutionarily ancient origin of the mechanisms governing higher-order 3D genome architecture (Dixon *et al.*, 2012).

The functional significance of TADs for proper gene regulation is highlighted by recent studies demonstrating that alterations in the regulatory landscape can give rise to Mendelian phenotypes. Chromosomal rearrangements – such as deletions, duplications, inversions, or translocations – can modify TAD structures, thereby altering the regulatory context of a gene. This may lead to changes in gene expression, a phenomenon known as the position effect, which can result in disease development (Chen *et al.*, 2009; Ibn-Salem *et al.*, 2014; Giorgio *et al.*, 2015).

The mechanisms underlying the position effect include the following (Kleinjan and van Heyningen, 1998):

- a chromosomal rearrangement may separate a promoter/transcriptional unit from a distant *cis*-regulatory element (such as an enhancer), preventing activation of the target gene and leading to its silencing;
- a rearrangement may juxtapose a gene next to an enhancer that normally regulates a different gene, causing aberrant gene activation;
- a translocation may place a gene in competition for regulatory elements normally controlling a disease-associated gene, thereby reducing its expression level;
- a rearrangement may juxtapose a euchromatic gene with a heterochromatic region, allowing heterochromatin to spread into the euchromatic region and silence the gene.

Although the position effect has received limited attention in microdeletion syndromes, it may contribute to – or even directly cause – patients' clinical phenotypes. In recent years, this pathogenic mechanism has been investigated in the context of NF1 (Ferrari *et al.*, 2017; Tritto *et al.*, 2024) and NF2 (Tritto *et al.*, 2022) microdeletion syndromes. These studies focused on the structural organization of chromatin into TADs within the chromosomal regions affected by deletions and on the regulatory elements encompassed within them. Analyses were performed through *in silico* approaches combined with quantitative Real-Time Polymerase Chain Reaction (qPCR) assays aimed at examining the expression levels of genes flanking the deletions (Fig. 11).

The first evidence supporting a position effect in the NF1 microdeletion syndrome was provided by the study of Ferrari *et al.* (2017), conducted on an atypical *de novo* 17q11.2 microdeletion identified in a 3-year-old NF1 patient (PT 171). This deletion resulted in the formation of a chimeric gene, *RNF135-SUZ12*, found to be overexpressed in PT 171 compared to the wild type *RNF135*, with which it shares the same promoter. In addition, quantitative expression level analysis revealed overexpression of *ADAP2*, a gene located approximately 12 kb upstream of the deletion, in PT 171 compared with healthy controls. The authors hypothesized that distal *cis*-acting regulatory elements might aberrantly influence the expression of genes with which they do not normally interact, as a result of conformational changes in chromatin architecture. Indeed, the deletion removes both TADs and TDBs, as shown by the analysis of the 17q11.2 chromosomal region performed using the 3D Genome Browser (Fig. 11A) (Ferrari *et al.*, 2017).

The overexpression of the chimeric *RNF135-SUZ12* gene, resulting from the position effect, may have contributed to the tumor phenotype observed in PT 171. Indeed, a clinical re-evaluation of PT 171 performed five years later revealed a higher number of cutaneous and subcutaneous neurofibromas compared with age-matched patients carrying typical *NF1* microdeletions. Since reduced expression of several targets of the transcriptional repressor PRC2 was also detected, it has been hypothesized that the chimeric protein may have acquired a gain-of-function effect within the PRC2 complex, compared with wild type SUZ12, potentially contributing to the early onset of neurofibromas in this patient (Tritto *et al.*, 2023b).

The same analytical approach was subsequently applied to 15 patients carrying the type-1 *NF1* microdeletion. *In silico* analysis revealed alterations in TAD organization and in the regulatory landscape of the 17q11.2 region (Fig. 11A), which were further confirmed by 4C-seq assays. Gene expression analysis demonstrated statistically significant differences between patients and healthy controls in seven out of twelve analyzed genes. Notably, potential correlations were identified between the hypoexpression of two genes located telomerically to the type-1 *NF1* deletion (*RHOT1* and *ZNF207*) and specific phenotypic traits observed in these patients (Tritto *et al.*, 2024).

The *RHOT1* (Ras Homolog Family Member T1) gene encodes MIRO1, a Rho GTPase involved in the formation of molecular contacts between mitochondrial and endoplasmic reticulum membranes, known as MERCs (Morlino *et al.*, 2014). Reduced expression levels of *RHOT1* have been associated with lymph node metastasis in pancreatic cancer patients (Li *et al.*, 2015). Since endocrine tumors frequently occur in NF1 patients (Khosrotehrani *et al.*, 2003; Jensen *et al.*, 2008), deregulated *RHOT1* expression could represent a risk factor for pancreatic tumorigenesis in these patients. Furthermore, as MERCs are implicated in synaptic processes (Shirokova *et al.*, 2020), *RHOT1* downregulation may also play a role in the atypical neurodevelopment observed in NF1 microdeletion patients (Tritto *et al.*, 2024).

The *ZNF207* (Zinc Finger Protein 207) gene encodes BuGZ protein, which binds spindle microtubules and regulates chromosome alignment during mitosis (Jiang *et al.*, 2014). *ZNF207* downregulation, achieved through siRNA-mediated knockdown in human Embryonic Stem Cells, has been shown to impair cell self-renewal and pluripotency by blocking differentiation of ectodermal lineages (Fang *et al.*, 2018). Therefore, reduced *ZNF207* expression has been proposed as a candidate mechanism contributing to the altered development of ectoderm-derived nervous tissue and epidermis, both of which are widely affected in NF1 microdeletion syndrome (Tritto *et al.*, 2024).

A position effect on the expression of genes flanking the deletion has also been identified in patients carrying 22q12 deletions (Tritto *et al.*, 2022). Unlike NF1 patients with type-1 deletions, in NF2 patients the disruption of TAD organization and the expression profiles of genes adjacent to the deletion are patient-specific. This variability arises from differences in deletion size and BP location, which uniquely alter the regulatory landscape of the 22q12 chromosomal region in each case (Fig. 11B). Consequently, possible correlations between deregulated gene expression and clinical phenotypes should be investigated on a patient-specific basis.

For instance, a potential correlation has been proposed between the overexpression of the *PIK3IP1* (Phosphoinositide-3-Kinase Interacting Protein 1)

gene – encoding a transmembrane protein that inhibits activation of the PI3K/Akt signaling pathway – observed in one NF2 microdeletion patient (PT 160) and the ischemic event he experienced at the age of three years (Tritto *et al.*, 2022). Consistently, overexpression of *Pik3ip1* has been reported in rat models of cerebral ischemia induced by middle cerebral artery occlusion. Moreover, the extent of ischemic damage can be mitigated through the use of miRNAs targeting *Pik3ip1* transcripts (Zhang *et al.*, 2020).

1.3.3 Pseudodominance of unmasked recessive variants

Beyond the position effect, another pathogenic mechanism that has been poorly investigated in microdeletion syndromes, yet should be considered when assessing genotype-phenotype correlations in microdeletion patients, is pseudodominance. This phenomenon consists in the expression of a recessive allele, resulting from the deletion of the corresponding dominant allele on the homologous chromosome. When the wild type allele is deleted and therefore no longer available to mask the recessive variant, the expression of recessive mutations can occur. These mutations, which under normal diploid conditions would have no phenotypic effect, become pathogenic because they are present in a hemizygous state (Schwartz *et al.*, 2018; Pebrel-Richard *et al.*, 2014; Brezo *et al.*, 2014; Jing *et al.*, 2021).

The unmasking of recessive variants through hemizygosity due to deletion of the corresponding allele can directly cause disease, as demonstrated by Schwartz *et al.* (2018) in two unrelated syndromic children with a clinical suspicion of Congenital Myasthenic Syndrome (CMS). Both patients carried a heterozygous deletion of comparable size in the chromosomal region 10q11.2. In addition, one patient harbored a rare hemizygous variant in the *CHAT* (Choline O-Acetyltransferase) gene, associated with CMS6 (OMIM #254210), while the other carried a rare hemizygous variant in the *SLC18A3* (Solute Carrier Family 18 Member A3) gene, associated with CMS21 (OMIM #617239). Both disorders exhibit autosomal recessive inheritance (Schwartz *et al.*, 2018).

Pseudodominance may also contribute to the variable expressivity observed among patients carrying a microdeletion, as reported by Pebrel-Richard *et al.* (2014). These authors described a patient with a 1.7 Mb deletion in the chromosomal region 16p11.2 who, in addition to the typical clinical features, exhibited neurodegenerative traits not usually associated with 16p11.2 microdeletion syndromes. This phenotypic variability was explained by the presence of an additional 1 kb deletion in the *CLN3* (CLN3 Lysosomal/Endosomal Transmembrane Protein, Battenin) gene, which lies within the 16p11.2 deletion region and is associated with juvenile neuronal ceroid lipofuscinosis (JNCL, OMIM #204200), a neurodegenerative disorder with autosomal recessive inheritance (Pebrel-Richard *et al.*, 2014).

Pseudodominance could likewise contribute to the broad phenotypic variability observed in patients with *NF1* and *NF2* microdeletions, although this mechanism has not yet been systematically investigated in these syndromes. In a previous study conducted by our group, this mechanism was explored in 19 patients carrying a type-1 *NF1* deletion, through Targeted Resequencing of a gene panel including loci located within the 17q11.2 region. No clear genotype-phenotype correlations were identified between unmasked recessive variants and the patients' clinical features. However, a rare variant – showing a minor allele frequency (MAF) < 0.01 – was detected in *RNF135* in one patient presenting with learning difficulties, overgrowth, and coarse facial features. The *RNF135* c.1245G>T (p.Trp415Cys) variant has previously been reported in three patients displaying Sotos-like features (Visser *et al.*, 2012). Nevertheless, its potential pathogenic relevance remains to be elucidated (Tritto *et al.*, 2024).

1.3.4 Modifier genes

Finally, another pathogenic mechanism that may contribute to the phenotypic variability, frequently observed among patients with microdeletion syndromes, is the presence of variants in so-called modifier genes. Although the number of gene alterations identified as primary causes of disease continues to increase, it is now evident that no single gene acts in isolation. Additional variants within modifier

genes across the genome may attenuate or exacerbate disease severity, thereby contributing to the observed phenotypic variability, even though they are not themselves sufficient to cause disease (Rahit and Tarailo-Graovac, 2020). The dependence of mutational effects on the genetic context in which they occur is known as epistasis, or genetic interaction. This concept refers to the occurrence of a phenotypic outcome that deviates from the expected effect when multiple mutations are combined (Domingo *et al.*, 2019).

Accordingly, in the context of microdeletion syndromes, a comprehensive understanding of the patient's phenotype requires consideration of how the primary genetic lesion interacts with the remainder of the genome. In particular, variants in modifier genes may alter the phenotype associated with a specific gene through genetic, biochemical, or functional interactions with one or more genes (Rahit and Tarailo-Graovac, 2020). In recent years, the advent of large-scale next-generation sequencing (NGS) technologies and bioinformatic tools for variant prioritization – based on the predicted functional role of the affected gene – has made it possible to analyze and evaluate modifier gene variants at the individual level, including in patients affected by microdeletion syndromes (Pinnaro *et al.*, 2020).

Typically, modifier genes belong to the same pathway or interactome as the gene harboring the primary pathogenic variant. Their variants are often classified as hypomorphic, occurring in the general population with low MAF due to negative selective constraints that prevent deleterious epistatic effects arising from the co-occurrence of multiple hypomorphic alleles (Deltas, 2018). In Noonan syndrome, which – like NF1 – is classified among the RASopathies, it has been demonstrated that the co-occurrence of a hypomorphic variant and a pathogenic variant, or of multiple hypomorphic variants within Ras pathway genes, can modulate or hyperactivate this signaling cascade, respectively (Longoni *et al.*, 2010; Ferrari *et al.*, 2020; Tritto *et al.*, 2023a). In 19 patients with type-1 *NF1* deletions, Targeted Resequencing was performed using a custom NGS panel including 16 neurofibromin interactors and 49 genes belonging to the Ras/MAPK pathway, leading to the identification of likely pathogenic rare variants in two Ras pathway genes, *RASAI* and *RAF1*, possibly contributing to phenotypic traits shown by patients (Tritto *et al.*, 2024).

The *RASAI* c.2656C>T (p.Pro886Ser) variant was identified in a patient displaying typical NF1 features of high severity, along with congenital hypothyroidism and facial hemangiomas, both uncommon findings in NF1. This *RASAI* variant may underlie the hemangiomas observed in the patient, in line with previously reported associations between *RASAI* variants and vascular malformation syndromes characterized by hereditary capillary malformations with or without arteriovenous malformations, arteriovenous fistulas, or Parkes Weber syndrome (Wooderchak-Donahue *et al.*, 2018; Tritto *et al.*, 2024).

The *RAF1* c.1811C>G (p.Ser604Cys) variant was found in a patient exhibiting a typical NF1 microdeletion phenotype including intellectual disability, dysmorphic features, and cerebrovascular anomalies. This *RAF1* variant may have amplified the Ras pathway dysregulation caused by the *NF1* microdeletion itself, thereby exacerbating the clinical severity observed in this patient (Tritto *et al.*, 2024).

Within the NF1 microdeletion syndrome, haploinsufficient genes included in the deletion interval, as well as flanking genes subject to position effects, are also regarded as potential modifier genes (Wang *et al.*, 2021). These genes may contribute both to the overall phenotypic severity in patients with typical deletions and to phenotypic variability among those carrying atypical deletions.

Similar to neurofibromin, Merlin participates in multiple cellular signaling pathways. Therefore, the variable expressivity observed among NF2 microdeletion patients could, in some cases, also result from variants in modifier genes acting within pathways that interact with Merlin. However, no supporting data have been reported to date.

Modifier genes have instead been hypothesized to reside in the 22q chromosome, in a 6.5 Mb region immediately telomeric to the *NF2* gene, as proposed by Bruder *et al.* (1999). These authors described a patient with intellectual disability and a severe form of NF2, carrying a 7.4 Mb 22q12 deletion, whose centromeric BP was comparable to that of two previously reported large deletions of similar size (700-800 kb) encompassing the entire *NF2* gene, but found in mildly affected NF2 patients (Bruder *et al.*, 1999).

1.4 Approaches for structural and functional characterization of microdeletions

In patients with a clinical suspicion of NF1 or NF2, especially when the phenotype is complex and suggestive of an underlying microdeletion, the mutational screening strategy relies primarily on MLPA and array-CGH analyses. For research purposes, to determine the precise genomic location and characterize the deletion BPs, several molecular techniques have been employed, including PCR, Long-Range PCR, and Sanger sequencing, which allow the amplification and sequencing of the DNA region containing the BPs (Venturin *et al.*, 2004a; Pasmant *et al.*, 2010; Bengesser *et al.*, 2014; Ferrari *et al.*, 2017).

In recent years, the advent of next-generation technologies has opened new perspectives for both the structural and functional characterization of microdeletions. In particular, Long-Read Sequencing (LRS) now allows the identification of microdeletions and the precise mapping of their BPs using a single analytical approach. At the same time, this technique can detect additional genetic variants or chromosomal alterations that may act as phenotypic modifiers (Akbari *et al.*, 2024; Showpnil *et al.*, 2024).

To assess the effects of microdeletions on the chromatin structure of the affected genomic region, several chromosome conformation capture (3C-based) techniques have been developed and recently applied in research settings. These approaches enable the investigation of higher-order chromatin organization and the potential disruption of TADs that could influence gene regulation across the deletion boundaries (Dimartino *et al.*, 2024; Maury *et al.*, 2023).

Finally, for the functional characterization of microdeletions, the use of patient-derived iPSCs has emerged as a powerful tool. The iPSC-based models provide a unique opportunity to investigate the cellular and molecular consequences of deletions in disease-relevant cell types and developmental contexts (Paranjape *et al.*, 2023; Wegscheid *et al.*, 2021).

1.4.1 Multiplex Ligation-dependent Probe Amplification

MLPA is a semi-quantitative molecular technique widely used for the detection of CNVs, including deletions and duplications, across multiple genomic regions simultaneously (Schouten *et al.*, 2002). The method is based on the hybridization of specific probe pairs to adjacent target sequences within the genome, followed by a ligation reaction and a PCR amplification using a single pair of universal primers. The amplified products are subsequently separated and quantified by capillary electrophoresis, allowing relative quantification – and the estimation of relative copy number changes across up to 50 loci in a single reaction – of each genomic region based on the comparison of probe peak intensities with those from a reference DNA sample (Fig. 12) (Hömig-Hölzel and Savola, 2012).

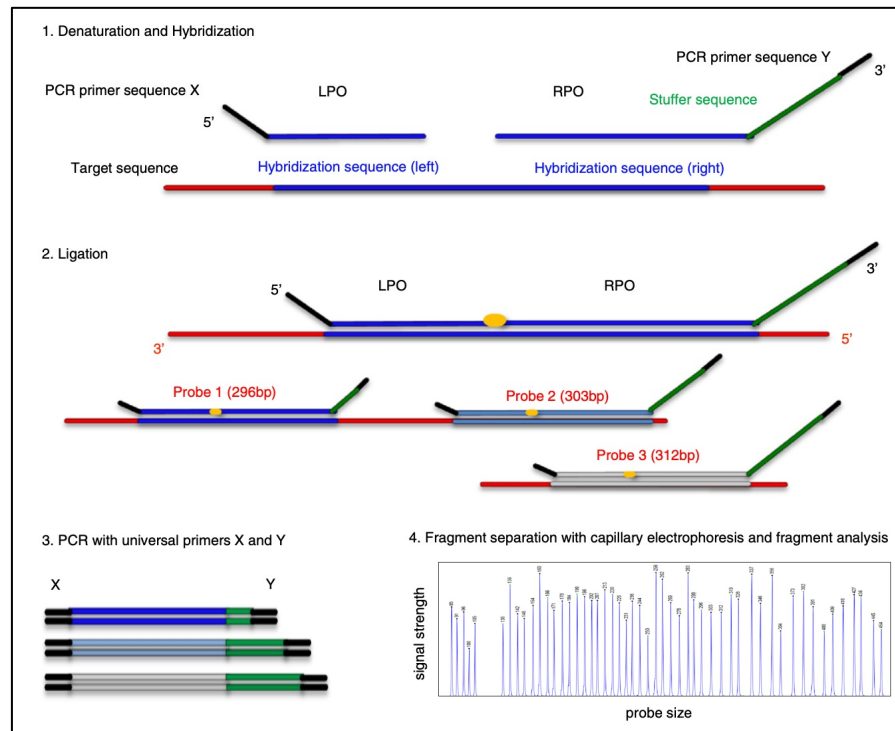


Figure 12. Schematic representation of MLPA technique. MLPA employs pairs of oligonucleotide probes – each consisting of a left (LPO) and a right (RPO) hemiprobe – that target adjacent sequences (blue) and a PCR primer sequence (black). The RPO includes a stuffer sequence (green) of variable length, which determines the final probe size. Following hybridization to denatured genomic DNA, the complementary hemiprobases are ligated to form complete PCR templates. These are then amplified using a universal PCR primer pair (X and Y). Due to differences in stuffer length, the resulting PCR products can be separated and quantified by capillary electrophoresis. Comparison of probe peak intensities with those from a reference DNA sample enables the estimation of relative copy number changes (adapted and modified from Hömig-Hölzel and Savola, 2012).

MLPA offers a cost-effective, rapid, and reliable approach to CNV detection, particularly suitable for diagnostic and research applications involving large or complex genes, chromosome regions, or entire chromosomes affected by common aneuploidies (Belvini *et al.*, 2017; Stuppia *et al.*, 2012; Schouten *et al.*, 2019;). In the study of microdeletions, MLPA serves as a valuable first-line or confirmatory tool. For instance, it allows the detection of both recurrent and non-recurrent *NF1* and *NF2* microdeletions, providing essential insights into mosaicism and the dosage of genes located within these genomic regions (Bianchessi *et al.*, 2015; Serra *et al.*, 2019; Pasmant *et al.*, 2018). Although MLPA does not reveal exact BP sequences or structural complexity, its scalability and robustness make it an indispensable assay for screening large cohorts of patients with suspected microdeletion syndromes (Hömig-Hölzel and Savola, 2012).

1.4.2 Array-based Comparative Genomic Hybridization

Array-CGH is a high-resolution genomic method developed to detect CNVs across the entire genome. It is based on the differential hybridization of fluorescently labeled test and reference DNA samples to microarrays containing thousands of genomic probes. Variations in fluorescence intensity ratios between the two samples reflect relative changes in DNA copy number, allowing precise identification of genomic gains and losses (Fig. 13) (Pinkel *et al.*, 1998; Ahn *et al.*, 2015). Depending on the probe density, array-CGH platforms can reach a resolution ranging from approximately 5 to 50 kb, far exceeding that of conventional cytogenetic methods. In addition to array-CGH, single nucleotide polymorphism (SNP) arrays represent an advanced platform that combines CNV detection with genotyping information. By analyzing allele-specific hybridization patterns, SNP arrays can identify not only copy number changes but also regions of loss of heterozygosity (LOH), uniparental disomy (UPD), and mosaicism, providing a more comprehensive overview of the genomic landscape (Rauch *et al.*, 2004).

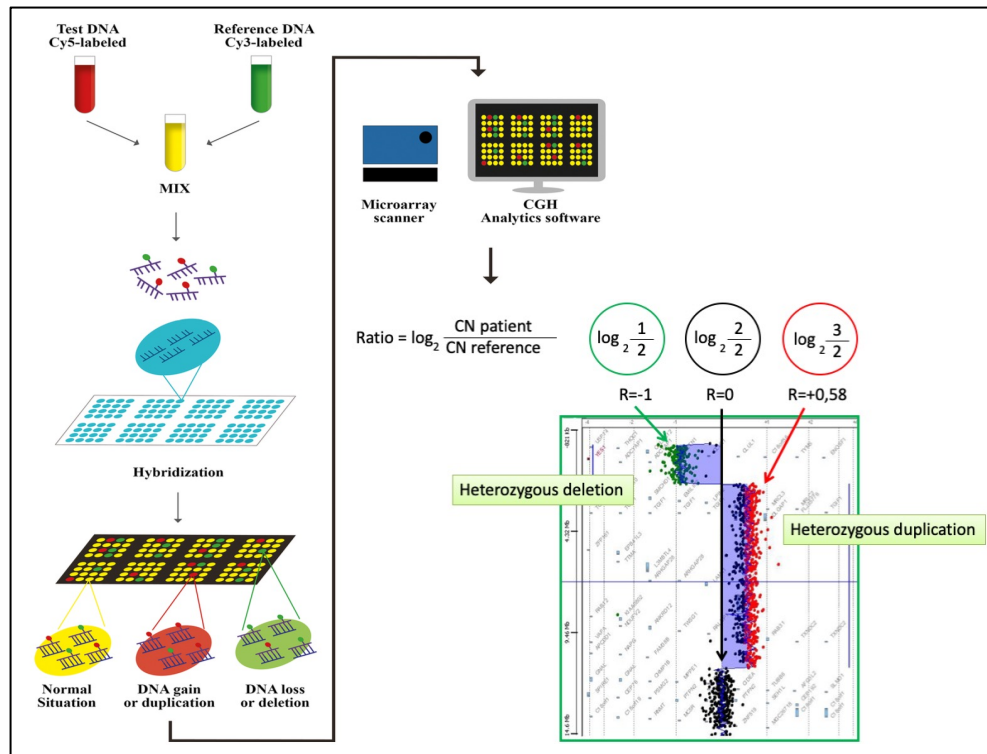


Figure 13. Schematic representation of array-CGH technique. The array-CGH procedure involves the extraction of genomic DNA from both the test (patient) and reference (control) samples, which are independently labeled with two distinct fluorophores, typically cyanine-5 (red) and cyanine-3 (green). The labeled DNAs are co-hybridized onto a microarray slide containing thousands of probes with known genomic positions, arranged in a defined grid. Following hybridization, the microarray scanner measures the fluorescence intensity of each probe, and analytical software calculates the $\log_2$ ratio (Cy5/Cy3) for every spot. CNVs are then inferred from the relative signal intensities: an increased signal from the test sample indicates a duplication or gain, whereas a reduced signal reflects a deletion or loss of genomic material. When both signals are balanced – producing a neutral color, such as yellow in the case of red and green fluorophores – it denotes a normal, unaltered genomic region (adapted and modified from Hömig-Hölzel and Savola, 2012).

Array-CGH has greatly advanced molecular cytogenetics by providing a genome-wide perspective of chromosomal CNVs at submicroscopic resolution. It is extensively applied in both clinical diagnostics and research to investigate developmental disorders, congenital anomalies, and oncogenic events (Davies *et al.*, 2005; Edelmann and Hirschhorn 2009). In the context of microdeletions, array-CGH enables accurate delineation of deletion size and gene content, offering valuable insights into genotype-phenotype correlations. Studies employing array-CGH in NF1 and NF2 syndromes have uncovered typical and atypical deletions (Pasmant *et al.*, 2009; Mantripragada *et al.*, 2006; Tritto *et al.*, 2022). Despite its

limitations in detecting balanced rearrangements, array-CGH remains a powerful tool for comprehensive CNV profiling in microdeletion syndromes.

1.4.3 Long-read Sequencing

LRS, also referred to as third-generation sequencing, encompasses a set of technologies that enable the direct reading of DNA fragments several kb in length, thereby overcoming many of the limitations associated with short-read platforms. Unlike second-generation sequencing, which requires DNA fragmentation and amplification, LRS captures long, continuous stretches of native DNA, providing a more comprehensive representation of the genome and its structural complexity (Warburton and Sebra, 2023). The two major platforms currently employed are Pacific Biosciences Single Molecule Real-Time (PacBio SMRT) sequencing (Fig. 14A) and Oxford Nanopore Technologies (ONT) (Fig. 14B).

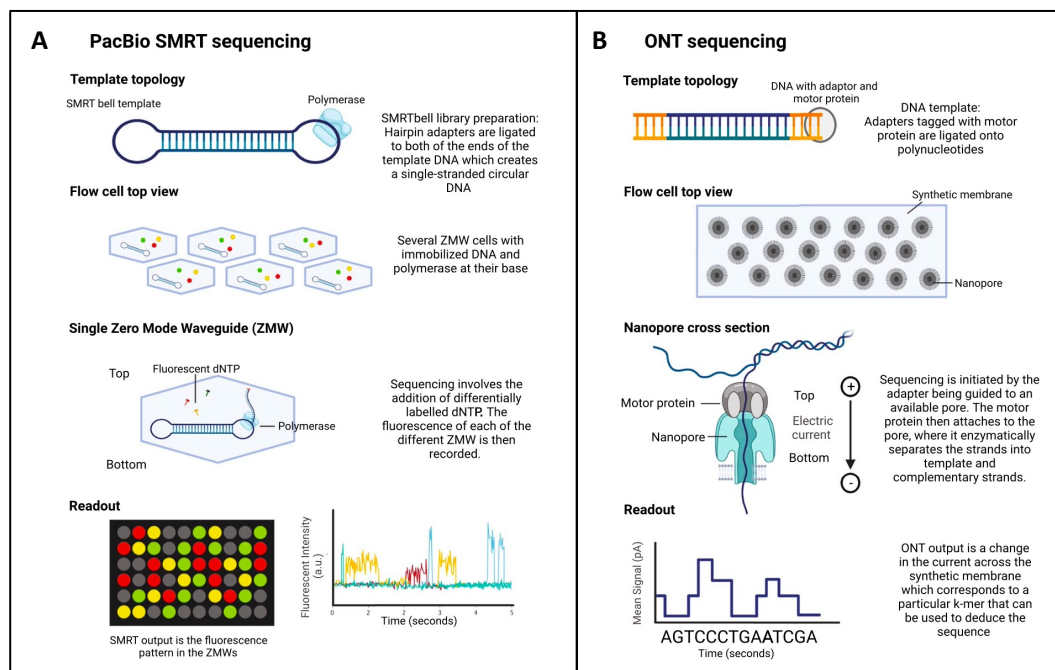


Figure 14. Schematic representation of LRS platforms. (A) PacBio SMRT sequencing detects the incorporation of fluorescently labeled nucleotides by a DNA polymerase immobilized in nanostructures called zero-mode waveguides, allowing real-time observation of DNA synthesis with high accuracy. (B) ONT measures changes in ionic current as single DNA or RNA molecules pass through biological nanopores embedded in a membrane, with the resulting electrical signals decoded into nucleotide sequences (adapted and modified from Oehler *et al.*, 2023).

LRS offers significant advantages in *de novo* genome assembly, haplotype phasing, and structural variant (SV) detection, including large insertions, deletions, inversions, and translocations that are often missed by short-read technologies. It also allows accurate resolution of repetitive and GC-rich regions, telomeres, centromeres, and segmental duplications, which are typically refractory to short-read mapping. Although historically limited by higher error rates and cost per base, recent advances in HiFi (high-fidelity) reads from PacBio and improved basecalling algorithms for ONT have markedly enhanced both the accuracy and throughput of LRS, enabling its integration into clinical and research applications such as rare disease diagnostics, cancer genomics, and epigenetic profiling (Eisfeldt *et al.*, 2025; Sakamoto *et al.*, 2020; Liu and Conesa, 2025).

For the investigation of microdeletions, LRS offers unique advantages. It allows the precise mapping of deletion BPs, detection of repetitive motifs contributing to genomic instability, and resolution of complex structural rearrangements involving multiple genomic loci. Importantly, precise BP characterization has potential clinical relevance, as it allows accurate definition of the genes and regulatory elements involved in the deletion (Sedlazeck *et al.*, 2018). This information can contribute to improved genotype-phenotype correlations, help stratify patients according to expected clinical severity, and inform prognosis in cases where BP position affects dosage-sensitive genes or modifies the risk of associated complications. In selected contexts, detailed BP mapping may also support personalized clinical surveillance and genetic counseling. In the study of NF1 and NF2, LRS improved the diagnostic accuracy (Zheng *et al.*, 2025; Perez-Becerril *et al.*, 2024). Moreover, the ability to phase alleles provides additional insights potentially useful for the study of differential allelic expression (DAE).

Although cost and error rates still limit widespread application, continuous technological improvements are establishing LRS as a reference tool for comprehensive SV analysis in microdeletion disorders.

1.4.4 Chromosome conformation capture techniques

Chromosome Conformation Capture (3C) and its derivatives – Circular Chromosome Conformation Capture (4C), Chromosome Conformation Capture Carbon Copy (5C), and High-throughput Chromosome Conformation Capture (Hi-C) – are molecular techniques developed to study the 3D organization of chromatin and its influence on gene regulation (Dekker *et al.*, 2002; Lieberman-Aiden *et al.*, 2009). These methods rely on formaldehyde crosslinking to stabilize physical interactions between genomic loci, followed by restriction digestion, proximity ligation, and sequencing of the ligated DNA fragments (Fig. 15). The resulting interaction maps reveal the spatial arrangement of chromatin and identify regions that physically interact within the nucleus (Ea *et al.*, 2015).

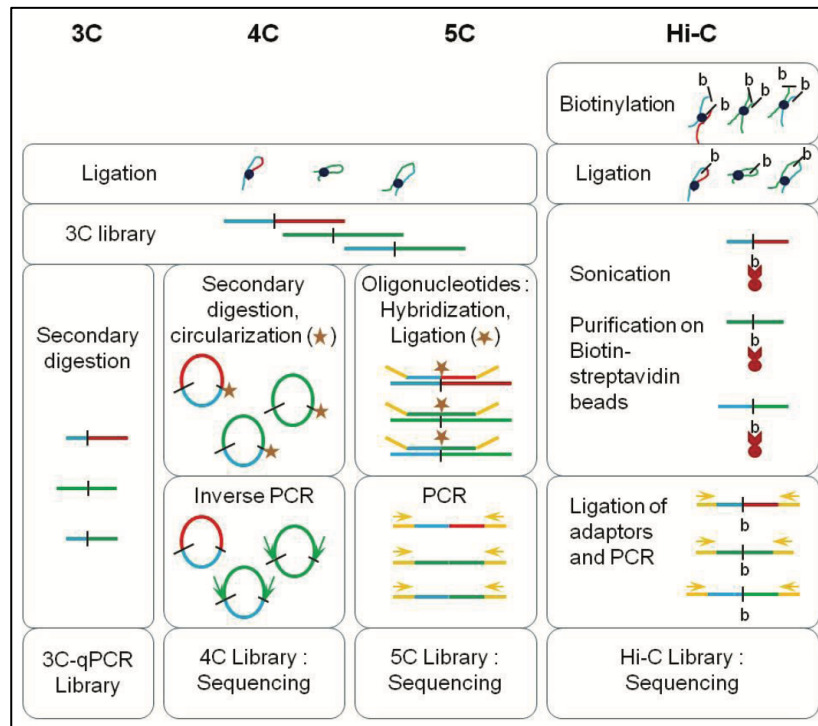


Figure 15. Schematic overview of 3C-derived techniques. All methodologies originating from the 3C protocol share a common experimental workflow that includes formaldehyde cross-linking of chromatin, enzymatic digestion of DNA, and subsequent ligation of spatially proximal fragments. However, each technique introduces distinct strategies to construct genomic libraries: secondary restriction digestion followed by quantitative PCR analysis in 3C-qPCR; fragment circularization and inverse PCR amplification in 4C; “carbon copy” amplification in 5C; and biotin labeling with purification on streptavidin-coated beads in Hi-C. The resulting ligation products are then quantified either through real-time qPCR, as in 3C-qPCR, or by high-throughput sequencing for the other approaches (adapted from Ea *et al.*, 2015).

In the study of microdeletions, chromosome conformation capture approaches are particularly relevant for investigating position effects. Deletions can disrupt TADs, abolish enhancer-promoter loops, or create ectopic regulatory contacts, thereby altering the transcriptional landscape of deletion flanking genes (Fraser *et al.*, 2015). Application of chromosome conformation capture techniques to patient-derived cells or iPSC models could allow the assessment of 3D genome reorganization in *NF1* and *NF2* microdeletions, as demonstrated by the application of the 4C-seq assay on peripheral blood mononuclear cells (PBMCs) from three patients with type-1 *NF1* deletion (Tritto *et al.*, 2024). By linking structural variation to gene regulation, these approaches provide an essential functional complement to DNA-level analyses.

1.4.5 Generation of iPSCs as disease model

Although model organisms are essential for recapitulating evolutionarily conserved pathogenic mechanisms, they do not always present the specific pathophysiological pattern found in humans. Therefore, in order to study the pathogenic mechanisms in microdeletion syndromes, the introduction of iPSCs, which can be differentiated into cell lines belonging to the tissues most affected by the diseases, has been fundamental (De Masi *et al.*, 2020).

The advent of iPSC technology has provided a transformative platform for modeling human diseases *in vitro* (Okano and Morimoto, 2022). Somatic cells reprogrammed through the expression of pluripotency-associated transcription factors (*OCT4*, *SOX2*, *KLF4*, and *c-MYC*) acquire the capacity to differentiate into multiple cell types, enabling the study of disease mechanisms in relevant cellular contexts (Takahashi and Yamanaka, 2006).

In the investigation of microdeletion syndromes, patient-derived iPSCs serve as a valuable resource for exploring the cellular and molecular effects of chromosome alterations. Differentiation into cell types affected by the disease allows the examination of tissue-specific phenotypes, transcriptomic profiles, and chromatin organization (Antony *et al.*, 2023; Shnaider *et al.*, 2019). These cell

models not only provide mechanistic insight but also constitute valuable tools for drug screening and the development of personalized therapeutic strategies for patients with microdeletion-associated disorders (Doss and Sachinidis, 2019).

Thus, iPSC-based models may represent a valuable tool for gaining deeper insight into the pathogenic mechanisms underlying NF1 and NF2 microdeletion syndromes. This approach has already proven effective in clarifying the consequences of *NF1* deletions, as shown by the generation of iPSC-derived cerebral organoids from a patient carrying a 17q11.2 microdeletion, which led to the identification of *CRLF3* as a critical regulator of neurodevelopmental defects (Wegscheid *et al.*, 2021). These findings highlight the potential of iPSC-derived systems to uncover gene-specific contributions to disease phenotypes and to advance our understanding of the complex molecular basis of microdeletion syndromes.

2. AIM

Neurofibromatosis type 1 (NF1) and Neurofibromatosis type 2 (NF2) are rare autosomal dominant disorders, primarily caused by point mutations or small indels in the *NF1* and *NF2* genes, respectively. Both syndromes affect multiple organs and are characterized by a predisposition to tumor development. In rare cases, patients present with microdeletions encompassing not only the causative gene but also flanking genes, often resulting in a more severe phenotype and increased variability of clinical expression. While *NF1* microdeletions, classified as typical or atypical based on BP location, have been extensively characterized in terms of gene content and molecular mechanisms, *NF2* microdeletions are rarer, highly variable, and poorly described due to the absence of recurrent BPs. In both syndromes, however, haploinsufficiency of the genes included in the deletions does not alone fully explain the variable expressivity and phenotypic severity observed.

The aim of my research project was to identify additional pathogenic mechanisms contributing to the complex phenotypes of NF1 and NF2 microdeletion patients. Regarding NF1, potential dysfunction of Polycomb Repressive Complex 2, including SUZ12, encoded by one of the genes affected by *NF1* deletions, was investigated in relation to tumor predisposition. In PT 171 carrying an atypical microdeletion, rare variants in modifier genes were also identified through WES. To generate models for functional studies, iPSCs were derived from a type-1 *NF1* deletion patient and PT 171. Regarding NF2, BPs and gene content of two novel microdeletions were characterized, and the chromatin organization and regulatory landscape of the 22q12 region were analyzed. Gene expression of deletion flanking genes was assessed by qPCR to explore potential position effects. LRS further identified variants in modifier genes and additional chromosomal alterations, which were correlated with patient-specific phenotypes.

The findings obtained in this study will guide future research aimed at clarifying genotype-phenotype correlations in patients carrying *NF1* and *NF2* microdeletions, with potential implications on diagnosis, prognosis, and genetic counseling, as well as on the identification of target genes for personalized pharmacological therapies.

3. MATERIALS AND METHODS

3.1 Human subjects

For the NF1 study conducted in this thesis, a cohort of 106 patients was recruited, including 95 individuals carrying intragenic *NF1* mutations, 10 patients harboring a type-1 *NF1* deletion, and one patient (PT 171) with an atypical *NF1* deletion. Eligible participants were identified by screening the electronic NF1 patient databases of Fondazione IRCCS Istituto Neurologico “Carlo Besta” and Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico in Milan. Clinical diagnosis was established according to the revised diagnostic criteria for Neurofibromatosis type 1 (Legius *et al.*, 2021). Clinical data were collected from medical records and reviewed by clinicians, including date of birth, sex, age at genetic testing, inheritance pattern, and NF1-related manifestations, such as café-au-lait macules, skinfold freckling, Lisch nodules, and cutaneous, subcutaneous, or plexiform neurofibromas. Additional data regarding visual impairment, epilepsy, cognitive deficits, neurological symptoms, skeletal and vascular abnormalities, OPG, and the presence of other central or peripheral neoplasms were also recorded. Furthermore, all patients underwent brain magnetic resonance imaging (MRI).

For the NF2 study, two patients (PT 568 and PT 690) carrying 22q12 microdeletions were included. These patients were identified from the NF2 patient database at Fondazione IRCCS Istituto Neurologico “Carlo Besta” and underwent comprehensive neurological, ophthalmological, and audiological assessments. The clinical diagnosis was made according to the revised diagnostic criteria for Neurofibromatosis type 2 (Plotkin *et al.*, 2022). Medical records were examined to collect data including age at onset of symptoms, age at diagnosis and at genetic testing, inheritance pattern, and NF2-associated clinical features, such as the presence of vestibular schwannomas, other cranial, spinal, or limb schwannomas, meningiomas, ophthalmic manifestations, cutaneous abnormalities, and neuropathy. Information about therapeutic interventions was also recorded. The number and distribution of schwannomas, meningiomas, and spinal tumors were

evaluated through brain and spinal MRI, while vascular abnormalities were assessed using magnetic resonance angiography (MRA) of the head.

All patients provided written informed consent to participate in the study and for the collection of biological samples, in accordance with the Declaration of Helsinki. The NF1 and NF2 studies were approved by the Ethics Committee of Fondazione IRCCS Istituto Neurologico “Carlo Besta” (approval numbers N°50-19/03/2018 and N°4-11/05/2022, respectively).

Finally, a control group consisting of 19 healthy donors was included to serve as a reference for comparative analyses.

3.2 Generation of induced Pluripotent Stem Cells

Four NF1 patients (PT 1051, PT 1664, PT 1749, and PT 171) were selected to generate iPSC models from their PBMCs. Peripheral blood samples (two 4-ml lithium heparin tubes per patient) were collected and provided by Dr. Eoli (Fondazione IRCCS Istituto Neurologico “Carlo Besta”) and by Dr. Natacci and Dr. Grilli (Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico). PBMCs were subsequently isolated and reprogrammed into iPSCs. Finally, individual iPSC colonies have been picked.

1. Isolation of PBMCs

PBMCs were isolated using the Ficoll density gradient method (density: 1.077 g/ml), which allows the separation of PBMCs – comprising lymphocytes and monocytes – from other blood components after centrifugation. During the process, plasma and platelets remain in the upper layer, while granulocytes and erythrocytes settle at the bottom of the tube. The PBMC isolation procedure included the following steps:

- Dilute 4 ml of peripheral blood 1:1 with phosphate-buffered saline (PBS) 1X (Euroclone) and carefully layer the mixture onto 4 ml of Ficoll® Paque Plus (Cytiva) in a 15 ml conical tube at room temperature (RT).

- Centrifuge at 1,800 rpm (ALC 4236-V1D Centrifuge) for 20 minutes at RT, with acceleration and brake turned off.
- Carefully aspirate, using a Pasteur pipette, the whitish ring-like layer at the interface between plasma and Ficoll – corresponding to the PBMC fraction – and transfer it into a new 15 ml conical tube.
- Add PBS 1X up to a final volume of 10 ml and centrifuge at 1,500 rpm for 10 minutes at RT.
- Discard the supernatant and resuspend the cell pellet in 2 ml of freezing medium consisting of fetal bovine serum (FBS; Euroclone) supplemented with 10% dimethyl sulfoxide (DMSO; Merck), which protects cells from lysis during the freezing process.
- Divide the suspension into two cryovials.

For each subject, four cryovials containing 1 ml of cell suspension were frozen at -80 °C in a cryobox filled with isopropanol to ensure gradual cooling. The isopropanol provides a controlled decrease in temperature of approximately 1 °C/min, promoting optimal cryopreservation and recovery of viable cells. Subsequently, frozen cryovials were transferred to a liquid nitrogen tank for long-term storage.

2. Reprogramming of PBMCs into iPSCs

In collaboration with Professor Ratti (Istituto Auxologico, Cusano Milanino, Milan), PBMCs from each patient were reprogrammed into iPSCs, following the procedure recommended by the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific), with minor modifications, as illustrated in Figure 16.

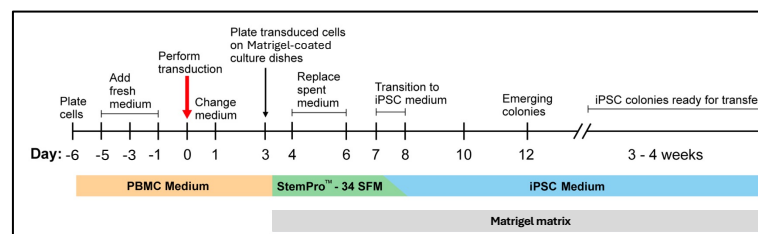


Figure 16. Timeline of the main steps involved in PBMC reprogramming into iPSCs. PBMCs are cultured and transduced with reprogramming vectors. The transduced cells are then plated on Matrigel-coated dishes, where they give rise to individual iPSC colonies that are subsequently picked (adapted and modified from CytoTune™-iPS 2.0 Sendai Reprogramming Kit user guide).

As shown in the timeline (Fig. 16), on day -6, PBMCs were thawed from one cryovial per patient according to the following procedure:

- Place the cryovial in a 37 °C water bath until partially thawed.
- Transfer the cells into a 15 ml conical tube and add dropwise 4 ml of PBMC medium supplemented with 40 µl of 100X penicillin-streptomycin solution (Pen/Strep; Euroclone) and 40 µl of RevitaCell™ Supplement 100X (Gibco).
- Centrifuge the tube at 1,000 rpm for 4 minutes at RT.
- Discard the supernatant, resuspend the pellet in 2 ml of PBMC medium, and plate the suspension in two wells of a 24-well plate.

The PBMC medium consisted of complete StemPro™-34 SFM Medium (Gibco) supplemented with four cytokines that promote hematopoietic cell survival, differentiation, and proliferation: Human Stem Cell Factor (SCF, also known as c-Kit Ligand; PeproTech), final concentration 100 ng/ml; Human Fms-related Tyrosine Kinase 3 Ligand (FLT3L; Gibco), final concentration 100 ng/ml; Human Interleukin-3 (IL-3; Gibco), final concentration 20 ng/ml; Human Interleukin-6 (IL-6; Gibco), final concentration 20 ng/ml (Table 4).

Table 4. Composition of the PBMC Medium in 50 ml

Reagent	Volume
StemPro™-34 SFM Medium	49.3 ml
Penicillin/Streptomycin 100X	500 µl
SCF	50 µl
FLT3L	50 µl
IL-3	50 µl
IL-6	50 µl

The cells were placed in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂. For the following five days, half of the medium was replaced daily with fresh complete PBMC medium.

On day 0 (Fig. 16), the cells were counted using the LUNA-II™ Automated Cell Counter (Logos Biosystems). A 10 µl mixture of cells and Trypan Blue (1:1 ratio) was prepared; this dye allows the distinction between viable and non-viable (blue-stained) cells.

For each sample, 500,000 cultured PBMCs were transduced using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit. This kit contains three vectors based on a modified, non-integrating form of the Sendai virus, which delivers and expresses the Yamanaka factors required for the reprogramming of somatic cells into iPSCs. These reprogramming vectors include KOS (encoding the polycistronic *KLF4-OCT3/4-SOX2*), hc-MYC (encoding the human gene *c-MYC*), and hKLF4 (encoding the human gene *KLF4*). The transduction volume for each virus is lot-dependent and specifies the recommended volume required to achieve the target multiplicity of infection (MOI) – 5 for KOS and hc-MYC, and 3 for hKLF4. The characteristics of the specific kit used in this study are summarized in Table 5.

Table 5. Transduction volume of CytoTune™ 2.0 reprogramming vectors

Component	Titer	MOI	Transduction volume
CytoTune™ 2.0 hKOS	1.2 x 10 ⁸ CIU/ml	5	25 µl
CytoTune™ 2.0 hc-MYC	1.1 x 10 ⁸ CIU/ml	5	27 µl
CytoTune™ 2.0 hKLF4	1.1 x 10 ⁸ CIU/ml	3	16 µl

The transduction was carried out following the steps below:

- Transfer the calculated volume of cell culture medium containing 500,000 cells into a 15 ml conical tube.
- Centrifuge at 1,500 rpm for 5 minutes at RT.
- Discard the supernatant and resuspend the cell pellet in 1 ml of PBMC medium supplemented with the transduction volume of each virus, as indicated in the kit datasheet (Table 5).
- Centrifuge at 2,250 rpm for 90 minutes at RT to enhance transduction efficiency.
- Gently resuspend the cells in the same medium, add 1 ml of fresh PBMC medium, and plate them into one well of a 12-well plate.

The cells were then incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

At day 1 (Fig. 16), CytoTune™ 2.0 Sendai viruses should be removed:

- Transfer the cells into a 15 ml conical tube.

- Centrifuge at 800 rpm for 5 minutes at RT.
- Discard the supernatant containing residual viruses and resuspend the pellet in 500 µl of PBMC medium.
- Plate the cells into one well of a 24-well plate.

At day 3 (Fig. 16), the cells need to be plated in a well of a 6-well plate, coated with Matrigel matrix (Corning), a solubilized basement membrane preparation obtained from the Engelbreth-Holm-Swarm mouse sarcoma, rich in extracellular matrix proteins such as laminin, collagen IV, heparan sulfate proteoglycans, and growth factors. The coating solution was prepared by diluting the Matrigel matrix in cold DMEM/F-12 (1:1) + GlutaMAX (Gibco). The amount of Matrigel to dilute in 25 ml of medium is lot-dependent and specified in the product datasheet. Once plated, the coating solution was allowed to polymerize for 30 minutes at 37 °C.

The plating procedure included the following steps:

- Transfer the content of each well into a 15 ml conical tube and centrifuge at 1,500 rpm for 5 minutes at RT.
- Discard the supernatant and resuspend the pellet in 2 ml of StemPro™-34 SFM Medium supplemented with 1% Pen/Strep.
- Plate the cells into the Matrigel-coated well of a 6-well plate after removing the Matrigel solution.

The cells were incubated at 37 °C and 5% CO₂. For the following 3 days, half of the medium was replaced daily with fresh StemPro™-34 SFM Medium containing 1% Pen/Strep.

At day 7 (Fig. 16), half of the StemPro™-34 SFM Medium was replaced with Essential 8™ Medium (E8) (Gibco), an iPSC-specific medium containing eight essential components required for stem cell growth and maintenance.

From day 8 (Fig. 16), every day, the entire volume of medium was replaced with fresh E8 Medium. The plates were observed daily under a phase-contrast microscope to monitor the appearance of emerging colonies indicative of successful reprogramming.

3. iPSC colony picking

By approximately day 22, individual iPSC colonies had reached an appropriate size for transfer. Colonies were manually picked using a 200 µl pipette and transferred onto 12-well Matrigel-coated culture plates containing E8 Medium.

3.3 iPSC maintenance and cryopreservation

For maintenance and expansion, iPSC colonies from PT 171 and PT 1051 were cultured in 6-well Matrigel-coated culture plates with E8 Medium for the first two weeks, and subsequently in StemFlex™ Medium (Gibco). StemFlex™ Medium contains essential nutrients, growth factors, and supplements that promote self-renewal while minimizing spontaneous differentiation of iPSCs.

The medium was replaced daily, and colonies were passaged whenever they reached approximately 70-80% confluence, following these steps:

- Remove the medium from the well and incubate the cells for 5 minutes in a 0.5 mM solution of ethylenediaminetetraacetic acid (EDTA) in PBS 1X (PBS/EDTA 0.5 mM).
- Remove the PBS/EDTA solution and mechanically detach the cells by pipetting 500 µl of medium onto a small area of the well.
- Transfer the cell suspension into a new Matrigel-coated well containing E8 or StemFlex™ Medium, up to a final volume of 1.6 ml.

Cells were maintained in an incubator at 37 °C in a humidified atmosphere with 5% CO₂.

For long-term storage, iPSC samples were cryopreserved as follows:

- Remove the medium from the well and incubate the cells for 5 minutes in PBS/EDTA 0.5 mM.
- Remove PBS/EDTA and mechanically detach the cells by pipetting 800 µl of iPSC Cryomedium (Gibco) supplemented with 8 µl of RevitaCell™ Supplement 100X.
- Transfer the cell suspension into a cryovial.

- Place the cryovial in a cryo-freezing container at -80 °C to ensure gradual cooling.
- Transfer the samples to a liquid nitrogen storage tank for long-term preservation.

3.4 Karyotype analysis

Karyotype analysis of PT 171 and PT 1051 iPSC lines was performed as follows. Cells were cultured in 60 mm Petri dishes for 48 hours prior to the procedure.

1. Metaphase arrest

- Five drops of 0.1 µg/ml Colcemid (Roche) were added to each Petri dish to arrest mitotic cells in metaphase by inhibiting spindle fiber formation.
- Cultures were incubated at 37 °C for 2 hours.

2. Cell suspension preparation

- The supernatant was removed and transferred to a 15 ml conical tube.
- The dish was washed with 2 ml of PBS/EDTA 0.5 mM and the volume was added in the tube.
- Cells were detached by incubation with 500 µl of trypsin-EDTA 1X in PBS (Euroclone) for 3-4 minutes.
- Cells were gently dissociated using a Pasteur pipette and transferred to the conical tube.
- Five ml of cold PBS 1X (4 °C) were added to inactivate trypsin.
- The suspension was centrifuged at 1,200 rpm for 10 minutes, the supernatant discarded, and the pellet gently resuspended.

3. Hypotonic treatment

- Five ml of pre-warmed 1% sodium citrate solution (Merck) were added to the cell suspension to induce swelling and membrane rupture.
- Samples were incubated at 37 °C for 20 minutes.

4. Pre-fixation

- A total of 500 µl of cold ethanol:acetic acid fixative (3:1 mixture of absolute ethanol and glacial acetic acid) was added to stabilize chromosomal structures, dehydrate the cytoplasm via ethanol, and reduce it via acetic acid.
- The suspension was centrifuged at 1,800 rpm for 10 minutes, the supernatant discarded, and the pellet gently resuspended.

5. Fixation

- Five ml of cold ethanol:acetic acid fixative were added, and samples were incubated at -20 °C for 45 minutes.
- The suspension was centrifuged at 2,000 rpm for 10 minutes, the supernatant discarded, and the pellet resuspended.
- Five ml of cold Carnoy's fixative (3:1 mixture of methanol and glacial acetic acid) were added, and samples were incubated at -20 °C for 30 minutes.
- The suspension was centrifuged at 2,000 rpm for 10 minutes, the supernatant discarded, and the pellet resuspended.
- Five ml of cold Carnoy's fixative were added, and samples were incubated at -20 °C for 20 minutes.
- The suspension was centrifuged at 2,000 rpm for 10 minutes, the supernatant discarded, and the pellet resuspended.

6. Slide preparation

- The cell suspension was adjusted with fixative to reach the desired turbidity.
- A few drops of the suspension were applied using a Pasteur pipette to slides pre-immersed in distilled water to form a thin aqueous film.
- The back of the slide was dried, and slides were briefly exposed to steam at 72 °C to spread metaphase chromosomes, then air-dried.
- Slides were examined under an inverted microscope (Olimpus IM).

7. Staining

- Slides were immersed in Quinacrine solution (Merck) to produce bright AT-rich and dark GC-rich bands under fluorescence (QFQ-banding).
- Samples were incubated for 10 minutes at RT, protected from light.

- Excess stain was rinsed off with McIlvaine buffer (pH 7), prepared from 0.1 M citric acid and 0.2 M disodium phosphate (Merck).
- Slides were mounted with coverslips.

8. Image acquisition

- Immersion oil was applied, and metaphase spreads were observed using a fluorescence microscope (Leica DMR).
- Images were captured with CromaWinPlus software (Tesi Imaging) for karyotype analysis.

3.5 *In vitro* spontaneous differentiation

The pluripotency of PT 171 and PT 1051 iPSC lines was evaluated by embryoid body (EB) formation and subsequent *in vitro* spontaneous differentiation into the three germ layers, followed by immunofluorescence (IF) staining for lineage-specific markers.

The procedure was performed as follows:

- Cells from a single well of a 6-well plate at 70-80% confluency were detached using PBS-EDTA 0.5 mM and resuspended in 3 ml of human embryonic stem cell (huES) Medium (Table 6) supplemented with 6 µl of ROCK inhibitor (ROCKi; Merck) and 6 µl of Fibroblast Growth Factor (FGF; Merck).
- The cell suspension was transferred to a 60 mm low-adhesion Petri dish pre-coated with poly-HEMA (10 mg/ml in ethanol 96%) and maintained at 37 °C in a humidified 5% CO₂ incubator.
- For seven days, EBs were collected daily using a Pasteur pipette into a 15 ml conical tube, centrifuged at 300 rpm for 3 minutes, the supernatant was discarded, and EBs were resuspended in 3 ml of fresh huES Medium containing ROCKi (1:500) and FGF (1:500), then replated in the same Petri dish.
- On day 8, EBs were collected into a 15 ml conical tube, centrifuged at 300 rpm for 3 minutes, the supernatant was discarded, and EBs were resuspended in 1 ml of E8 medium.

- EBs were plated onto six Matrigel-coated glass coverslips in a 24-well plate containing 500 µl of E8 medium per well, adding 2-3 drops of EB suspension per well.
- For seven days, the medium was replaced daily with fresh E8 medium and the plate was maintained at 37 °C in a 5% CO₂ incubator.
- On day 15, cells were analyzed by IF staining for germ layer-specific markers: α -fetoprotein (AFP; endoderm), desmin (mesoderm), and β III-tubulin (ectoderm).

Table 6. Composition of the huES Medium in 50 ml

Reagent	Volume
DMEM/F12 medium	38.2 ml
20% KnockOut Serum Replacement (KSR)	10 ml
Penicillin/Streptomycin 100X	500 µl
Glutamine	750 µl
Non-Essential Amino Acids 100X	500 µl
β -mercaptoethanol (1000X)	50 µl

3.6 Immunocytochemistry

To evaluate the pluripotency of PT 171 and PT 1051 iPSC lines and their capacity to spontaneously differentiate into the three germ layers, the expression of specific pluripotency and differentiation markers was assessed by IF staining.

The procedure was performed as follows:

1. Sample preparation and fixation

- iPSCs and EBs were cultured on Matrigel-coated glass coverslips placed in wells of 24-well plate containing 500 µl of E8 medium.
- Cells were washed once with 500 µl of PBS 1X and subsequently fixed with 300 µl of 4% paraformaldehyde (PFA; Santa Cruz Biotechnology) in PBS for 20 minutes at RT to preserve cellular architecture.
- Cells were washed three times with 300 µl of PBS 1X.

2. Permeabilization

- Cells were washed twice with 300 µl of 10% Normal Goat Serum in PBS (NGS/PBS), for 5 minutes at RT.
- Cells were incubated with 300 µl of cold methanol for 5 minutes at RT to increase the permeability of cell and nuclear membranes.
- Cells were permeabilized with 300 µl of 10% NGS/PBS supplemented with 0.3% Triton X-100 for 5 minutes at RT to facilitate antibody access by solubilizing lipid membranes.

3. Blocking

- Cells were incubated in 300 µl of blocking buffer (10% NGS/PBS) for 20 minutes at RT to minimize non-specific binding and reduce background fluorescence.

4. Primary and secondary antibody staining

- Primary antibodies, diluted in 70 µl of 10% NGS/PBS, were applied to the cells and incubated for 90 minutes at 37 °C.
- Cells were washed twice with 300 µl of 10% NGS/PBS for 5 minutes at RT.
- Cells were incubated with the corresponding secondary antibodies for 45 minutes at RT.
- Cells were washed twice with 300 µl of 10% NGS/PBS and twice with 300 µl of PBS 1X, for 5 minutes at RT.
- Coverslips were mounted on microscope slides using 8 µl of the nuclear staining DAPI II Countrestain (Abbott).
- The slides were dried overnight in the dark at RT and then stored at 4 °C.

5. Image acquisition

- Confocal images were acquired by the Imaging Facility Service of University of Milan (Segrate) using a Stellaris 8 DLS confocal microscope (Leica).
- Excitation wavelengths of 405 nm, 488 nm, and 555 nm were used for DAPI, Alexa Fluor 488, and Alexa Fluor 555, respectively.
- Z-stack images were collected with a step size of 0.5 µm.
- Image analysis was performed using LAS X software.

Antibodies used in IF staining assays are listed in Table 7.

Table 7. Antibodies used for immunocytochemistry

Category	Antibody	Dilution	Company
Pluripotency Markers	Mouse anti-Alkaline Phosphatase (AP)	1:200	Abcam
	Mouse anti-TRA-1-60	1:125	Invitrogen
Differentiation Markers	Mouse anti-Alpha-fetoprotein (AFP)	1:125	Invitrogen
	Mouse anti-Desmin	1:10	Chemicon Millipore
	Rabbit anti- β III-Tubulin	1:50	Abcam
Secondary antibodies	Alexa Fluor™ 488 goat anti-mouse IgG (H + L)	1:500	Life Technologies
	Alexa Fluor™ 555 goat anti-rabbit IgG (H + L)	1:500	Life Technologies

3.7 Nucleic acid extraction and quantification

RNA extraction was performed on iPSC lines derived from patients PT 171 and PT 1051, for line characterization, and on peripheral blood samples from nine healthy donors, for differential allelic expression analysis.

1. RNA extraction from iPSC lines

Total RNA was isolated from iPSC lines using the NucleoSpin® RNA Kit (Macherey-Nagel), which is based on a two-column silica membrane system, one for genomic DNA (gDNA) removal and the other for RNA purification. For each sample, cells were harvested from one well of a 6-well plate at 70-80% confluence.

The extraction protocol included the following steps:

- Remove the culture medium and incubate the cells for 5 minutes in PBS/EDTA 0.5 mM.
- Discard the PBS/EDTA solution and detach the cells mechanically by pipetting 800 μ l of PBS 1X.
- Transfer the cell suspension into a 1.5 ml microcentrifuge tube and centrifuge at 2,400 rpm (Eppendorf 5415 R Centrifuge) for 4 minutes.
- Discard the supernatant and resuspend the cell pellet in 350 μ l of Lysis Buffer LBP to homogenize and lyse the sample.

- Place a NucleoSpin® gDNA Removal Column in a 2 ml collection tube, transfer the homogenized sample onto the column, and centrifuge at 11,000 rcf for 30 seconds.
- Discard the column containing the capture DNA.
- Add 100 µl of Binding Solution BS to the flowthrough and mix thoroughly by pipetting.
- Place a NucleoSpin® RNA Plus Column in a 2 ml collection tube, load the sample, and centrifuge at 11,000 rcf for 15 seconds to bind the RNA to the column membrane.
- Add 200 µl of Wash Buffer WB1, centrifuge at 11,000 rcf for 15 seconds, discard the flowthrough with collection tube, and place the column into a new 2 ml collection tube.
- Add 600 µl of Wash Buffer WB2 to the column, centrifuge at 11,000 rcf for 15 seconds, discard the flowthrough, and place the column into the same collection tube.
- Add 250 µl of Wash Buffer WB2 to the column and centrifuge at 11,000 rcf for 2 minutes to completely dry the membrane.
- Transfer the column into a new RNase-free 1.5 ml microcentrifuge tube.
- Add 30 µl of RNase-free water to the column and centrifuge at 11,000 rcf for 1 minute to elute the RNA.
- Repeat the elution step with an additional 30 µl of RNase-free water to ensure complete RNA recovery.

2. RNA extraction from blood samples

Total RNA was isolated from peripheral blood samples using the Tempus™ Spin RNA Isolation Kit (Thermo Fisher Scientific). For each sample, 3 ml of whole blood were collected into Tempus™ Blood RNA Tubes containing 6 ml of stabilizing reagent, which lyses blood cells and preserves RNA integrity.

The extraction protocol included the following steps:

- Transfer the blood solution from the Tempus™ Blood RNA Tube into a 50 ml conical tube and add approximately 3 ml of PBS (Ca²⁺/Mg²⁺-free) 1X to bring the total volume to 12 ml.
- Vortex the closed tube vigorously for 30 seconds, then centrifuge at 3,000 rcf for 30 minutes at 4 °C.
- Carefully discard the supernatant, invert the conical tube on absorbent paper for 1-2 minutes, and blot any remaining droplets from the rim using clean absorbent paper.
- Add 400 µl of RNA Purification Resuspension Solution into the conical tube and briefly vortex to resuspend the RNA pellet located at the bottom.
- Insert the RNA Purification Filter into a waste collection tube and add 100 µl of RNA Purification Wash Solution to pre-wet the membrane.
- Add 400 µl of the resuspended RNA into the filter, centrifuge at 16,000 rcf for 30 seconds, remove the filter, discard the flowthrough, and reinsert the filter into the waste tube.
- Add 500 µl of RNA Purification Wash Solution 1 into the filter, centrifuge at 16,000 rcf for 30 seconds, remove the filter, discard the flowthrough, and reinsert the filter into the waste tube.
- Perform two washes with 500 µl of RNA Purification Wash Solution 2, centrifuging each time at 16,000 rcf for 30 seconds and discarding the flowthrough after each wash.
- Reinsert the filter into the waste tube and centrifuge at 16,000 rcf for 30 seconds to completely dry the membrane.
- Transfer the purification filter into a new collection tube, add 100 µl of Nucleic Acid Purification Elution Solution, incubate for 2 minutes at 70 °C, and centrifuge at 16,000 rcf for 30 seconds.
- Reload the eluate onto the same filter and centrifuge at 16,000 rcf for 2 minutes to maximize RNA recovery.
- Discard the filter and transfer approximately 90 µl of the RNA eluate into a new RNase-free 1.5 ml microcentrifuge tube.

In addition, gDNA from PT 171 (used for Whole Exome Sequencing) and gDNA from NF2 patients (used for array-CGH and LRS) were kindly provided by Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and Fondazione IRCCS Istituto Neurologico "Carlo Besta", respectively. Similarly, RNA samples from 106 NF1 patients, two NF2 patients, and ten healthy controls (used for expression studies) were provided by Fondazione IRCCS Istituto Neurologico "Carlo Besta".

Nucleic acid quantification was performed using the NanoDrop 1000 Spectrophotometer (Euroclone), which measures the absorbance of nucleic acid solutions at 260 nm, the wavelength at which nucleic acids exhibit maximum absorbance. In addition to determining concentration, the instrument also provides purity ratios by comparing absorbance values at different wavelengths, allowing the detection of potential contaminants. The A_{260}/A_{280} ratio indicates protein contamination, with optimal values ranging from 1.6 to 1.8 for DNA and 1.8 to 2.0 for RNA. The A_{260}/A_{230} ratio is used to evaluate contamination by carbohydrates or phenolic compounds (solvents), with an optimal value of approximately 2.2.

The instrument was initialized with 1 μ l of nuclease-free water and blanked using the elution buffer employed in the final step of the nucleic acid extraction procedure. The measurements were performed using 1 μ l of DNA or RNA solution. Table 8 reports the concentration (ng/ μ l) and purity ratios (A_{260}/A_{280} and A_{260}/A_{230}) for each DNA and RNA sample analyzed.

Table 8. Concentration and quality of each DNA or RNA sample analyzed

Nucleic acid	Sample ID	Classification	Concentration	A_{260}/A_{280}	A_{260}/A_{230}
DNA	PT 171	atypical NF1 MD	86.80	1.82	2.10
	PT 568	NF2 MD	415.00	1.80	2.10
	PT 690	NF2 MD	296.00	1.81	2.07
RNA	171 C1 P7 iPSCs	atypical NF1 MD	303.60	1.97	2.09
	171 C2 P10 iPSCs	atypical NF1 MD	136.30	2.05	1.79
	171 C2 P19 iPSCs	atypical NF1 MD	184.70	2.04	2.09
	171 C2 P24 iPSCs	atypical NF1 MD	71.70	2.05	1.75
	1051 C1 P8 iPSCs	NF1 MD	74.20	2.09	1.68
	1051 C4 P12 iPSCs	NF1 MD	61.20	2.08	0.91
	1051 C4 P20 iPSCs	NF1 MD	103.20	2.03	1.66
	273	NF1 IM	279.00	2.11	2.22
	290	NF1 IM	144.00	2.09	2.08
	323	NF1 IM	40.00	2.01	2.00
	356	NF1 IM	42.00	2.02	2.14
	384	NF1 IM	280.30	1.91	1.30
	412	NF1 IM	54.00	1.95	1.72
	501	NF1 IM	324.00	2.11	2.09
	507	NF1 IM	112.00	1.96	1.62
	524	NF1 IM	83.00	2.05	1.90
	558	NF1 IM	97.00	2.01	4.44
	603	NF1 IM	160.00	2.11	1.58

623	NF1 IM	144.80	2.08	2.05
663	NF1 IM	185.80	2.10	2.21
705	NF1 IM	217.00	2.08	2.15
733	NF1 IM	142.00	2.17	2.31
738	NF1 IM	95.20	2.15	1.38
744	NF1 IM	108.00	2.07	2.01
752	NF1 IM	125.50	2.07	2.08
765	NF1 IM	77.00	2.18	2.59
767	NF1 IM	160.10	2.09	2.12
809	NF1 IM	97.40	1.50	1.36
822	NF1 IM	97.40	1.94	1.23
858	NF1 IM	170.60	1.87	1.66
860	NF1 IM	79.50	1.89	1.30
876	NF1 IM	182.00	1.91	1.61
915	NF1 IM	142.60	2.00	1.53
936	NF1 IM	82.00	1.73	1.25
946	NF1 IM	158.50	1.95	1.13
966	NF1 IM	99.50	1.72	1.32
1020	NF1 IM	97.00	1.76	1.65
1194	NF1 IM	145.50	1.95	1.55
1213	NF1 IM	106.60	1.88	1.50
1238	NF1 IM	300.00	2.18	2.01
1328	NF1 IM	153.50	2.06	1.42
1353	NF1 IM	94.60	1.86	1.24
1358	NF1 IM	158.50	1.37	1.22
1428	NF1 IM	76.10	2.25	1.03
1435	NF1 IM	114.50	2.09	1.31
1452	NF1 IM	209.50	1.98	1.37
1455	NF1 IM	198.10	1.84	1.44
1467	NF1 IM	124.80	2.42	2.48
1490	NF1 IM	88.20	1.89	1.14
1491	NF1 IM	117.80	1.98	1.38
1500	NF1 IM	206.60	1.91	1.63
1504	NF1 IM	120.60	1.84	1.31
1533	NF1 IM	236.40	1.84	1.56
1542	NF1 IM	160.10	1.89	1.46
1566	NF1 IM	135.30	1.89	1.33
1577	NF1 IM	169.30	1.94	1.57
1590	NF1 IM	193.30	1.69	1.29
1594	NF1 IM	192.40	1.96	1.67
1601	NF1 IM	107.10	1.97	1.44
1608	NF1 IM	139.90	2.00	1.50
1871	NF1 IM	72.10	2.10	1.18
1228	NF1 IM	81.40	2.06	1.30
47B	NF1 IM	51.50	2.07	2.63
1436	NF1 IM	121.00	2.09	1.11
268	NF1 IM	98.00	2.18	2.62
319	NF1 IM	133.00	2.09	2.11
367	NF1 IM	102.00	2.24	2.59
368	NF1 IM	75.00	2.11	2.12
425	NF1 IM	101.00	2.07	2.33
509	NF1 IM	116.00	2.00	1.81
571	NF1 IM	119.80	2.05	2.20
584	NF1 IM	120.00	2.12	2.21
829	NF1 IM	178.40	1.55	1.89
834	NF1 IM	115.70	1.76	1.38
891	NF1 IM	142.00	2.10	2.25
981	NF1 IM	68.00	1.66	1.08
1099	NF1 IM	80.10	1.85	1.24
1145	NF1 IM	128.70	1.90	1.43
1242	NF1 IM	64.00	1.76	1.09
1263	NF1 IM	97.10	1.83	1.31
1367	NF1 IM	116.70	1.93	1.62
1382	NF1 IM	81.50	1.99	0.99
1450	NF1 IM	136.70	1.85	1.51
1493	NF1 IM	85.00	1.86	1.17
1498	NF1 IM	236.80	1.93	1.69
1521	NF1 IM	92.40	1.83	1.34
2207	NF1 IM	325.20	1.49	1.54
2281	NF1 IM	82.00	1.82	1.15
1136	NF1 IM	149.80	1.93	1.58
1140	NF1 IM	132.70	2.19	1.56
1139	NF1 IM	256.00	1.76	1.74
451	NF1 IM	190.00	1.80	1.94
494	NF1 IM	111.00	2.06	2.09
1153	NF1 IM	185.10	1.84	1.62
1202	NF1 IM	97.70	1.92	1.07
46B	NF1 IM	43.10	1.80	1.98
1434	NF1 IM	138.20	2.03	1.13
1931	NF1 IM	101.50	1.92	1.30
1271	NF1 IM	94.20	1.84	1.31
550	NF1 IM	142.00	2.10	2.16
1649	NF1 IM	56.30	1.88	0.96
1650	NF1 IM	55.10	1.91	0.97

307	NF1 MD	174.80	2.10	2.82
727	NF1 MD	185.60	2.15	2.31
641	NF1 MD	220.00	2.08	1.99
510	NF1 MD	171.00	2.09	2.09
924	NF1 MD	100.50	2.07	1.34
1051	NF1 MD	103.00	1.83	2.34
1376	NF1 MD	265.50	1.95	1.87
1464	NF1 MD	248.40	1.94	1.76
2331	NF1 MD	116.30	1.85	1.46
877	NF1 MD	191.40	1.91	1.51
PT 568	NF2 MD	165.20	1.85	1.46
PT 690	NF2 MD	125.50	1.82	1.50
WT 1	HD	115.60	1.73	1.36
WT 2	HD	232.00	1.69	1.81
WT 3	HD	105.10	1.84	1.45
WT 4	HD	100.60	1.84	1.38
WT 5	HD	125.40	1.83	1.44
WT 6	HD	102.80	1.93	1.35
WT 7	HD	154.90	1.76	1.58
WT 8	HD	184.80	1.80	1.66
WT 9	HD	57.20	1.82	0.91
WT 10	HD	56.20	1.80	1.04
WT 31	HD	190.00	2.05	2.16
WT 34	HD	124.70	2.05	2.13
WT 35	HD	108.10	2.02	2.23
WT 37	HD	162.80	2.05	2.13
WT 38	HD	164.70	2.01	2.09
WT 39	HD	135.30	2.05	2.32
WT 41	HD	132.90	2.04	2.27
WT 42	HD	167.70	2.05	2.15
WT 43	HD	72.50	2.06	1.92

HD, healthy donor; NF1 IM, patient with a *NF1* intragenic mutation; NF1 MD, patient carrying a *NF1* microdeletion; NF2 MD, patient carrying a *NF2* microdeletion

All DNA and RNA samples were stored at -20 °C and -80 °C, respectively.

3.8 Whole Exome Sequencing data analysis

Whole Exome Sequencing (WES) was performed, by an external service provider (Macrogen Europe), on gDNA extracted from peripheral blood of PT 171. The sample was prepared according to the provided instructions. An initial quality control assessment was conducted using the 2200 TapeStation system (Agilent Technologies) to evaluate DNA integrity, expressed as the DNA Integrity Number (DIN). Samples with a DIN value ≥ 7 were considered suitable for sequencing. A total of 500 ng of gDNA from PT 171, with a DIN value of 7.4, was submitted for sequencing. WES library preparation was carried out using the Agilent SureSelect V6 Exome + UTR kit, and sequencing was performed on the NovaSeq 6000 platform (Illumina).

Raw sequencing data were analyzed at Istituto di Tecnologie Biomediche (ITB), Consiglio Nazionale delle Ricerche (CNR), Segrate (Milan), by Dr. Mangano. The quality control checks on FASTQ files were performed using

FastQC, and BWA-MEM algorithm was applied to align the reads to the human reference genome (GRCh38/hg38). The obtained BAM files were uploaded to the BaseSpace (Illumina) platform, in which the DRAGEN Enrichment pipeline was applied. Variant annotation was performed using ANNOVAR (Wang *et al.*, 2010), and a list of annotated variants was generated and filtered based on default parameters.

For variant prioritization, in addition to an initial whole-exome analysis, specific attention was given to variants located in candidate genes belonging to selected categories (Table 9): (i) genes within the PT 171 *NFI* deletion region; (ii) genes flanking the PT 171 *NFI* deletion both centromerically and telomerically; (iii) genes involved in the Ras signaling pathway; and (iv) genes encoding proteins known to interact with neurofibromin. The list of Ras pathway genes was obtained from the National Cancer Institute database, with the addition of *LRP1* and *LZTR1* genes, which are known regulators of Ras signaling despite not being included in the reference list.

Table 9. Genes selected for variant prioritization analysis following WES

Classification	Genes
Genes inside PT 171 <i>NFI</i> deletion	<i>COPRS, EVI2A, EVI2B, NFI, OMG, RAB11FIP4, RNF135, SUZ12, UTP6</i>
Genes located centromerically to PT 171 <i>NFI</i> deletion	<i>ABHD15, ADAP2, ALDOC, ANKRD13B, ATAD5, BLMH, CORO6, CPD, CRLF3, CRYBA1, DHRS13, EFCAB5, ERAL1, FAM222B, FLOT2, FOXN1, GIT1, GOSR1, IFT20, KIAA0100, MYO18A, NEK8, NSRP1, NUFIP2, PHF12, PIGS, PIPOX, POLDIP2, PROCA1, RAB34, RPL23A, SARM1, SDF2, SEZ6, SGK494, SLC13A2, SLC46A1, SLC6A4, SPAG5, SSH2, SUPT6H, TAOK1, TBC1D29, TEFM, TIAF1, TLCD1, TMEM199, TMEM97, TMIGD1, TNFAIP1, TP53I13, TRAF4, UNC119, VTN</i>
Genes located telomerically to PT 171 <i>NFI</i> deletion	<i>ASIC2, C17orf75, CDK5R1, LRRC37B, MYO1D, PSMD11, RHBDL, RHOT1, SPACA3, TMEM9, ZNF207</i>
Genes involved in the Ras pathway	<i>AKT1, AKT2, AKT3, ALK, APAF1, ARAF, ARHGAP35, ARHGEF2, BARD1, BRAF, BRCA1, BRCA2, BRIP1, BUB1, CASP3, CASP7, CASP8, CBL, CBLB, CBLC, CCNA1, CCNA2, CCND1, CCND2, CCND3, CDC25A, CDC6, CDK2, CDK4, CDK6, CDKN1A, CNKSR1, CNKSR2, CYTH2, DAB2IP, DHFR, DUSP1, DUSP2, DUSP3, DUSP4, DUSP5, DUSP6, E2F1, E2F2, E2F3, E2F7, ECT2, EGFR, EIF4EBP1, EIF4EBP2, EIF4EBP3, ELK1, ERBB2, ERF, ETS1, ETS2, EXOC1, EXOC2, EXOC3, EXOC4, EXOC5, EXOC6, EXOC7, EXOC8, FANCA, FANCC, FGFR1, FGFR2, FGFR3, FGFR4, FLT3, FNTA, FNTB, FOS, FOSL1, GRB10, GRB2, HRAS, ICMT, INPP4B, INSR, INSR, IRS1, IRS2, JUN, KRAS, KSR1, KSR2, LRP1, LZTR1, MAP2K1, MAP2K2, MAPK1, MAPK3, MCM3, MCM4, MCM5, MCM6, MCM7, MDM2, MET, MLST8, MRAS, MTOR, MYB, MYC, NF1, NFE2L2, NFKB1, NOX1, NRAS, PAK1, PAK2, PAK3, PAK4, PDGFRA, PDGFRB, PDPK1, PEA15, PEBP1, PIK3CA, PIK3CD, PIK3CG, PIK3R1, PIK3R2, PIK3R3, PIK3R5, PIK3R6, PIN1, PLCE1, PLXNB1, PPP1CA, PREX2, PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2, PRKAG3, PTEN, PTPN11, RAC1, RAC2, RAC3, RAD52, RAF1, RALA, RALB, RALBP1, RALGAP1, RALGAP2, RALGAP3, RAPGEF1, RAPGEF2, RASA1, RASA2, RASA3, RASAL1, RASAL2, RASAL3, RASGRF1, RASGRF2, RASGRP1, RASGRP2, RASGRP3, RASGRP4, RASSF1, RASSF10, RASSF2, RASSF3, RASSF4, RASSF5, RASSF6, RASSF7, RASSF8, RASSF9, RB1, RCE1, RGL1, RGL2, RGL3, RHEB, RHOA, RHOB, RHOC, ROCK1, ROCK2, ROS1, RPS6KA1, RPS6KA2, RPS6KA3, RPS6KA6, RPS6KB1, RPS6KB2, RPTOR, SAV1, SCRIB, SHC1, SHC2, SHC3, SHC4, SHOC2, SOS1, SOS2, SPRED1, SPRED2, SPRED3, SPRY1, SPRY2, SPRY3, SPRY4, STK11, STK3, STK4, TBK1, TFDP1, TFDP2, TIAM1, TIAM2, TK1, TP53, TP73, TSC1, TSC2, TYMS, UNG, VAV1, YAP1</i>
Genes encoding neurofibromin interactors	<i>APP, CALM1, CASK, CDC5L, DYNC1H1, GRIN1, GRIN2B, MAPK3, PML, SDC1, SDC2, SDC3, SDC4, SUMO1, YWHAB, YWHAZ</i>

For the investigation and characterization of genetic variants, several bioinformatic tools and online databases were employed to obtain comprehensive information on gene function and structure, the predicted effects of each variant, and potential clinical correlations. The following resources were consulted:

- GeneCards (<https://www.genecards.org>), a comprehensive database integrating clinical, transcriptomic, proteomic, and functional data for human genes, used to assess the biological function of the proteins under investigation (Stelzer *et al.*, 2016).
- Ensembl Genome Browser (<https://www.ensembl.org/index.html>), a genomic browser providing detailed information on vertebrate genomes, employed to retrieve transcript-specific data for the analyzed genes (Newman *et al.*, 2018).
- St. Jude Pecan (<https://pecan.stjude.cloud/variants/proteinpaint>), a platform designed to identify and visualize mutations associated with pediatric cancers. The ProteinPaint tool was specifically used to map each variant along the amino acid sequence and within the corresponding protein's functional domains.
- String (<https://string-db.org/cgi/>), a database of known and predicted protein-protein interactions, including both physical and functional associations, used to explore potential interactors of the studied proteins (Szklarczyk *et al.*, 2025).
- Uniprotkb (<https://www.uniprot.org/uniprotkb>), a resource used to perform multiple sequence alignments across different species, in order to assess the degree of evolutionary conservation of amino acid residues within the analyzed proteins (Boutet *et al.*, 2007).
- Franklin (<https://franklin.genoox.com/clinical-db/home>), an integrated platform for variant classification and clinical interpretation, employed to support the annotation and pathogenicity assessment of the identified variants.
- ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), a public archive reporting the relationships between genetic variants and their associated phenotypes, used to investigate genotype-phenotype correlations (Landrum *et al.*, 2016).
- OMIM (<https://www.omim.org/>), a compendium of human genes and genetic phenotypes that is freely available and updated daily (Amberger and Hamosh, 2017).

3.9 Array-CGH analysis

The array-CGH analysis was performed, in collaboration with Dr. Redaelli at Fondazione IRCCS San Gerardo dei Tintori (Monza), on 500 ng of gDNA from two patients (PT 568 and PT 690) and two reference samples (a male and a female, respectively gender-matched with the patients) using Agilent SurePrint G3 Human CGH + SNP 4x180K Microarray kit (Agilent Technologies). This platform provides a median overall probe spacing of approximately 25 kb, with increased resolution of about 5 kb within ISCA (International Standards for Cytogenomic Arrays) regions.

The procedure was performed as follows:

1. Sample preparation

The volumes of gDNA (corresponding to 500 ng) and nuclease-free water required to reach a final reaction volume of 20 µl were calculated individually for each sample, according the following formulas:

$$x \text{ µl of gDNA} = \frac{500 \text{ ng}}{\text{initial gDNA concentration} \left(\frac{\text{ng}}{\text{µl}} \right)}$$

$$\text{µl of nuclease free water} = 20 \text{ µl} - x \text{ µl of gDNA}$$

For each sample, the calculated volumes of gDNA and nuclease-free water were added into a 200 µl PCR tube.

2. Enzymatic digestion

A Digestion master mix was prepared as shown in Table 10.

Table 10. Composition of Digestion master mix (per sample)

Reagent	Volume
10X Restriction Enzyme Buffer	2.6 µl
BSA	0.2 µl
Alu I	0.5 µl
Rsa I	0.5 µl
Nuclease-free water	2.2 µl

For each sample, 6 µl of Digestion master mix were added to the tube and the mixture was incubated in a thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories) under the conditions described in Table 11.

Table 11. Composition of reaction mix and incubation conditions for the enzymatic digestion

Reaction mix		Thermal cycler conditions	
Reagent	Volume	Time	Temperature
gDNA	20 µl	120'	37 °C
Digestion master mix	6 µl	20'	65 °C
		∞	4 °C

To assess digestion efficiency, 2 µl of digested gDNA from each sample were mixed with 2 µl of H₂O and 1 µl of loading dye, and run alongside an undigested DNA control on a 2% agarose gel at 135 V for 15 minutes.

3. Fluorescent labeling of gDNA

Two Labeling master mixes were prepared as shown in Table 12: one for target samples labeled with Cy5, and the other for reference samples labeled with Cy3.

Table 12. Composition of Labeling master mix (per sample)

Reagent	Volume
5X Reaction Buffer	10 µl
10X dNTPs	5 µl
Cyanine 3-dUTP or Cyanine 5-dUTP	3 µl
Exo (-) Klenow	1 µl
Nuclease-free water	2 µl

For each sample, 5 µl of Random Primer and 21 µl of Labeling master mix were added to the tube and the mixture was incubated under the conditions described in Table 13.

Table 13. Composition of reaction mix and incubation conditions for the fluorescent labeling of gDNA

Reaction mix		Thermal cycler conditions	
Reagent	Volume	Time	Temperature
Digested gDNA	24 µl	3'	98 °C
Random Primer	5 µl	~10'	4 °C
Labeling master mix	21 µl		

4. Purification of labeled gDNA

A purification column was placed into a collection tube, and 330 µl of 1X TE buffer (pH 8.0) were added to the column. To each tube containing 50 µl of DNA, 100 µl of 1X TE buffer were added, and the entire volume (150 µl) was transferred onto the purification column. The samples were then centrifuged at 14,000 rcf for 10 minutes at RT. The eluate was discarded, and 480 µl of 1X TE buffer were added to each column, followed by centrifugation at 14,000 rcf for 10 minutes at RT. The eluate was discarded, and the column was inverted into a new 2 ml collection tube. A final centrifugation was performed at 1,000 rcf for 1 minute at RT to collect the purified sample.

The purified samples were quantified using a NanoDrop 2000 UV-VIS spectrophotometer (Thermo Fisher Scientific). After blanking with 1.5 µl of 1X TE, 1.5 µl of labeled gDNA were analyzed to measure the absorbance at A₂₆₀ nm (DNA), A₅₅₀ nm (CY-3), and A₆₅₀ nm (CY-5). The labeling efficiency, specific activity, and yield were determined using the following formulas:

$$\text{Labeling efficiency (\%)} = \frac{340 \times \text{pmol dye}/\mu\text{l}}{\text{ng gDNA}/\mu\text{l} \times 1000} \times 100$$

$$\text{Specific activity (pmol}/\mu\text{g}) = \frac{\text{pmol dye}/\mu\text{l}}{\mu\text{g gDNA}/\mu\text{l}}$$

$$\text{Yield } (\mu\text{g}) = \frac{\text{gDNA concentration } \left(\frac{\text{ng}}{\mu\text{l}}\right) \times \text{sample volume } (\mu\text{l})}{1000 \left(\frac{\text{ng}}{\mu\text{g}}\right)}$$

5. Hybridization setup

Each target sample was paired with a reference sample matched by sex, concentration, and labeling yield, to ensure equimolar hybridization. The reference sample volume was adjusted according to the following proportionality:

$$V_{\text{final}} = \frac{V_{\text{initial}} \times R_{\text{final}}}{R_{\text{initial}}}$$

where:

- V_{initial} = total volume of target sample
- V_{final} = volume of reference sample to be used
- R_{initial} = yield of reference
- R_{final} = yield of target

Once the required amounts of target and reference DNA were calculated, the samples were combined, and, if necessary, 1X TE buffer was added to adjust the total volume of each sample to at least 39 μl , as required for the 4-pack microarray. Hybridization reactions were prepared on ice and then incubated in the thermal cycler as described in Table 14.

Table 14. Composition of hybridization reaction mix and incubation conditions

Reaction mix		Thermal cycler conditions	
Reagent	Volume	Time	Temperature
Target + Reference samples	39 μl	3'	98 °C
Cot-1 DNA (1.0 mg/ml)	5 μl	30'-70'	37 °C
10X aCGH Blocking Agent	11 μl		
2X HI-RPM Hybridization Buffer	55 μl		

A volume of 100 μl of each hybridization mix was dispensed onto microarray slide, covered with microarray coverslip, and placed into the hybridization chamber. The chamber was incubated into a vacuum oven (Shel Lab) at 67 °C, with shaking at 20 rpm for 24 hours.

6. Post-hybridization washes

Hybridization chamber was disassembled. The microarray slide and coverslip were quickly immersed in Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1 and separated. The microarray slide was transferred sequentially through two wash solutions, as described in Table 15.

Table 15. Post-hybridization washing steps

Reagent	Time	Temperature	Shaking
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	5'	RT	350 rpm
Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	1'	37 °C	No

7. Microarray scanning and data analysis

The microarray slide was placed in a slide holder and scanned using the Agilent DNA Microarray Scanner G2505C. Data extraction was performed using Agilent Feature Extraction and copy number changes were analyzed by CytoGenomics Software v5.2.0.2 (ADM-2 algorithm; Agilent Technologies).

3.10 Long-Range PCR assays

To refine the genomic region encompassing the 22q12 deletion BPs in PT 690, Long-Range PCR assays were performed on the patient's gDNA. Subsequently, Nested and Semi-Nested Long-Range PCR assays were conducted using primers mapping within the PCR product obtained from the initial amplification.

The procedure consisted of the following steps:

1. Primer design and preparation

Primers were designed using the Primer3web version 4.1.0 software (<http://primer3.ut.ee/>), and their sequences are reported in Table 16. To ensure that the designed sequences were unique within the human genome, the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) provided by the National Center for Biotechnology Information (NCBI) was used. Possible secondary structures or complementarity between forward and reverse primers were assessed using the

OligoAnalyzer™ Tool (Integrated DNA Technologies;
<https://eu.idtdna.com/pages/tools/oligoanalyzer>).

Table 16. Sequences of forward (F) and reverse (R) primers designed for Long-Range PCR assays

Primer ID	Primer sequence (5' to 3')	Primer Nested sequence (5' to 3')
MIRb_F	CATGCTGGAATGCAGTGGTG	ATACAGGGGTGAGCCACCAT
MER68_F	GAAGTGCCATTTACCAGGGC	CCACCCCGAGTTATGAGGAT
AluSx (1)_F	TCTCTGACCCACCAACCCCTA	CCAAGGCAGGTGGATCACTT
MER90_1_F	TGCAGACGCACCATCTTTAAG	ACCACCCCTACCCTAACTGATA
MER90_2_F	AGATACCCAATCACAGCGGG	TTCTCTTGCCCTTTTCGCCCA
L1MC4a_F	TTACAGGTGTGAACCGTCCC	GGTTCACACTGTGCAACACAT
AluSx (2)_F	TGGTGAAACCCTGTCTCTACT	CAAGCGATTCTCCTGCCTCATC
AluSx (3)_F	ACCCCTGGCTAATCTTCGTAT	CCTCCTGAGTAGCTGGGATT
MER53 (1)_R	GGAAGGAAGGGATGTGAGCT	CCTGGGTGTCTTCACTTCAC
L2c_1_R	TGTTGGTCAGGCTGGTCTCA	CTAGAAGTAGGGCTGCCAGA
L2c_2_R	CTACCTCAGCCTCCTGAGTA	GCCTCCCAGATTACTGAGGT
MER53 (2)_R	ACTAGAAGTAGGGCTGCCAG	GCCTCCCAGATTACTGAGGT
MER90_R	AGCCACTATGTGACCATGATG	GCCCTCGTCCAAATCACCTA
L1MC4a_R	GCCAGCCCAGTCAATCAGTT	GGCTGAAGCTCTAAGGTTCC
MER58B_R	GGGCCATAGAGTAAATACTTCA	ATGGGTGTGACTGAAATGAACA
L1MB3_1_R	GAATAGTGGTTGGCAGTGGC	AACAGTTCTGGAGGTAGATGG
L1MB3_2_R	CGACAGAGAGACCCTGTCTT	AACCAGGAGCCACTTGGTCA
AluJb_R	GTGGCTAGGGGGAAGAAGAA	ACAGTTCTGGAGGTAGATGGT
MIR_R	GCCAACAGCACAGTTCCTGA	ATGCCAGTGCCTTACCTGC

The primers were supplied in lyophilized form (Merck). Each lyophilized oligonucleotide was dissolved by adding a volume of nuclease-free water (in μl) equivalent to its amount in nmol, to obtain an initial concentration of 1 mM. Subsequently, two serial dilutions were prepared: an intermediate dilution of 100 μM and a working dilution of 10 μM , both adjusted to a final volume of 30 μl . All primers were stored at $-20\text{ }^{\circ}\text{C}$ until use.

2. Long-Range PCR setup

The Long-Range PCR was performed using the TaKaRa LA Taq DNA Polymerase kit (TaKaRa LA Taq), which enables the amplification of DNA fragments up to 35 kb in length through a series of thermal cycling reactions. The composition of the reaction mixture and the corresponding thermal cycling conditions are summarized in Table 17.

Table 17. Composition of Long-Range PCR reaction mix and incubation conditions

Reaction mix		Thermal cycler conditions		
Reagent	Volume	Time	Temperature	No. cycles
10x LA PCR Buffer II (Mg ²⁺ plus)	5 µl	1'	94 °C	} 30
dNTP mix (2.5 mM)	8 µl	10''	98 °C	
TaKaRa LA Taq (5 U/µl)	0.5 µl	30''	60.5 °C	
Primer F (10 µM)	1.2 µl	10'	68 °C	
Primer R (10 µM)	1.2 µl	10''	72 °C	
DNA/PCR product	300 ng/0.5 µl	∞	10 °C	
Nuclease-free water	up to 50 µl			

3. Gel electrophoresis

To assess the success of the Long-Range PCR reaction, the amplified products were separated on a 0.8% agarose gel prepared in 1X TBE buffer. DNA fragments were visualized using EuroSafe Nucleic Acid Stain 20000X (Euroclone), an intercalating dye. For each sample, a mix containing 5 µl of PCR product and 2 µl of Gel Loading Dye Blue 6X (BioLabs) was prepared. The loading buffer consisted of a glycerol solution, which facilitates sample deposition into the wells, and the bromophenol blue, a tracking dye that allows visualization of electrophoretic migration. The 1kb DNA Ladder (Promega) was used as a molecular weight marker to estimate the size of the amplified fragments. Electrophoresis was carried out at 80 V for 90 minutes. PCR products were visualized under ultraviolet light using the Transilluminator Essential V6 (UVITEC™ Cambridge).

3.11 Nanopore sequencing data analysis

In collaboration with Dr. Mangano and Dr. Bordoni (ITB, CNR), a LRS was performed on gDNA from PT 568 and PT 690, using the Oxford Nanopore Technologies (ONT).

The sequencing library was prepared starting from 1 µg of gDNA using the ligation sequencing kit SQK-LSK114 (ONT). Five hundred femtomoles of the final

library were loaded onto a PromethION2 Solo flow cell (R10.4.1) and the data were collected for three days.

Raw sequencing data were acquired in POD5 format using MinKNOW and base-calling was performed using Dorado (v0.9.0). The resulting FASTQ reads were aligned to the human reference genome (GRCh38/hg38) using minimap2 (Li, 2018). Coverage profiles were computed using mosdepth (Pedersen and Quinlan, 2018), and variant calling was performed using the Human variation workflow implemented by Epi2me (<https://github.com/epi2me-labs/wf-human-variation>).

After prioritization process, candidate small variants, CNVs, and SVs were manually visualised using the Integrative Genomics Viewer (IGV, v2.18.0) (Robinson *et al.*, 2011) and their functional characterization was performed using several bioinformatic tools and online databases, as previously described for WES data analysis.

3.12 *In silico* analysis of the 22q12 chromosomal region

Following the results obtained from the array-CGH and subsequent LRS analyses, an *in silico* investigation of repeated motifs within the regions encompassing the 22q12.1q12.2 deletion BPs was performed using the RepeatMasker Track (Repeating Elements by RepeatMasker) of the UCSC Genome Browser (<https://genome.ucsc.edu/>) (Zweig *et al.*, 2008). To assess the degree of homology between the repeated sequences mapped within the regions including the centromeric and telomeric BPs, the Global Alignment Track of the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Sayers *et al.*, 2019) was employed.

The DECIPHER (v11.29, <https://www.deciphergenomics.org/>) (Firth *et al.*, 2009) and OMIM (<https://www.omim.org/>) (Amberger and Hamosh, 2017) databases were used to investigate the genes included in the 22q12.1q12.2 deletions. In addition, the potential haploinsufficiency of the deleted genes was predicted using the pLI score reported in the gnomAD v2.1.1 database (<https://gnomad.broadinstitute.org/>) (Karczewski *et al.*, 2020).

To evaluate potential position effect, TADs and regulatory elements mapped within the 22q12 region – including the microdeletions identified in PT 568 and PT 690 and their flanking regions – were analyzed using the Human GRCh38/hg38 assembly as reference genome. The genomic interval considered for this analysis (chr22:27,400,000-31,640,000) corresponded to the first non-deleted TADs, upstream of the PT 568 microdeletion and downstream of the PT 690 microdeletion. Hi-C interaction data, corresponding to TAD structures, detected in the GM12878 cell line, were visualized using the 3D Genome Browser 2.0 (<http://3dgenome.fsm.northwestern.edu/>) (Wang *et al.*, 2018). The study of regulatory elements was conducted using the VISTA Enhancer Browser (<https://enhancer.lbl.gov/>) (Visel *et al.*, 2007) to identify enhancers, and CTCFBSDB 2.0 (<http://insulatordb.uthsc.edu/>) (Ziebarth *et al.*, 2013) to detect predicted insulators. Two custom tracks were added to the UCSC Genome Browser to visualize the relative positions of the identified regulatory elements.

3.13 Reverse transcription

A total of 500 ng of RNA from each sample collected for this study (Table 8) was reverse transcribed into complementary DNA (cDNA), using the Maxima H Minus cDNA Synthesis Master Mix with dsDNase (Thermo Fisher Scientific). The volume of RNA solution required for the reverse transcription reaction was calculated individually for each sample, according to the following formula:

$$x \text{ } \mu\text{l of RNA} = \frac{500 \text{ ng}}{\text{initial RNA concentration} \left(\frac{\text{ng}}{\mu\text{l}} \right)}$$

The protocol consists of two main steps:

- Degradation of residual double-strand DNA (dsDNA). Remaining dsDNA in the RNA sample was digested using the dsDNase enzyme. For this first step, a reaction mix was prepared and incubated in a dry bath (Merck) for 2 minutes at 37 °C, followed by immediate chilling on ice (Table 18).

Table 18. Composition of dsDNA removal reaction mix and incubation conditions

Reaction mix		Dry bath condition	
Reagent	Volume	Time	Temperature
10X dsDNase Buffer	1 µl	2'	37 °C
dsDNase	1 µl		
RNA	x µl		
Nuclease-free water	up to 10 µl		

- Reverse transcription of RNA into cDNA. In the second step, a separate reaction mix was prepared and added to the first mix to synthesize cDNA using the reverse transcriptase enzyme. The reactions were performed in the thermal cycler according to a specific temperature program (Table 19).

Table 19. Composition of reverse transcription reaction mix and incubation conditions

Reaction mix		Thermal cycler conditions	
Reagent	Volume	Time	Temperature
First mix	10 µl	10'	25 °C
Maxima cDNA H Minus		30'	50 °C
Synthesis Master Mix (5X)	4 µl	5'	85 °C
Nuclease-free water	6 µl	∞	4 °C

The cDNA samples obtained from the reverse transcription were stored at -20 °C.

3.14 Real-Time PCR assays

All gene expression analyses performed in this study were conducted on cDNA obtained by reverse transcription of RNA from peripheral blood of the subjects.

To investigate the PRC2 dysregulation, the expression levels of genes encoding PRC2 components (*SUZ12*, *EZH2*, and *EED*) and PRC2 target genes (*CDKN2B*, *MECP2*, *PSMD11*, *CDKN1B*, *NFKB2*, and *EIF3A*), together with *TBP* gene used

as housekeeping control for normalization, were evaluated in 95 patients carrying intragenic *NF1* variants and 10 patients with type-1 *NF1* deletions (Table 8).

The expression of the haploinsufficient *AP1B1* gene, included within the PT 568 and PT 690 deletions, was analyzed in patients and eight healthy controls. To assess a potential position effect, the expression of genes flanking the PT 568 and PT 690 deletions was examined in these patients and in 10 healthy donors. Twelve 22q12 genes, reported in the GTEx portal (<https://gtexportal.org>) as having an expression level higher than 2 TPM in whole blood, were selected for this analysis, in which the *EIF4A2* gene was used as housekeeping control for normalization.

The procedure for the expression analysis consisted of the following steps:

1. Primer design and preparation

The primers were designed, quality-checked, and reconstituted exactly as described in the “Long-Range PCR assays” section. In addition, to ensure specificity for cDNA, at least one primer of each pair was designed to span an exon-exon junction. The analyzed genes, their classification, and the sequence of the corresponding primers used for qPCR assays are reported in Table 20.

Table 20. Sequences of forward (F) and reverse (R) primers designed for qPCR assays

Classification	Gene target	Primer ID	Primer sequence (5' to 3')
22q12 chromosome region genes	<i>AP1B1</i>	<i>AP1B1</i> qPCR_F	CCTGGTCACACTGCTGTCA
		<i>AP1B1</i> qPCR_R	TCAGGCCTTTCTGCACGAT
	<i>PITPNB</i>	<i>PITPNB</i> qPCR_F	GTCAAGTTGAACCAAGCAGACT
		<i>PITPNB</i> qPCR_R	GCCTCTCTTGGTCTTGACTG
	<i>HSCB</i>	<i>HSCB</i> qPCR_F	GCGTCTTGTCACCCAGATT
		<i>HSCB</i> qPCR_R	GGAGCTTTAGAAGGTACAGTC
	<i>MORC2</i>	<i>MORC2</i> qPCR_F	GCTCCAAAACCTGTGCAATTC
		<i>MORC2</i> qPCR_R	TTGATGTCTATGTCTCTCTGC
	<i>SELENOM</i>	<i>SELENOM</i> qPCR_F	GATGAGCCTCCTGTTGCCTC
		<i>SELENOM</i> qPCR_R	TCATCCCCCGCAGGTCTCTA
	<i>RNF185</i>	<i>RNF185</i> qPCR_F	GCAGGACAGCACTTTCGAG
		<i>RNF185</i> qPCR_R	ACCACTGATGTAAACACGGC
	<i>LIMK2</i>	<i>LIMK2</i> qPCR_F	CTCTTGCTTCCGGTGTTTCCAG
		<i>LIMK2</i> qPCR_R	GCAGTAGAGCTTCCCATCC
	<i>PIK3IP1</i>	<i>PIK3IP1</i> qPCR_F	GGGTACAAGCATTCTCTGTC
		<i>PIK3IP1</i> qPCR_R	GTGTGCCAGAAACAGCCTC
	<i>PATZ1</i>	<i>PATZ1</i> qPCR_F	GCCTGATCACTTGAACGGAC
		<i>PATZ1</i> qPCR_R	GTCTCGGGTGGCAAAAAG
	<i>DRG1</i>	<i>DRG1</i> qPCR_F	CGTCAAGTCATTGCAGTGGC
		<i>DRG1</i> qPCR_R	TAATGCCTCCCTTGTCTTCT
	<i>EIF4ENIF1</i>	<i>EIF4ENIF1</i> qPCR_F	AGGATAGTAGATCCACGAGAG
		<i>EIF4ENIF1</i> qPCR_R	CGAAGCCCATCACTATCTTTC
	<i>SF11</i>	<i>SF11</i> qPCR_F	GAGTATTTCCCTCTAAAGCCA
		<i>SF11</i> qPCR_R	CACTCGTGCTGGAAAACCC
	<i>PISD</i>	<i>PISD</i> qPCR_F	GCACCGATGTCTGGGATTAC
		<i>PISD</i> qPCR_R	TAAGGGCTGTAGGGATTGGC

PRC2 component genes	<i>SUZ12</i>	<i>SUZ12</i> qPCR_F	TGCAGCTTACGTTTACTGGT
		<i>SUZ12</i> qPCR_R	GGTCAGGATTCCAAAGGCACC
	<i>EZH2</i>	<i>EZH2</i> qPCR_F	TACCAGCATTTGGAGGGAGC
		<i>EZH2</i> qPCR_R	CCTGCTTCCCTATCACTGTC
	<i>EED</i>	<i>EED</i> qPCR_F	ACTGGCACAGTAAAGAAGGAG
		<i>EED</i> qPCR_R	CATCAGCATCAGCATCCACG
PRC2 target genes	<i>CDKN2B</i>	<i>CDKN2B</i> qPCR_F	GAATGCGCGAGGAGAACAAG
		<i>CDKN2B</i> qPCR_R	CCATCATCATGACCTGGATCG
	<i>MECP2</i>	<i>MECP2</i> qPCR_F	GCTCCAGGAAGTGGCAGAGG
		<i>MECP2</i> qPCR_R	CGTTTCTGGGAATGGCCTG
	<i>PSMD11</i>	<i>PSMD11</i> qPCR_F	CATCGACATCCTCCACTCCA
		<i>PSMD11</i> qPCR_R	CCAGTCTTTGCCAGGAGAGA
	<i>CDKN1B</i>	<i>CDKN1B</i> qPCR_F	CTCTGAGGACACGCATTGG
		<i>CDKN1B</i> qPCR_R	GAGTAGAAGAATCGTCGGTTG
	<i>NFKB2</i>	<i>NFKB2</i> qPCR_F	TTCTGAAGGCTGGTGCTGAC
		<i>NFKB2</i> qPCR_R	AAGTGAGGTCAAGAGGCGTG
	<i>EIF3A</i>	<i>EIF3A</i> qPCR_F	ATTAGTCAAGCCAGGTGGATG
		<i>EIF3A</i> qPCR_R	TCCTTGTCATTCTCTCCCG
Housekeeping genes	<i>TBP</i>	<i>TBP</i> qPCR_F	CGGCTGTTTAACCTCGCTTC
		<i>TBP</i> qPCR_R	CACACGCCAAGAAACAGTGA
	<i>EIF4A2</i>	<i>EIF4A2</i> qPCR_F	GGTCAGGTCAAGTCGTGTT
		<i>EIF4A2</i> qPCR_R	CCCCTCTGCCAATTCTGTG

2. Real-Time PCR setup

The Real-Time PCR (or quantitative PCR, qPCR) assays were performed using the GoTaq® qPCR Master Mix kit (Promega), stored at -20 °C. This kit contains all the reagents required for amplification, including the SYBR® Green fluorescent dye. SYBR® Green intercalates into double-strand DNA during amplification, allowing real-time detection of the PCR product. The reactions were set up in 384-well plates, with the reaction mixture prepared and incubated according to the protocol summarized in Table 21.

Table 21. Composition of qPCR reaction mix and incubation conditions

Reaction mix		Thermal cycler conditions		
Reagent	Volume	Time	Temperature	No. cycles
Master mix	5 µl	2'	50 °C	} 40
Primer F (10 µM)	0.2 µl	10'	95 °C	
Primer R (10 µM)	0.2 µl	15''	95 °C	
cDNA	2.5 µl	1'	60 °C	
Nuclease-free water	2.1 µl	∞	4 °C	

Prior to addition to the reaction mix, cDNA samples were diluted 1:3 with nuclease-free water. Each sample was analyzed in technical triplicate. In addition, to exclude the presence of contaminants, a negative control (no-template control,

NTC) was included for each primer pair, consisting of a reaction mixture identical to that of the samples but without cDNA. The qPCR reactions were performed using the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific).

3. Statistical analysis

For each sample, the mean Cycle threshold (Ct) value obtained from the three technical replicates was calculated. Gene expression levels were determined for each sample using the $2^{-\Delta Ct}$ method (ΔCt = mean Ct of target gene - mean Ct of housekeeping gene). For all analyzed genes, for each sample group, outliers were identified and excluded using the Tukey Fences test ($k = 1.5$). Subsequently, the mean, standard deviation (SD), standard error of the mean (SEM), and 95% confidence interval (CI) were calculated.

The sample groups included: (i) for PRC2 expression analysis, 95 patients carrying intragenic *NFI* variants and 10 patients with type-1 *NFI* deletions; (ii) for *APIBI* expression analysis, two biological replicates from PT 568, two biological replicates from PT 690, and eight healthy controls; and (iii) for position effect analysis, 10 healthy donors.

For the PRC2 study, the results obtained in the analyzed patients were also compared with those previously obtained from the biological triplicate of PT 171 and 10 healthy controls (Tritto *et al.*, 2023b). Statistical analyses were performed using one-way ANOVA followed by Tukey's comparison test. For the *APIBI* expression analysis, comparisons between means were performed using the independent samples Student's *t*-test, assuming equal variances.

All statistical analyses were carried out using the Statistics Kingdom software (<https://www.statskingdom.com/>). Results were considered statistically significant when the *p*-value was < 0.05 .

A statistical analysis could not be conducted for the position effect study due to the unavailability of patient RNA required to perform biological duplicates or triplicates.

3.15 Polymerase Chain Reaction assays

In this study, several Polymerase Chain Reaction (PCR) assays were performed for different purposes: to exclude Mycoplasma contamination by analyzing the culture medium of PT 171 and PT 1051 iPSC lines; to verify the clearance of Sendai reprogramming vectors and the expression of pluripotency markers by analyzing cDNA from the same cell lines; to amplify the deletion junction fragments of NF2 patients PT 568 and PT 690 using gDNA; and to identify healthy controls carrying the same heterozygous SNPs detected in PT 568 and PT 690, within genes affected by position effect, by testing their cDNA for future DAE analyses.

All PCR assays were carried out following the steps described below.

1. Primer design and preparation

To amplify the junction fragments of the deletions identified in patients PT 568 and PT 690, one primer pair was designed for each patient, consisting of a forward primer located upstream and a reverse primer located downstream of the deletion. The primers were designed, quality-checked, and reconstituted exactly as described in the “Long-Range PCR assays” section. Additional primer pairs were designed on cDNA upstream and downstream of two and three SNPs identified in PT 568 and PT 690, respectively, following the same procedures detailed in the “Real-Time PCR assays” section. The primers used for the remaining PCR assays were already available in the laboratory.

The sequences and the annealing temperature (T_a) of all forward and reverse primers employed in the various PCR assays are listed in Table 22.

Table 22. Sequences of forward (F) and reverse (R) primers used for PCR assays

Analysis	Target	Primer ID	Primer Sequence (5' to 3')	T_a
Assessment of Sendai reprogramming vector loss	<i>KLF4</i>	<i>KLF4_cDNA_F</i>	TTCCTGCATGCCAGAGGAGCCC	54 °C
		<i>KLF4_cDNA_R</i>	AATGTATCGAAGGTGCTCAA	
	<i>KOS</i>	<i>KOS_cDNA_F</i>	ATGCACCGCTACGACGTGAGCGC	54 °C
		<i>KOS_cDNA_R</i>	ACCTTGACAATCCTGATGTGG	
	<i>c-MYC</i>	<i>c-MYC_cDNA_F</i>	TAACTGACTAGCAGGCTTGTCG	54 °C
		<i>c-MYC_cDNA_R</i>	TCCACATACAGTCCTGGATGATGATG	
	<i>SeV</i>	<i>SeV_cDNA_F</i>	GGATCACTAGGTGATATCGAGC	54 °C
		<i>SeV_cDNA_R</i>	ACCAGACAAGAGTTTAAGAGATATGTATC	

Evaluation of pluripotency marker expression	<i>SOX2</i>	<i>SOX2_cDNA_F</i>	GGGAAATGGGAGGGGTGCAAAAGAGG	60 °C
		<i>SOX2_cDNA_R</i>	TTGCGTGAGTGTGGATGGGATTGGTG	
	<i>OCT3/4</i>	<i>OCT3/4_cDNA_F</i>	GACAGGGGGAGGGGAGGAGCTAGG	60 °C
		<i>OCT3/4_cDNA_R</i>	CTTCCCTCCAACCAGTTGCCCAAAC	
	<i>Nanog</i>	<i>Nanog_cDNA_F</i>	CAGCCCTGATTCTTCCACCAGTCC	60 °C
		<i>Nanog_cDNA_R</i>	GTTCTGGAACCAGGTCTTCACCTG	
Amplification of deletion junction fragments	PT 568 deletion-flanking region	BPcent568_gDNA_F	GTTAGCACATACACACCCCG	57 °C
		BPtel568_gDNA_R	AGGCTCCACTTCTACGATCC	
	PT 690 deletion-flanking region	BPcent690New_gDNA_F	TTCACAGTTACCGTATGCCAG	57 °C
		BPtel690New_gDNA_R	ATAACCAGGCAGCAATTAGGG	
SNP screening for DAE analysis	<i>MORC2</i> c.378C>G	cDNA_ <i>MORC2</i> ex5-7_F	ATGCTGCCAGTGTGATCCAG	57 °C
		cDNA_ <i>MORC2</i> ex5-7_R	CAGTGCGGAATGGAGAGTAC	
	<i>MORC2</i> c.1437A>G	cDNA_ <i>MORC2</i> ex14-16_F	TCCTGGAGCCTACACACAAC	57 °C
		cDNA_ <i>MORC2</i> ex14-16_R	AGGATCAGGGTTCATGGAGC	
	<i>LIMK2</i> c.103G>A	cDNA_ <i>LIMK2</i> ex2-3_F	GAAGATGTCTGGAGGTGTCC	57 °C
		cDNA_ <i>LIMK2</i> ex2-3_R	GAGCACCCATGACAGAACTC	
	<i>SFI1</i> c.1646G>A	cDNA_ <i>SFI1</i> ex15-18_F	AAGAGCAACACGTTTCCACAG	57 °C
		cDNA_ <i>SFI1</i> ex15-18_R	TCCTCTCTGTTCTGAGCCCT	
	<i>SFI1</i> c.3260T>C	GcDNA_ <i>SFI1</i> ex29_F	CCTCAGAAGCCACAGGAAC	55 °C
		cDNA_ <i>SFI1</i> ex31_R	GTCTGGTAGTGCAGTAGTTG	

2. PCR setup

For the Mycoplasma detection assay, the Mycoplasma PCR Detection Kit (abm®), stored at -20 °C, was used. This kit allows the detection of more than 200 Mycoplasma strains with high specificity and sensitivity. It provides a ready-to-use MasterMix containing all necessary reaction components, a pre-formulated Primer Mix, and a positive control. The reaction mixture was prepared in a final volume of 25 µl according to the manufacturer's instructions. The detailed composition of the reaction mix and the thermal cycling conditions used are reported in Table 23.

Table 23. Composition of Mycoplasma PCR detection reaction mix and incubation conditions

Reaction mix		Thermal cyclor conditions		
Reagent	Volume	Time	Temperature	No. cycles
BlasTaq™ 2X PCR MasterMix	12.5 µl	3'	95 °C	} 35
Primer Mix	1 µl	15''	95 °C	
Test Sample, Positive Control, or NTC	2.5 µl	15''	55 °C	
Nuclease-free water	9 µl	15''	72 °C	
		1'	72 °C	
		∞	4 °C	

The remaining PCR assays were performed using the GoTaq® G2 DNA Polymerase kit (Promega), stored at -20 °C. The reaction mixture components and the conditions set on the thermocycler to allow amplification of the target regions are reported in Table 24.

Table 24. Composition of GoTaq® G2 DNA Polymerase reaction mix and incubation conditions for PCR assays

Reaction mix		Thermal cycler conditions		
Reagent	Volume	Time	Temperature	No. cycles
5X GoTaq® Reaction Buffer	5 µl	5'	95 °C	} 35
dNTPs (10 mM)	2.5 µl	30''	95 °C	
GoTaq® G2 DNA Polymerase (5 U/µl)	0.25 µl	30''	x (see Table 22)	
Primer F (10 µM)	0.6-3.75 µl	30''	72 °C	
Primer R (10 µM)	0.6-3.75 µl	5'	72 °C	
cDNA/gDNA	2-5 µl/40 ng	∞	4 °C	
Nuclease-free water	up to 25 µl			

For all PCR assays, the amplification reaction included an initial denaturation step at 95 °C, allowing complete separation of the DNA double strands, followed by an annealing step at a temperature specific for each primer pair, enabling primer binding to the complementary DNA template sequences. This was followed by an extension step at 72 °C, the optimal temperature for Taq polymerase activity. These three temperature steps were repeated for 35 cycles to achieve amplification of the target fragments.

3. Gel electrophoresis

To assess the success of the PCR assays, the amplified products were separated by agarose gel electrophoresis, as previously described in the “Long-Range PCR assays” section. The only modification to the previous protocol consisted in running the samples on a 1.5% agarose gel at 130 V for 30 minutes.

3.16 Sanger sequencing

Before setting up a sequencing reaction, the PCR products were purified. This procedure was performed by incubating the amplified products at 37 °C with a mixture containing Exonuclease I (ExoI, New England BioLabs), which degrades residual PCR oligonucleotides, and Shrimp Alkaline Phosphatase (SAP, New England BioLabs), which inactivates the remaining deoxynucleotide triphosphates (dNTPs) by acting on their phosphate groups. Subsequently, the enzymes were inactivated by heating the samples to 80 °C. The composition of the reaction mix and the thermocycler conditions used for PCR product purification are reported in Table 25.

Table 25. Composition of reaction mix and incubation conditions for PCR product purification

Reaction mix		Thermal cycler conditions	
Reagent	Volume	Time	Temperature
SAP (1 U/μl)	0.8 μl	25'	37 °C
ExoI (20 U/μl)	0.4 μl	15'	80 °C
PCR product	$1 \leq x \leq 4$ μl	∞	4 °C
Nuclease-free water	up to 7 μl		

For the sequencing reactions, set up in 96-well plates with a final volume of 10 μl per sample, the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) was used. This kit contains the DNA polymerase along with both labeled dideoxynucleotide triphosphates (ddNTPs) and unlabeled dNTPs. The composition of the reaction mix and the thermocycler conditions used for the PCR product sequencing are reported in Table 26.

Table 26. Composition of reaction mix and incubation conditions for PCR product sequencing

Reaction mix		Thermal cycler conditions		
Reagent	Volume	Time	Temperature	No. cycles
BigDye® 5X Buffer	0.6 µl	1'	96 °C	} 25
BigDye	0.4 µl	15''	96 °C	
Primer (10 µM)	0.5 µl	10''	50 °C	
PCR purified	7 µl	2'	60 °C	
Nuclease-free water	1.5 µl	∞	4 °C	

The sequencing reaction was purified using the BigDye XTerminator™ Purification Kit (Thermo Fisher Scientific). To each 10 µl sample, 36 µl of SAM™ Solution and 6 µl of X-Terminator™ Solution were added to facilitate precipitation of nonspecific fragments, and the final volume was adjusted to 60 µl with nuclease-free water. Empty wells of the same run were also brought to 60 µl with nuclease-free water. The plate was incubated on a shaker for 30 minutes at 2,500 rpm, followed by centrifugation for 1.5 minutes (Sigma 2-16P), and subsequently loaded onto an ABI-3130xl DNA Genetic Analyzer (Thermo Fisher Scientific) with 16 capillaries. Electropherograms were visualized using Chromas Lite software (Technelysium Pty Ltd).

4. RESULTS

4.1 Identification of potential mechanisms contributing to phenotypic severity and variability in NF1 microdeletion patients

The severity and complexity of the phenotype observed in patients affected by NF1 microdeletion syndrome have been historically attributed to the haploinsufficiency of genes within the deleted region. This relationship has been supported by genotype-phenotype correlations identified through functional studies for some of these genes (e.g., *SUZ12* and *ADAP2*), as well as by the dosage sensitivity of five genes (*ATAD5*, *NF1*, *OMG*, *RAB11FIP4*, and *SUZ12*), each showing a pLI value greater than 0.9.

In recent years, we proposed additional pathogenic mechanisms to influence the phenotypic variability and severity shown by the NF1 microdeletion patients, including variants in modifier genes, correlated with specific clinical traits manifested by patients, and the position effect on genes flanking the deleted region, validated through the 4C-seq functional study (Ferrari *et al.*, 2017; Tritto *et al.*, 2024). Moreover, in PT 171, who carries an atypical *NF1* deletion generating the chimeric gene *RNF135-SUZ12*, we previously identified a dysfunction of the PRC2 transcriptional repressive complex, of which *SUZ12* is a core subunit. This alteration was potentially associated with the tumor predisposition phenotype exhibited by PT 171 (Tritto *et al.*, 2023b).

Therefore, part of the research presented in this thesis aimed to further elucidate the pathogenic mechanisms underlying the phenotypic severity and variability of NF1 microdeletion syndrome. To gain a better understanding of the molecular basis of PRC2 dysregulation and its potential contribution to the clinical phenotype, the analysis of PRC2 component and target genes was extended to (i) a cohort of patients carrying the type-1 *NF1* deletion, which results in the complete loss of one *SUZ12* allele, and (ii) a cohort of patients with intragenic *NF1* mutations, who share with the microdeletion patients the hyperactivation of the Ras signaling pathway, also potentially related to PRC2 activity.

In addition, the molecular basis of the complex phenotype observed in PT 171 was further explored through the assessment of variants in candidate modifier genes identified by WES analysis.

Finally, to establish reliable cellular models for functional studies aimed at elucidating and validating the pathogenic mechanisms driving NF1 microdeletion syndrome, patient-specific iPSC lines were generated.

4.1.1 Expression analysis of PRC2 component and target genes in NF1 patients

In a previous study, a qPCR-based expression analysis performed on cDNA retrotranscribed from peripheral blood RNA of PT 171 and 10 healthy donor (HD) controls revealed in our patient increased expression of *EZH2*, encoding the PRC2 catalytic subunit, along with reduced expression of *SUZ12* and *EED*, encoding the other two PRC2 core subunits, and of five out of seven PRC2 target genes analyzed. The reduced expression of PRC2 target genes suggested an enhanced transcriptional repression mediated by PRC2, which was hypothesized to result from either the incorporation of the RNF135-SUZ12 chimeric protein into the complex, the reduced dosage of wild type *SUZ12*, or the upregulation of *EZH2* potentially associated with Ras hyperactivation (Tritto *et al.*, 2023b).

Building upon these preliminary findings, we extended the qPCR expression analysis of the three PRC2 core component genes (*SUZ12*, *EZH2*, and *EED*) and six PRC2 target genes (*CDKN2B*, *MECP2*, *PSMD11*, *CDKN1B*, *NFKB2*, and *EIF3A*) to two additional NF1 patient cohorts: (i) 10 patients with the type-1 *NF1* deletion (NF1 MD), resulting in the complete loss of one *SUZ12* allele; and (ii) 95 patients carrying intragenic *NF1* mutations (NF1 IM), in which *SUZ12* is not genetically altered, but Ras pathway hyperactivation is shared with microdeletion patients.

The qPCR results, obtained analyzing the cDNA retrotranscribed from peripheral blood RNA of the newly patients, were compared with the previously generated dataset from three biological replicates of PT 171 and from 10 HD

controls. Statistical significance was assessed using one-way ANOVA, followed by Tukey's multiple comparison test (Table 27).

Table 27. Statistical analysis of qPCR data for the expression of PRC2 component and target genes

Gene	Samples	N	$2^{-(\Delta Ct)}$ mean	SD	SEM	95% CI		One-way ANOVA - Tukey's Multiple Comparison Test adjusted <i>p</i> -value			
						Lower	Upper	vs HD	vs NF1 IM	vs NF1 MD	vs PT 171
<i>SUZ12</i>	HD	10	12.060	1.206	0.381	11.197	12.923	-	<0.0001	0.2243	0.6407
	NF1 IM	92	29.989	9.533	0.993	28.015	31.964	<0.0001	-	<0.0001	<0.0001
	NF1 MD	10	4.609	2.336	0.739	2.938	6.280	0.2243	<0.0001	-	0.9993
	PT 171	3	5.331	0.251	0.145	4.708	5.954	0.6407	<0.0001	0.9993	-
<i>EZH2</i>	HD	10	0.191	0.029	0.009	0.170	0.212	-	<0.0001	0.0036	0.9590
	NF1 IM	91	3.155	0.851	0.089	2.977	3.332	<0.0001	-	<0.0001	<0.0001
	NF1 MD	8	1.516	0.663	0.234	0.961	2.070	0.0036	<0.0001	-	0.2049
	PT 171	3	0.453	0.239	0.138	-0.142	1.047	0.9590	<0.0001	0.2049	-
<i>EED</i>	HD	10	5.153	0.755	0.239	4.612	5.693	-	<0.0001	<0.0001	0.0022
	NF1 IM	93	3.297	0.589	0.079	3.176	3.418	<0.0001	-	<0.0001	0.6075
	NF1 MD	10	1.977	0.499	0.158	1.620	2.334	0.0001	<0.0001	-	<0.0001
	PT 171	3	3.726	0.242	0.140	3.124	4.329	0.0022	0.6075	<0.0001	-
<i>CDKN2B</i>	HD	10	0.043	0.007	0.002	0.038	0.049	-	<0.0001	<0.0001	0.0007
	NF1 IM	89	0.01	0.005	0.0005	0.010	0.012	<0.0001	-	<0.0001	0.0121
	NF1 MD	10	0.028	0.019	0.006	0.014	0.042	<0.0001	<0.0001	-	0.8185
	PT 171	3	0.024	0.0004	0.0002	0.023	0.025	0.0007	0.0121	0.8185	-
<i>MECP2</i>	HD	9	1.232	0.138	0.046	1.125	1.338	-	<0.0001	0.0291	0.7490
	NF1 IM	92	0.788	0.167	0.024	0.754	0.823	<0.0001	-	0.0014	0.0091
	NF1 MD	10	1.007	0.259	0.082	0.822	1.192	0.0291	0.0014	-	0.7776
	PT 171	3	1.116	0.062	0.036	0.961	1.270	0.7490	0.0091	0.7776	-
<i>PSMD11</i>	HD	8	3.192	0.165	0.058	3.053	3.330	-	0.0007	0.9379	0.6224
	NF1 IM	29	2.192	0.715	0.133	1.920	2.464	0.0007	-	0.0023	0.5095
	NF1 MD	10	3.027	0.475	0.150	2.687	3.367	0.9379	0.0023	-	0.8402
	PT 171	3	2.699	0.033	0.019	2.617	2.782	0.6224	0.5095	0.8402	-
<i>CDKN1B</i>	HD	8	11.220	0.839	0.297	10.518	11.921	-	<0.0001	<0.0001	<0.0001
	NF1 IM	93	3.720	0.899	0.093	3.535	3.906	<0.0001	-	0.7929	<0.0001
	NF1 MD	8	4.014	0.282	0.099	3.779	4.250	<0.0001	0.7929	-	<0.0001
	PT 171	3	7.840	0.646	0.373	6.236	9.446	<0.0001	<0.0001	<0.0001	-
<i>NFKB2</i>	HD	10	22.959	5.158	1.631	19.269	26.648	-	<0.0001	<0.0001	0.3202
	NF1 IM	29	1.458	0.403	0.075	1.305	1.612	<0.0001	-	0.9256	<0.0001
	NF1 MD	8	2.030	0.569	0.201	1.555	2.506	<0.0001	0.9256	-	<0.0001
	PT 171	3	25.597	0.597	0.345	24.113	27.082	0.3202	<0.0001	<0.0001	-
<i>EIF3A</i>	HD	9	124.697	16.307	5.436	112.162	137.232	-	<0.0001	<0.0001	0.0183
	NF1 IM	26	56.937	11.960	2.345	52.106	61.767	<0.0001	-	<0.0001	<0.0001
	NF1 MD	9	20.666	4.596	1.532	17.133	24.199	<0.0001	<0.0001	-	<0.0001
	PT 171	3	100.654	3.783	2.184	91.258	110.051	0.0183	<0.0001	<0.0001	-

N, number of samples per group after excluding outliers identified by Tukey's test; SD, standard deviation; SEM, standard error of the mean; CI, confidence interval; HD, healthy donor controls; NF1 IM, patients with intragenic *NF1* mutations; NF1 MD, patients with type-1 *NF1* deletion; PT 171, patient 171.

The qPCR results are shown in Figure 17, where the mean expression levels of each gene in the different groups were normalized to those of the HD controls, set to 1.

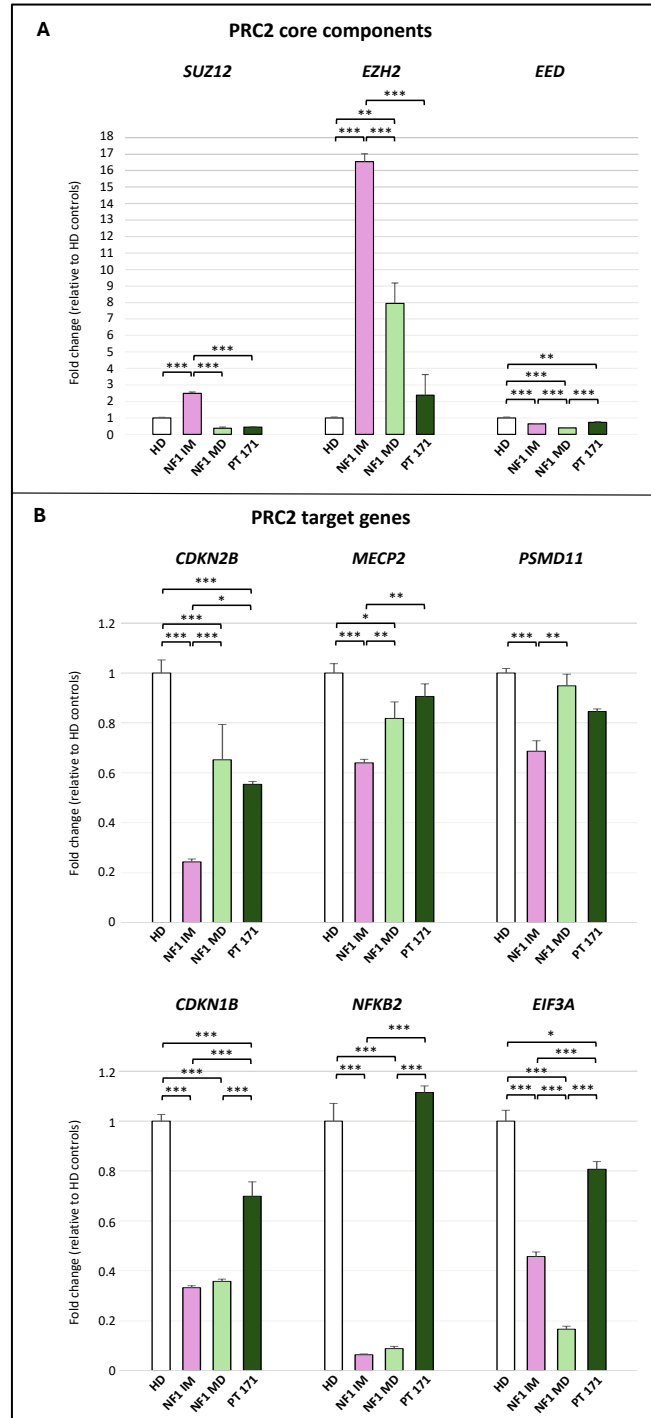


Figure 17. Expression levels of PRC2 component and target genes. (A) Among the PRC2 core component genes, *SUZ12* was overexpressed in NF1 IM patients and hypoexpressed in NF1 microdeletion patients compared with HD controls, whereas *EZH2* was overexpressed and *EED* hypoexpressed in all NF1 patient groups relative to HD controls. (B) All PRC2 target genes were hypoexpressed in all NF1 patient groups compared with HD controls, except for *NFKB2* in PT 171. For all analyses, mean expression levels were normalized to HD controls (set to 1) and are presented as mean $\pm$ SEM. * p -adjusted <0.05 , ** p -adjusted <0.01 , *** p -adjusted <0.001 , one-way ANOVA, followed by Tukey's multiple comparison test.

Regarding the genes encoding PRC2 components (Fig. 17A), *SUZ12* expression was significantly increased by 2.5-, 6.5-, and 5.5-fold in NF1 IM patients compared with HD controls, NF1 MD patients, and PT 171, respectively. In contrast, a greater than 50% reduction in *SUZ12* expression was detected in both NF1 MD patients and PT 171 compared with HD controls, although this decrease did not reach statistical significance. *EZH2* expression was 16.5-, 8-, and >2-fold higher in NF1 IM patients ($p < 0.0001$), NF1 MD patients ($p < 0.01$), and PT 171 (not significant), respectively, compared with HD controls. A statistically significant difference in *EZH2* expression was also observed between NF1 IM patients and NF1 microdeletion patients. *EED* expression was reduced by ~30-60%, with statistical significance, in all NF1 patient cohorts compared with HD controls. Notably, NF1 MD patients showed a significantly stronger reduction in *EED* expression than the other NF1 groups.

Regarding the PRC2 target genes (Fig. 17B), a trend of reduced expression was observed in all NF1 patient groups compared with HD controls, with the only exception of *NFKB2* in PT 171. Specifically, all six genes, five genes (*CDKN2B*, *MECP2*, *CDKN1B*, *NFKB2*, and *EIF3A*), and three genes (*CDKN2B*, *CDKN1B*, and *EIF3A*) showed a statistically significant reduction in NF1 IM patients, NF1 MD patients, and PT 171, respectively, compared with HD controls. Except for *EIF3A*, the lowest expression levels were consistently detected in NF1 IM patients.

Notably, comparing the NF1 microdeletion patients, PT 171 displayed significantly higher expression than NF1 MD patients for three out of six genes (*CDKN1B*, *NFKB2*, and *EIF3A*). Moreover, when re-evaluating the expression levels of PRC2 component and target genes by considering all NF1 microdeletion patients as a single group, PT 171 emerged as an outlier compared with the NF1 MD patients with a type-1 *NF1* deletion for two out of the nine genes analyzed (*NFKB2* and *EIF3A*) (Fig. 18).

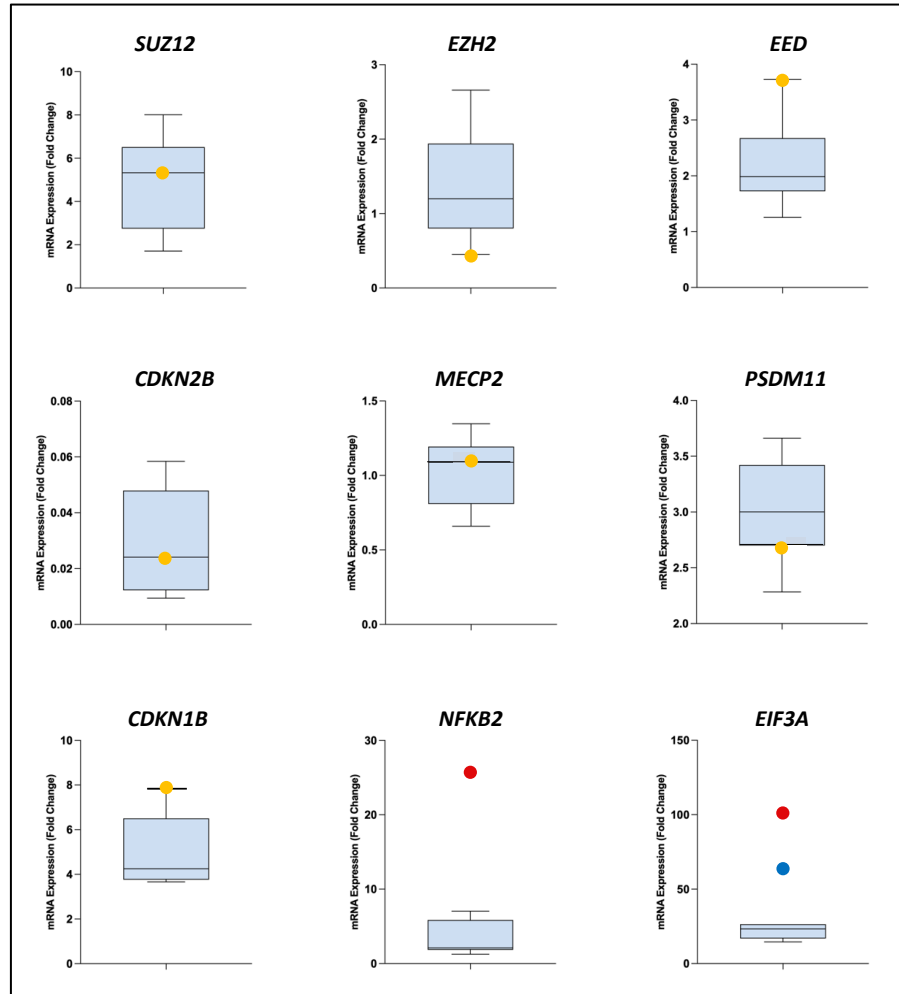


Figure 18. Distribution of expression levels of PRC2 component and target genes in NF1 deletion patients. PT 171, shown as a yellow dot, was plotted within the NF1 MD cohort. The lower and upper edges of each box indicated the first quartile (25th percentile) and third quartile (75th percentile), respectively, and the line inside the box represented the median (50th percentile). Whiskers denoted the minimum and maximum values, excluding outliers, which were displayed as points outside the whisker range. Blue and red dots marked NF1 MD and PT 171 outliers identified by the Tukey fences method ($k = 1.5$).

4.1.2 Identification of variants in modifier genes in PT 171

To identify potential genetic variants contributing to the clinical phenotype variability observed in PT 171, a WES analysis was performed by an external service provider (Macrogen Europe), and the raw sequencing data were analyzed by Dr. Mangano (ITB, CNR). Following quality control of FASTQ files and alignment of reads to the human reference genome (GRCh38/hg38), the resulting BAM files were processed using the DRAGEN Enrichment pipeline on the Illumina

BaseSpace platform. The DRAGEN Enrichment Sequencing Report for the PT 171 sample is summarized in Table 28.

Table 28. DRAGEN Enrichment Sequencing Report for PT 171 WES data

Total PF reads	Total aligned reads	Total aligned bases	Mean target coverage depth	Total small variants identified	Total SVs identified
70,034,154	59,727,388	8,842,800,009	64.6x	81,230 SNVs 5,047 insertions 5,155 deletions	142 SV insertions 127 SV deletions 1 SV tandem duplication 35 SV breakends

PF, passing filter; SNVs, single nucleotide variants; SVs, structural variants.

Variant annotation was carried out using ANNOVAR, which yielded 91,946 annotated variants following an initial filtering process based on default settings. For variant prioritization, the annotated dataset was further refined according to the following criteria (Fig. 19): (i) variants failing the “PASS” quality filter were removed; (ii) intronic, intergenic, UTR, non-coding RNA, upstream, and downstream variants were excluded; (iii) synonymous and non-frameshift variants were excluded; (iv) variants with a $MAF \geq 1\%$ (based on EUR.sites.2015_08 and ExAC_NFE datasets) were excluded; and (v) variants classified as benign, likely benign, or of uncertain significance (InterVar) were discarded. Only one variant, c.823C>T (p.Arg275Trp) in the *PRKN* gene, successfully passed the entire filtering workflow (Fig. 19).

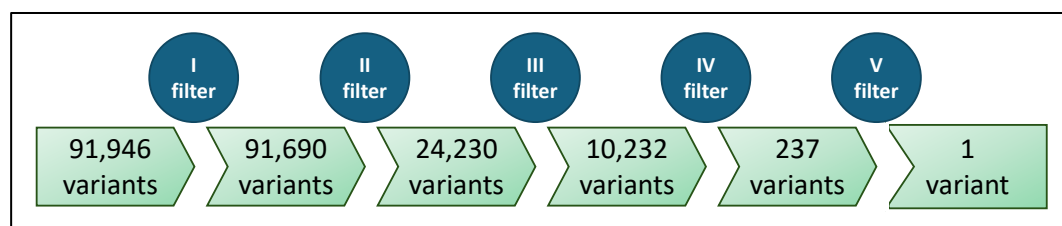


Figure 19. Variant prioritization workflow applied to WES data from PT 171. A stepwise filtering strategy was implemented based on: (I) variant quality, (II) genomic location, (III) predicted impact on transcription/protein function, (IV) MAF, and (V) clinical significance prediction. Starting from 91,946 annotated variants, only one variant successfully passed all filtering steps.

A second variant prioritization analysis was performed on the full set of 91,946 annotated variants, with the aim of identifying potential variants that, although not

rare, could exert a deleterious effect in combination with the *NFI* microdeletion present in the patient. To this purpose, the same filtering criteria applied in the previous analysis were used, except for the MAF threshold, which was increased to 5%, and the inclusion of a gene-based filtering step. Specifically, this analysis focused on candidate genes belonging to selected functional categories (Table 9), including: (i) genes located within the *NFI* deletion region of PT 171, (ii) genes flanking the *NFI* deletion on both the centromeric and telomeric sides, (iii) genes involved in the Ras signaling pathway, and (iv) genes encoding proteins known to interact with neurofibromin. No variants passed the complete filtering workflow.

Following the prioritization analysis, the c.823C>T (p.Arg275Trp) variant in the *PRKN* gene was functionally characterized to evaluate its potential role as a genetic modifier contributing to the complex phenotype observed in PT 171.

The *PRKN* gene, located on chromosome 6q26, encodes multiple transcript isoforms (22 reported), with the canonical transcript spanning 4,178 bp across 12 exons. The resulting protein is composed of 465 aa and functions as part of a multiprotein E3 ubiquitin ligase complex, mediating substrate recognition and targeting for proteasomal degradation.

The c.823C>T (p.Arg275Trp) variant of *PRKN* (NM_004562.3) was detected in PT 171 in the heterozygous state. This variant, associated with the rs34424986 identifier, has a MAF of 0.002 in the European population and is classified as pathogenic according to InterVar (ACMG/AMP 2015 guidelines). It results in the substitution of arginine with tryptophan at codon 275. This variant is located in the RING finger 1 domain of the encoded protein sequence (Fig. 20).

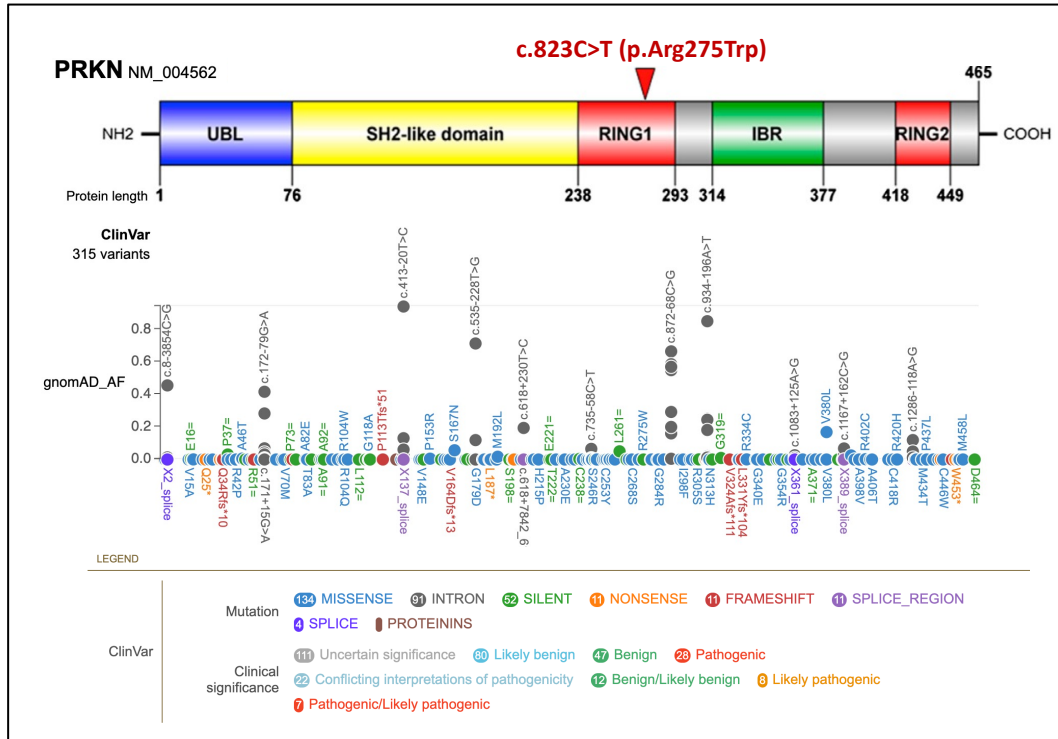


Figure 20. PRKN encoded protein and associated variants. The PRKN encoded protein is shown at the top, with distinct colors marking the functional domains. The lower panel displays the gene variants reported in ClinVar. The red arrowhead highlights the transcript region and residue position affected in PT 171. The image was adapted from the St. Jude Pecan browser and Xiong *et al.*, 2015.

Phylogenetic conservation analysis of the PRKN protein sequence, performed using UniProtKB Align tool, confirmed conservation of the wild type arginine residue at position 275 across human, mouse, and rat orthologs (Fig. 21).

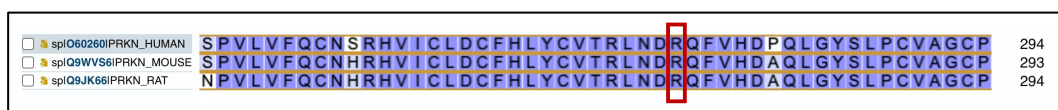


Figure 21. Sequence alignment of PRKN orthologs. Amino acid sequences of human, mouse, and rat PRKN were aligned using UniProtKB. The conserved arginine (R) residue, replaced by tryptophan (W) in PT 171, is indicated by a red box.

According to ClinVar and Franklin databases, focused on genotype-phenotype correlations, this variant has been previously associated with neurological and oncological conditions, including Parkinson's disease, ovarian cancer, lung cancer, and autism spectrum disorders.

4.1.3 Generation and characterization of NF1 patient-specific iPSC models

To better investigate and validate the pathogenic mechanisms proposed to explain the severe and heterogeneous phenotype observed in patients with NF1 microdeletion syndrome, we generated iPSC lines from two patients carrying *NF1* microdeletions: one with a type-1 *NF1* deletion (PT 1051) and one with an atypical deletion (PT 171). Additionally, to discriminate the specific contribution of the deletion from the Ras pathway hyperactivation present in all NF1 patients, the generation of an iPSC line from a patient with an intragenic *NF1* mutation (PT 1749, carrying the *NF1* variant c.5470A>T, p.Ile1824Phe) was also performed.

In collaboration with Professor Ratti's group (Istituto Auxologico), the iPSC lines were generated from patients' PBMCs using a commercially available reprogramming kit based on modified, non-integrating Sendai virus vectors expressing the Yamanaka factors *KLF4*, *OCT3/4*, *SOX2*, and *c-MYC*. Following reprogramming, 7, 5, and 12 colonies derived from PT 1051, PT 171, and PT 1749, respectively, were manually picked (Fig. 22).

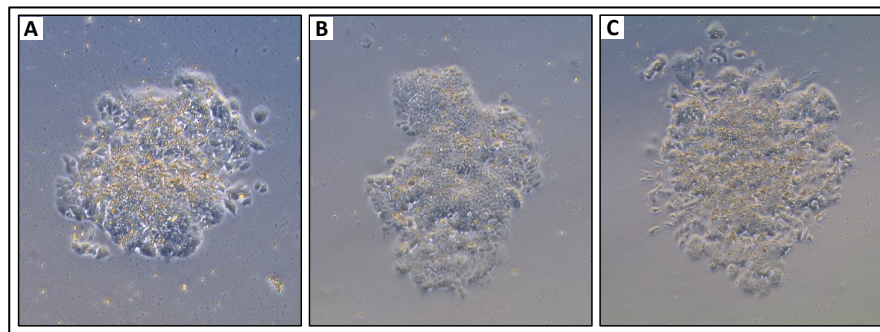


Figure 22. iPSC colonies derived from NF1 patients. Representative bright-field microscopy images of reprogrammed colonies from (A) PT 1051, (B) PT 171, and (C) PT 1749. At the colony-picking stage, all lines exhibited the typical human pluripotent stem cell morphology, including compact structure and well-defined borders.

After the first passages in culture, two clones from PT 1051 (1051_C1 and 1051_C4) and two from PT 171 (171_C1 and 171_C2) retained typical pluripotent stem cell morphology and were subsequently expanded and characterized. In contrast, all colonies derived from PT 1749 underwent spontaneous differentiation into fibroblast-like cells upon passaging, indicating inefficient reprogramming.

Therefore, a second attempt to generate iPSCs from a patient with an intragenic *NF1* mutation was performed using PBMCs from an additional patient (PT 1664, carrying the *NF1* variant c.1467dupT, p.Tyr1824fs). However, this attempt also failed to generate stable iPSC lines.

Morphological and functional characterization was carried out on the 1051_C1, 1051_C4, 171_C1, and 171_C2 iPSC clones.

Karyotype analyses were performed on the four iPSC lines between culture passages 8 and 16 (P8-P16) using QFQ-banding, achieving a resolution of 400 bands per haploid set. A normal male chromosomal complement (46,XY) was observed in all 20 metaphases examined per line, with no evidence of additional chromosomal abnormalities (Fig. 23). As expected, *NF1* microdeletions are below the resolution threshold of standard karyotyping.

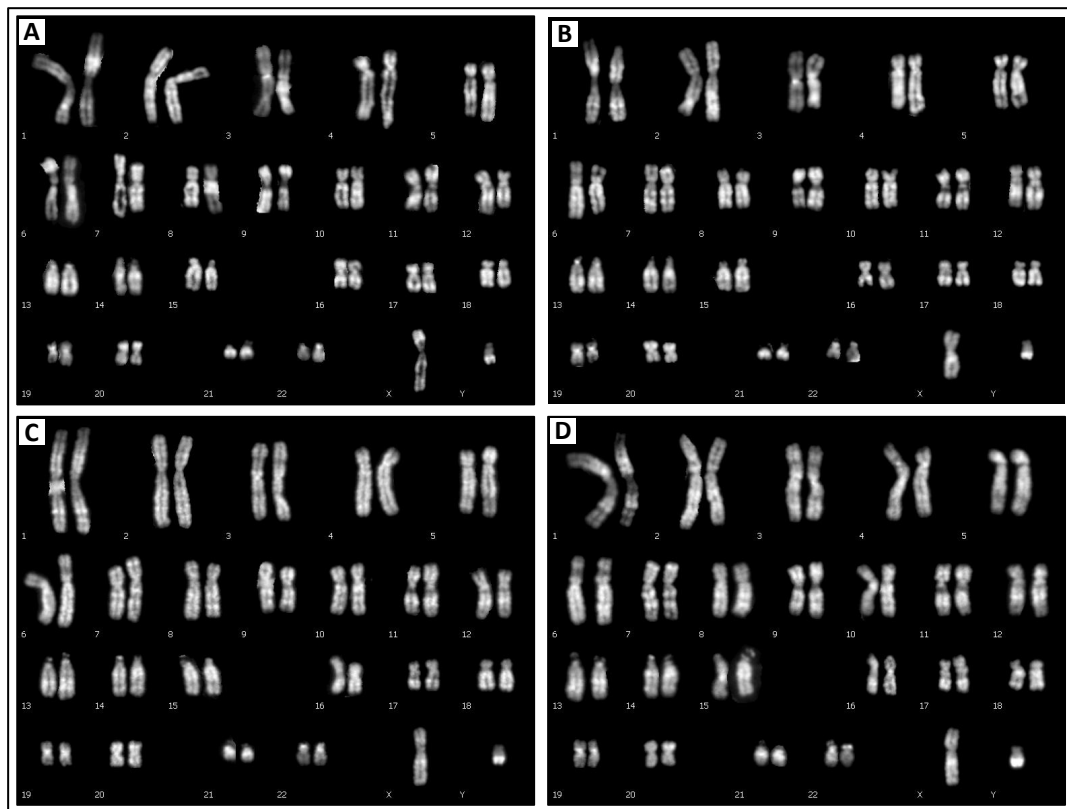


Figure 23. QFQ-banded karyotype analysis of *NF1* microdeletion patient-derived iPSC clones. (A) 1051_C1 (P8), (B) 1051_C4 (P12), (C) 171_C1 (P16), and (D) 171_C2 (P9) iPSC clones exhibited a normal male chromosomal complement (46,XY), as shown in fluorescence microscopy images. As expected, *NF1* microdeletions are not detectable at this resolution.

Pluripotency of the four iPSC clones was verified by assessing the expression of canonical stemness markers *SOX2*, *OCT3/4*, and *Nanog* via PCR on cDNA (Fig. 24).

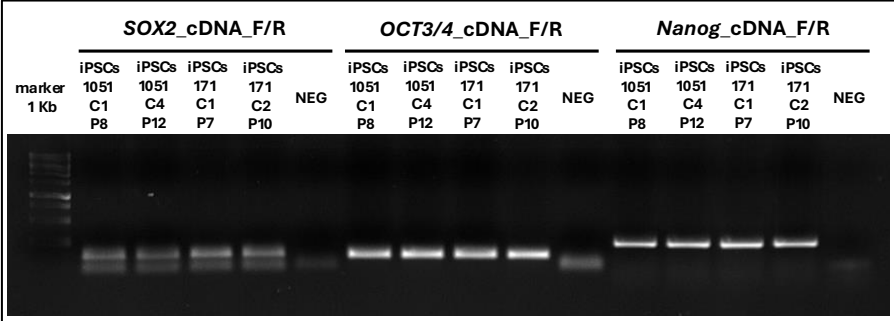


Figure 24. Pluripotency marker expression in NF1 microdeletion patient-derived iPSC clones. The 1051_C1 (P8), 1051_C4 (P12), 171_C1 (P7), and 171_C2 (P10) iPSC clones showed robust expression of the pluripotency markers *SOX2*, *OCT3/4*, and *Nanog*, as assessed by PCR on cDNA.

The expression of pluripotency markers was further confirmed in the 1051_C4 and 171_C2 iPSCs by IF analysis of alkaline phosphatase (AP) and TRA-1-60 (Fig. 25).

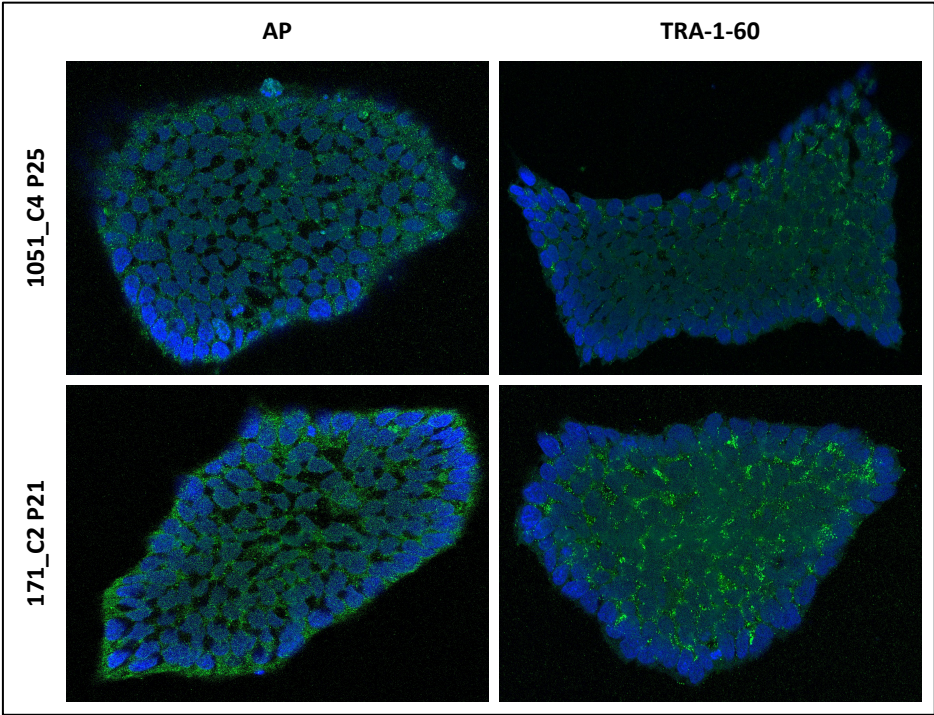


Figure 25. IF analysis of pluripotency markers in NF1 microdeletion patient-derived iPSC clones. The 1051_C4 (P25) and 171_C2 (P21) iPSC clones expressed the intracellular marker AP (green) and the cell surface marker TRA-1-60 (green), as shown by IF images. Cell nuclei were stained with DAPI (blue).

The potential spontaneous differentiation into the three germ layers of 1051_C4 and 171_C2 iPSCs was assessed by *in vitro* embryoid body formation, followed by IF staining for lineage-specific markers: α -fetoprotein (AFP, endoderm), desmin (mesoderm), and β III-tubulin (ectoderm). Both analyzed iPSC clones demonstrated the capacity to differentiate into derivatives of the three germ layers (Fig. 26).

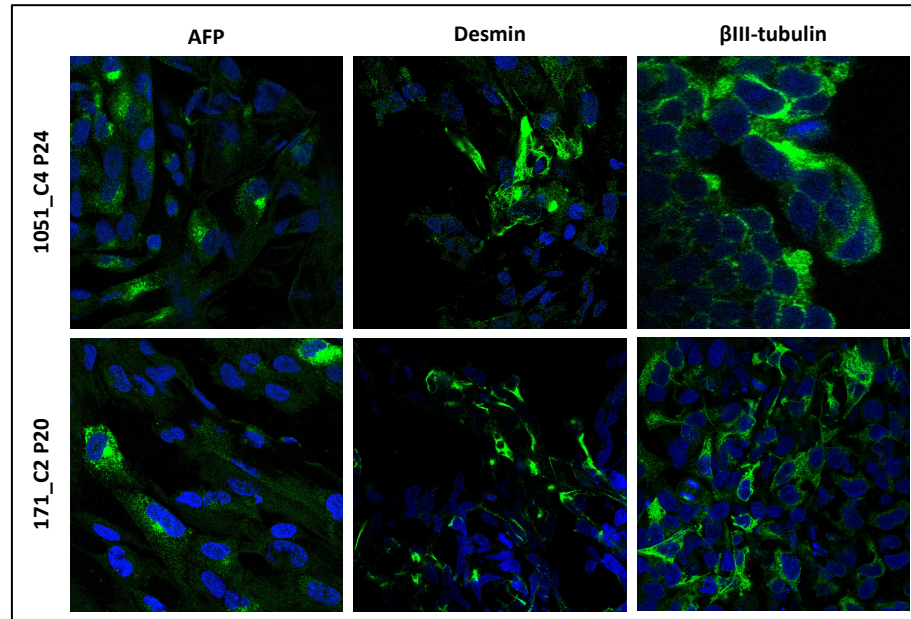


Figure 26. IF analysis of germ layer markers in NF1 microdeletion patient-derived iPSC clones. Following *in vitro* embryoid body formation, the 1051_C4 (P24) and 171_C2 (P20) iPSC clones spontaneously differentiated into derivatives of the three germ layers. Lineage-specific markers were detected by IF staining: AFP (endoderm, green), desmin (mesoderm, green), and β III-tubulin (ectoderm, green). Cell nuclei were stained with DAPI (blue).

To confirm the loss of Sendai virus reprogramming vectors, PCR assays were performed on cDNA from 171_C2 and 1051_C4 iPSCs using primers specific for *KLF4*, *KOS*, *c-MYC*, and *SeV*. A negative iPSC control (F02_C1), a positive control represented by PBMCs immediately after reprogramming (CTRL+), and a no-template reaction (NEG) were included in the assay. The 1051_C4 line (P20) showed complete elimination of all vectors, whereas the 171_C2 line (P19) still retained *c-MYC* and *SeV* (Fig. 27A). To promote clearance, 171_C2 iPSCs were incubated at 39 °C for 5 days, as recommended in the reprogramming protocol. A second incubation cycle was required, after which complete loss of Sendai vectors was confirmed (Fig. 27B).

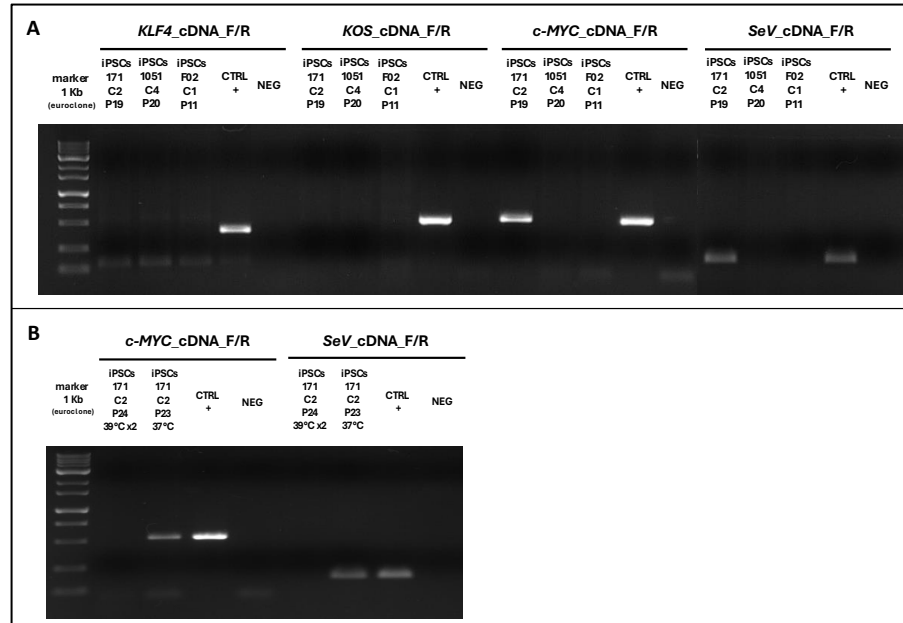


Figure 27. Clearance of Sendai virus vectors in NF1 microdeletion patient-derived iPSC clones. (A) The 1051_C4 (P20) iPSC clone showed complete absence of Sendai vectors, comparable to the negative control F02_C1 (P11). In contrast, the 171_C2 (P19) iPSC clone still retained *c-MYC* and *SeV*, similar to the positive control (CTRL+). (B) Following two rounds of incubation at 39 °C, the 171_C2 (P24) clone achieved complete clearance of Sendai vectors, whereas the 171_C2 (P23) clone, not subjected to heat treatment, still retained the vectors.

Finally, all iPSC cultures were verified to be free of Mycoplasma contamination (Fig. 28).

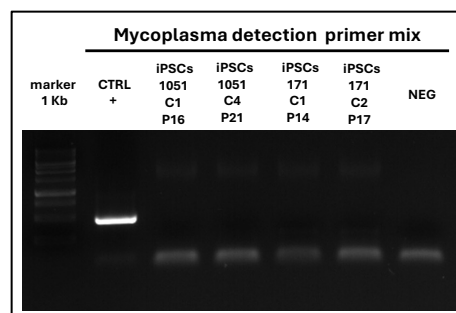


Figure 28. Mycoplasma detection in NF1 microdeletion patient-derived iPSC clones. The 1051_C1 (P16), 1051_C4 (P21), 171_C1 (P14), and 171_C2 (P17) iPSC clones tested negative for Mycoplasma contamination, as compared with the positive control (CTRL+).

Overall, the characterization of PT 171 and PT 1051 iPSC lines confirmed their ability to self-renew and differentiate, validating them as robust *in vitro* cell models for investigating the pathogenic mechanisms underlying NF1 microdeletion syndrome.

4.2 Identification of potential mechanisms contributing to phenotypic severity and variability in NF2 microdeletion patients

Building on the findings obtained from the characterization of NF1 microdeletion syndrome – where deletion size, gene content, and additional pathogenic mechanisms emerged as key contributors to phenotypic variability – we extended our investigation to another tumor-predisposition condition in which chromosomal rearrangements can occur: NF2.

NF2 microdeletions exhibit marked heterogeneity in terms of genomic size, BP localization along chromosome 22q12, and gene content beyond *NF2*, due to the absence of recurrent BP regions. Their actual frequency remains unknown, and only a limited number of cases have been described in the literature. Moreover, detailed structural characterization is frequently incomplete because diagnostic workflows typically rely on MLPA and array-CGH – approaches that do not allow precise BP mapping nor a comprehensive assessment of deleted gene content.

Given the broad phenotypic variability observed in patients with 22q12 microdeletions, we collaborated with Dr. Eoli (Fondazione IRCCS Istituto Neurologico “Carlo Besta”) to recruit NF2 patients carrying such genomic lesions detected by MLPA, with the objective of elucidating their genetic and epigenetic pathogenic effects. In a previous study, we characterized three microdeletions of distinct sizes, identified a candidate gene potentially contributing to cognitive impairment, and demonstrated a position effect on the expression of flanking genes (Tritto *et al.*, 2022).

However, due to the intrinsic structural heterogeneity of these rearrangements, expanding the patient cohort is crucial both to confirm shared pathogenic mechanisms and to identify patient-specific features.

Therefore, the second part of this thesis focused on the comprehensive characterization of two newly identified 22q12 microdeletions. The primary goal was BP definition to infer the underlying molecular mechanism. To explore potential genotype-phenotype correlations, we investigated not only the genes

encompassed within the deletion intervals, but also the epigenetic impact on neighboring genes, as well as additional genomic variants or chromosomal alterations that may act as modifier factors contributing to the complexity of the clinical phenotype.

4.2.1 Clinical description

To investigate the pathogenic mechanisms associated with *NF2* microdeletions, two patients – PT 568 and PT 690 – clinically evaluated by Dr. Eoli (Fondazione IRCCS Istituto Neurologico “Carlo Besta”, Milan), were enrolled in the study. Both patients were sporadic cases carrying large deletions encompassing the *NF2* region.

PT 568 is a 22-year-old male with an unremarkable family history. Corpus callosum hypoplasia was diagnosed prenatally, and both motor and cognitive developmental delays were observed. At the age of 4 years, he underwent surgical correction of hypospadias. Brain and spinal MRI at 6 years revealed corpus callosum hypoplasia, focal cortical dysplasia, and mild ventricular enlargement, but no spinal lesions. Nine years later, MRI showed the initial onset of bilateral vestibular schwannomas (VSs). *NF2* was diagnosed at 15 years.

At 18 years, brain MRI with gadolinium contrast demonstrated further enlargement of bilateral VSs and the appearance of multiple cranial nerve schwannomas involving the left trigeminal, right oculomotor, and glossopharyngeal nerves. Spinal MRI revealed multiple asymptomatic schwannomas (C1-C2, D7-D8, and left psoas muscle) and right convex scoliosis. Audiometric testing detected mild sensorineural hearing loss. At age 19, due to mild VS enlargement and progressive hearing decline, the patient started Bevacizumab treatment (7.5 mg/kg every three weeks), which was discontinued two years later due to severe proteinuria despite disease stabilization. Genetic testing identified a large *de novo NF2* region deletion.

At his last clinical assessment, he exhibited mild intellectual disability, ocular hypertelorism, a long neck, right convex scoliosis, bilateral nystagmus, and mild hearing impairment.

PT 690 was a woman who died at 37 years of age from cardiogenic shock. At 3 years old, she underwent surgical correction for pulmonary flow obstruction and a ventricular septal defect. At age 13, progressive hearing loss prompted brain MRI with gadolinium, which revealed bilateral VSs and tetraventricular hydrocephalus. NF2 was diagnosed at 14 years. One year later, due to tumor progression and instability, gross total resection of both VSs was performed, resulting in severe hearing impairment. MRI follow-up showed bilateral cerebellar malacia, residual VSs, and persistent hydrocephalus.

During adolescence, she developed apathy, childlike behavior, and progressive speech difficulties, becoming able to answer only “yes” or “no” by age 18. In adulthood, she underwent removal of left-hand and left-submandibular schwannomas. From age 26, she experienced seizures (treated with phenobarbital) and progressive limb weakness. At 36 years, she required bilateral support to walk. Brain MRI with gadolinium revealed enlargement of VSs – particularly on the right side with Meckel’s cave extension – together with a left hypoglossal schwannoma and mass effect on the pons and medulla. Spinal MRI showed multiple bilateral schwannomas (C1-C3) causing cervical myelopathy, and additional schwannomas at D8, D9, L3-L4, and in the caudal roots. Electromyography revealed axonal and demyelinating polyneuropathy of both upper and lower limbs. MRI of the right thigh identified a plexiform schwannoma, which, along with cervical schwannomas, was surgically removed. Genetic testing identified a large *de novo* NF2 region deletion.

At her last clinical assessment, she presented with a faint café-au-lait spot on the left thigh and multiple subcutaneous schwannomas on both hands, left wrist, and left shoulder. She was unable to speak, to maintain the standing position, and to walk. She exhibited bilateral optic neuropathy, nystagmus, bilateral anacusis with VII nerve palsy, left hypoglossal paralysis, severe hyposthenia, and dysmetria of the limbs.

The clinical features of both NF2 microdeletion patients are summarized in Table 29.

Table 29. Clinical features of NF2 microdeletion patients

Feature	PT 568	PT 690
Gender	Male	Female
Sporadic/familial	Sporadic	Sporadic
Age at first symptom	1	3
Age at diagnosis	15	14
Current age	22	-
Bilateral VSs	Yes	Yes
Non-VS intracranial schwannoma	Yes	Yes
Spinal schwannoma	Yes	Yes
Other tumors	No	No
Hearing loss	Mild bilateral	Severe bilateral
Cranial nerve palsy	No	Yes
Seizures	No	Yes
Gait disturbance	No	Yes
Scoliosis	Yes	No
Cataract	Yes	No
Hyperpigmentation	No	Yes
Skin lumps	No	Yes
Subcutaneous schwannomas	No	Yes
Corpus callosum hypoplasia	Yes	No
Focal cortical dysplasia	Yes	No
Mental disability	Yes	Yes
Dysmorphic features	Yes	No
VS surgery	No	Yes
Spinal surgery	No	Yes
Bevacizumab therapy	Yes	No

VS, vestibular schwannoma.

4.2.2 Identification of 22q12 microdeletions

Following the clinical evaluation, mutational screening was performed at Fondazione IRCCS Istituto Neurologico “Carlo Besta”, on gDNA extracted from peripheral blood of PT 568 and PT 690. Intragenic mutations were first excluded using a targeted NGS panel on the Ion Torrent platform, which included the *NF2* gene and the Schwannomatosis-related *LZTR1* and *SMARCB1* genes. Subsequently, the screening for large deletions involving the *NF2* gene was carried out.

MLPA analysis revealed the presence of a deletion encompassing the 22q12 chromosomal region in both patients (Fig. 29).

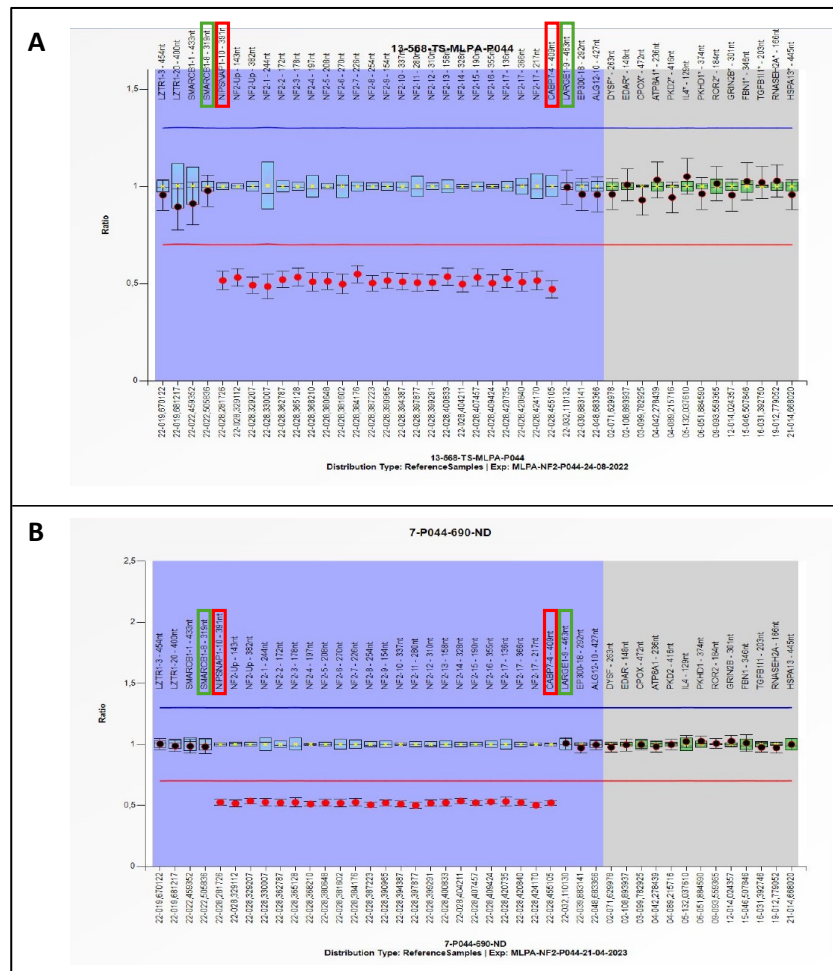


Figure 29. MLPA analysis results of NF2 microdeletion patients. The figure shows the 22q12 chromosomal deletion identified in PT 568 (A) and PT 690 (B). The last and first not in-loss probes before and after the deleted region, respectively, are highlighted in green, while the first and last in-loss probes are shown in red.

The MLPA profiles of PT 568 (Fig. 29A) and PT 690 (Fig. 29B) showed identical patterns. The centromeric BP was mapped within a 5,722,088 bp region, located between the last proximal not in-loss probe (08294-L20837 of P044 NF2 C1), in the exon 8 of the *SMARCB1* gene, and the first in-loss probe (02580-L02042), located in exon 10 of the *NIPSNAP1* gene. The telomeric BP was mapped within a 3,655,028 bp region, between the last in-loss probe (03317-L31857), in the exon 4 of the *CABP7* gene, and the first distal not in-loss probe (12460-L13461), in the exon 9 of the *LARGE1* gene.

Given the limited resolution of MLPA, high-resolution analyses were required to accurately define the microdeletion BPs.

4.2.3 Definition of the 22q12 microdeletion extent

To more precisely define the extent of the 22q12 microdeletions identified in PT 568 and PT 690, a high-resolution array-CGH analysis was performed in collaboration with Dr. Redaelli (Fondazione IRCCS San Gerardo dei Tintori), using a 180K CGH+SNP microarray on gDNA extracted from patients' peripheral blood. The array-CGH results (Fig. 30) defined the genomic coordinates of the deletions as follows:

- PT 568: arr[GRCh38] 22q12.1q12.2(27792105x2,27807049_30858596x1,30894707x2)
- PT 690: arr[GRCh38] 22q12.1q12.2(28760409x2,28785970_31147501x1,31173878x2)

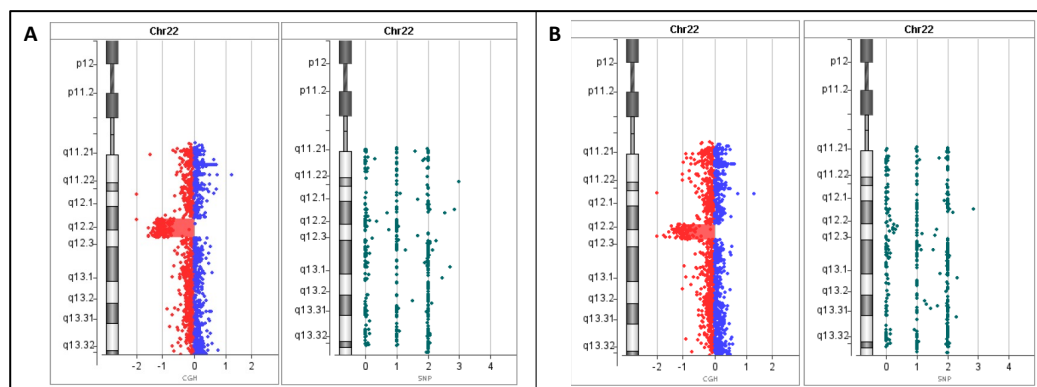


Figure 30. Array-CGH and SNP array results of NF2 microdeletion patients. The figure shows the array-CGH (left) and SNP array (right) profiles obtained using a 180K CGH+SNP microarray, revealing that the deletions on chromosome 22 of PT 568 (A) and PT 690 (B) extend across the 22q12.1-q12.2 region.

For PT 568 (Fig. 30A), array-CGH results revealed a microdeletion spanning from 3,051,548 bp to 3,102,601 bp. The centromeric BP was mapped within a 14,944 bp region, encompassing the first exon of the *MNI* gene (NM_002430.3) and an adjacent intergenic region. The telomeric BP was localized within a 36,111 bp interval, between introns 2 and 12 of the *OSBP2* gene (NM_030758.4).

In PT 690 (Fig. 30B), the array-CGH analysis defined a 22q12 microdeletion ranging from 2,361,532 bp to 2,413,468 bp. The centromeric BP was located within a 25,561 bp region that included an intergenic segment and the first exons (up to intron 4) of the *CCDC117* gene (NM_173510.4). The telomeric BP mapped within a 26,377 bp region involving an intergenic sequence and the *RNF185* gene (NM_152267.4), spanning from its 5' UTR to intron 1.

Overall, these high-resolution analyses enabled a more accurate characterization of the *NF2* deletions in both patients, pinpointing the affected chromosomal region to 22q12.1q12.2. Nevertheless, the precise deletion BPs could not be resolved.

4.2.4 *In silico* analysis of sequences containing breakpoints

Since the 22q12.1q12.2 deletions identified in the two *NF2* patients might have originated from recombination events between partially homologous sequences, an *in silico* analysis of the BP regions, defined by array-CGH, was performed to identify potential candidate sequences.

For each patient, the entire chromosomal region containing the centromeric BP was aligned with the sequence including the telomeric BP using the Global Alignment Track available in BLAST. This analysis provided the percentage of sequence identity between the two aligned regions, which was 32% for PT 568 and 52% for PT 690 (Table 30). By visualizing the alignment between the entire regions encompassing the centromeric and telomeric BPs, subregions displaying higher homology were identified and subsequently realigned to determine their specific percentage of sequence identity. The two additional alignments performed for PT 568 showed sequence identities of 65% and 56%, whereas the seven alignments

carried out for PT 690 displayed sequence identities ranging from 78% to 84% (Table 30).

Table 30. Percentage of sequence identity between chromosomal regions containing the centromeric and telomeric BPs of the 22q12.1q12.2 deletions in PT 568 and PT 690

Patient	Alignment	Centromeric BP region (hg38)	Telomeric BP region (hg38)	Sequence identity
PT 568	between entire regions	chr22:27,792,106-27,807,049	chr22:30,858,596-30,894,706	32%
	between selected regions	chr22:27,795,871-27,795,938	chr22:30,868,397-30,868,470	65%
		chr22:27,804,844-27,804,923	chr22:30,889,450-30,889,535	56%
PT 690	between entire regions	chr22:28,760,410-28,785,970	chr22:31,147,501-31,173,877	52%
	between selected regions	chr22:28,760,876-28,761,042	chr22:31,148,175-31,148,340	83%
		chr22:28,764,573-28,764,651	chr22:31,151,697-31,151,776	81%
		chr22:28,767,775-28,767,896	chr22:31,154,672-31,154,794	84%
		chr22:28,770,059-28,770,259	chr22:31,157,614-31,157,814	83%
		chr22:28,770,890-28,771,175	chr22:31,158,429-31,158,715	83%
		chr22:28,782,456-28,782,547	chr22:31,170,528-31,170,619	84%
		chr22:28,782,456-28,782,716	chr22:31,170,528-31,170,789	78%

Furthermore, an *in silico* analysis of repetitive elements – commonly involved in chromosomal rearrangements – was performed within the BP regions. This analysis was conducted using the UCSC Genome Browser’s RepeatMasker Track, which allows for the identification of several classes of repetitive sequences, including short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs), long terminal repeat elements (LTRs), DNA transposons, simple repeats (micro-satellites or short tandem repeats, STRs), and satellite repeats. As shown in Figure 31, the BP regions of both 22q12.1q12.2 deletions display a high density of repetitive elements, consistent with the hypothesis of a recombination-mediated mechanism.

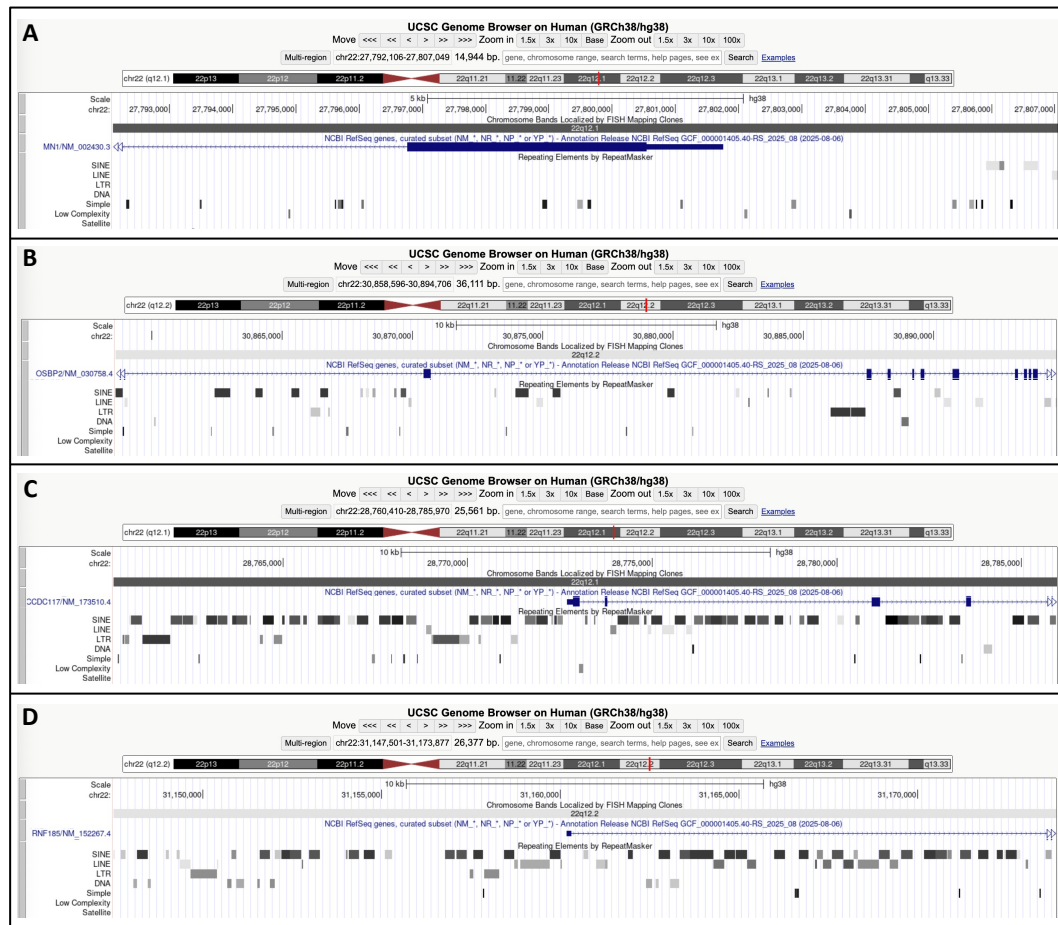


Figure 31. Chromosomal regions containing the centromeric and telomeric BPs of the 22q12.1q12.2 deletions in PT 568 and PT 690. (A) Centromeric BP region of PT 568; (B) telomeric BP region of PT 568; (C) centromeric BP region of PT 690; (D) telomeric BP region of PT 690. Each panel displays the selected chromosomal region, cytogenetic band, and annotated genes and repetitive sequences. The regions containing the centromeric and telomeric BPs of the 22q12.1q12.2 deletions in PT 568 and PT 690 are enriched in repetitive sequences, including SINEs, LINEs, LTRs, and STRs.

Pairwise comparisons were performed between each repetitive sequence mapped in the centromeric BP region and those identified in the telomeric region. For PT 568, all alignments showed sequence identity below 60%, preventing the identification of specific repeat motifs likely involved in the rearrangement event. Conversely, the PT 690 analysis revealed multiple pairs of repeats with homology exceeding 80%, suggesting the possible involvement of these sequences in a NAHR mechanism underlying the deletion.

4.2.5 Identification and characterization of 22q12.1q12.2 microdeletion breakpoints

To precisely characterize the BPs of the patients' 22q12.1q12.2 deletions and identify both the exact gene content of each deletion and the specific sequences potentially involved in their formation, thereby clarifying the underlying pathogenic mechanism, sequencing of the deletion junction fragment was required.

Initially, based on the pairs of sequences showing the highest degree of homology identified in PT 690, Long-Range PCR assays were set up on gDNA using primer pairs comprising a forward primer located upstream of a selected sequence within the centromeric BP region and a reverse primer downstream of a highly homologous sequence within the telomeric BP region (Table 16). However, Sanger sequencing of the obtained PCR products did not allow the recovery of the junction fragment, yielding only nonspecific amplicons.

Given the complexity of the 22q12 region, which is enriched in repetitive sequences, LRS was then performed on the gDNA extracted from the patients' peripheral blood to identify the exact deletion BPs. The LRS was carried out at ITB-CNR using the ONT, where Dr. Mangano processed the raw sequencing data. Following quality control of FASTQ files and alignment of reads to the human reference genome (GRCh38/hg38), the resulting BAM files were analyzed using the wf-human-variation nextflow workflow provided by ONT.

According to the LRS results, PT 568 carried a 22q12.1q12.2 deletion of 3,067,802 bp (chr22:27,805,772-30,873,574, GRCh38/hg38) (Fig. 32A). The centromeric BP mapped within an intergenic region between the *MNI* and *PITPNB* genes. The telomeric BP was located in intron 4 of the *OSBP2* gene (NM_030758.4), leading to the loss of its 5' region since the gene is transcribed from the forward DNA strand. For PT 690, LRS identified a 22q12.1q12.2 deletion of 2,405,034 bp (chr22:28,763,833-31,168,867, GRCh38/hg38) (Fig. 32B). The centromeric BP fell within an intergenic region between the *HSCB* and *CCDC117* genes, while the telomeric BP was mapped to intron 1 of the *RNF185* gene (NM_152267.4), resulting in the loss of its 5' portion.

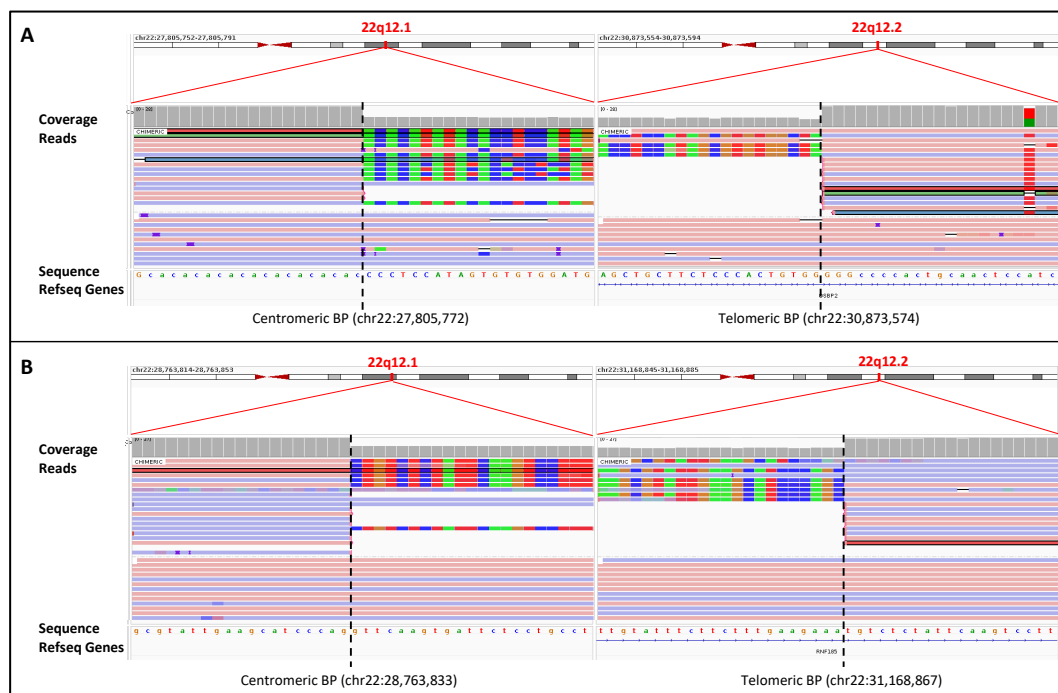


Figure 32. Identification of the 22q12.1q12.2 deletion junction fragments in PT 568 and PT 690 by LRS. Read alignment revealed the centromeric and telomeric BPs of the 22q12.1q12.2 deletions in PT 568 (A) and PT 690 (B), identified through chimeric reads mapping upstream and downstream of the deleted regions. The Integrative Genomics Viewer (IGV) visualization shows the cytoband, coverage profile, read alignment, sequence, and genes surrounding the 22q12 deletion BPs (indicated by black dashed lines). The coverage profile (in grey) indicates the presence of two copies before and after the deletion BPs, and a single copy within the deleted region. The chimeric reads spanning the BPs are highlighted by a misaligned colored end.

To validate the LRS findings, the junction fragments derived from the PT 568 and PT 690 22q12.1q12.2 deletions were amplified by PCR assays (Fig. 33), using primer pairs comprising a forward primer located upstream of the centromeric BP and a reverse primer downstream of the telomeric BP (Table 22).

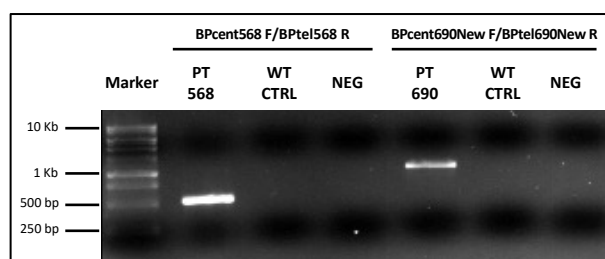


Figure 33. PCR amplification of the 22q12.1q12.2 deletion junction fragments of PT 568 and PT 690. A specific PCR product corresponding to the deletion junction fragment was obtained from gDNA of PT 568 and PT 690, confirming the presence of the 22q12.1q12.2 deletion. No amplification product was detected in a wild type control (WT CTRL) sample, consistent with the absence of the deletion.

Subsequently, the PCR products were sequenced by Sanger sequencing. The BPs identified by LRS were confirmed. However, in both patients, Sanger sequencing revealed the presence of a short insertion – 30 bp in PT 568 and 1 bp in PT 690 – between the centromeric and telomeric BPs identified through LRS (Fig. 34).

A	CCATAGTGTGTGGATGTCAGCACACACACACACACACACATACAC CTCCATAGTGTGTGGCTATGTCAGCACACACACACACACACATAT ACACCTCCATAGCGTGTGGCTATGTCAGCACACACACACACACAC	Centromeric BP region (CA) _n Intergenic sequence
	ACACATATACACCTCCATAGCGTGTGGCTA	Insertion
B	GGGCCCCACTGCAACTCCTTCCCACTCCCCAGCCCCATCCTGACTGT GATGTGTGCGGGGATGCTCCTAGCATCCCCTAGCGCCCGTGCCTGCA TTAGGGAAATCTCTACTGCCACTGGCTCCTCGGACAGGGCAGACCCT	Telomeric BP region (CCCCAT) _n <i>OSBP2</i> intron 4
C	CAGGCCAGGAGTTAGAGACAAGCCTGGGCAACATAGCAAGACCCCAT CTCAAGTTCTTTTTTTTTTTTTTTTTTAGATAGTCTCGCTCTGTTGCC CAGGCTGTAGTGCAATGGTGTGGTCTAAGCGTATTGAAGCATCCCAG	Centromeric BP region AluSx1 Intergenic sequence
	TGTCTCTATTCAAGTCCTTTGCCCCCCCCGCCACCACCACCCCTTTA AAAATTTTTTTTGAGACAGAGTCTCTCTCTCTTGGCCAGGCTGGA GTGCAGTGGTGGAATCTGGGCTCTGCAACCTCCACCTCCTGGGTTCA	Insertion
D		Telomeric BP region L1MB3 <i>RNF185</i> intron 1

Figure 34. Characterization of the 22q12.1q12.2 deletion junction fragments. (A) Sequence analysis of the PT 568 deletion junction fragment revealed that the centromeric BP mapped within a (CA)_n repeated motif located in an intergenic region, while the telomeric BP fell within a (CCCCAT)_n repeat in *OSBP2* intron 4. A 30 bp insertion was identified between the two aligned sequences. (B) In PT 690, the centromeric BP was located within an AluSx1 repeat mapped to an intergenic region, whereas the telomeric BP was included in a L1MB3 repeated element within *RNF185* intron 1. A 1 bp insertion was detected between the two aligned sequences.

An *in silico* analysis was then performed to identify, within the BP regions, repeated motifs that might have contributed to the formation of the 22q12.1q12.2 microdeletions. In PT 568, the centromeric and telomeric BPs fell into the STRs (CA)_n and (CCCCAT)_n, respectively (Fig. 34A). In PT 690, the centromeric BP was located within a SINE AluSx1 element, while the telomeric BP was mapped within a LINE L1MB3 element (Fig. 34B).

Alignment analysis of the repeated motifs mapped in the centromeric and telomeric BP regions of each patient revealed a sequence identity of approximately 50%, suggesting that NHEJ or microhomology-based mechanisms, such as

microhomology-mediated end-joining (MMEJ) or fork stalling and template switching and microhomology-mediated break-induced replication (FoSTeS/MMBIR), may represent the underlying mechanisms responsible for the 22q12.1q12.2 deletions.

4.2.6 Analysis of the gene content within the 22q12.1q12.2 microdeletions

To explore whether genes deleted in addition to *NF2* might contribute to the complex clinical phenotypes observed in PT 568 and PT 690, an *in silico* analysis of the gene content within the 22q12.1q12.2 deletions was performed. The pLI score – ranging from 0 to 1 and indicating the probability that a gene is intolerant to loss-of-function variants – was used to predict the potential haploinsufficiency of genes encompassed by the deletions. Furthermore, possible genotype-phenotype correlations were assessed using data from the Online Mendelian Inheritance in Man (OMIM) database.

The deletion identified in PT 568 encompassed 44 protein-coding genes, 38 of which were listed in OMIM (Table 31). Among these, eight genes (*TTC28*, *CCDC117*, *ZNRF3*, *EWSR1*, *AP1B1*, *NF2*, *MTMR3*, and *SF3A1*) showed a pLI score between 0.9 and 1, indicating high intolerance to loss-of-function alterations (Fig. 35). The PT 690 deletion included 47 protein-coding genes, 41 of which were reported in OMIM (Table 31). Among them, eight genes (*CCDC117*, *ZNRF3*, *EWSR1*, *AP1B1*, *NF2*, *MTMR3*, *SF3A1*, and *MORC2*) displayed pLI scores greater than 0.9 (Fig. 35).

Table 31. Gene content of 22q12.1q12.2 microdeletions

Patient	Gene content
PT 568	<i>PITPNB</i> , <i>TTC28</i> , <i>CHEK2</i> , <i>HSCB</i>
PT 568 and PT 690	<i>CCDC117</i> , <i>XBPI</i> , <i>ZNRF3</i> , <i>C22orf31</i> , <i>KREMEN1</i> , <i>EMID1</i> , <i>RHBDD3</i> , <i>EWSR1</i> , <i>GAS2L1</i> , <i>RASL10A</i> , <i>AP1B1</i> , <i>RFPL1</i> , <i>NEFH</i> , <i>THOC5</i> , <i>NIPSNAP1</i> , <i>NF2</i> , <i>CABP7</i> , <i>ZMAT5</i> , <i>UQCR10</i> , <i>ASCC2</i> , <i>MTMR3</i> , <i>HORMAD2</i> , <i>LIF</i> , <i>OSM</i> , <i>CASTOR1</i> , <i>TBC1D10A</i> , <i>SF3A1</i> , <i>CCDC157</i> , <i>RNF215</i> , <i>SEC14L2</i> , <i>MTFP1</i> , <i>SEC14L3</i> , <i>SEC14L4</i> , <i>SEC14L6</i> , <i>GAL3ST1</i> , <i>PES1</i> , <i>TCN2</i> , <i>SLC35E4</i> , <i>DUSP18</i> , <i>OSBP2</i>
PT 690	<i>MORC2</i> , <i>TUG1</i> , <i>SMTN</i> , <i>SELENOM</i> , <i>INPP5J</i> , <i>PLA2G3</i> , <i>RNF185</i>

Genes reported in OMIM database are shown in bold.

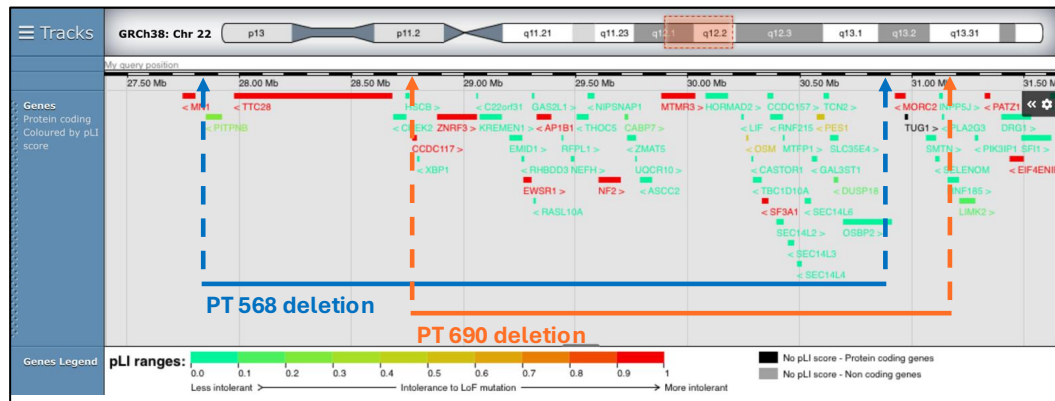


Figure 35. Gene content of the 22q12.1q12.2 deletions and haploinsufficient genes. The figure, obtained from the DECIPHER database, shows the chromosomal region encompassing the deletions of PT 568 (delimited by blue dashed lines) and PT 690 (delimited by orange dashed lines), together with the genes mapped within these intervals. Genes are color-coded according to their pLI score: green for genes with $pLI < 0.5$, orange for genes with $0.5 \leq pLI \leq 0.9$, and red for genes with $pLI > 0.9$. PT 568 and PT 690 22q12.1q12.2 deletions involve 44 and 47 protein-coding genes, respectively. Among these genes, 8 genes in PT 568 and PT 690 show a pLI score greater than 0.9.

Among the haploinsufficient genes included in the patients' deletions, *APIB1* loss-of-function has previously been associated with MEDNIK-like syndrome (mental retardation, enteropathy, deafness, neuropathy, ichthyosis, keratoderma), an autosomal recessive disorder characterized by variable clinical features, including intellectual disability (Alsaif *et al.*, 2019). Since both PT 568 and PT 690 presented cognitive impairment, a feature generally uncommon in NF2 patients, the presence of possible pathogenic variants in the *APIB1* non-deleted allele as well as its expression levels were investigated.

Variant analysis performed on LRS data revealed only variants of uncertain significance or benign, located in the second allele of the *APIB1* gene (Table 32).

Table 32. *APIBI* gene variants identified by LRS

Patient	Position	Nucleotide variation (NM_001127.4)	rs	MAF (GnomAD)	Clinical significance (ClinVar/Franklin)
PT 568	Intron 1	c.-28+5961C>G	rs174773	0.0424	Benign
	Intron 2	c.37+372_37+373dupGT	rs200885223	0.1566	Benign
	Intron 2	c.38-349G>C	rs174769	0.3828	Benign
	Intron 6	c.716+235G>C	rs4823033	0.8339	Benign
PT 690	Intron 1	c.-28+9677A>G	rs174771	0.3837	Benign
	Intron 1	c.-28+9390G>T	rs174772	0.1669	Benign
	Intron 1	c.-28+5961C>G	rs174773	0.0424	Benign
	Intron 1	c.-28+4529A>G	rs174774	0.0425	Benign
	Intron 2	c.37+372_37+373delGT	rs200885223	0.1759	Benign
	Intron 2	c.38-349G>C	rs174769	0.3828	Benign
	Intron 3	c.143+173T>C	rs570731	0.3806	Benign
	Intron 6	c.716+235G>C	rs4823033	0.8339	Benign
	Exon 8	c.2349G>A (#)	rs174765	0.5198	Benign
	Intron 9	c.1156-114A>G	rs737883	0.3632	Benign
	Intron 11	c.1437+1428G>A	rs715589	0.1820	Benign
	Intron 11	c.1437+2423C>G	rs558341728	0.0005	VUS
	Intron 11	c.1438-2356T>C	rs174767	0.3001	Benign
	Intron 15	c.2020-188G>A	rs3747146	0.1926	Benign
	Intron 16	c.2163+182C>T	rs6006098	0.3985	Benign
	Intron 16	c.2163+283A>C	rs174766	0.3178	Benign
	Intron 16	c.2164-226T>C	rs98069	0.3369	Benign
	Intron 17	c.2309+252G>A	NA	NA	NA
	Intron 17	c.2310-949T>A	rs9613864	0.1228	Benign
	Intron 21	c.2767-184A>G	rs2071578	0.2958	Benign
	Downstream	c.*2753T>C	NA	NA	VUS
	Downstream	c.*2908C>T	rs9613863	0.1315	Benign
	Downstream	c.*3649C>T	rs174764	0.3295	Benign

MAF, Minor Allele Frequency; NA, not available; rs, Reference SNP ID; VUS, variant of uncertain significance; #, synonymous change.

APIBI expression levels were evaluated by qPCR on cDNA retrotranscribed from peripheral blood RNA of eight HD controls and from two biological samples obtained from PT 568 and PT 690. Both patients showed a marked reduction (approximately 60-70%) in *APIBI* expression compared to controls (Table 33 and Fig. 36), supporting the hypothesis that *APIBI* haploinsufficiency may contribute to the cognitive impairment observed in the patients. However, the absence of pathogenic variants in the second allele likely explains the lack of the full clinical spectrum typically associated with MEDNIK-like syndrome.

Table 33. Statistical analysis of qPCR data for the *AP1B1* expression

Samples	N	$2^{-(\Delta Ct)}$ mean	SD	SEM	95% CI		Student's <i>t</i> -test <i>p</i> -value PT vs HD
					Lower	Upper	
HD	8	0.037	0.006	0.002	0.032	0.042	
PT 568	2	0.014	0.003	0.002	-0.012	0.040	0.0009
PT 690	2	0.012	0.002	0.001	-0.003	0.026	0.0005

CI, confidence interval; N, number of samples per group after excluding outliers identified by Tukey's test; SD, standard deviation; SEM, standard error of the mean; HD, healthy donor controls.

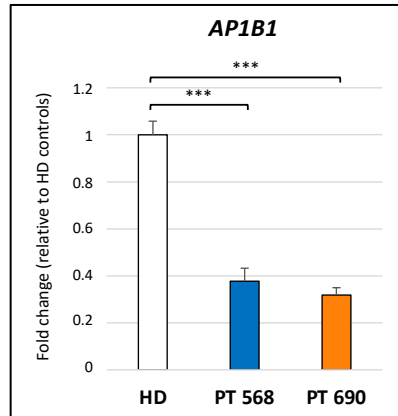


Figure 36. Expression level of the *AP1B1* non-deleted allele. Quantitative expression analysis ($2^{-(\Delta Ct)}$) of *AP1B1* in peripheral blood of PT 568 and PT 690 revealed a statistically significant reduction of approximately 60% and 70%, respectively, compared with eight healthy donors (HD). For each patient, the mean value of two biological replicates is shown $\pm$ SEM, while for controls the mean $\pm$ SEM of the group is reported. All values were normalized to the HD group mean. *** $p < 0.001$, Student's *t*-test.

4.2.7 Characterization of the regulatory landscape of the 22q12 region

To assess whether the 22q12.1q12.2 deletions identified in PT 568 and PT 690 could lead to a position effect on flanking genes – namely, a dysregulation of their expression due to changes in the surrounding regulatory context following chromosomal rearrangement – the organization of TADs and regulatory elements within the 22q12 region was investigated. TADs are regulatory domains that maintain proper gene expression by promoting promoter-enhancer and promoter-silencer interactions within the same domain, while restricting contacts between elements located in different TADs through boundary (TDB) regions enriched in

insulators. Alterations in TAD architecture may therefore enable the establishment of novel promoter-regulatory element interactions, reshaping the regulatory landscape and ultimately affecting gene expression.

The *in silico* analysis of the topological organization of the chromosomal region encompassing and flanking the 22q12.1q12.2 deletions was performed using the 3D Genome Browser 2.0. This tool integrates data from Hi-C experiments – an extension of the 3C method that identifies genome-wide long-range DNA-DNA interactions – conducted on multiple cell lines. For each dataset, the browser provides graphical representations in the form of heatmaps, where the x-axis indicates the genomic coordinates of the region of interest, and the color gradient from white to red reflects the number of DNA-DNA interactions detected (white: no interaction; deep red: more than 1000 interactions). Highly interactive chromosomal areas, visualized as “red triangles” on the heatmaps, represent TADs, which are separated by narrow TDBs characterized by a sharp decrease in chromatin contacts.

In addition, *in silico* analyses of the regulatory elements within the 22q12 region were performed by consulting the VISTA Enhancer Browser and CTCFBSDB 2.0 databases. VISTA Enhancer Browser reports non-coding human and murine sequences that have been experimentally validated in transgenic mice to act as enhancers, after being selected for analysis based on their extreme conservation among vertebrates or on epigenomic evidence (ChIP-Seq) supporting their identification as putative enhancers. The CTCFBSDB 2.0 database provides experimentally determined (via ChIP-Seq) or computationally predicted binding sites for CTCF (CCCTC-binding factor), an evolutionarily conserved transcriptional regulator that binds specific DNA sequences. Depending on the genomic context, CTCF may function as a transcriptional activator or repressor, or as a chromatin insulator – that is, a sequence capable of shielding genes from inappropriate *cis*-regulatory influences originating from neighboring genomic regions.

Specifically, for the present analysis, TAD organization was visualized in the 3D Genome Browser 2.0 focusing on the chromosomal interval chr22:27,400,000-

31,640,000 (GRCh38/hg38), which includes the deletions of PT 568 and PT 690 and approximately 0.5 Mb of flanking sequences upstream and downstream (Fig. 37). To simultaneously visualize also the genes and regulatory elements included in the region of interest, two custom tracks were added to the UCSC Genome Browser, one for VISTA enhancers and one for CTCF-binding sites, previously identified. The identification codes (IDs) and genomic coordinates of the regulatory elements identified through the *in silico* analysis are reported in Table 34.

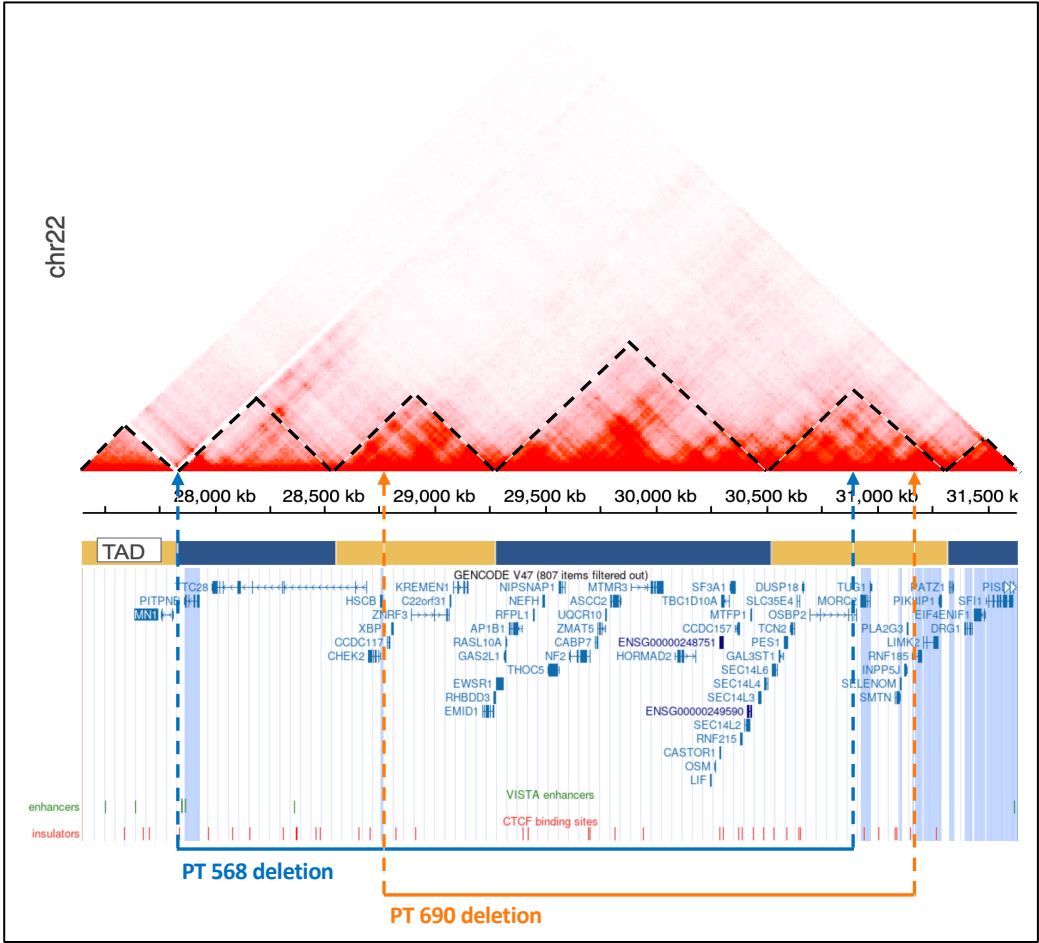


Figure 37. TADs, genes, and regulatory elements mapping within the 22q12 genomic region. The heatmap displays 22q12 TADs, represented as red triangles outlined by black dashed lines, with their chromosomal positions indicated by yellow and blue bars. TAD visualization was obtained using the 3D Genome Browser 2.0 based on Hi-C data from blood-derived GM12878 lymphoblastoid cell line. The UCSC Genome Browser track below shows genes (blue), VISTA enhancers (green), and CTCF-binding sites (red). Genes analyzed by qPCR for expression studies are highlighted in light blue. Vertical dashed lines indicate the patients' deletion BPs. The PT 568 deletion encompassed four TADs, including 44 protein-coding genes, three out of six enhancers mapped in the region, and 30 CTCF-binding sites. The PT 690 deletion affected three TADs, involving 47 protein-coding genes, no enhancers, and 24 CTCF-binding sites.

Table 34. Regulatory elements mapping in 22q12 region

Category	ID	Genomic coordinates (hg38)	PT 568 deletion	PT 690 deletion
Enhancer	hs2757.0	chr22:27,494,577-27,495,250	out	out
Insulator	EMBL_M2	chr22:27,579,972-27,579,980	out	out
Enhancer	hs2415.0	chr22:27,632,266-27,633,748	out	out
Insulator	EMBL_M2	chr22:27,666,885-27,666,893	out	out
Insulator	EMBL_M2	chr22:27,693,031-27,693,039	out	out
Insulator	EMBL_M2	chr22:27,829,254-27,829,262	in	out
Enhancer	hs1271.0	chr22:27,842,325-27,843,730	in	out
Enhancer	hs2876.0	chr22:27,842,924-27,843,240	in	out
Insulator	EMBL_M2	chr22:27,963,744-27,963,752	in	out
Insulator	EMBL_M2	chr22:28,073,240-28,073,248	in	out
Insulator	EMBL_M2	chr22:28,147,906-28,147,914	in	out
Insulator	EMBL_M2	chr22:28,302,225-28,302,233	in	out
Enhancer	hs1645.0	chr22:28,350,709-28,352,407	in	out
Insulator	EMBL_M2	chr22:28,362,386-28,362,394	in	out
Insulator	EMBL_M2	chr22:28,364,353-28,364,361	in	out
Insulator	EMBL_M2	chr22:28,451,510-28,451,518	in	out
Insulator	EMBL_M2	chr22:28,469,049-28,469,057	in	out
Insulator	EMBL_M2	chr22:28,646,000-28,646,008	in	out
Insulator	EMBL_M2	chr22:28,694,508-28,694,516	in	out
Insulator	EMBL_M2	chr22:28,814,980-28,814,988	in	in
Insulator	EMBL_M2	chr22:28,902,873-28,902,881	in	in
Insulator	EMBL_M2	chr22:29,392,698-29,392,706	in	in
Insulator	MIT_LM7	chr22:29,412,943-29,412,962	in	in
Insulator	EMBL_M2	chr22:29,687,939-29,687,947	in	in
Insulator	EMBL_M2	chr22:29,697,370-29,697,378	in	in
Insulator	EMBL_M2	chr22:29,810,333-29,810,341	in	in
Insulator	EMBL_M2	chr22:29,938,149-29,938,157	in	in
Insulator	EMBL_M1	chr22:30,286,993-30,287,006	in	in
Insulator	EMBL_M2	chr22:30,301,867-30,301,875	in	in
Insulator	EMBL_M2	chr22:30,370,491-30,370,499	in	in
Insulator	REN_20	chr22:30,387,499-30,387,518	in	in
Insulator	EMBL_M1	chr22:30,387,502-30,387,515	in	in
Insulator	EMBL_M2	chr22:30,438,029-30,438,037	in	in
Insulator	MIT_LM2	chr22:30,485,555-30,485,573	in	in
Insulator	EMBL_M2	chr22:30,532,217-30,532,225	in	in
Insulator	EMBL_M2	chr22:30,590,910-30,590,918	in	in
Insulator	EMBL_M2	chr22:30,645,219-30,645,227	in	in
Insulator	EMBL_M2	chr22:30,652,008-30,652,016	in	in
Insulator	EMBL_M2	chr22:30,940,992-30,941,000	out	in
Insulator	EMBL_M2	chr22:31,005,716-31,005,724	out	in
Insulator	MIT_LM23	chr22:31,082,821-31,082,840	out	in
Insulator	EMBL_M2	chr22:31,088,017-31,088,025	out	in
Insulator	EMBL_M2	chr22:31,152,592-31,152,600	out	in
Insulator	EMBL_M2	chr22:31,267,566-31,267,574	out	out
Enhancer	hs2240.0	chr22:31,623,379-31,627,836	out	out

As shown in Figure 37, the PT 568 deletion encompassed four TADs (outlined by black dashed triangles in the figure), three of which were entirely removed and one partially deleted. The PT 690 deletion affected three TADs, two of which were partially deleted and one fully removed. Six enhancers were identified within the same region. In PT 568, three enhancers mapped inside the microdeletion, two were

located centromeric to it, and one mapped to the telomeric side. For PT 690, all six enhancers were located outside the deleted interval, with five positioned centromeric and one telomeric to the deletion. A total of 39 CTCF-binding sites were detected within the 22q12 genomic region, of which 30 and 24 were encompassed by the PT 568 and PT 690 microdeletions, respectively (Fig. 37 and Table 34).

Therefore, the structural rearrangement affecting the TAD organization of the 22q12 genomic region, by disrupting conventional DNA-DNA interactions and simultaneously establishing novel, potentially inappropriate contacts, could alter the regulation of the genes flanking the deletion. This may occur through the creation of a new regulatory landscape composed of *cis*-acting elements, such as enhancers, silencers, and insulators, mapped within the surrounding genomic region.

4.2.8 Evaluation of the position effect on genes flanking the 22q12.1q12.2 microdeletions

To assess the hypothesized position effect on the expression of genes flanking the 22q12.1q12.2 deletions of PT 568 and PT 690, qPCR assays were performed to investigate possible aberrant expression profiles of the deletion-flanking genes. To select which genes to analyze, among those located within the non-deleted regions of the TADs encompassing the patients' deletion BPs as well as within the first flanking TADs not affected by the deletions, the GTEx Portal database was queried. This database provides quantitative expression data for all human genes based on RNA-Sequencing (RNA-Seq) analyses performed on multiple human tissues collected from donors. Specifically, genes showing ubiquitous expression and a sufficiently high expression level in peripheral blood (≥ 2 TPM, transcripts per million) were selected, since the cDNA used for the qPCR assays was retrotranscribed from RNA extracted from peripheral blood.

Among the twelve selected genes (highlighted in light blue in Fig. 37), two (*PITPNB* and *HSCB*) were mapped in the centromeric region of the PT 690

microdeletion, a gene-poor area, whereas the other ten genes were located in the gene-rich telomeric region of the PT 568 microdeletion (*MORC2*, *SELENOM*, *RNF185*, *LIMK2*, *PIK3IP1*, *PATZ1*, *DRG1*, *EIF4ENIF1*, *SFII*, and *PISD*). The last seven genes were also located telomeric to the PT 690 microdeletion.

The qPCR results, obtained from the analysis of cDNA retrotranscribed from peripheral blood RNA of PT 568, PT 690, and ten HD controls, are summarized in Table 35 and illustrated in Figure 38.

Table 35. Statistical analysis of qPCR data for the expression of 22q12.1q12.2 deletion flanking genes

Gene	Samples	N	$2^{-(\Delta Ct)}$ mean	SD	SEM	95% CI	
						Lower	Upper
<i>PITPNB</i>	HD	10	0.078	0.022	0.007	0.063	0.094
	PT 690	1	0.261				
<i>HSCB</i>	HD	10	0.004	0.002	0.001	0.003	0.006
	PT 690	1	0.013				
<i>MORC2</i>	HD	8	0.063	0.057	0.020	0.016	0.111
	PT 568	1	0.185				
<i>SELENOM</i>	HD	9	0.065	0.059	0.019	0.019	0.110
	PT 568	1	0.006				
<i>RNF185</i>	HD	9	0.284	0.218	0.072	0.117	0.452
	PT 568	1	0.853				
<i>LIMK2</i>	HD	10	0.234	0.157	0.049	0.122	0.346
	PT 568	1	0.373				
	PT 690	1	0.508				
<i>PIK3IP1</i>	HD	10	0.185	0.038	0.012	0.158	0.212
	PT 568	1	0.599				
	PT 690	1	0.816				
<i>PATZ1</i>	HD	9	0.089	0.076	0.025	0.030	0.148
	PT 568	1	0.101				
	PT 690	1	0.028				
<i>DRG1</i>	HD	7	0.856	0.689	0.260	0.219	1.493
	PT 568	1	5.077				
	PT 690	1	2.501				
<i>EIF4ENIF1</i>	HD	9	0.067	0.045	0.015	0.032	0.101
	PT 568	1	0.249				
	PT 690	1	0.069				
<i>SFII</i>	HD	6	0.196	0.021	0.008	0.175	0.218
	PT 568	1	0.092				
	PT 690	1	0.021				
<i>PISD</i>	HD	10	0.146	0.072	0.023	0.094	0.197
	PT 568	1	0.115				
	PT 690	1	0.259				

CI, confidence interval; N, number of samples per group after excluding outliers identified by Tukey's test; SD, standard deviation; SEM, standard error of the mean; HD, healthy donor controls.

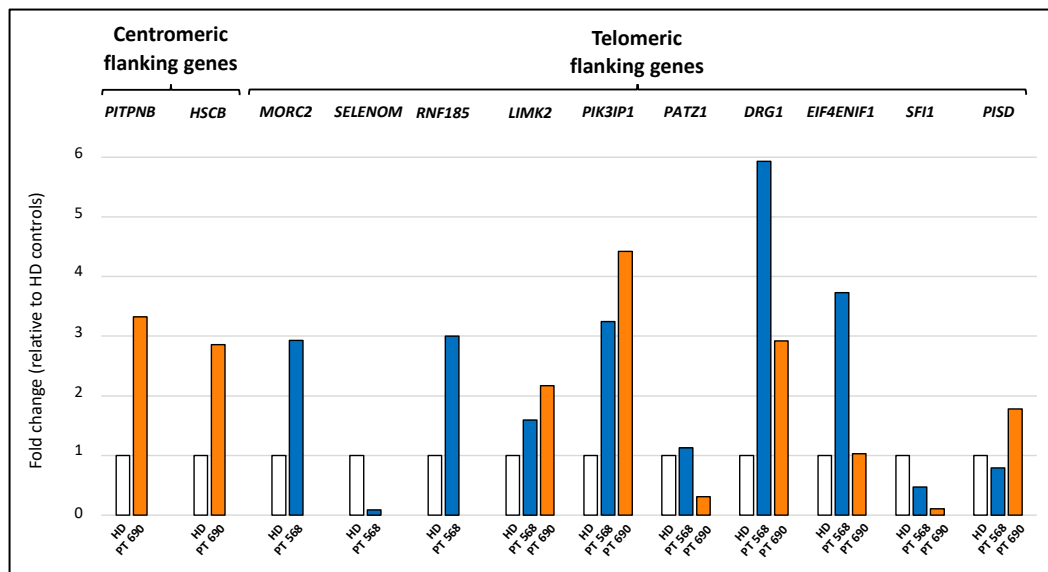


Figure 38. Expression profiles of selected genes flanking the 22q12.1q12.2 deletions of PT 568 and PT 690. The histogram illustrates the quantitative expression levels ($2^{-\Delta C_t}$) in the peripheral blood of two genes centromeric to the PT 690 deletion and ten genes telomeric to the PT 568 deletion, seven of which are also telomeric to the PT 690 deletion. For each patient, expression values are reported relative to the mean of ten healthy donor (HD) controls. Both patients exhibited altered expression profiles compared with HD controls.

The qPCR analysis revealed an increased expression of the telomeric genes *MORC2*, *RNF185*, *LIMK2*, *PIK3IP1*, *DRG1*, and *EIF4ENIF1*, together with a reduced expression of the telomeric genes *SELENOM* and *SFI1* in PT 568 compared with HD controls. PT 690 exhibited an increased expression of the *PITPNB* and *HSCB* genes, located centromeric to the deletion, as well as an overexpression of the *LIMK2*, *PIK3IP1*, *DRG1*, and *PISD* genes within the telomeric region. Moreover, PT 690 showed reduced expression of the *PATZ1* and *SFI1* telomeric genes compared with HD controls (Fig. 38). Due to the limited availability of patient RNA samples, qPCR assays could not be performed on biological triplicates, preventing statistical testing of the observed differences between patients and controls.

The gene expression profiles observed therefore support the hypothesis of a position effect on genes located within the TADs immediately adjacent to the 22q12.1q12.2 deletions in the peripheral blood of PT 568 and PT 690.

To clarify whether this position effect alters gene expression *in cis* – affecting only the allele flanking the deletion – or *in trans* – affecting both alleles – differential allelic expression (DAE) analyses are required.

LRS data provide variant phasing information, allowing discrimination between heterozygous variants located *in cis* with the deletion and those located *in trans*. Therefore, to perform DAE assays, heterozygous SNPs with a MAF greater than 20% in the general population and mapped within the genes subjected to the position effect were searched in PT 568 and PT 690 using LRS data. This analysis identified two heterozygous SNPs in the *MORC2* gene of PT 568, and one SNP in the *LIMK2* gene and two SNPs in the *SFII* gene of PT 690 (Table 36).

Table 36. Heterozygous SNPs mapped within the genes subjected to the position effect of PT 568 and PT 690

Patient	Gene (Ensembl transcript ID)	Gene expression	Heterozygous SNP	rs	MAF (1000G)	Allele <i>in cis</i> with deletion
PT 568	<i>MORC2</i> (ENST00000397641.8)	increased expression	c.378C>G	rs2072132	0.35244	C
		increased expression	c.1437A>G	rs3747151	0.46785	A
PT 690	<i>LIMK2</i> (ENST00000331728.9)	increased expression	c.103G>A	rs5997917	0.2270	G
	<i>SFII</i> (ENST00000400288.7)	reduced expression	c.1646G>A	rs2006771	0.3518	A
		reduced expression	c.3260T>C	rs12171042	0.4297	T

Since DAE assays require healthy controls carrying the same heterozygous SNPs, nine HDs were screened by PCR on cDNA, followed by Sanger sequencing of the PCR products. As reported in Table 37, seven controls were found to be heterozygous for one or more of the identified SNPs. At present, DAE analysis is ongoing by digital PCR at the Laboratory of Professor Rosanna Asselta, Humanitas University, Milan.

Table 37. Screening of HD controls for the identified SNPs

SNP	HD genotype								
	WT31	WT34	WT35	WT37	WT38	WT39	WT41	WT42	WT43
<i>MORC2</i> c.378C>G	C/G	C/C	C/G	G/G	C/G	C/C	C/C	C/G	C/C
<i>MORC2</i> c.1437A>G	A/G	A/A	A/G	G/G	A/G	NA	A/A	A/G	A/A
<i>LIMK2</i> c.103G>A	G/G	G/G	G/G	G/G	G/A	G/G	G/G	G/G	G/A
<i>SFII</i> c.1646G>A	A/A	G/A	G/G	G/A	G/A	A/A	NA	NA	G/G
<i>SFII</i> c.3260T>C	T/T	T/C	T/T	T/C	T/T	T/T	T/T	T/T	C/C

NA, not available

4.2.9 Identification of variants in modifier genes and additional Copy-Number Variations

To explore the potential contribution of modifier genes to the complex clinical phenotypes observed in the two NF2 patients carrying 22q12.1q12.2 microdeletions, the single nucleotide variants (SNVs), indels, and additional CNVs identified across their whole genomes through LRS were analyzed.

The summary reports generated by the Human Alignment Workflow and the Human Variation Workflow, provided by ONT, for PT 568 and PT 690 are presented in Table 38.

Table 38. LRS data summary report from the Human Alignment and Human Variation workflows for PT 568 and PT 690

Patient	Total reads	Total aligned reads	Total aligned bases	Mean coverage	Total small variants identified	Total CNVs identified
PT 568	6,901,061	6,555,207	57,569,587,002	18.474x	4,446,921 SNVs 730,343 indels	2 duplications 6 deletions
PT 690	17,348,003	17,214,923	132,449,329,193	42.899x	4,301,266 SNVs 749,244 indels	4 duplications 3 deletions

CNVs, Copy-Number Variations; indel, short insertions/deletions; SNVs, single nucleotide variants.

For small variant prioritization, the annotated dataset was further refined following a stepwise filtering strategy (Fig. 39): (i) variants classified as benign, likely benign, or of uncertain significance according to ClinVar database were discarded; (ii) variants with a MAF $\geq 1\%$ in the GnomAD database were excluded; (iii) variants supported by fewer than five sequencing reads were also removed. Only two variants per patient successfully passed the complete filtering workflow (Fig. 39 and Table 39).

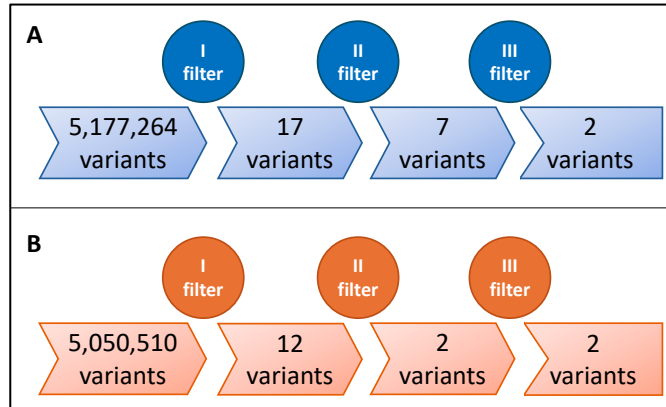


Figure 39. Variant prioritization workflow applied to LRS data from PT 568 and PT 690. A stepwise filtering strategy was applied to prioritize small variants of PT 568 (A) and PT 690 (B) based on: (I) clinical significance, (II) MAF, and (III) number of supporting reads. Only two variants for each patient passed all filtering steps.

Table 39. Gene variants identified after the prioritization process

Patient	Gene (RefSeq)	Position	Nucleotide variation (protein change)	Effect	rs
PT 568	<i>PDE6A</i> (NM_000440.3)	exon 17	c.2053G>A (p.Val685Met)	missense	rs121909835
	<i>HNF1A</i> (NM_000545.8)	exon 4	c.864delG (p.Pro291fs)	frameshift	rs762703502
PT 690	<i>EVC</i> (NM_153717.3)	exon 7	c.922_923delGA (p.Glu308fs)	frameshift	rs1729200967
	<i>TMEM70</i> (NM_017866.6)	intron 1	c.317-2A>G	splice-site variant	rs183973249

To assess the potential impact of the CNVs identified by LRS (Table 38), their gene content was analyzed. First, only CNVs encompassing genes reported in the OMIM database were selected for further investigation. Subsequently, to determine whether these genes are dosage-sensitive, the pLI score was used for genes included in deletions, and the pTriplo score for those included in duplications. This analysis allowed the identification of one potentially pathogenic deletion in each patient (Table 40).

Table 40. Potentially pathogenic CNVs identified by LRS in PT 568 and PT 690

Patient	Type	Localization	Gene content (pLI)
PT 568	Deletion	chr15:71,808,000-71,900,000	<i>NR2E3</i> (0), <i>MYO9A</i> (1)
PT 690	Deletion	chr21:31,475,000-31,562,000	<i>TIAM1</i> (0.99)

Correlations between the selected variants and CNVs and the clinical phenotypes observed in the patients were investigated by consulting ClinVar, OMIM, and DECIPHER databases. Notably, the *EVC* gene frameshift variant c.922_923delGA (p.Glu308fs), identified in PT 690, has been previously associated with Ellis-van Creveld syndrome, an autosomal recessive disorder characterized by multiple clinical features, including congenital heart defects (Da Silva *et al.*, 2023). Interestingly, PT 690 exhibited heart defects, a phenotype generally uncommon among NF2 patients.

To explore the possibility that the phenotype might result from biallelic involvement of the *EVC* gene, additional variants potentially affecting the second allele were searched among those not retained after the filtering process of PT 690 LRS data. Since LRS data provide variant phasing information, it was possible to identify among the *EVC* variants exactly those located *in trans* with the c.922_923delGA (p.Glu308fs) variant. Five alterations were identified in the second allele of the *EVC* gene (NM_153717.3), including three missense SNVs, one splice-site SNV, and one intronic indel (Table 41). However, all these variants were predicted to have no pathogenic effects. This evidence may explain the lack in PT 690 of the full clinical spectrum typically associated with Ellis-van Creveld syndrome.

Table 41. Additional *EVC* gene variants identified in PT 690 by LRS analysis

Position	Nucleotide variation (protein change)	Effect	rs	MAF (gnomAD)	Clinical significance (ClinVar/Franklin)
exon 6	c.772T>C (p.Tyr258His)	missense	rs6414624	0.2793	benign
exon 10	c.1346C>A (p.Thr449Lys)	missense	rs2302075	0.8332	benign
exon 12	c.1727G>A (p.Arg576Gln)	missense	rs1383180	0.3208	benign
intron 15	c.2305-8T>C	splice-site variant	rs1031919	0.2789	benign
intron 8	c.1098+244_1098+310del	-	rs1560330268	0.1467	benign

5. DISCUSSION

5.1 Challenges in understanding variable expressivity and severity in NF1 microdeletion patients

Patients affected by the NF1 microdeletion syndrome display a more severe clinical phenotype compared to those with classical NF1 caused by intragenic *NF1* mutations. Moreover, these individuals exhibit remarkable variability in the expression of diagnostic features (Venturin *et al.*, 2005; Kehrer-Sawatzki and Cooper, 2021), suggesting the involvement of multiple pathogenic mechanisms, which were therefore investigated in the present study.

Previous studies have demonstrated that both the severity and complexity of the phenotype are influenced by the haploinsufficiency of genes encompassed within the deletion interval. In comparison with the general NF1 population, microdeletion patients show a higher incidence of MPNSTs, a phenotype associated with the loss of one allele of *ATAD5* and *SUZ12* (Pasmant *et al.*, 2010; Kehrer-Sawatzki *et al.*, 2017). Cardiac malformations have been correlated with the hemizyosity of the *ADAP2*, *SUZ12*, and *UTP6* genes (Venturin *et al.*, 2014; Serra *et al.*, 2019), whereas overgrowth, craniofacial dysmorphisms, intellectual disability, and increased tumor predisposition have been linked to pathogenic variants or deletions affecting *RNF135* and *SUZ12*, encoding a core subunit of the PRC2 complex (Ferrari *et al.*, 2017; Douglas *et al.*, 2007; Imagawa *et al.*, 2018).

Although these functional studies have provided valuable genotype-phenotype correlations, as well as computational analyses have predicted dosage-sensitive genes (*ATAD5*, *NF1*, *OMG*, *RAB11FIP4*, and *SUZ12*, all with pLI scores >0.9), the overall clinical severity observed in NF1 microdeletion patients cannot be fully explained by the cumulative effects of gene haploinsufficiency alone. This evidence supports the hypothesis of a more complex pathogenic landscape.

In this context, our group recently proposed that position effect may represent an additional mechanism contributing to the distinctive phenotype of patients with the type-1 *NF1* microdeletion. Building upon previous evidence of position effect-driven transcriptional dysregulation in genes flanking the atypical *NF1* deletion

identified in PT 171 (Ferrari *et al.*, 2017), this phenomenon was subsequently investigated in a larger cohort sharing the same type-1 *NF1* deletion (Tritto *et al.*, 2024). *In silico* analyses revealed alterations in TAD organization and perturbations in the regulatory landscape of the 17q11.2 region (Fig. 11A), findings that were further validated through 4C-seq assays. Gene expression analyses confirmed statistically significant differences between patients and controls in seven out of twelve flanking genes examined. Notably, correlations were proposed between the dysregulation of specific position effect-sensitive genes and particular clinical manifestations. For instance, *RHOT1* downregulation was suggested as a potential risk factor for pancreatic tumorigenesis and as a possible contributor to the atypical neurodevelopmental features observed in *NF1* microdeletion patients, while reduced *ZNF207* expression was implicated in the abnormal development of ectoderm-derived tissues, such as the nervous system and epidermis, both of which are commonly affected in this syndrome (Tritto *et al.*, 2024).

Beyond phenotypic severity, *NF1* microdeletion syndrome is also characterized by striking interindividual variability, with diagnostic features that may be variably present or absent and complications that can arise unpredictably, thereby complicating prognostic assessment (Viskochil, 2002). Even among individuals carrying identical deletions, marked differences in phenotypic expression have been observed, potentially influenced by environmental factors, gene haploinsufficiency, and each individual's unique genetic background.

Although no specific environmental or infectious factors have been identified to date (Viskochil, 2002), haploinsufficiency itself has been suggested to contribute to phenotypic variability. The stochastic silencing of the single remaining allele or monoallelic gene expression may result in transient or stable fluctuations in gene dosage, respectively, which could modulate the expressivity of the syndrome (Johnson *et al.*, 2019).

The influence of genetic background is supported by the observation that intrafamilial variability is generally lower than interfamilial variability, consistent with the sharing of functional polymorphisms that may act as genetic modifiers (Viskochil, 2002). In our previous study involving a large cohort of patients

carrying the type-1 *NF1* microdeletion, NGS was employed to identify potential variants in modifier genes. Notably, a correlation was observed between the *RASAI* c.2656C>T (p.Pro886Ser) variant and the occurrence of facial hemangiomas – an uncommon manifestation in NF1 – suggesting that modifier genes may underlie at least part of the phenotypic variability typical of this condition (Tritto *et al.*, 2024).

Based on these premises, the research project presented in this thesis aimed to explore additional modifier genes and pathogenic mechanisms that may contribute to the phenotypic severity and variability observed in NF1 microdeletion syndrome. By investigating these poorly characterized mechanisms, the study sought to expand our understanding of the etiopathogenesis of NF1 microdeletion syndrome and to provide a foundation for the identification of novel molecular targets that could inform the development of personalized therapeutic approaches.

5.1.1 Identification of PRC2 as additional player involved in NF1 pathogenesis

The dysfunction of the PRC2 transcriptional repressive complex, of which SUZ12 is a core component, was previously identified in PT 171 (Tritto *et al.*, 2023b), who carried an atypical *NF1* deletion generating the chimeric *RNF135-SUZ12* gene (Ferrari *et al.*, 2017). This patient exhibited a higher burden of neurofibromas compared to age-matched individuals carrying atypical *NF1* deletions reported in the literature (Kehrer-Sawatzki *et al.*, 2021; Tritto *et al.*, 2023b). The altered PRC2 activity was proposed to be associated with the tumor predisposition phenotype observed in PT 171 (Tritto *et al.*, 2023b).

In the present study, to determine whether the dysregulated expression of PRC2 component and target genes observed in PT 171 resulted from a gain-of-function effect of the chimeric RNF135-SUZ12 protein within the PRC2 complex, from a loss of *SUZ12* function, or alternatively from hyperactivation of the Ras signaling pathway – which could account for the increased expression of *EZH2*, encoding the catalytic subunit of PRC2 – the analysis was extended to two additional NF1 patient cohorts: one carrying the canonical type-1 *NF1* microdeletion, resulting in the

complete loss of one *SUZ12* allele, and another with intragenic *NF1* mutations, who share Ras pathway hyperactivation with microdeletion patients. For expression analyses, the Tukey Fences test was applied within each patient cohort to exclude outliers, with the purpose of identifying expression changes shared by the majority of patients and potentially reflecting common molecular mechanisms. However, given the well-known high variable expressivity of NF1, it cannot be ruled out that some of the excluded outliers may represent biologically meaningful manifestations of the broad phenotypic spectrum associated with NF1.

Gene expression analyses performed on peripheral blood samples revealed that, among genes encoding PRC2 core components, *SUZ12* was overexpressed in patients with intragenic *NF1* mutations and hypoexpressed in NF1 microdeletion patients compared to healthy controls, whereas *EZH2* was consistently overexpressed and *EED* hypoexpressed across all NF1 cohorts (Fig. 17A). Moreover, a general downregulation of PRC2 target genes was observed in all NF1 patient groups relative to controls, with the exception of *NFKB2* in PT 171 (Fig. 17B).

These findings indicate that PRC2 dysregulation represents a shared molecular feature among all NF1 patients, likely reflecting a secondary consequence of Ras pathway hyperactivation – a hallmark of NF1 – that may indirectly promote *EZH2* overexpression. Notably, the expression of both *EZH2* and *SUZ12* is under the regulatory control of the E2F1 transcription factor (Lee *et al.*, 2015), a key regulator involved in cell cycle progression and apoptosis (Ertosun *et al.*, 2016), whose activity is enhanced through the Ras pathway hyperactivation (Berkovich and Ginsberg, 2001).

The downregulation trend of PRC2 target genes observed across NF1 cohorts suggests an overall increase in PRC2-mediated repressive activity. However, notable differences emerged between groups. The most pronounced downregulation of PRC2 targets was detected in patients harboring intragenic *NF1* mutations, who also exhibited higher expression levels of both *EZH2* and *SUZ12*. This expression pattern may indicate a greater abundance of catalytically active PRC2 complexes in these individuals. Conversely, microdeletion patients displayed

significant *SUZ12* expression reduction despite *EZH2* upregulation, consistent with the complete or partial loss of one *SUZ12* allele, which could impair PRC2 complex stability and activity. Evidence from *Drosophila* and mammalian systems has demonstrated that EZH2 contains a SET domain responsible for H3K27 methyltransferase activity, but the enzyme exerts a lower activity in isolation due to an auto-inhibitory conformation. Only upon binding to SUZ12 and EED does PRC2 acquire full catalytic competence, initiating H3K27me3 deposition that propagates repressive chromatin domains through a feedforward loop (Lee *et al.*, 2018; Oksuz *et al.*, 2018; Park *et al.*, 2021). Importantly, the C-terminal VEFS domain of SUZ12 is essential for the assembly of a stable and enzymatically active PRC2 complex (Højfeldt *et al.*, 2018). Thus, the enhanced repression of PRC2 target genes observed in all NF1 patients is likely driven by increased *EZH2* expression, but may be further potentiated in patients with intragenic *NF1* mutations, who also exhibit *SUZ12* upregulation, unlike microdeletion patients.

When comparing microdeletion patients, PT 171 displayed expression patterns consistent with those of the type-1 *NF1* microdeletion group in seven out of nine analyzed genes (Fig. 18), further suggesting that the chimeric RNF135-SUZ12 protein may not be the primary determinant of PRC2 dysfunction in this patient. However, approximately half of the PRC2 target genes were upregulated in PT 171 compared to patients with the type-1 *NF1* deletion, implying a relatively reduced PRC2 repressive activity, which could be attributed to a partial functional impact of the chimeric protein on PRC2 activity.

Overall, increased PRC2 activity across all NF1 cohorts may contribute to the pathogenesis of neurofibromatosis. Aberrant H3K27 methylation has been widely implicated in tumorigenesis (Liu and Liu, 2022; Chase and Cross, 2011). *EZH2* overexpression has been documented in several malignancies, including prostate (Varambally *et al.*, 2002), breast (Kleer *et al.*, 2003), lung (Watanabe *et al.*, 2008), gastric (Matsukawa *et al.*, 2006), bladder (Arisan *et al.*, 2005), and hepatocellular carcinoma (Sudo *et al.*, 2005), where it correlates with poor prognosis and reduced overall survival. Notably, elevated *EZH2* expression has also been reported in MPNSTs from NF1 patients (Pemov *et al.*, 2019).

The precise molecular mechanisms underlying the crosstalk between Ras pathway hyperactivation and PRC2 dysfunction, as well as the contribution of this dysregulation to NF1 pathogenesis, remain to be elucidated. Although our findings demonstrate altered expression of PRC2 components and several target genes in the peripheral blood of NF1 patients, further investigations are required to determine whether similar transcriptional patterns are present in disease-relevant tissues, such as the nervous system and Schwann cell-derived tumors.

5.1.2 Evaluation of pseudodominance and modifier gene contributions to the PT 171 phenotype

The variability in the clinical phenotype observed among patients with *NF1* microdeletions may be influenced not only by the haploinsufficiency of *NF1* and co-deleted genes, but also by the presence of additional gene variants affecting:

- Modifier genes, potentially accounting for specific phenotypic traits unique to individual patients.
- Genes located within the deletion interval, which may contribute through a mechanism of pseudodominance.
- Genes flanking the *NF1* deletion, whose deleterious effects could be exacerbated by dysregulated expression resulting from position effect.
- Genes involved in the Ras signaling pathway, which may act synergistically with *NF1* loss to further enhance Ras pathway hyperactivation.
- Genes encoding proteins that interact with neurofibromin, potentially amplifying the functional impairment of the protein.

Given that PT 171 displayed a particularly severe tumor phenotype for his age (Tritto *et al.*, 2023b), the potential contribution of additional genetic variants was explored through WES analysis. The identified variants were evaluated using two complementary prioritization strategies. The first consisted of a general screening for pathogenic and likely pathogenic variants with a MAF < 1% across the entire exome. The second analysis focused on specific genes of interest, including those located within the patient's deletion interval, genes flanking the deletion, genes

belonging to the Ras signaling cascade, and genes encoding known neurofibromin interactors. For this targeted analysis, a more permissive MAF threshold of < 5% was applied to include variants that might exert additive effects in the context of the already pathogenic 17q11.2 deletion.

Only one variant emerged from the general analysis: the heterozygous c.823C>T (p.Arg275Trp) substitution in the *PRKN* (*PARK2*) gene (NM_004562.3), reported as pathogenic in both ClinVar and Franklin databases (Fig. 19 and Fig. 20). This variant has previously been associated with autosomal recessive juvenile parkinsonism when present in homozygosity or compound heterozygosity (Lesage *et al.*, 2008; Regoni *et al.*, 2024), and with young-onset Parkinson's disease even in the heterozygous state (Jankovic *et al.*, 2018). Functional studies have demonstrated that the mutant protein forms intracellular aggregates, fails to degrade its substrates, and leads to mitochondrial dysfunction (Sriram *et al.*, 2005). Moreover, the c.823C>T (p.Arg275Trp) *PRKN* variant has been implicated as a susceptibility factor in tumorigenesis, having been identified in a family with a hereditary form of lung cancer (Xiong *et al.*, 2015), as well as in somatic form in glioblastoma multiforme, colorectal, and lung cancers (Veeriah *et al.*, 2010).

To date, none of the clinical phenotypes previously associated with *PRKN* variants have manifested in PT 171, who is currently 11 years old. Nonetheless, this variant may represent a potential risk factor for the future development of related clinical manifestations and should be re-evaluated in the context of ongoing clinical follow-up.

The candidate gene analysis did not reveal additional variants. Therefore, based on current evidence, the patient's phenotype appears to result from the combined effects of the 17q11.2 deletion, the consequent hyperactivation of the Ras signaling pathway, and the observed alteration of the PRC2 complex.

5.1.3 Prospects for iPSC-based disease models of NF1 patients

The iPSCs have emerged as an invaluable tool for disease modeling, offering unique opportunities to investigate human pathophysiology within a controlled *in*

vitro environment. By reprogramming somatic cells into a pluripotent state, iPSCs retain the donor's genetic background while acquiring the capacity to differentiate into virtually any cell lineage. This feature allows researchers to reproduce disease-relevant cellular phenotypes and to explore the molecular mechanisms driving pathological processes directly in human-derived cells (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007).

In the context of disease modeling, iPSC-based systems overcome several limitations of conventional animal models, which often fail to fully recapitulate human-specific aspects of disease onset and progression. Furthermore, iPSC-derived cells provide a powerful platform for drug discovery and toxicological testing, enabling the assessment of therapeutic responses in a patient-specific manner and paving the way toward personalized medicine (Inoue *et al.*, 2014; Rowe and Daley, 2019).

In recent years, several iPSC-based models of NF1 have been generated, either by reprogramming somatic cells obtained from patients carrying intragenic *NF1* mutations (Xin *et al.*, 2024) or from individuals with the 17q11.2 microdeletion (Wegscheid *et al.*, 2021), as well as by engineering control human iPSC lines using CRISPR/Cas9 technology (Anastasaki *et al.*, 2020). iPSCs from NF1 patients can be derived from a variety of somatic sources, including hair follicle keratinocytes, dermal fibroblasts, urine-derived epithelial cells, umbilical cord blood, and PBMCs. Once established, these lines can be efficiently differentiated into multiple cell types relevant to NF1 pathology through the use of specific growth factors, cytokines, and extracellular matrix components.

This versatility allows the generation of *in vitro* models that reflect the multifaceted manifestations of NF1. For example, iPSC-derived Schwann cells are instrumental for investigating the mechanisms underlying neurofibroma and MPNST formation, whereas neuronal derivatives provide valuable tools to study the molecular basis of cognitive impairment, autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), and motor dysfunction associated with NF1. Moreover, neurons, astrocytes, and microglia can be employed to

reconstruct the tumor microenvironment in models of optic pathway and brainstem gliomas (Wegscheid *et al.*, 2018).

Anastasaki and colleagues, for instance, generated seven iPSC lines carrying distinct *NF1* mutations and subsequently differentiated them into 2D neural progenitor cells, astrocytes, and 3D cerebral organoids. This approach enabled the identification of mutation-specific effects on human central nervous system cells and tissues (Anastasaki *et al.*, 2020). Similarly, the generation of iPSC-derived cerebral organoids from a patient harboring a 17q11.2 microdeletion led to the identification of *CRLF3* as a critical regulator of neurodevelopmental abnormalities (Wegscheid *et al.*, 2021).

In the present work, to further investigate and validate the pathogenic mechanisms proposed to explain the severe and variable phenotype exhibited by patients with NF1 microdeletion syndrome, we sought to generate patient-specific iPSC lines. To date, we have successfully established an iPSC line from PT 171, a particularly relevant case in our study, characterized by an unusually severe tumor phenotype and the presence of an atypical *NF1* deletion resulting in the formation of the chimeric *RNF135-SUZ12* gene (Ferrari *et al.*, 2017; Tritto *et al.*, 2023b). In addition, we generated an iPSC line from PT 1051, carrying the canonical type-1 *NF1* deletion. This model is especially valuable for representing the classical NF1 microdeletion syndrome, as the 1.4 Mb type-1 deletion is the most recurrent, occurring in approximately 70-80% of all NF1 microdeletion patients, and encompasses the *NF1* type-2 and type-3 deletion intervals (Kehrer-Sawatzki and Cooper, 2021). Therefore, an iPSC model derived from a type-1 *NF1* deletion patient provides a relevant system to study the common pathogenic mechanisms underlying the majority of NF1 microdeletion cases.

Attempts were also made to generate iPSC lines from two patients with intragenic *NF1* mutations. However, these reprogramming efforts were unsuccessful. Future work will aim to repeat these experiments, as generating iPSC lines from patients with intragenic *NF1* mutations is essential to fully elucidate the mechanisms specifically associated with *NF1* deletions. A direct comparison between iPSC models derived from deletion carriers and those from patients with

intragenic mutations will allow us to distinguish the specific contribution of the deletion from the shared Ras pathway hyperactivation that characterizes all NF1 patients.

The iPSC models generated in this study, along with those from patients with intragenic *NF1* mutations, will be of great value for several future research directions. Firstly, iPSC lines derived from patients with distinct *NF1* genetic alterations can be differentiated into the main NF1-affected cell lineages, enabling comprehensive omics-based investigations – such as transcriptomic, proteomic, and phosphoproteomic analyses – to identify molecular pathways preferentially associated with specific genotypes. Such studies will also deepen our understanding of PRC2's contribution to NF1 pathogenesis, as omics profiling in cell type-specific contexts (e.g., iPSC-derived Schwann cells, which represent the cellular component of neurofibromas and MPNSTs) could provide a complete overview of PRC2 targets and activity patterns, particularly in light of its tumorigenic role when dysregulated.

Moreover, iPSC lines derived from patients with intragenic *NF1* mutations could be used for CRISPR/Cas9-mediated knock-in correction to revert the mutation and restore physiological Ras pathway regulation. According to the hypotheses proposed in this thesis, such correction would be expected to reduce *EZH2* expression and normalize PRC2 activity. These experiments would therefore clarify whether *EZH2* overexpression indeed represents a downstream consequence of Ras pathway hyperactivation.

Finally, NF1 patient-derived iPSC models will serve as a powerful platform to validate the proposed epigenotype/genotype-phenotype correlations through functional assays. For instance, in differentiated iPSCs from microdeletion patients, the expression of genes previously identified as being subject to position effect dysregulation could be assessed in specific cellular contexts. Gene expression could then be experimentally modulated using CRISPR-based systems – such as CRISPR interference (CRISPRi) to repress, or CRISPR activation (CRISPRa) to enhance gene expression (Huang *et al.*, 2018) – to determine whether normalization of expression levels can rescue disease-associated phenotypes.

Altogether, these models will constitute a fundamental resource for dissecting the molecular and epigenetic complexity of NF1 microdeletion syndrome.

5.2 Challenges in understanding variable expressivity and severity in NF2 microdeletion patients

Whereas *NF1* microdeletions have been extensively studied and characterized, very little is known about the rare *NF2* microdeletions – including their frequency, for which no reliable estimates are currently available. The few cases reported in the literature suggest that patients carrying large-scale *NF2* microdeletions generally display a more severe and complex phenotype than individuals with intragenic *NF2* mutations (Barbi *et al.*, 2002; Kluwe *et al.*, 2005; Said *et al.*, 2011; Davidson *et al.*, 2012; Bosson *et al.*, 2016; Breckpot *et al.*, 2016; Bruder *et al.*, 1999; Bruder *et al.*, 2001). These patients often present with early-onset manifestations of classical NF2 symptoms, frequently accompanied by additional clinical features such as congenital heart defects, cleft palate, velopharyngeal insufficiency, seizures, and intellectual disability (Bruder *et al.*, 1999; Halliday *et al.*, 2019b; Trizuljak *et al.*, 2023).

In contrast to the 17q11.2 microdeletions associated with NF1 microdeletion syndrome, or to other pathogenic microdeletions and microduplications on chromosome 22 (such as those responsible for Cat Eye, DiGeorge, Walker-Warburg, and 22q13.3 deletion syndromes), in which specific LCRs mediate the generation of these chromosomal rearrangements through NAHR (Shaikh *et al.*, 2001; Torres-Juan *et al.*, 2007; Lupski and Stankiewicz, 2005), *NF2* microdeletions are characterized by non-recurrent BPs. This is due to the presence, within the 22q12 region, of multiple classes of repetitive elements, including Alu, SINE, and LINE sequences.

The lack of recurrent BPs results in a highly variable size and gene content among 22q12 microdeletions. These deletions can range from approximately 100 kb to several megabases in length, encompassing from a single gene to nearly one hundred genes in addition to *NF2* itself (Trizuljak *et al.*, 2023). Moreover, the

structural characterization of these rearrangements is often incomplete, as standard diagnostic workflows primarily rely on MLPA and array-CGH analyses, both techniques that lack the resolution required for precise BP mapping and for an exhaustive assessment of the deletion gene content (Tritto *et al.*, 2022).

Consequently, the limited number of reported cases, the pronounced heterogeneity of the deletions, and the incomplete clinical and molecular characterization of affected individuals have thus far precluded the establishment of clear genotype-phenotype correlations for *NF2* deletion carriers. The pathogenic mechanisms underlying the severity and variability of the *NF2* microdeletion phenotype therefore remain largely unexplored.

Some studies have proposed preliminary correlations between the haploinsufficiency of specific genes co-deleted with *NF2* and distinct clinical features. These correlations have typically been inferred by identifying the minimal common deleted region among patients exhibiting shared clinical traits, although functional validation is still lacking. For instance, the *MNI* gene, located 1.8 Mb upstream of *NF2*, has been proposed as a candidate gene responsible for cleft palate (Beck *et al.*, 2015; Breckpot *et al.*, 2016), while *DEPDC5*, located approximately 2 Mb downstream of *NF2*, has been implicated in seizure disorders (Trizuljak *et al.*, 2023). We have previously proposed *AP1B1*, mapped 215 kb upstream of *NF2*, as a potential contributor to cognitive impairment (Tritto *et al.*, 2022). Additionally, the *CHEK2* gene, located 900 kb upstream of *NF2* and encoding a cell-cycle checkpoint regulator, may act synergistically with *NF2* in predisposing *NF2* microdeletion patients to multiple tumor types (Stolarova *et al.*, 2020).

As well as discussed for *NF1*, the phenotypic variability observed among patients carrying 22q12 microdeletions may also be explained by other genetic/epigenetic mechanisms, at least in part, such as the pseudodominance – a mechanism in which recessive alleles on the homologous (non-deleted) chromosome become unmasked within the deleted interval, thereby contributing to disease expression. This phenomenon has been invoked to explain atypical or additional clinical features in syndromic patients with microdeletions (Schwartz *et al.*, 2018; Pebrel-Richard *et al.*, 2014; Brezo *et al.*, 2014; Jing *et al.*, 2021).

In a recent study, for the first time in NF2, we proposed and demonstrated the existence of an additional pathogenic mechanism potentially contributing to the complex phenotype of patients with 22q12 microdeletions, namely the position effect on the expression of genes flanking the deletion (Tritto *et al.*, 2022).

Taken together, these findings suggest that a deeper investigation of the common and peculiar pathogenic mechanisms underlying the clinical phenotype of NF2 microdeletion patients is still essential.

Based on these premises, the research project presented in this thesis aimed to characterize two novel 22q12 microdeletions identified in NF2 patients exhibiting complex phenotypes. In both cases, the clinical presentation included not only the classical manifestations of NF2 – such as vestibular schwannomas, hearing loss, meningiomas, and ependymomas – but also additional, patient-specific features, including intellectual disability and congenital heart malformations. Since the genomic context presents high density of repetitive elements, the application of conventional PCR methods for the identification of deletion junction fragment failed for the two patients. The exact localization of the deletion BPs was obtained through the application of LRS technology, inferring the underlying molecular mechanism. To identify possible genotype-phenotype correlations, we then analyzed not only the gene content within each deletion but also additional variants or CNVs detected by LRS that could contribute to phenotypic variability. Finally, we evaluated the potential position effect on the expression of genes flanking the identified deletions.

5.2.1 Application of Long-Read Sequencing for a comprehensive analysis of 22q12 microdeletions

The 22q12 deletions identified in patients PT 568 and PT 690 had been previously detected through MLPA, according to current guidelines for *NF2* mutation screening. However, this assay provides reliable coverage only within the *NF2* gene, while the regions flanking the gene are poorly represented. As a result, for these patients, MLPA data only indicated broad genomic intervals – spanning

This stepwise approach highlights the limitations of conventional diagnostic techniques in resolving complex genomic rearrangements and underscores the decisive contribution of LRS in achieving nucleotide-level BP definition. The advent of LRS technologies, such as Oxford Nanopore, used in this study, and PacBio HiFi, has profoundly improved the structural characterization of genomic rearrangements, particularly in regions enriched in repetitive elements. In contrast to short-read sequencing and array-based approaches, which often fail to resolve complex or repetitive genomic architectures, LRS enables the direct and continuous sequencing of long DNA fragments, thereby allowing the accurate delineation of deletion junction fragments (Xu *et al.*, 2025; Showpnil *et al.*, 2024).

This enhanced capability is particularly relevant for the 22q12 region, which harbors a high density of repetitive elements, including SINE, LINE, LTR, STR, and Alu sequences, that pose significant challenges for both BP prediction and amplification of deletion junction fragments (Tritto *et al.*, 2022). Within this complex genomic context, the application of LRS has proven crucial in overcoming the ambiguity introduced by the highly repetitive landscape of chromosome 22q12. Traditional methods such as MLPA or array-CGH can only infer the approximate boundaries of deletions, whereas short-read sequencing frequently fails to uniquely align reads within homologous repeat regions, resulting in incomplete or erroneous BP mapping. By contrast, the extended read lengths and improved mappability of long-read data allow the traversal of repetitive regions and enable the precise identification of non-recurrent and structurally complex BPs with high accuracy (Warburton and Sebra, 2023).

This level of precision is crucial for elucidating the molecular basis of *NF2* microdeletions. The accurate identification of BP junctions, providing insight into the sequences mediating the rearrangement, allows the inference of the underlying mutational mechanisms, such as NAHR, NHEJ, or replication-based repair processes.

In the present study, our findings suggest that NHEJ or MMEJ repair pathways, as well as replication-based processes such as FoSTeS/MMBIR, may represent the mechanisms responsible for the two 22q12.1q12.2 deletions analyzed. This

hypothesis is supported by the low degree of sequence homology (approximately 50%) observed between the repetitive motifs mapped at the centromeric and telomeric BP regions of each patient, namely, the STRs (CA)_n and (CCCAT)_n in PT 568, and the SINE AluSx1 and the LINE L1MB3 elements in PT 690. Additional evidence comes from the detection of short insertions at the BP junctions – 30 bp in PT 568 and 1 bp in PT 690 – which are consistent with imperfect end-joining events characteristic of NHEJ/MMEJ repair pathways or with replication-based mechanisms such as FoSTeS/MMBIR.

Both NHEJ and MMEJ, as well as replication-based processes such as FoSTeS/MMBIR, have been proposed as mechanisms underlying the formation of non-recurrent genomic deletions (Lee *et al.*, 2007; Marey *et al.*, 2016; You *et al.*, 2013). In the NHEJ pathway, double-strand DNA breaks are repaired through the direct ligation of DNA ends, often accompanied by the loss of DNA fragments of variable length and the insertion of small additional nucleotides at the junction site (Gu *et al.*, 2008). MMEJ is an error-prone repair mechanism that relies on the alignment of short microhomologous sequences internal to the break ends prior to ligation, and typically results in deletions or insertions that mark the original break site (Sfeir and Symington, 2015). FoSTeS/MMBIR represents a replication-associated mechanism triggered by replication fork stalling or collapse, in which the disengaged DNA strand switches templates using short regions of microhomology, potentially generating complex genomic rearrangements, including deletions, duplications, and insertions (Zhang *et al.*, 2009). Therefore, all these mechanisms appear compatible with the BP features observed in our study. Conversely, NAHR can be excluded, as this mechanism requires a high degree of sequence homology (> 95%) and is typically mediated by recombination between LCRs (Marey *et al.*, 2016).

Taken together, these observations support the hypothesis that repetitive elements within the *NF2* locus act as hotspots of genomic instability, predisposing the 22q12 region to non-recurrent rearrangements mediated by error-prone DNA repair or replication-associated mechanisms. This intrinsic structural fragility may explain the heterogeneity and sporadic occurrence of *NF2* microdeletions, further

highlighting the importance of high-resolution approaches such as LRS for their accurate characterization.

The precise identification of deletion BPs also enables the accurate determination of the gene content within each deleted interval. This information is essential for establishing robust genotype-phenotype correlations, as the inclusion or exclusion of specific genes beyond *NF2* itself can markedly influence both the clinical severity and the spectrum of associated manifestations. Such correlations are particularly relevant in *NF2* microdeletion patients, where the wide heterogeneity in deletion size and gene content contributes to the variability in disease expression among individuals (Trizuljak *et al.*, 2023). A detailed molecular definition of the deleted region through LRS can therefore improve prognostic assessment and support personalized clinical management and follow-up, facilitating the early detection and monitoring of comorbidities or complications linked to the loss of additional genes.

In the context of identifying potential genotype-phenotype correlations relevant for prognostic assessment and personalized medicine, another major advantage provided by LRS in *NF2* microdeletion patients is its ability to detect additional genetic variants and CNVs that may contribute to the clinical phenotype.

Although the relatively high error rate and current cost of LRS still limit its implementation in routine clinical diagnostics, this technology represents a transformative tool for the accurate structural characterization of *NF2* deletions. With a single assay, it enables the comprehensive detection of both chromosomal rearrangements and additional alterations in potential modifier genes, thereby offering an all-encompassing view of the patient's genomic landscape (Perez-Becerril *et al.*, 2024).

5.2.2 Study of the chromatin structure alteration and position effect associated with 22q12 microdeletions

In the present study, the position effect caused by the 22q12.1q12.2 deletions identified in patients PT 568 and PT 690 was investigated. As a first step, an *in*

silico analysis was performed to assess the topological organization and regulatory landscape of the 22q12 region. As shown in Figure 37, the microdeletion in PT 568 removes three TADs completely and one partially at the telomeric end, while the PT 690 microdeletion involves three TADs, two of which are only partially affected. The 22q12 region comprises six enhancers – three of which fall within the PT 568 deletion – and thirty-nine CTCF-binding sites, indicative of chromatin insulator elements (Dixon *et al.*, 2012), of which 30 and 24 are deleted in PT 568 and PT 690, respectively. These findings suggest the establishment of non-canonical chromatin interactions between the TADs located upstream and downstream of the deletions, potentially leading to altered regulatory contacts across the locus.

TADs are chromosomal domains within which DNA sequences frequently engage in long-range physical interactions, providing the structural framework that supports the regulatory architecture of the genome (Dixon *et al.*, 2012; Ea *et al.*, 2015). Regulatory elements such as enhancers and silencers typically exert their activity by physically contacting promoters within the same TAD (Nora *et al.*, 2013; Nora *et al.*, 2017). Consequently, structural rearrangements that disrupt TAD boundaries can profoundly alter the regulatory context of a genomic region. Such reorganization may result in disease-associated position effects, driven by the inappropriate adoption or loss of enhancer-promoter interactions, or by competition among neighboring genes for shared regulatory elements (Chen *et al.*, 2009; Ibn-Salem *et al.*, 2014; Giorgio *et al.*, 2015).

Although the *in silico* analysis of TADs was performed using Hi-C data from the lymphoblastoid cell line GM12878 on 3D Genome Browser 2.0 (Fig. 37), it is noteworthy that the topological organization of the chromosomal region under investigation appears to be conserved across different cell types (Fig. 41). As shown in Figure 41, which displays Hi-C-derived heatmaps from multiple human cell lines – including a lymphoblastoid cell line (GM12878) (Fig. 41A), a dorsolateral prefrontal cortex-derived (DLPFC) cell line (Fig. 41B), iPSC-derived neurons (NPC-Derived_Neurons_D868D_1) (Fig. 41C), and mesenchymal stem cells (H1-MS) (Fig. 41D) – the region affected by the 22q12.1q12.2 deletions identified in

patients PT 568 and PT 690 shows a highly similar topological organization across all cell types.

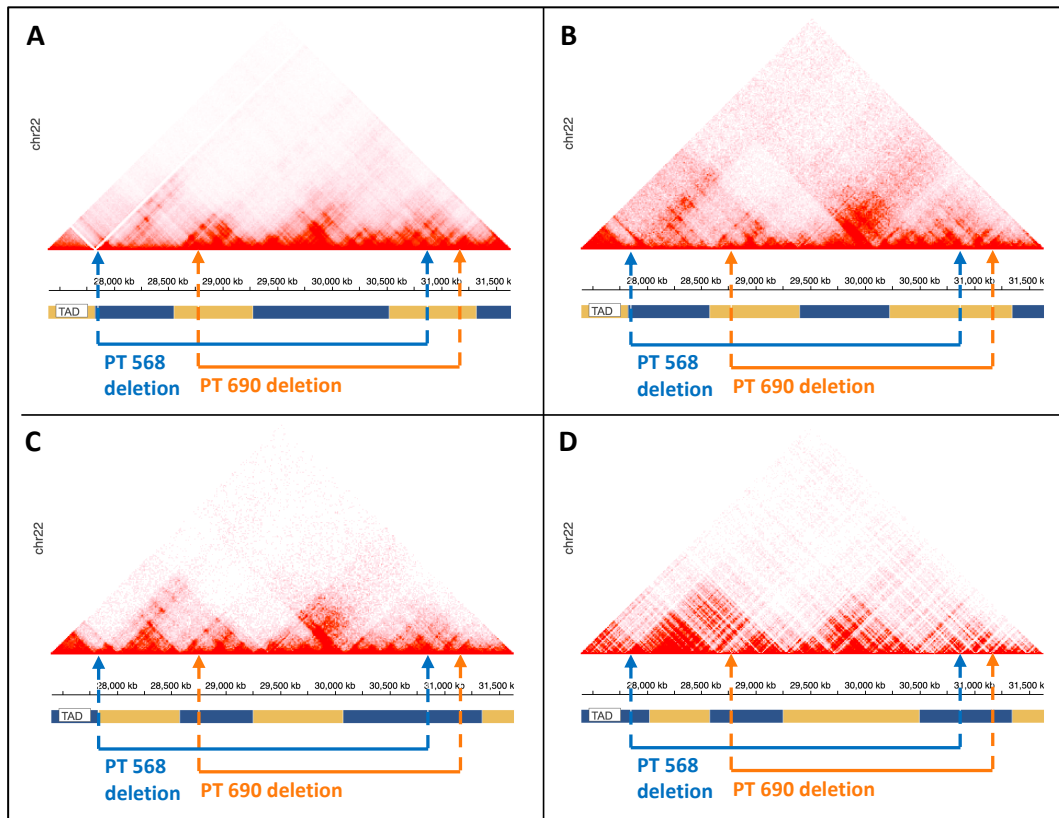


Figure 41. Topological organization of the chromosomal region harboring the 22q12.1q12.2 deletions identified in patients PT 568 and PT 690 across different cell lines. Hi-C heatmaps generated using 3D Genome Browser 2.0 illustrate large-scale chromosomal interactions defining TADs within the 22q12 region. Data are shown for four human cell lines: (A) GM12878 lymphoblastoid cells; (B) DLPFC cell line derived from dorsolateral prefrontal cortex tissue; (C) NPC-Derived_Neurons_D868D_1 iPSC-derived neurons; and (D) H1-MSC mesenchymal stem cells. Deletion BPs of PT 568 and PT 690 are indicated by vertical dashed lines. The overall topological organization of the region is conserved across all analyzed cell lines.

Based on these premises, we hypothesized that the conformational changes in chromatin induced by the 22q12.1q12.2 deletions could impact gene expression. To test this, the expression levels of selected genes flanking the *NF2* deletions in the two patients were quantified by qPCR. The results revealed altered expression profiles for most of the analyzed genes in both patients compared with healthy controls (Fig. 38). Although it cannot be excluded that one or more genes may be subject to inter-individual variability unrelated to the microdeletion, these findings

suggest the presence of a position effect, likely resulting from the establishment of a novel regulatory configuration following chromatin remodeling.

The analysis was performed on RNA extracted from peripheral blood, thus capturing this mechanism specifically in that tissue. Nevertheless, since the selected genes are ubiquitously expressed and the TADs of the 22q12 region appear to be conserved across different cell lines, including those of the nervous system (Fig. 41), a tissue particularly affected in NF2, it is plausible that similar expression alterations may occur in other tissues as well.

To elucidate the molecular mechanisms underlying the observed position effect, further chromatin organization studies will be necessary (Fig. 15). Given the number of deregulated genes and regulatory elements identified within the 22q12 region, Hi-C analysis would represent a powerful next step. Hi-C, the most comprehensive evolution of chromosome conformation capture techniques, enables genome-wide mapping of physical interactions between chromosomal regions that are spatially proximal in the 3D nuclear space, even when distant in the linear genome (Šimková *et al.*, 2024). Application of this method would allow the identification of novel TADs formed as a consequence of the deletions, as well as non-canonical long-range interactions between promoters, enhancers, and other regulatory elements, thereby elucidating the molecular basis of the detected position effect.

In a previous study from our group on patients with NF1 microdeletion syndrome, 4C-seq assays functionally validated a position effect associated with the type-1 *NF1* deletion (Tritto *et al.*, 2024). Interestingly, this analysis revealed chromatin remodeling not only in the genomic regions flanking the deletion, as expected, but also within the deleted interval on the homologous non-deleted chromosome. This observation suggests the involvement of *trans*-acting mechanisms, possibly mediated by cross-talk between the two homologous chromosomes 17. Building on these findings, we aimed to determine whether the position effect identified in NF2 microdeletion patients influences gene expression solely *in cis* – affecting the allele flanking the 22q12.1q12.2 deletion – or also *in trans*, impacting both alleles.

To this end, digital PCR assays are currently underway to evaluate allele-specific differential expression of selected deletion flanking genes showing deregulated expression. Unlike qPCR, digital PCR provides higher sensitivity and allele-specific absolute quantification using probes designed at the level of specific heterozygous polymorphisms (Basu, 2017). For these assays, heterozygous SNVs with a high MAF identified by LRS – which also determined their phasing relative to the deletion (*cis* or *trans*) – will be exploited (Table 36 and Table 37). These experiments will provide insights into whether the position effect acts locally or involves broader regulatory interactions extending beyond the deleted allele.

Together, these findings underscore the importance of investigating the 3D organization of the genome as a key determinant of gene regulation, as the alterations in chromatin architecture may represent an additional layer of complexity influencing the phenotypic variability observed in NF2 microdeletion patients.

5.2.3 Investigation of potential epigenotype/genotype-phenotype correlations in NF2 microdeletion patients

Given the complex clinical phenotypes observed in the two NF2 microdeletion patients analyzed in this study – characterized by the presence of both classical NF2 manifestations and additional, less common clinical features such as intellectual disability (in both patients) and cardiac abnormalities (in PT 690) – genotype-phenotype correlations were explored based on the pathogenic mechanisms investigated.

First, once the precise BPs of the 22q12.1q12.2 deletions were defined, the gene content of each deletion was analyzed. To estimate gene haploinsufficiency, we considered the pLI score (probability of being loss-of-function intolerant) derived from the ExAC database (Lek *et al.*, 2016). The deletions identified in PT 568 and PT 690 encompassed eight genes with $pLI \geq 0.9$, seven of which were shared (*CCDC117*, *ZNRF3*, *EWSR1*, *AP1B1*, *NF2*, *MTMR3*, and *SF3A1*) and one unique to each deletion (*TTC28* in PT 568 and *MORC2* in PT 690), suggesting that

their haploinsufficiency is likely to have pathological consequences and may collectively contribute to the complex clinical phenotype of these patients. Nevertheless, the potential contribution of the remaining 36 and 39 genes included in the PT 568 and PT 690 deletions, respectively – though not predicted to be loss-of-function sensitive – cannot be excluded *a priori*, as they might still modulate disease severity.

Subsequently, the analysis focused on identifying specific genotype-phenotype correlations between deleted genes and the clinical features observed in these patients. Since both PT 568 and PT 690, as well as two previously characterized NF2 microdeletion patients (PT 160 and PT 366) from our cohort (Tritto *et al.*, 2022), presented with intellectual disability, we hypothesized that this phenotype could be associated with a gene commonly deleted among these cases. By comparing the gene content of the four deletions, we defined the smallest region of overlap (Table 42), encompassing 13 genes (highlighted in red) including two, in addition to *NF2*, predicted to be loss-of-function intolerant (in bold).

Table 42. Gene content of 22q12 deletions in PT 568, PT 690, PT 160, and PT 366

Patients	Deletion gene content
PT 568	<i>PITPNB</i> , <i>TTC28</i> , <i>CHEK2</i> , <i>HSCB</i>
PT 568 - PT 690	<i>CCDC117</i> , <i>XBPI</i>
PT 568 - PT 690 - PT 160	<i>ZNRF3</i> , <i>C22orf31</i> , <i>KREMEN1</i>
PT 568 - PT 690 - PT 160 - PT 366	<i>EMID1</i> , <i>RHBDD3</i> , <i>EWSR1</i> , <i>GAS2L1</i> , <i>RASL10A</i> , <i>AP1B1</i> , <i>RFPL1</i> , <i>NEFH</i> , <i>THOC5</i> , <i>NIPSNAP1</i> , <i>NF2</i> , <i>CABP7</i> , <i>ZMAT5</i>
PT 568 - PT 690 - PT 160	<i>UQCR10</i> , <i>ASCC2</i> , <i>MTMR3</i> , <i>HORMAD2</i> , <i>LIF</i> , <i>OSM</i> , <i>CASTOR1</i> , <i>TBC1D10A</i> , <i>SF3A1</i> , <i>CCDC157</i> , <i>RNF215</i> , <i>SEC14L2</i> , <i>MTFPI</i> , <i>SEC14L3</i> , <i>SEC14L4</i> , <i>SEC14L6</i> , <i>GAL3ST1</i> , <i>PES1</i> , <i>TCN2</i> , <i>SLC35E4</i> , <i>DUSP18</i> , <i>OSBP2</i>
PT 690	<i>MORC2</i> , <i>TUG1</i> , <i>SMTN</i> , <i>SELENOM</i> , <i>INPP5J</i> , <i>PLA2G3</i> , <i>RNF185</i>

Genes with pLI ≥ 0.9 are shown in bold; genes included in the smallest region of overlap are displayed in red.

Among the genes within the smallest region of overlap, *AP1B1* emerged as a compelling candidate for association with the intellectual disability phenotype. *AP1B1* encodes the β -subunit of the adaptor protein complex 1 (AP-1), which regulates intracellular membrane trafficking and ensures the correct localization and function of the copper transporter ATP7A, essential for copper absorption and

secretion. Loss-of-function mutations in *APIBI* disrupt the localization of transmembrane proteins and impair ATP7A activity, and when present biallelically, cause MEDNIK-like syndrome, a disorder characterized by intellectual disability, enteropathy, sensorineural deafness, peripheral neuropathy, ichthyosis, and keratoderma (Alsaif *et al.*, 2019).

To assess the potential involvement of *APIBI* in the intellectual disabilities of our patients, both the presence of loss-of-function variants and the expression level of the remaining allele were examined. Variant analysis using LRS data revealed no deleterious variants, including intronic changes classified in ClinVar as benign or VUS, in both patients, and a single exonic variant, c.2349G>A (NM_001127.4), only in PT 690 (Table 32). The last variant has also been previously detected in PT 160 and PT 366; however, it is a synonymous benign variant with a MAF $\approx$ 50% (Tritto *et al.*, 2022). qPCR analysis showed an approximately 60% reduction in *APIBI* expression in PT 568 and PT 690 relative to healthy controls (Fig. 36). The *APIBI* screening results therefore support the presence of one functional allele, consistent with the absence of the full MEDNIK-like phenotype in these patients.

Nonetheless, haploinsufficiency of *APIBI* may still contribute to the neurological manifestations. Indeed, when comparing the gene content and phenotypes of 22 patients with *NF2* microdeletions – including the two patients presented here, three cases from our previous study (Tritto *et al.*, 2022), and 17 cases reported by Trizuljak *et al.* (2023) – loss of *APIBI*, found in 18 cases, is accompanied by intellectual disability in 89% of them (Fig. 42). Three additional patients presented cognitive impairment despite retention of *APIBI* (Fig. 42); however, it cannot be ruled out that *APIBI* expression is deregulated by a position effect, or that other genetic or regulatory factors contribute to the cognitive phenotype. Further functional studies, such as those using patient-derived iPSCs differentiated into neurons, will be necessary to clarify the role of *APIBI* in neurological impairment among *NF2* microdeletion patients.

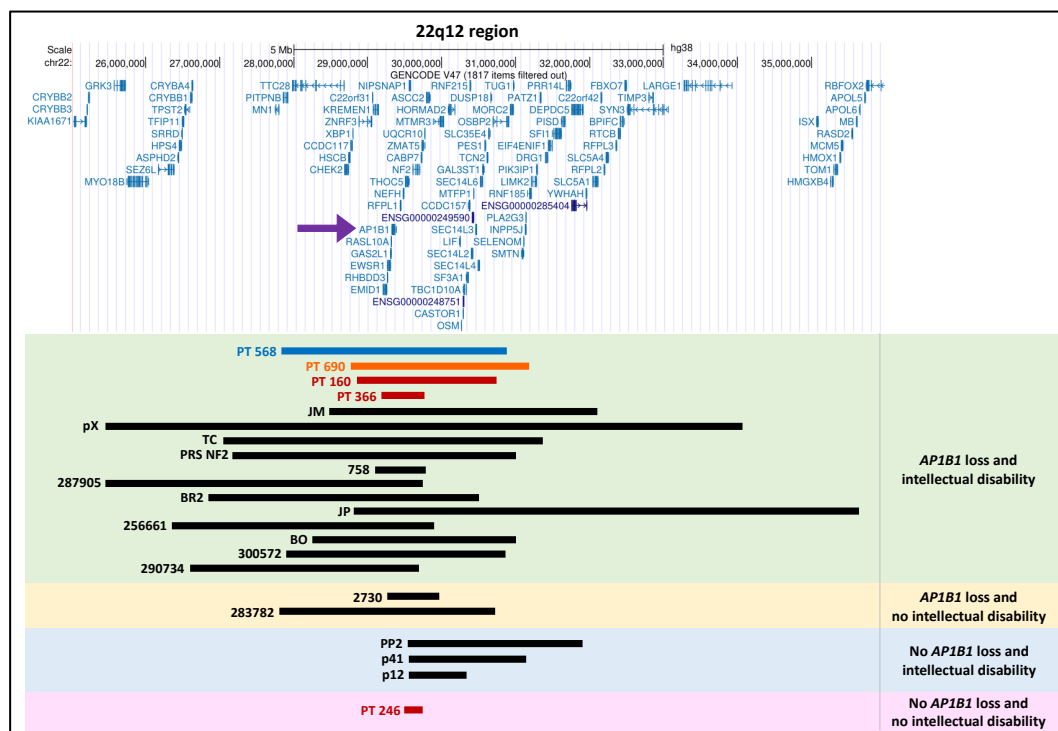


Figure 42. Correlation between *AP1B1* hemizyosity and intellectual disability in patients with 22q12 deletions. The upper panel shows the genes mapped within the 22q12 region, including *AP1B1* (indicated by the purple arrow). The lower panel illustrates the deletions detected in PT 568 (blue), PT 690 (orange), three previously characterized patients from our cohort (red), and 17 cases reported in the literature (black). Deletions are highlighted with a green box when *AP1B1* is deleted and intellectual disability is present, with a yellow box when *AP1B1* is deleted but no intellectual disability is observed, with a light-blue box when intellectual disability occurs without *AP1B1* involvement, and with a pink box when neither *AP1B1* loss nor intellectual disability is detected.

Another atypical feature for NF2 identified in our cohort was the presence of cardiac anomalies, detected exclusively in PT 690. Since this patient's deletion extends further into the telomeric region of *NF2* than the other cases, the gene content of this additional segment was examined. Seven genes were identified within this region (Table 42); however, none have previously been associated with cardiac malformations. This observation suggests that the cardiac phenotype of PT 690 is unlikely to be solely attributable to the loss of genes within the deleted 22q12 region.

Interestingly, based on LRS results, a possible genotype-phenotype correlation emerged between the cardiac abnormalities of PT 690 and a pathogenic frameshift variant in the *EVC* gene, c.922_923delGA (p.Glu308fs). This variant introduces a

premature stop codon predicted to cause truncation and nonsense-mediated decay of the protein and has been reported in the homozygous state in patients with Ellis-van Creveld syndrome (Shamseldin *et al.*, 2020), a recessive disorder frequently associated with congenital heart defects (Da Silva *et al.*, 2023). To explore potential biallelic involvement of *EVC*, additional variants *in trans* were searched among the unfiltered LRS data for PT 690. Five variants were found, but none were predicted to be pathogenic (Table 41). Considering that PT 690 does not display all hallmark features of Ellis-van Creveld syndrome, such as postaxial polydactyly or thoracic malformations, it remains possible that heterozygous loss-of-function variant of *EVC* contributes specifically to the cardiac phenotype. Future functional studies using iPSC-derived cardiomyocytes from PT 690 could provide mechanistic insight into this potential association.

An additional pathogenic mechanism explored in this thesis that may contribute to the complex phenotype of NF2 microdeletion patients is the position effect. In peripheral blood, altered expression was detected for multiple genes: in PT 568, *MORC2*, *RNF185*, *LIMK2*, *PIK3IP1*, *DRG1*, and *EIF4ENIF1* were upregulated, while *SELENOM* and *SFII* were downregulated; in PT 690, *PITPNB*, *HSCB*, *LIMK2*, *PIK3IP1*, *DRG1*, and *PISD* were upregulated, whereas *PATZ1* and *SFII* showed reduced expression (Fig. 38). Although these transcriptional changes did not directly correlate with specific clinical traits, they confirm the presence of a position effect resulting from *NF2* microdeletions.

A limitation of this approach lies in its tissue specificity: expression analyses were performed on peripheral blood, while deregulation mediated by position effect may occur predominantly in disease-relevant tissues. Therefore, future studies should extend this analysis to iPSC-derived cell types most affected in NF2 pathology, such as Schwann cells. These iPSC models will also enable the investigation of genes expressed specifically in these tissues, which have not yet been evaluated by qPCR as they are not expressed in blood. Among these, *MNI* is of particular interest, as its deregulation has been implicated in the malignant transformation of meningiomas (Zhang *et al.*, 2014; Ragel and Jensen, 2005).

In conclusion, establishing clear genotype-phenotype correlations that fully account for the complex clinical manifestations observed in NF2 microdeletion patients remains a major challenge. This difficulty stems from the rarity of such cases, the variable sizes and non-recurrent BPs of deletions, and the limited number of studies providing detailed molecular characterization, including precise BP definition and complete gene content analysis. Expanding the characterization of 22q12 microdeletions in a larger patient cohort, integrating both deleted and flanking genes, and performing functional studies will be essential to validate candidate genotype-phenotype correlations. Such efforts may ultimately enable the identification of novel therapeutic targets, paving the way toward personalized medicine approaches aimed at alleviating the pathological manifestations associated with *NF2* microdeletions.

6. CONCLUSIONS AND PERSPECTIVES

The results obtained in this study allow the following conclusions to be drawn:

- The severity and variability of the clinical phenotypes observed in patients carrying *NF1* and *NF2* microdeletions strongly indicate that the pathogenesis of both syndromes extends beyond the mere haploinsufficiency of the *NF1* and *NF2* genes, respectively.
- A dysregulation of the PRC2 repressive complex and its activity was detected in NF1 patients, both with microdeletions and intragenic mutations. This alteration is likely driven by Ras pathway hyperactivation and may contribute to the tumorigenic phenotype characteristic of these patients.
- Two iPSC lines were successfully generated as disease models from NF1 microdeletion patients, one carrying the type-1 *NF1* deletion and the other an atypical deletion, providing a valuable tool for future functional and molecular studies.
- The precise BPs and gene content of two *NF2* microdeletions were defined by the application of Long-Read Sequencing. This technology also enabled the identification of additional variants and CNVs in the patients, allowing for the establishment of genotype-phenotype correlations.
- Through the analysis of the topological organization and regulatory landscape of the 22q12 region, it was possible to predict alterations in the 3D chromatin structure and regulatory interactions associated with *NF2* deletions, leading to a position effect that impacts the expression of several deletion flanking genes.
- By providing more detailed epigenotype/genotype-phenotype correlations, the results of this work have potential implications for diagnosis, prognosis, and counseling of patients carrying *NF1* and *NF2* microdeletions, as well as for the development of personalized therapeutic strategies.
- Overall, this work represents a pilot study, opening new perspectives on the identification of pathogenic mechanisms in microdeletion syndromes. The findings suggest that the phenotype may result not only from the loss of the deleted genes themselves but also from deregulation of neighboring genes and the contribution of variants in modifier genes.

To validate the hypotheses formulated in this thesis and to further elucidate the mechanisms underlying the pathogenesis of patients carrying *NF1* and *NF2* microdeletions, several future research directions are envisioned:

- To better understand the contribution of deleted genes to the phenotype of NF2 microdeletion patients, it will be necessary to expand the patient cohort, combining detailed molecular characterization of deletion BPs and gene content with comprehensive clinical profiling.
- To obtain additional disease models, new iPSC lines will be generated from NF1 and NF2 patients carrying both intragenic mutations and microdeletions. These models will serve as a platform for mechanistic and therapeutic studies.
- To validate the proposed pathogenic mechanisms and epigenotype/genotype-phenotype correlations, functional studies will be performed on patient-derived iPSCs differentiated into disease-relevant cell types, such as Schwann cells and neurons. These studies will integrate gene-editing approaches, chromosome conformation capture techniques, and Omics analyses.

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7.2 Sitography

- 3D Genome Browser 2.0: <http://3dgenome.fsm.northwestern.edu/>
- BLAST: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>
- ClinVar: <https://www.ncbi.nlm.nih.gov/clinvar/>
- CTCFBSDB 2.0: <http://insulatordb.uthsc.edu/>
- DECIPHER: <https://www.deciphergenomics.org/>
- Ensembl genome browser: <http://www.ensembl.org/index.html/>
- Epi2me: <https://github.com/epi2me-labs/wf-human-variation>
- Franklin: <https://franklin.genoox.com/clinical-db/home>
- GeneCards: <https://www.genecards.org>
- gnomAD v.2.1.1: <https://gnomad.broadinstitute.org/>

- GTEx Portal: <https://www.gtexportal.org/>
- NCBI: <http://www.ncbi.nlm.nih.gov/pubmed/>
- OligoAnalyzer™ Tool: <https://eu.idtdna.com/pages/tools/oligoanalyzer>
- OMIM: <http://www.omim.org/>
- Primer3web version 4.1: <http://primer3.ut.ee/>
- PubMed: <https://pubmed.ncbi.nlm.nih.gov/>
- St. Jude Pecan; <https://pecan.stjude.cloud/variants/proteinpaint>
- Statistics Kingdom: <https://www.statskingdom.com/>
- String: <https://string-db.org/cgi/>
- UCSC Genome Browser: <https://genome.ucsc.edu/>
- Uniprotkb: <https://www.uniprot.org/uniprotkb>
- VISTA Enhancer Browser: <https://enhancer.lbl.gov/>