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IN VITRO AND *IN VIVO* MODELLING OF CORNELIA DE LANGE SYNDROME CAUSATIVE MUTATIONS: WNT ACTIVATION AS A POSSIBLE THERAPEUTIC APPROACH

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All’importanza delle collaborazioni
e delle nuove conoscenze

SOMMARIO

La sindrome di Cornelia de Lange (CdLS) è una rara malattia genetica che colpisce ogni distretto del corpo, compreso il sistema nervoso centrale, causando un ritardo variabile del neurosviluppo. Le malformazioni causate da questa sindrome derivano da mutazioni a carico di geni del complesso delle coesine (complesso proteico coinvolto nel controllo della coesione dei cromatidi fratelli) e da un'alterata regolazione di *pathway* molecolari durante lo sviluppo, tra cui il *pathway* canonico di WNT, che risulta essere meno attivo.

Al fine di osservare un miglioramento del fenotipo, ho indagato i potenziali effetti positivi dell'utilizzo di cloruro di litio come attivatore del *pathway* canonico di WNT, avvalendomi di due modelli differenti: cellule staminali pluripotenti indotte di origine umana (hiPSCs) e *Drosophila melanogaster*.

Per simulare un'aploinsufficienza delle coesine, le hiPSCs differenziate in precursori neurali (hNPC), sono state trattate con uno specifico inibitore della proteina HDAC8, nota per essere implicata nella patogenesi della CdLS. Dopo essere state trattate con cloruro di litio, queste cellule hanno mostrato una migliorata capacità di differenziamento attraverso il *lineage* neuronale.

Esemplari adulti di *Drosophila melanogaster* portatori di un allele *loss-of-function* a carico di un gene delle coesine, dopo essere stati allevati su cibo supplementato con litio come attivatore del *pathway* canonico di WNT, hanno mostrato un recupero statisticamente significativo della morfologia dei *mushroom bodies*, una struttura del sistema nervoso centrale importante per l'apprendimento olfattivo e la memoria.

I risultati derivanti da questo progetto di dottorato supportano ulteriormente l'ipotesi che una perturbazione del *pathway* canonico di WNT, causato da mutazioni a carico delle coesine, giochi un ruolo centrale nell'eziopatogenesi della CdLS. Questa teoria è corroborata dal recupero consistente del fenotipo nei modelli sperimentali grazie alla somministrazione di cloruro di litio e apre la strada a possibili strategie terapeutiche, fortemente necessarie per la CdLS.

ABSTRACT

Cornelia de Lange Syndrome (CdLS) is a rare genetic disorder affecting almost any organ including the central nervous system, inducing a variable neurodevelopmental delay. CdLS malformations derive from mutations in cohesin complex genes (protein complex involved in the cohesion control of sister chromatids) and deregulation of developmental pathways, inclusive of the canonical WNT pathway, which results less active.

In order to ameliorate the phenotype, I have explored possible ameliorative effects of the canonical WNT pathway chemical activation using lithium in two different models: human induced pluripotent stem cells (hiPSCs) and *Drosophila melanogaster.*

For mimicking cohesins haploinsufficiency, hiPSCs differentiated in neural precursor cells (hNPC) were treated with a specific HDAC8 inhibitor, protein known to be implicated in CdLS pathogenesis. Upon treatment with lithium chloride, cells showed improved differentiation capabilities through the neuronal lineage.

Drosophila melanogaster adults carrying a loss-of-function allele in a cohesins gene and reared on food supplemented with lithium as activator of canonical WNT pathway, showed a significant rescue in mushroom bodies morphology, a central nervous system structure important for the olfactory learning and memory.

This PhD project's results further support the hypothesis that disruption in canonical WNT pathway, caused by cohesins mutations, plays a central role in CdLS etiopathogenesis. This theory is corroborated by the consistent phenotype rescue by lithium administration in experimental models, paving the way for new possible and urgently needed therapeutic strategies.

LIST OF ABBREVIATIONS

ANKRD11 – Ankyrin Repeat Domain 11
APC – Adenomatous Polyposis Coli
ASD – Autism Spectrum Disorder
βTrCP – β-Transducin repeat-Containing Protein
BdLS – Brachmann-de Lange syndrome
BRD4 – Bromodomain Containing 4
CdLS – Cornelia de Lange Syndrome
cffDNA – cell-free fetal DNA
CNS – Central Nervous System
CK1α – Casein Kinase 1 α
CTCF – CCCTC-binding Factor
DCA – Deoxycholic Acid
DPBS – Dulbecco's Phosphate-Buffered Saline
DSB – Double Strand Break
DVL – Dishevelled
EDTA – Ethylenediaminetetraacetic Acid
EMS – Ethyl Methanesulfonate
EtOH – Ethanol
FZD – Frizzled
GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase
GFP – Green Fluorescent Protein
GSK3β – Glycogen Synthase Kinase 3 β
HDAC8 – Histone Deacetylase 8
hiPSCs – human induced Pluripotent Stem Cells
hNPC – human Neural Precursor Cells
LRP5/6 – Low-density-lipoprotein-Related Protein 5/6
NGS – Next Generation Sequencing
NIPBL – Nipped-B Like Protein
NMR – Nuclear Magnetic Resonance
PBS – Phosphate-Buffered Saline
PBT – Phosphate-Buffered Saline + Triton X-100
PCP – Planar Cell Polarity

PFA – Paraformaldehyde

PhD – Doctor of Philosophy

RAD21 - RAD21 Cohesin Complex Component

SMC1A – Structural Maintenance of Chromosomes 1A

SMC3 – Structural Maintenance of Chromosomes 3

TCF/LEF – T-Cell Factor/Lymphoid Enhancer Factor

WABS – Warsaw Breakage Syndrome

WES – Whole Exome Sequencing

WNT – Wingless-related integration site

WREs – WNT Responsive Elements

THESIS EVALUATION

The work presented in this thesis was reviewed and evaluated by the three following independent reviewers:

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RESEARCH INTEGRITY STATEMENT

This PhD project contains data that were produced and analyzed according to the principles of “The European Code of Conduct for Research Integrity” (ALLEA - All European Academies, Berlin 2018):

RELIABILITY to guarantee research quality;

HONESTY to produce complete, clear and unbiased data;

RESPECT for colleagues, society and environment;

ACCOUNTABILITY in coordination, publication supervision and tutoring.

This PhD project respects the open access key: research results have been and will be presented to conferences to establish an open dialogue with other researchers.

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1. INTRODUCTION

1.1 Cornelia de Lange Syndrome (CdLS)

Cornelia de Lange syndrome (CdLS; OMIM #122470, #300590, #610759, #614701, #300882) was first described by Winfried Robert Clemens Brachmann in 1916 [1], a German physician who wrote about the distinct features of the disease from a 19-year-old patient. As a matter of fact, this syndrome was previously known as Brachmann-de Lange syndrome (BdLS). Then, the same syndrome was better described by Cornelia Catharina de Lange, a Dutch pediatrician, in 1933 [2] after whom the disorder has been named Cornelia de Lange syndrome.

Cornelia de Lange syndrome is a rare genetic disorder that affects the development, and characterized by dysmorphic features, distinctive physical abnormalities and behavioral characteristics. These clinical features can vary widely among affected individuals and range from relatively mild to severe phenotypes.

The prevalence was assessed to be around 1 in 40,000 - 100,000 live births [3], [4] but it is now estimated to be more common, between 1 in 10,000 - 30,000 live births [5]. However, the exact number is still unclear as this condition is probably underdiagnosed, since affected individuals with mild or uncommon features may never be recognized as CdLS patients.

1.2 CdLS clinical presentation

A combination of signs and symptoms defines the classic CdLS phenotype: slow growth before and after birth leading to short stature; intellectual disability that is usually moderate to severe; and abnormalities of bones in the arms, hands, and fingers. CdLS patients also have distinctive facial features, including arched eyebrows that often meet in the middle (synophrys), long eyelashes, low-set ears, a small and upturned nose, and small and widely spaced teeth (Fig. 1). Some patients are born with a cleft palate. Moreover, seizures, heart defects, and eye problems have also been reported in people with this condition.

Many affected individuals present behavioral problems typical of autism spectrum disorders (ASD). Additional signs and symptoms can include excessive body hair (hypertrichosis), an unusually small head (microcephaly), hearing loss, and problems with the gastrointestinal tract (i.e. duodenal atresia, annular pancreas, imperforate anus, Meckel diverticulum and congenital diaphragmatic hernia) [6].

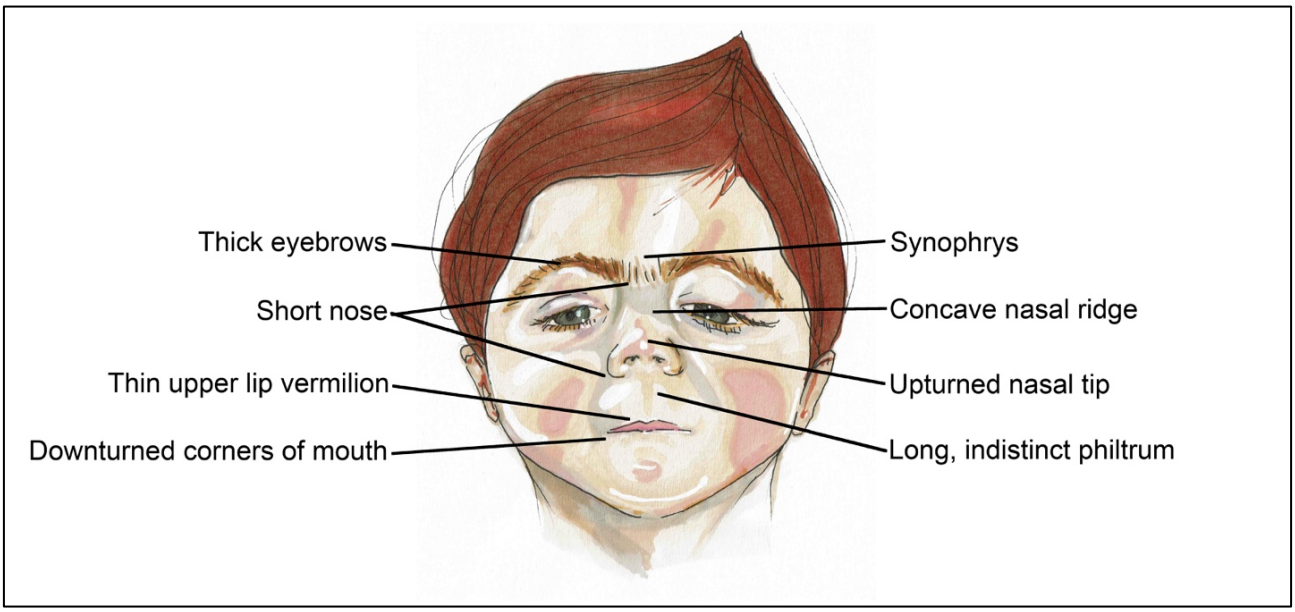

Figure 1 – Typical *facies* of CdLS patients. Typical facial features of a CdLS patient carrying a mutation in *NIPBL* gene. Typical facial dysmorphisms, together with other signs and symptoms, help the clinicians with the diagnosis.

The clinical features of patients can differ according to the pathogenetic variants in one of the seven known causative genes. In fact, different mutations can give rise to distinctive phenotypic signs, but sometimes the same variant can result in different phenotypes (Tab. 1), complicating the genotype-phenotype correlation for this syndrome.

CdLS is defined as a multisystemic condition since affected patients can present abnormalities in almost any anatomical district. Patients are reported to manifest clinical issues regarding: central nervous system (mental retardation, language delay, hypertonicity), eyes (ptosis, myopia, astigmatism, synophrys, long curly eyelashes, optic atrophy), ears (sensorineural hearing loss, conductive hearing loss to due otitis media), cardiovascular (congenital heart defect), respiratory lung (congenital diaphragmatic hernia, pneumonia), gastrointestinal tract (gastroesophageal reflux, pyloric stenosis), genitourinary system (cryptorchidism, hypoplastic male genitalia, structural anomalies of the renal tract, absent/poor corticomedullary differentiation, vesicoureteral reflux, small or ectopic kidney, renal cyst), skeletal apparatus (phocomelia, limited elbow extension, dislocation of the radial head, cleft lip/palate, short neck, microcephaly, brachycephaly).

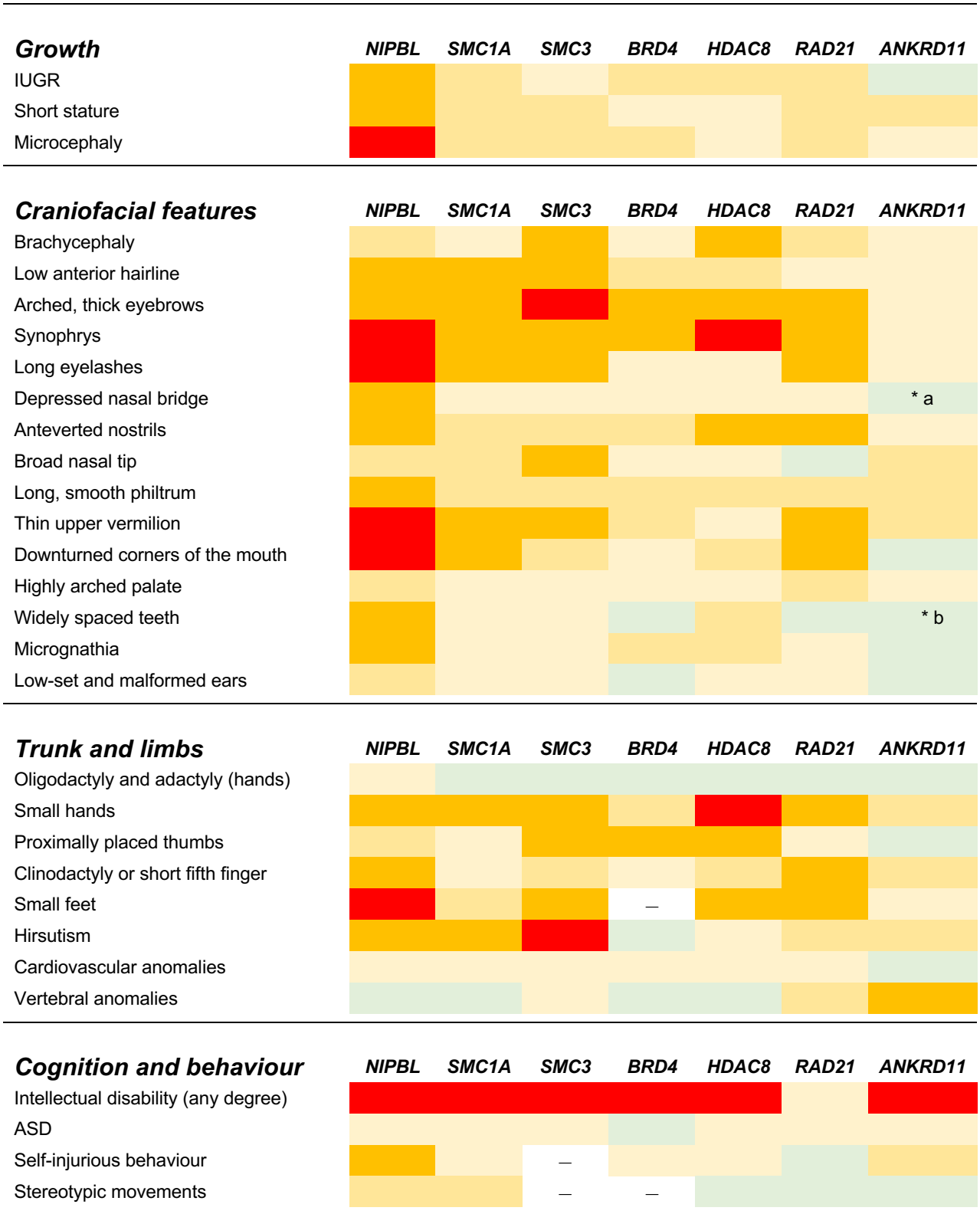

Table 1 – Comparison of the main clinical findings in individuals with molecularly confirmed CdLS (modified from Kline *et al.*, 2018) [6]. Red: ≥90%; dark orange: 70–89%; light orange: 50–69%; yellow: 20–49%; light green: <20%. – Not reported. * a: Prominent nasal bridge. * b: Macrodontia (larger than normal teeth).

1.3 CdLS genetics and causative genes

The genetic cause of the syndrome has been established for approximatively 84-85% of the affected patients [6], but a portion (about 15-16%) does not still have a genetic diagnosis accounting for CdLS (Fig. 2).

CdLS is now defined as a spectrum since some characteristics can overlap with other similar syndromes with non-classic phenotype arising from mutations in genes involved in chromatin regulation, most commonly those involving the cohesin complex [7]–[12].

So far, 7 genes are known to be implicated in the pathogenesis of CdLS: *NIPBL, SMC1A, SMC3, RAD21, HDAC8, BRD4* and *ANKRD11*. Mutations in these genes usually occur *de novo* in heterozygosity (homozygous conditions are not compatible with life); but further analyses are still required to investigate new possible candidate genes.

Some patients carrying discrete mutations in *AFF4*, *EP300*, *KMT2A*, *NAA10*, *SWI/SNF* complex genes and *TAF6* display CdLS-like features, leading to hypothesize other genes as responsible for symptoms and signs of CdLS.

Even if the molecular bases of CdLS are not fully understood yet, it is known that the cohesin complex (a protein multimeric complex important for chromatid cohesion, DNA repair, gene expression, development, and genome integrity) plays a fundamental role in CdLS pathogenesis, as a regulator of gene expression.

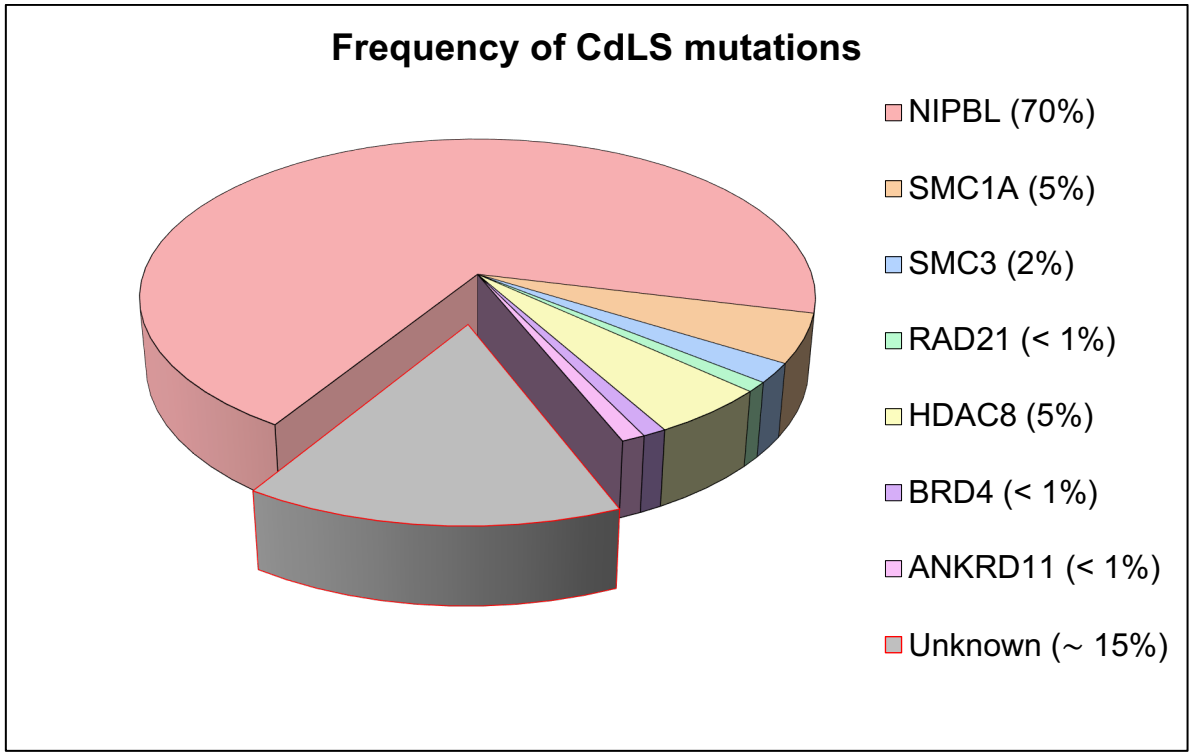

Figure 2 – Percentual frequency of mutations in CdLS patients. Frequency is reported in the pie chart starting with mutations in *NIPBL* gene, first discovered as causative for CdLS, followed by the other genes unravelled. A big portion of CdLS patients (about 15% indicated by the grey slice), are still lacking a known genetic cause.

1.3.1 *NIPBL*

- Cytogenetic band: 5p13.2

More than half of CdLS patients (~70%) carry a mutation in *NIPBL* (Nipped-B-Like Protein) gene (OMIM #608667 – CDLS1) [7], [8] which encodes for the homolog protein of the *Drosophila melanogaster Nipped-B* gene product, and fungal *Scc2*-type sister chromatid cohesion proteins. NIPBL (also known as delangine) plays an important role in the loading of the cohesin complex onto the DNA. It forms a heterodimeric complex (also known as cohesin loading complex) with MAU2/SCC4 which mediates the loading of the cohesin core onto chromatin [13], [14]. This protein also plays a role in cohesin loading at sites of DNA damage [15].

Pathogenetic variants in *NIPBL* are present in heterozygosity as loss-of-function mutations leading to an haploinsufficiency. The reduction of NIPBL protein levels of 15-30% has been reported to be sufficient for causing CdLS [7], [8]. The wild-type *NIPBL* allele is believed to compensate haploinsufficiency by upregulation, but some cell types or particular developmental processes are more sensitive than other regarding *NIPBL* dosage. Some

mutations can also interfere with the interaction of NIPBL with MAU2 or other partner proteins in specific functions [16]. Mutations regarding *NIPBL* gene usually lead to the most sever phenotype in patients (Fig. 3).

1.3.2 *SMC1A*

- Cytogenetic band: Xp11.22

Mutations in *SMC1A* (Structural Maintenance of Chromosomes 1A) (OMIM #300040 – CDLS2) account for approximately 5% of CdLS cases [9], [10], [17]. SMC1A is one of the core proteins of the cohesin complex. Heterozygous missense mutations in *SMC1A* are thought to interfere with the structure of the cohesin complex subunits and with its function, thus causing CdLS [10], [18]. Patients with mutations in *SMC1A* gene usually display milder symptoms and fewer structural abnormalities than *NIPBL*-mutated ones (Fig. 3), however, all patients manifest some degree of cognitive defect [10], [19] suggesting that *SMC1A* plays a crucial role during brain development in the embryo. Interestingly, *SMC1A* does not undergo X chromosome inactivation [20]; indeed, in some cases, females have been reported to be less affected than males [9], [21].

1.3.3 *SMC3*

- Cytogenetic band: 10q25.2

Mutations in *SMC3* (Structural Maintenance of Chromosomes 3) (OMIM #606062 – CDLS3) are uncommon in CdLS patients (2%) and usually missense changes occur [22], maybe due to the fact that loss-of-functions variants are less tolerated, but also nonsense mutations have been reported [22].

The protein encoded by *SMC3* gene is a central component of cohesin complex. SMC3 with its partner SMC1A, forms a ring within which sister chromatids can be trapped in.

SMC3 mutations were also identified in patients with signs similar to CdLS (intellectual disability, short stature and congenital anomalies) who do not fully met the clinical diagnostic criteria of non-classic CdLS [22], [23].

1.3.4 *RAD21*

- Cytogenetic band: 8q24.11

RAD21 (RAD21 Cohesin Complex Component) (OMIM #606462 – CDLS4) mutations also cause CdLS [11] and patients with these pathogenetic variants have even milder symptoms than those carrying *SMC1A* mutations (Fig. 3).

The protein RAD21 is encoded by a gene highly conserved from the *rad21* present in the yeast *Schizosaccharomyces pombe.* This protein, part of the cohesin complex main core, is involved in the repair of DNA double-strand breaks and in chromatid cohesion. In particular, it is a nuclear phospho-protein that becomes hyperphosphorylated during the cell cycle M phase, showing a strong association specifically at the centromere region. In accordance with its important role in DNA damage response, cell cultured from patients exhibited a high sensitivity to radiation [11].

To date, only 23 patients with *RAD21* variants have been observed, which means that *RAD21* mutations account only for a small percentage of CdLS causes (less 1%). Mutations regarding *RAD21* include heterozygous deletions and missense mutations (dominant negative or loss-of-function mutations).

The small number of reported CdLS patients with *RAD21* mutations precludes any detailed genotype-phenotype correlation, albeit they usually display non-classical CdLS phenotype [11], [23], [24].

1.3.5 *HDAC8*

- Cytogenetic band: Xq13.1

To date, individuals with *HDAC8* (Histone Deacetylase 8) (OMIM #300882 – CDLS5) variants have been reported [23], [25]–[29], accounting for about 5% of the cases.

HDAC8 gene encodes for a protein responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Moreover, it regulates epigenetic repression and plays an important role in transcriptional regulation, cell cycle progression and developmental events. HDAC8 protein is also involved in the deacetylation of cohesin complex protein SMC3 regulating the release of cohesin complexes from chromatin [30].

Interestingly, the phenotypical characteristics of *HDAC8* mutated patients can vary widely and is typically non-classic, even though some individuals meet classical CdLS criteria. *HDAC8* is located on the X chromosome but, in contrast to *SMC1A*, can be inactivated [28], therefore female carrying *HDAC8* mutated alleles can be either affected or completely healthy, depending on lyonization occurrence.

1.3.6 *BRD4*

- Cytogenetic band: 19p13.12

BRD4 (Bromodomain Containing 4) is one of the last genes described as causative for CdLS [6], it was first implicated in CdLS when a *de novo* mutation (deletion that included *BRD4)* was identified in an individual with an non-classical CdLS phenotype. Following the first patient description, *BRD4* targeted sequencing determined *de novo* intragenic variants [12].

BRD4 gene encodes for a chromatin reader protein that plays a key role in transmission of epigenetic memory across cell divisions and transcription regulation by recognizing and binding acetylated histones.

Through mass-spectrometry NIPBL was identified as the prevalent interacting protein with BRD4, and mutations in *BRD4* gene demonstrated to diminish its interaction with acetylated histone while retaining NIPBL [12], suggesting that sequestration of NIPBL could underlie the pathogenic mechanism.

The number of patients carrying *BRD4* mutations is too small to speculate on a possible common phenotype.

1.3.7 *ANKRD11*

- Cytogenetic band: 16q24.3

To date, only five *de novo ANKRD11* (Ankyrin Repeat Domain 11) variants have been described in patients that displayed a non-classic CdLS phenotype [23], [31], and some additional variants have been identified in research cohorts [6]. In fact, mutations in *ANKRD11* are usually linked to another rare genetic disease, KBG syndrome (named after the initials of the last names of the first three families identified in 1975 [32]). KBG syndrome patients' features partially overlap with CdLS since they are characterized by short stature,

developmental abnormalities of the limbs, vertebrae, extremities, and/or underdevelopment of the bones of the skeleton. Craniofacial dysmorphism may also be present and most individuals have some degree of developmental delay or intellectual disability [33].

ANKRD11 is a chromatin regulator which modulates histone acetylation and gene expression in neural precursor cells. It interacts with HDACs to the p160 coactivators/nuclear receptor complex to inhibit ligand-dependent transactivation [34], and it has a role in proliferation and development of cortical neural precursors [35].

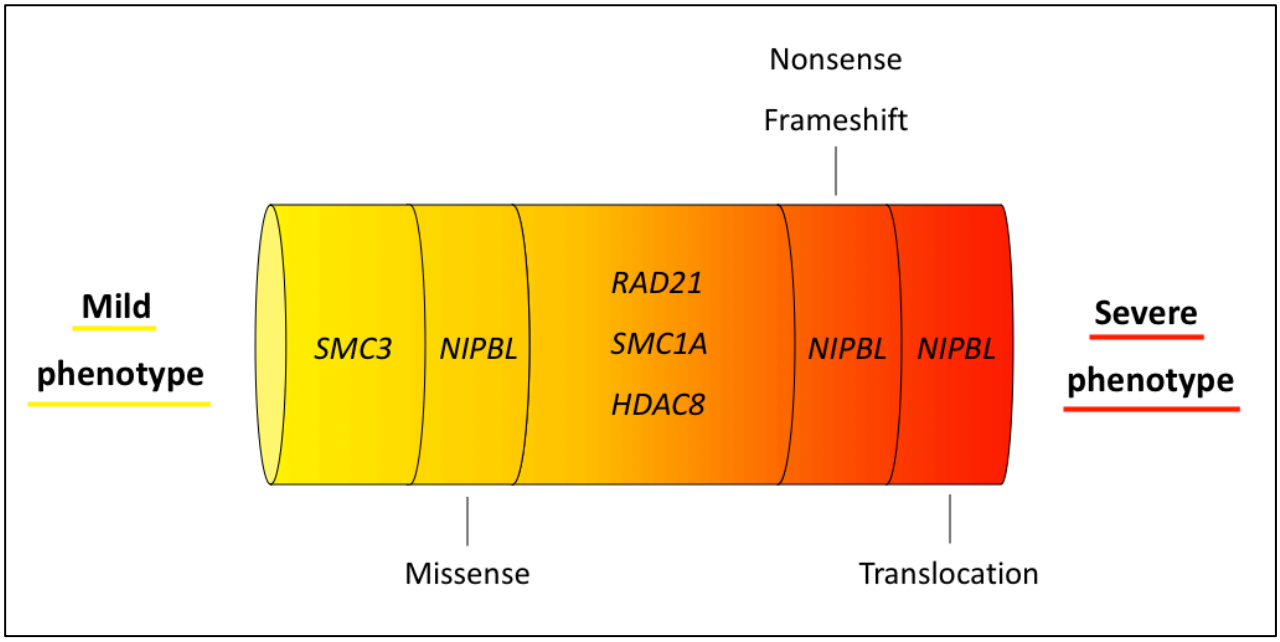

Figure 3 – Genotype-phenotype correlation in Cornelia de Lange syndrome (modified from Sarogni et al. 2019) [36]. The correlation between a mutated gene and its clinical outcomes is not easy to foresee. The image shows the importance not only to identify the mutated gene, but also the type of mutation which is reflected on the protein alterations.

1.3.8 Other genes

In the last years, with the use of Whole Exome Sequencing (WES) applying Next Generation Sequencing (NGS) techniques, several additional genes have been identified but these variants were detected in patients with borderline non-classical CdLS phenotype. Patients displaying CdLS-like phenotype, but not fully meeting all the clinical diagnostic criteria, can manifest features that overlap with aspects of other syndromes and chromatin disorders [37]. On the other hand, patients diagnosed with a chromatin disorder (e.g. Wiedemann-Steiner syndrome, Rubinstein-Taybi syndrome, Coffin-Siris syndrome) can display CdLS

clinical signs [38]–[40]. This clinical overlapping may be due to the shared functional network between cohesins and chromatin-associated complexes.

To date, novel good candidate genes are*: AFF4* [41]*, EP300* [40]*, KMT2A* [38], [39]*, NAA10* [42]*, SETD5* [39]*, SWI/SNF* complex genes [39]*, TAF6* [38]; but further studies are needed in order to associate the pathological variants with CdLS [43].

1.4 CdLS diagnosis and management

To date, CdLS is defined as a spectrum with classical and non-classical phenotype. In 2018 Kline and colleagues defined the cardinal features considered to be the most characteristic for CdLS, and suggestive features, which can help with the diagnosis but are less specific (Tab. 2) [6].

Cardinal features (2 points each if present)	**Suggestive features** (1 points each if present)
• Synophrys and/or thick eyebrows • Short nose, concave nasal ridge and/or upturned nasal tip • long and/or smooth philtrum • Thin upper lip vermilion and/or downturned corners of mouth • Hand oligodactyly and/or adactyly • Congenital diaphragmatic hernia	• Global developmental delay and/or intellectual disability • Prenatal growth retardation (<2 SD) • Postnatal growth retardation (<2 SD) • microcephaly (prenatally and/or postnatally) • Small hands and/or feet • Short fifth finger • Hirsutism

Table 2 – Cornelia de Lange clinical features (Kline et al. 2018) [6].

Based on these features the consensus [6] developed the criteria to diagnose CdLS:

> *A score of ≥11 indicates classic CdLS if at least three cardinal features are present; a score of 9–10 indicates non-classic CdLS if at least two cardinal features are present; a score of ≥4 is sufficient to warrant molecular testing for CdLS if at least one cardinal feature is present; a score below <4 is insufficient to indicate such testing. A score of ≥11 confirms the diagnosis of CdLS regardless of whether a pathogenic variant in one of the known genes can be found.*

At a molecular level, the CdLS spectrum has been associated with molecular abnormalities affecting genes involved in chromatin regulation, most commonly those involving the cohesin complex.

Prenatal diagnosis is indicated when parents are known to likely carry genetic alterations in CdLS genes, and this usually happens because of a child born with CdLS in a previous pregnancy from the same parents. Most frequently, on the other hand, there is no family history of mutations in CdLS genes, but ultra-sonography screening usually shows features suggestive of CdLS. The most common features are: symmetric intrauterine growth restriction (IUGR) that usually occurs in the second trimester (80% of cases); limb anomalies (66% of cases); increased nuchal thickness (51% of cases) and abnormal facial profile (50% of cases, such as micrognathia and prominent maxilla) [44], [45]. Other reported features include diaphragmatic hernia (28% of cases) and cardiac malformation (15% of cases) [44].

When there is the suspect of CdLS pregnancy, the clinician should discuss with parents about pros and cons of prenatal studies and molecular tailored tests. Molecular tests can be performed on chorionic villous samples, amniocentesis or cell-free fetal DNA (cffDNA), and provide for sequencing of single CdLS causative gene. In the last years, with the advent of NGS panel, testing all known causative genes in a single time is possible, allowing the identification of *de novo* variants in families without a previous CdLS child. Panel sequencing is the most effective way for detecting causative variants in any of the genes known to cause CdLS, and first-line molecular testing should use a panel that contains at least the seven known CdLS genes. Most diagnostic laboratories include several additional genes that can cause a phenotype resembling CdLS. However, comparison with both biological parental samples is essential to interpret the large number of variants whose pathogenicity may be difficult or impossible to determine, thus precluding the meaningful use of this approach in routine practice at the present [6]. Owing to the complexity of the molecular findings, prenatal testing for CdLS outside of a known familial pathogenic variant remains challenging. Results

interpretation of these test is difficult because pathogenicity is not always easy to determine, especially with the presence of an undetectable mosaicism that frequently occurs in CdLS [46]. If sequencing does not detect pathogenetic variants, a study aimed at detecting mosaicism should be considered, using samples other than blood. If this further test will be negative as well, testing for deletions or duplications of *NIPBL* using multiplex ligation-dependent probe amplification (MLPA) should be considered (Fig. 4).

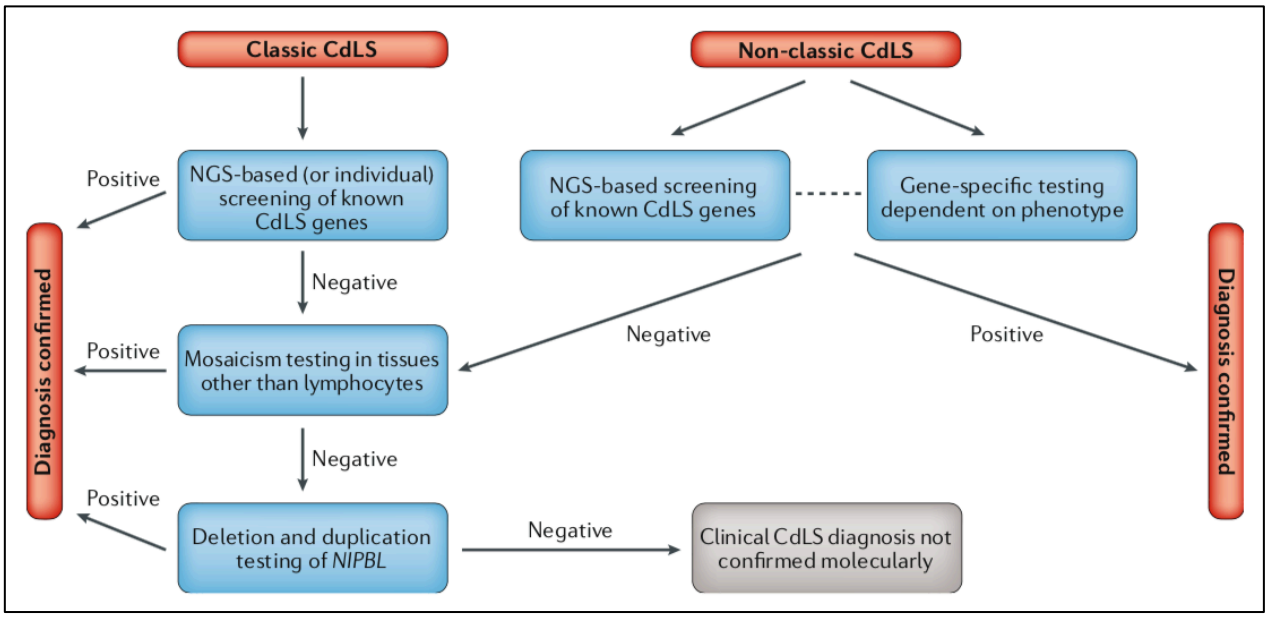

Figure 4 – CdLS molecular diagnostic pathways (Kline *et al.*, 2018) [6]. Schematic representation of the diagnostic pathway used for the identification of Cornelia de Lange syndrome in patients.

1.5 Cohesin complex

CdLS is the most common among the so-called "cohesinopathies", pathologies arising from molecular defects in the cohesin complex. The other known cohesinopathies are: (I) Robert syndrome (RBS) arising from mutations in *ESCO2* gene, component of the machinery of the cohesin complex; and (II) Warsaw Breakage Syndrome (WABS) arising from mutations in *DDX11* gene, that encodes for a DNA helicase.

Cohesins are a ring-shaped multimeric complex that consists of four core subunits (SMC1A, SMC3, RAD21, STAG) and other factors (PSD5, WAPL) [47], [48]. This complex is loaded onto the DNA by the protein NIBPL with its partner MAU2. For cohesion establishment acetylation-deacetylation of SMC proteins by ESCO and HDAC8 respectively plays a crucial role (Fig. 5).

Cohesin complex is pivotal to regulate most aspects of cellular biology, including chromosome segregation, maintenance of genome stability, regulation of gene expression, chromatin structure and genome organization [49]–[52].

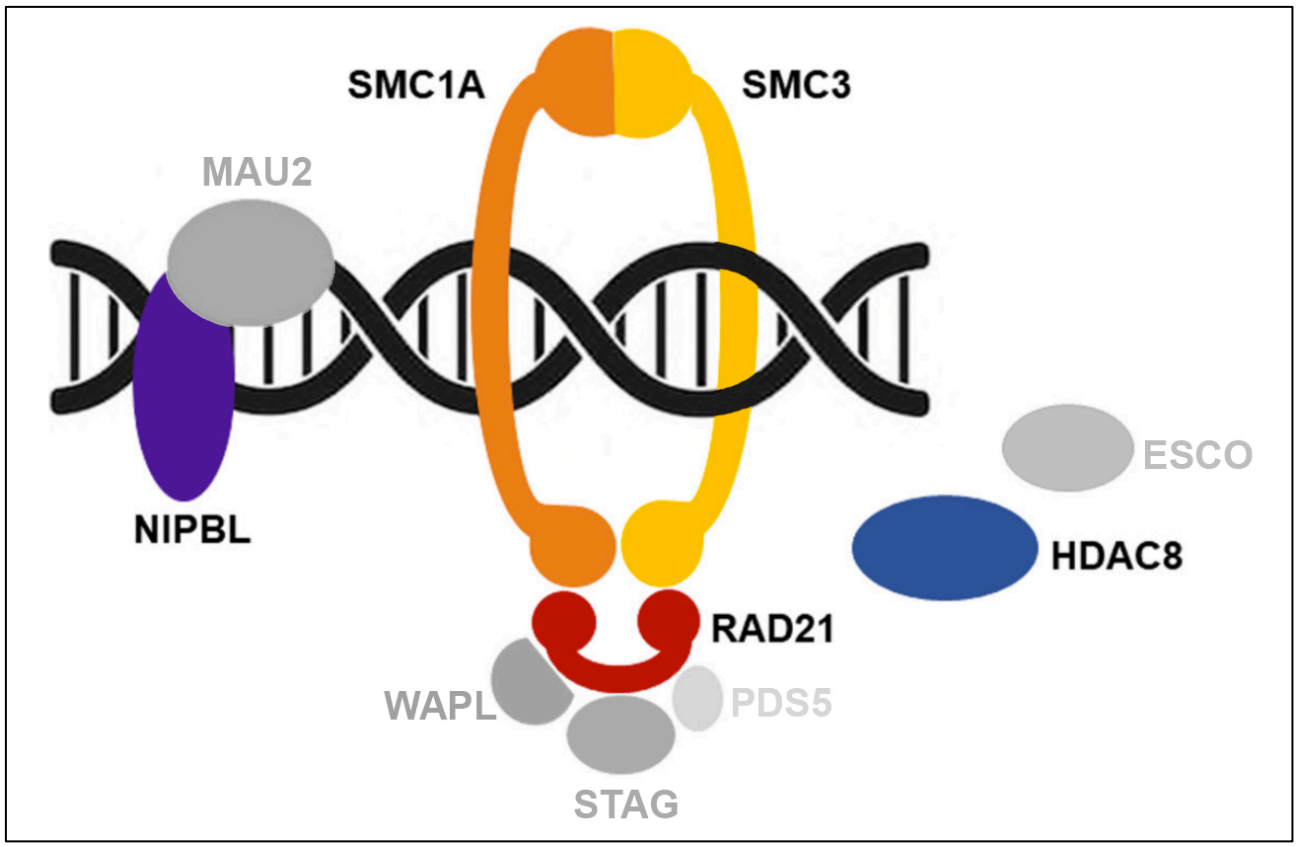

Figure 5 – Cohesin complex and associated factors (modified from Sarogni et al. 2019) [36]. The main proteins of the cohesin complex involved in the pathogenesis of CdLS are showed in the drawing. NIPBL (purple) is the cohesin ring's loader onto the DNA; SMC1A/SMC3 (orange and yellow) are the main components of the cohesin ring; RAD21 (red) closes the ring binding both to SMC1A and SMC3; HDAC8 (blue) is an associated factor that works in combination with ESCO (gray) to deacetylate and acetylate SMC3 respectively. The other factors MAU2, WAPL, STAG and PDS5 (grey) are important for the proper functioning of the complex but, to date, mutations in genes encoding for these proteins have not been associated to CdLS.

Three main functions have been described for this intracellular machinery: (I) cohesion between replicated sister chromatids and chromosome segregation; (II) repair of damaged DNA; (III) regulation of gene expression in both proliferating and post-mitotic cells.

These three different functions, taken together, can be summarized in a main general activity: promote long-range interactions between distant genomic regions, that is to say, the ability to create topological links between two segments of the chromatin fiber.

1.5.1 Sister chromatids cohesion and chromosome segregation

The firstly described function of cohesin complex (after which this complex was named) is the capability of entrapping the DNA strand and binding together the two sister chromatids during the S phase of cell cycle till the mitosis occurs. To date, it is not clear the exact mechanism by which the cohesins ring recognizes and binds to the replicated DNA, leaving the question open to different hypothesis and molecular models [53], [54]. Moreover, it is also unclear how cohesins loaded on unreplicated DNA allows DNA synthesis to proceed during S phase in the cell cycle, given the big dimension of the replisome.

1.5.2 DNA repair

In *S. pombe* it was observed that mutations in cohesins leads to high sensitivity to γ-irradiation and it was the first evidence for a role in maintaining genome integrity and in repairing damaged DNA [55]. Essentially, the role of cohesins in DNA repair is to put together the two sister chromatids so that double-strand break (DSB) on one sister can be fixed using the other sister as a template through homologous recombination. Moreover, it has been demonstrated that cohesins are essential to initiate DSB repair process by bringing damaged and intact strands in close vicinity, but does not play a role in the next step of actual DNA repair [56].

1.5.3 Gene expression regulation

Since cohesins were found to be highly expressed also in non-dividing tissue as the central nervous system (CNS) [57], in the last years another non-canonical role has been described for cohesin complex: the regulation of gene expression. Now it is known that cohesins regulate gene expression with tissue-specific transcription factors and/or in combination with the CCCTC-binding factor (CTCF) insulator protein [50], [58] (Fig. 6). This non-canonical role in transcriptional control and the consequent altered expression of multiple developmental genes is thought to be the main cause of cohesinopathies.

Translation process too has been shown to be indirectly regulated by cohesins. In budding yeast and humans, cohesins have been shown capable of augmenting translational capacity by increasing transcription of rRNA [59].

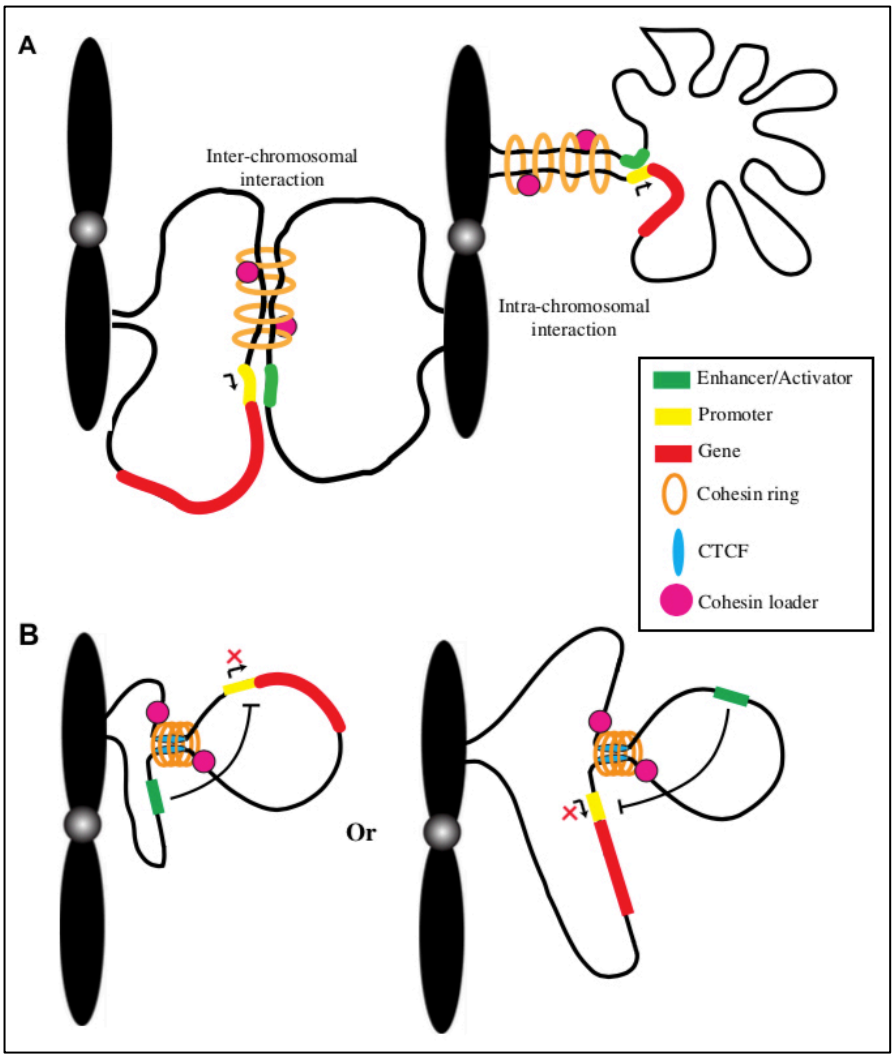

Figure 6 – Cohesin complex role in transcriptional regulation (modified from Mehta *et al.*, 2013) [56]. (A) Mechanism of cohesin-mediated transcription activation: cohesin entraps intra/inter chromosomal DNA duplexes otherwise located in a remote chromosomal locus. With this mechanism, cis-acting enhancers come into close proximity to the promoters through cohesin and allow to switch on the transcription of a gene (as shown by the black arrow). Similarly, entrapment of a cis-acting silencer close to a promoter can impose transcriptional suppression of the corresponding gene (not shown). (B) Mechanism of CTCF and cohesin mediated transcription repression: cohesin stabilizes the chromatin loops formed by CTCF. Formation of the loops blocks the interaction between the enhancers/activators and the promoters, leading to suppression of transcription. Similarly, entrapment of silencer sequences within the loop can activate the transcription of a gene (not shown).

1.6 Canonical WNT pathway

Wingless-related integration site (WNT) signalling pathway is an ancient and evolutionarily conserved pathway which regulates crucial cellular processes including cell fate determination, organogenesis during embryonic development, normal adult homeostasis, motility, polarity, neural patterning, and stem cell renewal [60]. Alterations regarding this

pathway have been associated to a number of CNS diseases [61], including CdLS. A regulation of the canonical WNT pathway by cohesins during development have already been shown by our group in zebrafish embryos and in fibroblasts from CdLS patients [62]. In other models of disease, such as autosomic recessive microcephaly, a decrement in the expression of genes regulated by WNT pathway showed alteration in CNS, defects that managed to be rescued by overexpression of β-catenin [63]. But several other animal models carrying alteration in the expression of WNT-regulated genes showed a phenotype partially overlapping with our CdLS model: zebrafish *masterblind* mutant, which develops reduction of the telencephalon, optic vesicles, and an abnormal antero-posterior patterning, was found to be a spontaneous *axin1* mutant [64], [65]. Mouse model carrying mutations in *Hesx1*, *Six3*, and *Tcf3* genes [66]–[68] have been described with comparable phenotype at the antero-posterior patterning of the CNS during embryonic development. Some other evidence suggests a possible regulation of the WNT pathway by cohesins in non-dividing cells. In the *Drosophila melanogaster* model, SMC3 appears important to control expression of *Flamingo/Starry night*, the orthologs of vertebrate *Celsr*, which is known to transduce the WNT signalling cascade to control cell polarity [69]. It is interesting to note that this WNT-cohesin regulation might be two-way, since the known upregulation of β-catenin appears to control expression of *SMC3* in colorectal cancer [70].

Hence, our hypothesis consists of a downregulation of canonical WNT pathway in CdLS, as a result of mutations in cohesin complex genes, as the underlying mechanism for CNS developmental abnormalities.

WNT genes encode for 19 glycoproteins highly conserved across species that are secreted upon a process of post-translational acetylation by porcupine, a membrane associated O-acyl transferase. Acetylation leads to palmitoylation, step required for the WNT protein release and binding to its receptors frizzled (FZD).

WNT ligands can activate different intracellular cascades, resulting in a large number of cellular responses.

WNT pathway has been divided into 2 different categories depending on the mechanism of the intracellular cascade [71], [72]: canonical WNT pathway (Wnt/β-catenin dependent pathway) and non-canonical WNT pathway (β-catenin independent pathway). The non-canonical pathway has been further divided in two additional branches: the planar cell polarity (PCP) and the Wnt/calcium pathways [60] (Fig. 7).

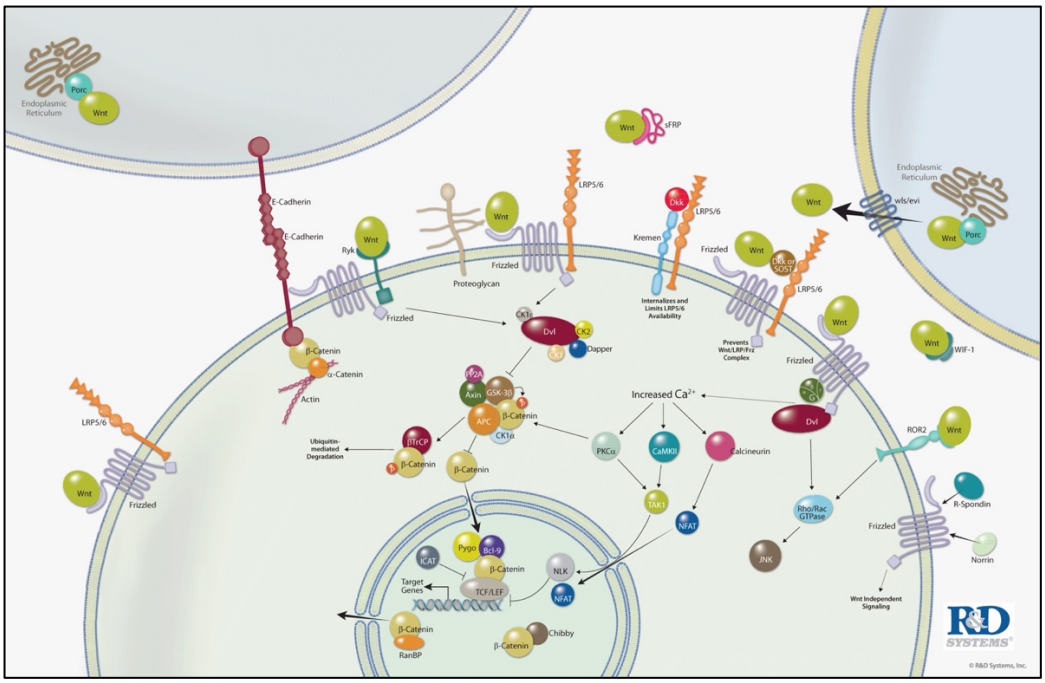

Figure 7 – Overview of WNT signalling pathways (modified form R&D System, Inc.). The scheme illustrates the many possible ways by which WNT protein can bind its ligand Frizzled, or be blocked by specific proteins, and the plethora of intracellular responses.

For the purpose of this thesis, we will focus only on canonical WNT pathway, which is so far the one and only impaired in CdLS.

The canonical WNT pathway's main player is the β-catenin. When WNT ligands are not bound to the membrane receptor FZD (Fig. 8, Wnt OFF), the level of intracellular β-catenin is low because this protein is sent to proteasomal degradation by the so called "destruction complex". This "destruction complex" consists of two kinases: casein kinase 1 α (CK1α), glycogen synthase kinase 3 β (GSK3β**),** and two scaffold proteins: axis inhibition (Axin), adenomatous polyposis coli (APC). β-catenin is phosphorylated by CK1α at serine 45, 33, 37 and by GSK3β at threonine 41. At this point, the E3 ubiquitin ligase β-transducin repeat-containing protein (βTrCP) tags the β-catenin with the ubiquitin and sent it to proteasomal degradation [73]. This mechanism does not allow the nuclear translocation of the β-catenin and therefore Groucho repressor and histone deacetylases act together by condensing the chromatin and inhibiting gene transcription [74].

On the other hand, when WNT ligands bind to their plasma membrane receptor (Fig. 8, Wnt ON), there is an activation of the intracellular cascade which allows β-catenin to translocate into the nucleus. For the pathway activation WNT proteins need both FZD family receptors and the co-receptors low-density-lipoprotein-related protein 5/6 (LRP5/6), which must be

phosphorylated for the receptor activation. Following receptor activation, the intracellular protein dishevelled (DVL) is phosphorylated, leading to Axin de-phosphorylation and the migration of the “destruction complex” to the plasma membrane allowing the β-catenin to accumulate and stabilize in the cytoplasmatic compartment. This accumulation allows β-catenin to translocate into the nucleus to act as a transcription factor [75].

Many players interact during the activation of canonical WNT pathway. Targeting these proteins with specific chemical compounds can activate (or inhibit) the pathway itself. Molecules such as: lithium [76], [77], BIO [78] or CHIRR-99021 [79] inhibit the protein GSK3β, part of the destruction complex, thus resulting in a pathway activation. Deoxycholic acid (DCA) [80] can facilitate β-catenin translocation from the cytoplasm to the nucleus with a mechanism not fully understood. IQ-1 [81], by targeting the subunit of the serine/threonine phosphatase PP2A, prevents β-catenin from being tagged for proteasomal degradation.

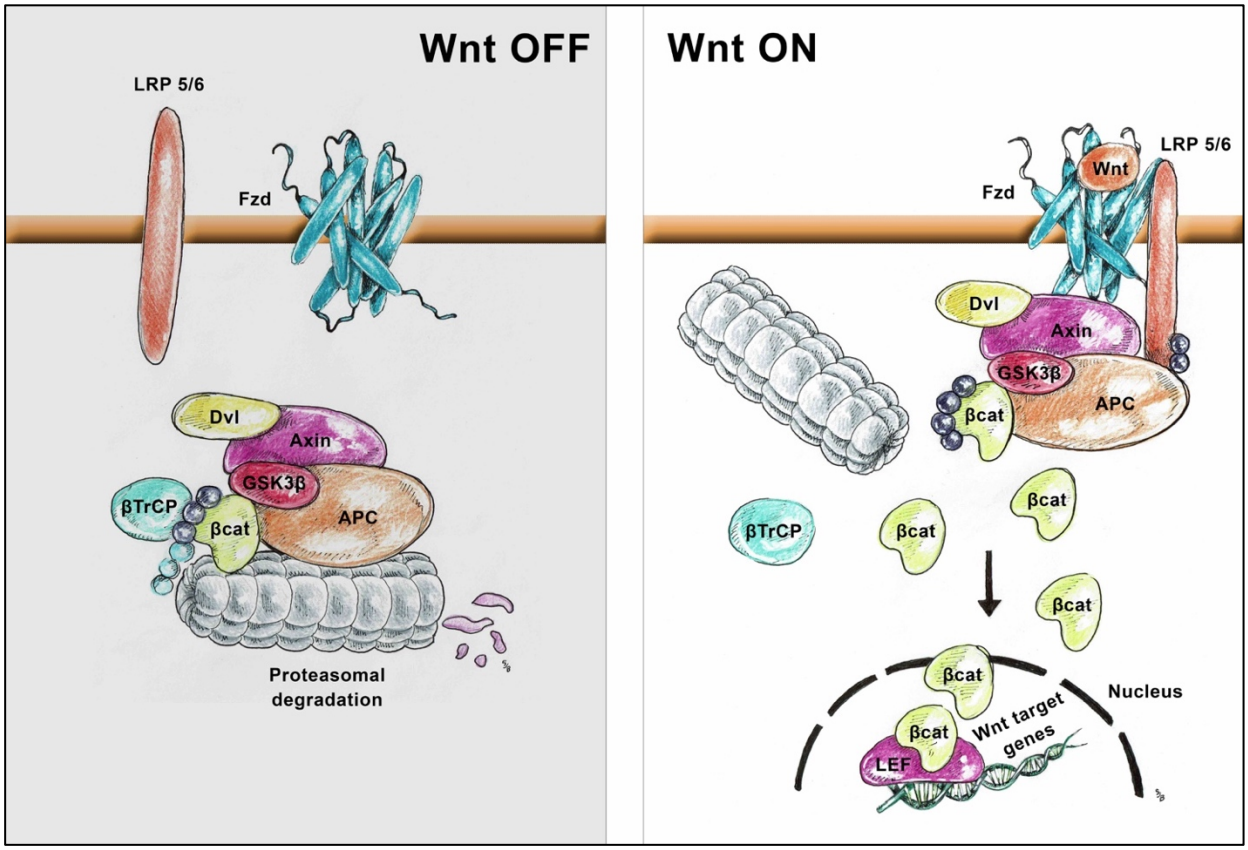

Figure 8 – Canonical WNT pathway activation (Avagliano *et al.*, 2017) [82]. (Left panel) The absence of WNT signalling induce the proteasomal degradation of β-catenin, mediated by the destruction complex (left panel). The binding of WNT to its membrane receptor Frizzled induces an inactivation of the destruction complex, therefore β-catenin is free to translocate into the cell nucleus and act as a transcription factor (right panel).

The exact mechanism by which β-catenin translocases from the cytoplasm into the nucleus remains unclear. Nucleoporins usually do not allow the passage of proteins larger than 40 kDa [83], moreover β-catenin does not display any nuclear localization sequence (NLS) that usually permits the passage of bigger proteins by binding to Importin-α/Importin-β, interactors of the nuclear pore complex (NPC) [84]. To date, several models have been proposed for the translocation of β-catenin into the nucleus but none of them has been confirmed [83]–[92].

When β-catenin reaches the nuclear localization, it has two main functions: I) facilitating the recruitment of "mediator complex" proteins essential for the RNA Polymerase II-mediated transcription [93] and II) helping the chromatin opening by recruiting histone acetyltransferases (HAT), which in turns facilitates transcription factor to target their promoters [94].

β-catenin is composed of 12 Armadillo repeats (Fig. 9) necessary for the binding of several partner proteins transducing the activation of WNT target genes.

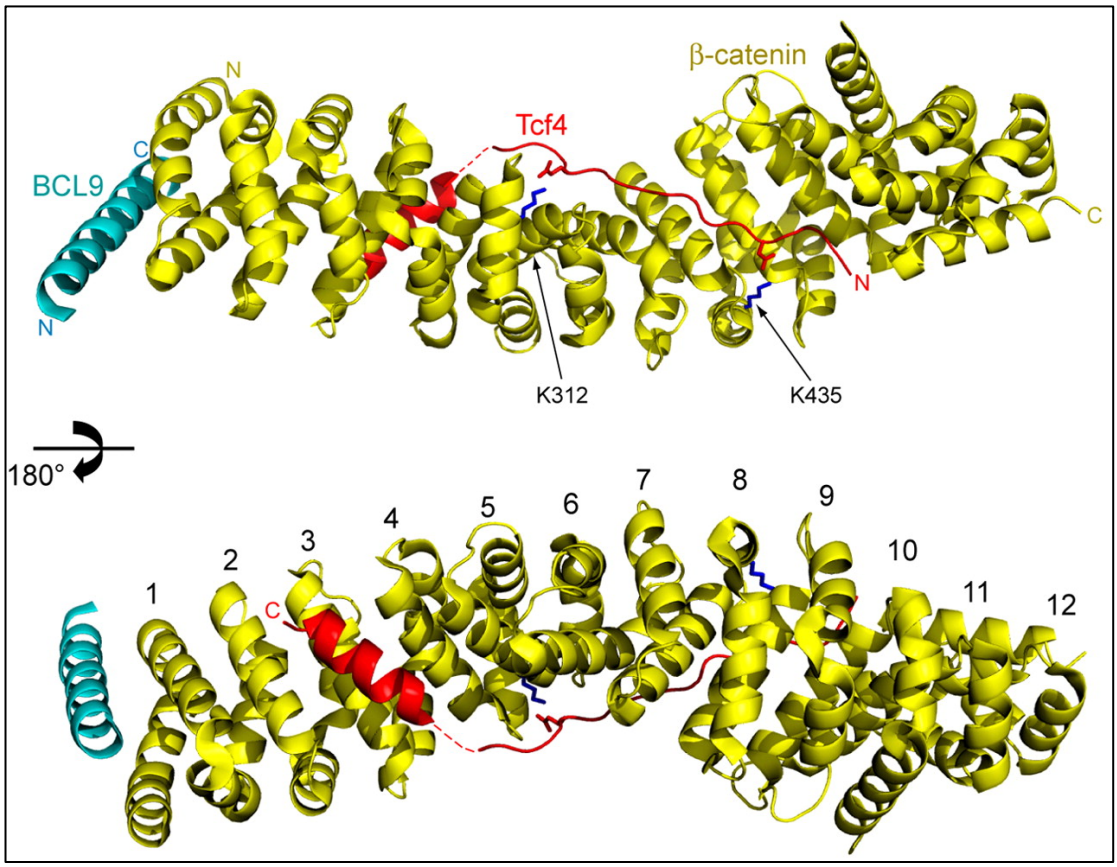

Figure 9 – β-catenin protein structure (Xu *et al.,* 2007) [95]. Crystal structure of the β-catenin armadillo repeat domain in complex with Tcf4 (red) and BCL9 (cyan), two of the most common transcription factors that bind β-catenin. In blue residues of charged lysines are shown (K312, K435).

The most known and well characterized among these partner proteins is T-Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) that mediate WNT-responsive transcription. This family of proteins (four in human: TCF1, TCF3, TCF4, LEF1) is highly conserved and bind to WNT Responsive Elements (WREs). They can also work as scaffold for the recruitment and positioning of other factors involved in WNT-mediated transcriptional regulation.

To date, the best model representing how β-catenin acts as a transcription factor binding to WREs is the one proposed by Fiedler and colleagues [96]: the WNT "enhanceosome" (Fig. 10). This is a complex made up by several proteins that help β-catenin to recognize TCF/LEF sequences for transcription of all *WNT* target genes. In order to be fully active, however, the enhanceosome must contain (I) a chromatin remodeler, (II) a protein for the communication between the enhancer and promoter regions of *WNT* target genes, (III) a protein that recruits mediator complex components to promote general transcription, and (IV) a protein that regulates the interaction between the repressor TLE and TCF/LEF [97].

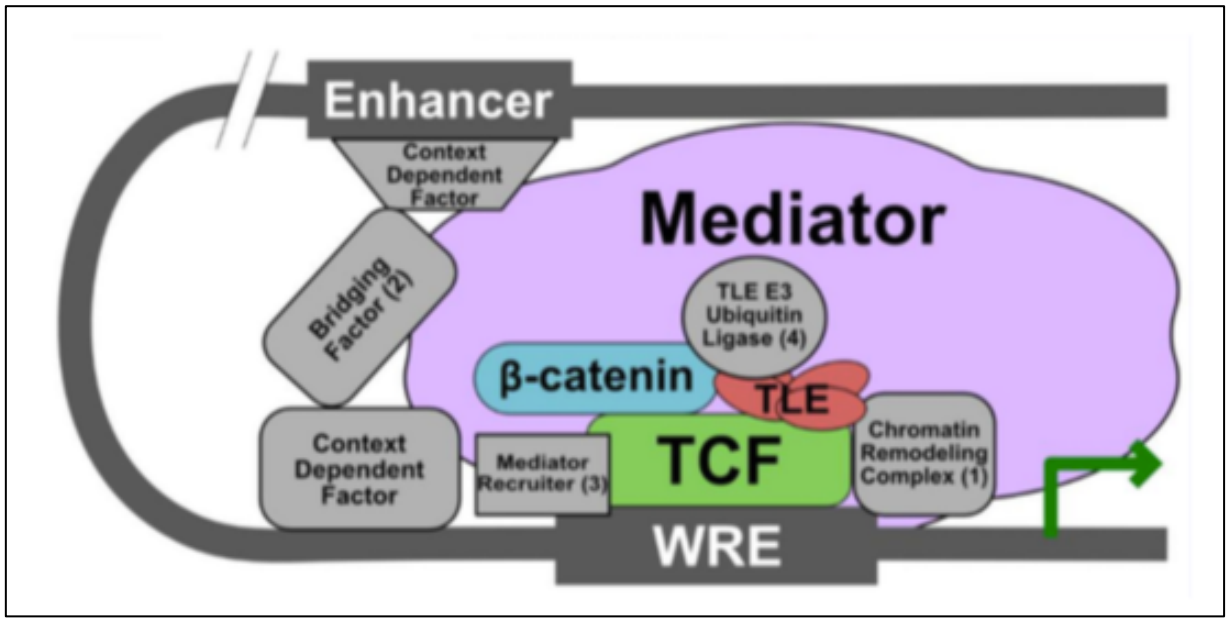

Figure 10 – WNT enhanceosome model (Anthony *et al.*, 2020) [97]. One of the many proposed models of WNT enhanceosome with a β-catenin-TCF/LEF core complex and other components that are context dependent. In this model at least four additional components are required to form a functional enhanceosome: (1) a chromatin remodelling complex (e.g., SET-1, a histone methyltransferase, and CBP/p300, a histone acetyltransferase) that promotes gene transcription; (2) a bridging factor to link enhancer regions to the WRE and which may coordinate context-dependent factors (e.g., BCL9); (3) a mediator recruiter (e.g., β-catenin); (4) an E3 ubiquitin ligase that promotes Gro/TLE dissociation from TCF/LEF.

More than 80 genes are known, so far, to be direct or indirect target of canonical WNT pathway activation in human. These genes are involved in many embryonic processes such as body axis patterning, cell fate specification, cell proliferation and cell migration. In addition, canonical WNT pathway also governs a plethora of biological processes in the adult organism such as maintenance of adult tissue homeostasis, regulation of stem cell self-renewal, cell proliferation, differentiation, and apoptosis [98]. For the purpose of this project one of the target gene I took in consideration most is *CyclinD1* (*CCND1*). Cyclin D1 is required for progression through the G1 phase of the cell cycle. During the G1 phase, it is synthesized rapidly and accumulates in the nucleus, then it is degraded as the cell enters S phase [99].

1.7 CdLS experimental models

1.7.1 *Drosophila melanogaster*

Despite the evolutionary divergence, over three-quarters of genes linked to human diseases present *Drosophila* homologs. Canonical WNT pathway conservation through evolution, a short generation time, a smaller genome, and unique genetic tools make *Drosophila melanogaster* a good model to recapitulate genetic and *in vivo* features of CdLS. In 2016, Dorsett demonstrated how *Drosophila* can be used as a model to recapitulate Cornelia de Lange syndrome [100], and how this animal model can be used to increase understanding of genetic syndromes caused by mutations with broad effects on gene transcription and exploited to develop novel therapies [101], [102].

1.7.2 *Mus musculus*

Mouse is one of the most used animal models to study human diseases but, unfortunately, mice with mutations in cohesins genes do not fully recapitulate the human characteristics of CdLS.

Heterozygous mice for a gene-trap mutation in *Nipbl* exhibited some defects characteristic of CdLS, including small size, craniofacial anomalies, microbrachycephaly, heart defects, hearing abnormalities, delayed bone maturation, reduced body fat, and high mortality (75–80%) during the first weeks of life [103]. However, no limb defects or clear behavioural signs were present.

Hdac8 mutant mice obtained by Haberland and colleagues, were viable until embryonic day 18.5 (E18.5), although they showed a small reduction in body size and weight. Immediately after birth, however, mutants showed deficiencies in movement, signs of hypoxia, were often neglected by their mothers and usually died within 4-6 h. They observed hemorrhage in the brains, and in some severe cases a herniation of brain and other soft tissue through the top of the skull [104].

Heterozygous mice for *Brd4* weighed 25%-10% less than their wild-type littermates throughout the prenatal period and after birth. The general growth restriction observed is likely to be due to a reduction in the proliferation rate. A few weeks after birth, the heterozygotes were easily recognizable because of the abnormal shape of their heads, this recognizable *facies* was due to a shorter incisive bone as well as a shorter and bent nasal bone. The mandible was also reduced in length. Various morphological abnormalities were seen in the skin, liver, testis (epididymal duct and vas deferens), and brain of heterozygotes. In a sample population of 34 heterozygotes between 6 weeks and 6 months of age, four individuals had one or both eyes reduced in size and seven had a cataract [105].

1.7.3 human induced Pluripotent Stem Cells (hiPSCs)

The possibility to derive and handle hiPSCs directly from patients' samples has opened the path to a series of studies aiming to uncover several aspects of human development and pathology (Fig. 11). Several research groups have published differentiation protocols that, starting from pluripotent undifferentiated cells, allow to obtain a wide range of fully mature cells [106]. Through these processes, the investigation of otherwise unexplored *in vitro* basic cell mechanisms peculiar of human development is finally achievable. Regarding neural differentiation, many efforts have been spent to set up protocols that generate a wide range of neural population [107]–[110]. This step is crucial to investigate specific mechanisms underlying the onset of neurological and neurodevelopmental disorders such as Cornelia de Lange syndrome.

Mills and colleagues [111] generated hiPSCs from primary skin cells collected from four CdLS probands carrying heterozygous mutations in the *NIPBL* gene and characterized them in the heart lineage.

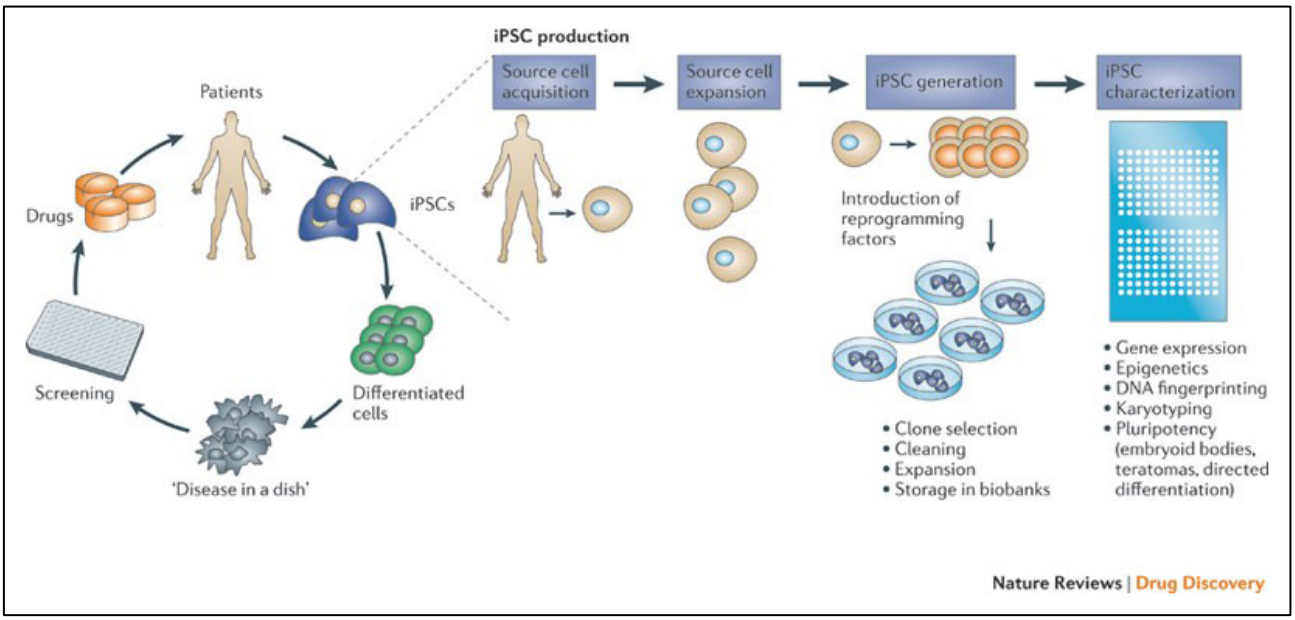

Figure 11 – An hiPSCs technology platform for drug discovery and development (Grskovic *et al.*, 2011) [112]. In this diagram on the left, the drug discovery process starts with the patient samples used for the derivation of human induced pluripotent stem cells (hiPSCs), followed by directed differentiation of these cells into cells that have a crucial role in the studied disease. The hallmark of the technology, that makes it valuable for drug discovery, is the ability to recapitulate pivotal aspects of the disease and create a "disease in a dish" model for drug screening. On the right, a schematic diagram of the hiPSCs production process is shown. Starting with source cell acquisition from reliable patient sources with informed consent, fibroblasts are expanded and hiPSCs are derived; the process is followed by a fully characterization of the hiPSCs, their expansion and the possible storage in a biobank.

2. AIM

Cornelia de Lange syndrome is a rare genetic disorder for which only clinical management of symptoms is available. One of the main issues halting advancement in possible therapeutic approach is that, as true for many mendelian genetic conditions, molecular mechanisms underlying the etiopathogenesis are not fully elucidated. Therefore, a deeper understanding of CdLS biology is urgently required for better defining the disease and develop much needed targeted therapies.

To this end, I took advantage of human induced pluripotent stem cells which are a versatile tool to model CdLS *in vitro*. In order to recapitulate the human pathogenesis of CdLS carrying *HDAC8* loss-of-function mutations, I mimicked the *HDAC8* shutdown through chemical treatment with a specific inhibitor: PCI-34051.

Moreover, canonical WNT pathway is one of the key players in CNS development and it has been previously shown to be downregulated in CdLS models. This pathway is highly conserved during evolution making *Drosophila melanogaster* a good model to recapitulate genetic and *in vivo* features of CdLS. Exploiting this modelling system, allowed to study the syndrome in a complex organism, focusing on its well characterised CNS.

The final goal of my PhD project was to exploit the above mentioned *in vitro* and *in vivo* CdLS model systems to study and further unravel the molecular biology of the disease and at the same time, to identify new potential chemical compounds that could be the starting point to envisage possible improve future CdLS treatments.

3. MATERIALS AND METHODS

3.1 Cellular models

3.1.1 Human induced Pluripotent Stem Cells (hiPSCs)

For the *in vitro* experiments, commercially available hiPSCs were used ("Human Episomal iPSC Line" - Gibco™ #A18945). These cells were derived from $CD34^+$ cord blood using a three-plasmid, seven-factor (SOKMNLT; SOX2, OCT4 (POU5F1), KLF4, MYC, NANOG, LIN28, and SV40L T antigen) EBNA-based episomal system. This hiPSCs line is considered to be zero footprint as there was no integration into the genome from the reprogramming event. They also have a normal karyotype and endogenous expression of pluripotent markers (Oct4, Sox2, Nanog, Oct4, SSEA4, TRA-1-60 and TRA-1-81).

These cells were cultured on Geltrex-coated (Gibco™ A1413301) 6 multiwell in Essential 8 medium (Gibco™ #A1517001) and medium was replaced every day after thawing cells. Passaging and dissociation were performed using Ultrapure EDTA diluted at 0,5 mM (Invitrogen™ #15575020) in DPBS without Ca^{++} and Mg^{++} (Gibco™ #14190144). hiPSCs were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere.

3.1.2 Human Neural Precursor cells (hNPC)

For the generation of hNPC a commercially available kit was used ("PSC Neural Induction Medium" - Gibco™ #A1647801). Briefly, I started with 70-80% confluency high quality hiPSCs (with minimal or no differentiated colonies) and I passed these cells as described above to reach a seeding density of $3x10^5$ hiPSCs/well. After 24 hours, I changed the medium from Essential 8 to PSC Neural Induction Medium and I kept changing it every two days. After 1 week, cells were almost 100% confluent, ready to be harvested and expanded.

3.1.3 Differentiation through neuronal lineage

hNPCs were treated with a specific home-made medium (Neuronal differentiation medium) enriched with factors to induce the differentiation through the neuronal lineage. Neuronal differentiation medium contains: Neurobasal Medium (Gibco™, #21103049), GlutaMAX Supplement 1X (Gibco™, #35050061), MEM Non-Essential Amino Acids 1X (Gibco™, #11140035), B-27 Supplement 1X (Gibco™, #17504044), BDNF 20 ng/mL, GDNF 20 ng/mL

and L-ascorbic acid 200 µM. Cells were plated on laminin-coated plastic (10 µg/mL) and medium was changed every 2/3 days.

3.1.4 Cells treatments

Cells were treated with two different chemical compounds: LiCl (canonical WNT pathway activator) and PCI-34051 (HDAC8 inhibitor). LiCl was dissolved in water, used as a vehicle, at a final concentration of 3 mM. PCI-34051 was dissolved in DMSO, used as a vehicle, at a final concentration of 10 µM and 20 µM. Treatments were freshly added every 48/72h concurrently with medium changing.

3.1.5 Immunofluorescence

Immunofluorescent stainings were performed in order to check the correct hNPC generation from hiPSCs and to evaluate the difference in neuronal maturation.

Briefly, medium was carefully removed, and cells were fixed with paraformaldehyde (PFA) 4% for 15 minutes at room temperature (RT). After three washes with Phosphate-Buffered Saline (PBS), cells were permeabilised with PBS Triton X-100 (PBT) 0,5 % for 15 minutes and then aspecific sites were blocked with PBT 0,3 % + Fetal Bovine Serum (FBS) 2 %, for 1 hour at RT. Incubation with the primary antibody (Tab. 3) was performed over-night at 4°C. The following day, cells were washed and incubated with secondary antibody (Tab. 4) for 2 hours. Nuclei staining was performed with Hoechst 33342. Cells were visualized in PBS + Sodium Azide 10 mM.

Primary Antibodies		
Target	**Host**	**Dilution**
Nestin	Mouse	1:300
Sox2	Rabbit	1:200
Tuj1	Rabbit	1:500

Table 3 – Primary antibodies for immunofluorescence stainings.

Secondary Antibodies			
Target	**Conjugation**	**Host**	**Dilution**
Mouse	488	Goat	1:500
Mouse	568	Donkey	1:500
Rabbit	488	Goat	1:500
Rabbit	568	Donkey	1:500

Table 4 – Secondary antibodies for immunofluorescence stainings.

3.1.6 RNA extraction from cells

RNA was extracted from hNPC treated with chemical compounds, both while proliferating and differentiating in neuronal cells. Commercially available EuroGold Trifast™ Kit (Euroclone #EMR507100) was used following the manufacturer's protocol. Briefly, cells were harvested using 1 mL of Trifast solution scraping the bottom of the multiwell. The solution was incubated 5 minutes at room temperature, then vigorously hand-shaken for 15 seconds after addition of 0,2 mL of chloroform and another 10 minutes at room temperature. Samples were centrifuged at 12.000g for 15 minutes at 4°C and upper aqueous phase were collected. RNA was precipitated with 500 µL of isopropanol followed by an incubation on ice for 15 minutes and samples centrifugation at 12.000g for 15 minutes at 4°C. Supernatant was carefully removed and RNA pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 7.500 g at 4°C. Finally, RNA was air-dried and resuspended in RNase-free water.

3.1.7 Real-Time qPCR

First strand cDNA was synthesized using SensiFAST™ cDNA Synthesis Kit (Bioline #BIO-65054) following manufacturer's protocol. 3 genes of interest were analysed (*BDNF*, *CCND1*, *NIPBL*) using TB Green Premix Ex Taq (Tli RNase H Plus) (Takara Bio Inc. #RR420A) and the Applied Biosystems StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The *GAPDH* expression was tested in parallel with genes of interest as a housekeeping gene. The list of primers is reported in Tab. 5. Output data were analysed using StepOnePlus™ Software (Applied Biosystem) and Prism 7 (GraphPad).

Homo sapiens qPCR primers	
NIPBL_Fw	GGCAGCACAGATGAATGAAAG
NIPBL_Rv	CTTGCAATTTGTGGTCGATCTT
CCND1_Fw	CTGGAGGTCTGCGAGGAA
CCND1_Rv	GGGGATGGTCTCCTTCATCT
BDNF_Fw	CATCCGAGGACAAGGTGGCTTG
BDNF_Rv	GCCGAACTTTCTGGTCCTCATC
GAPDH_Fw	GAGTCAACGGATTTGGTCGT
GAPDH_Rv	TTGATTTTGGAGGGATCTCG

Table 5 – Primers used for *Homo sapiens* qPCR analyses.

3.2 *Drosophila melanogaster*

3.2.1 *Drosophila* husbandry

Flies were cultured in plastic vials on common food medium, which consist of cornmeal, yeast, molasses, agar, propionic acid, tegosept and water at 25°C. For all experiments, mutants and relative controls were grown at the same time with the same batch of food preparation.

3.2.2 Genetics

Drosophila lines used in this study were provided by the Bloomington Drosophila Stock Center or by our collaborators:

- *yw*
- *yw; Nipped-B^{407}, pw^{+} / CyO, Kr-GPF* [101], [102], [113], [114]
- *Nipped-B^{NC138} cn^{1} bw^{1} / SM1* (Bloomington Stock Center #7163) [115], [116]
- *w; 201Y-Gal4, UAS-GFP* (Kind gift from Florence Besse, Institute of Biology Valrose)

The allele *Nipped-B*^{407} was created by gamma ray and is classified as loss-of-function allele. The allele *Nipped-B*^{NC138} was created by mutagenesis with ethyl methanesulfonate (EMS). The driver *201Y-Gal4* is expressed in the *Drosophila* mushroom bodies, especially the gamma lobes.

3.2.3 Flies treatments

Flies were grown on food with different concentrations of WNT activators. Lithium chloride (LiCl) was added to reach the desired concentration in the food after the cooking process, when the temperature was under 60°C. The final concentrations used were 100 mM, 200 mM for LiCl (using water as solvent vehicle). These doses have been chosen according to previous experiment reported in scientific literature [117]–[120]. Adult flies indicated as parental generation (P) were reared on food in plastic vial for one week at 25°C, then they were discarded in order to collect only the offspring indicated as first generation (F1). Following this protocol, food and drug were ingested both by the parental generation and by the larvae which keep feeding through all their stages, that in turn become the F1 generation of flies.

3.2.4 Immunofluorescence of adult brains

The protocol for brain dissection and staining was optimized from what is reported in the literature [121]. Drosophila brains from F1 adult flies were dissected in PBS added with 1% Triton-X 100, then fixed in PFA 4% for 1 hour and stained with mouse antibody anti-FasII (clone 1D4; Developmental Studies Hybridoma Bank) for 2 nights. This was followed by incubation with Alexa-488 secondary antibody for 1 hour. Among the steps, samples were washed on mild agitation. Brain samples were then whole mounted in glycerol based mounting media. Signals were visualized using a fluorescence microscope (Leica DMRB) and acquired using a digital camera (Leica DFC480) or using a confocal microscope (Nikon A1R).

3.2.5 Generation of GFP-labelled mushroom bodies *Drosophila* strain

I managed to exploit the *Drosophila melanogaster* homologous recombination and Gal4-UAS system, in order to create flies mutated in *Nipped-B*^{407}, with the expression of Green

Fluorescent Protein (GFP) in the mushroom bodies. Briefly, I crossed virgin females carrying *Nipped-B*^{407} allele with males carrying the driver for mushroom bodies (201Y-Gal4) and the reporter (UAS-GFP). From the offspring I took the recombinogenic females and I performed single crosses to balance the second chromosome where the recombination may have occurred, and I selected only the flies in which the recombination actually occurred. I back-crossed these males with virgin females of the original *Nipped-B*^{407} stock to check whether the homozygous lethality for *Nipped-B*^{407} was still present. Finally, I cleaned up the second chromosome from the *kr-GFP* present in the original stock, and I obtained the recombinant flies with the following genotype:

- *yw; Nipped-B*^{407}*, pw*$^{+}$*, 201Y-Gal4, UAS-GFP / CyO*

3.2.6 RNA extraction from tissues

RNA was extracted from whole Drosophila using a commercial kit (RNeasy Mini Kit - Qiagen) and following the manufacturer's protocol. For every biological sample (n=3) 14 flies were used (7 males and 7 females).

3.2.7 Real-time qPCR

Real-time qPCR analyses were performed after retro-transcription of 2 ng of RNA template with SuperScript VILO cDNA Synthesis Kit (Invitrogen™ 11754050). 3 genes of interest were analysed *(arm, en, wg)* using SsoFastTM EvaGreen® Supermix (BioRad) and CFX96 Touch Real-Time PCR Detection System (BioRad #172-5202). The *RpL32* expression was tested in parallel with genes of interest as a housekeeping gene. The list of primers is reported in Tab. 6. Output data were analysed using CFX Manager Software (BioRad) and Prism 7 (GraphPad).

Drosophila melanogaster qPCR primers	
arm_Fw	TCTGCTGCAACGAAACAACG
arm_Rv	CTGCATCCGAAAGATTGCGG
en_Fw	TATCGCCGCACTTCAAAAGC
en_Rv	TTTACAGAGCGGTTGCAAGC
RpL32_Fw	ACAGGCCCAAGATCGTGAAG
RpL32_Rv	CTTGCGCTTCTTGGAGGAGA
wg_Fw	TGTTGTGTCCCATGATTGCC
wg_Rv	TGCGTATGCCGATATTGCTG

Table 6 – Primers used for *Drosophila melanogaster* qPCR analyses.

3.3 Data analysis

qPCR data for hNPCs and *Drosophila melanogaster* tissues were analyzed with student's unpaired t-test. Mushroom bodies morphology data in *Drosophila melanogaster*, blinded scored by two operators, were analyzed using Fisher exact test. For all the analyses, $p \leq 0.05$ (*) was set as statistically significant, $p \leq 0.01$ (**), $p \leq 0.005$ (***). Graphs were realized using Prism 7 (Graphpad) and figures were assembled using Adobe Photoshop CC.

4. RESULTS

4.1 Inhibition of HDAC8 in proliferating hNPC

Proliferating hNPC derived from commercial hiPSCs were treated with the HDAC8 inhibitor PCI-34051 at two concentrations (10 μM and 20 μM) according to manufacturer's datasheet. Cells were treated for 6 days and acquired images (Fig. 12) suggest that no visible cytotoxic effects are present until 48h-72h, but cells treated with the higher dose (20 μM) display cellular distress/death. Moreover, cells display a more differentiated morphology (flat and elongated cells) with a dose-dependent trend.

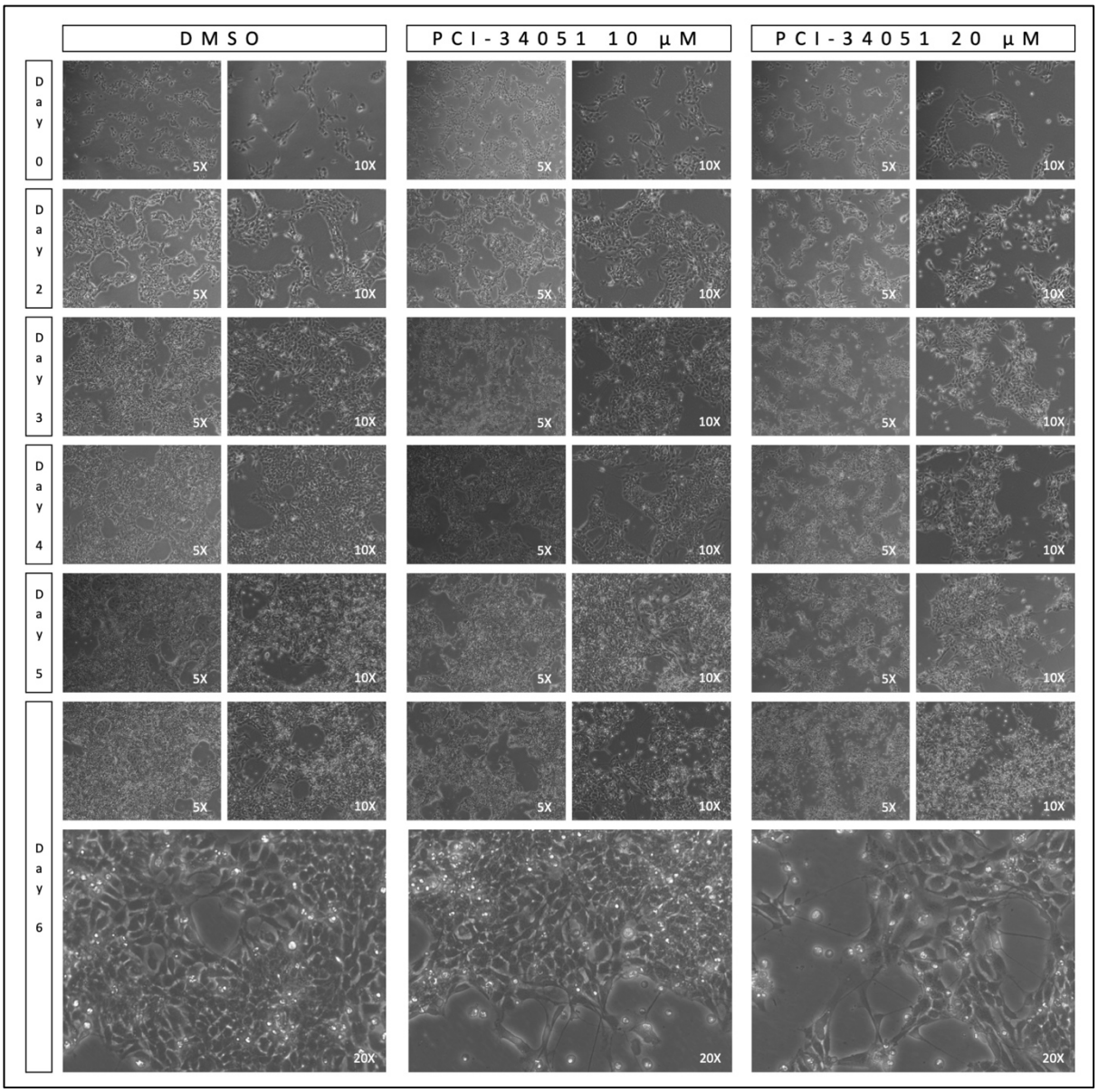

Figure 12 – Treatments of proliferating hNPC with HDAC8 inhibitor (PCI-34051). Panel shows proliferating hNPCs treated with DMSO, PCI-34051 10 μM and PCI-34051 20 μM at three magnifications (5X, 10X and 20X). Images were acquired at 6 time points: day 0 (0 h), day 2 (48 h), day 3 (72 h), day 4 (96 h), day 5 (120 h), day 6 (144 h).

I evaluated the expression of the three genes: *NIPBL*, *CCND1* and *BDNF,* to assess cohesin function, WNT pathway activation and neuronal differentiation. Real-time qPCR analyses highlighted that *NIPBL* expression was unaffected by treatments, but *CCND1* (target of canonical WNT pathway) and *BDNF* (known factor for growth and differentiation of new neurons) expression was increased (Fig. 13), due to a compensation mechanism that provide for an upstream impairment in the cohesins mechanism. In this specific case the inhibition of the auxiliary factor HDAC8, which interacts with the cohesin complex and allows the recycling of SMC3 core protein.

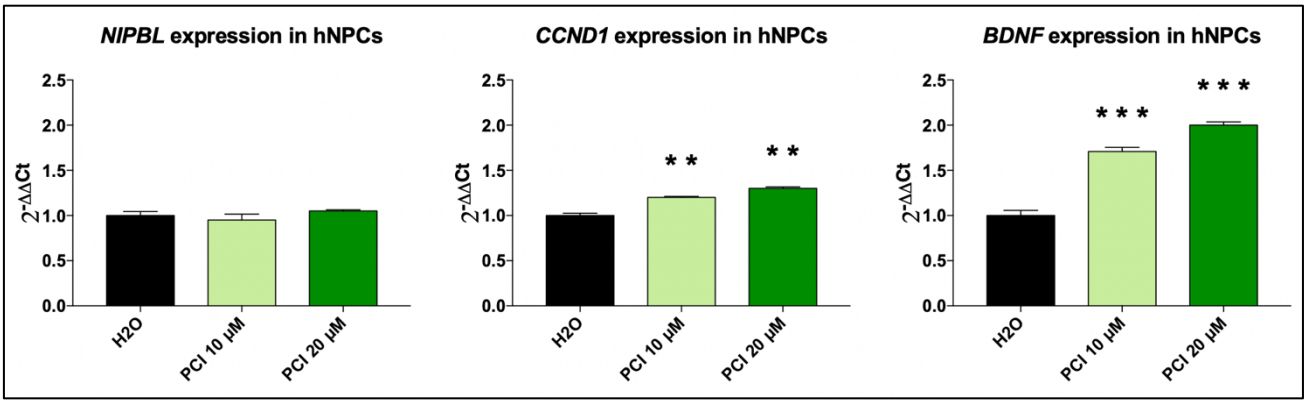

Figure 13 – Real-time qPCR analyses of proliferating hNPCs. Panel shows gene expression for *NIBPL*, *CCND1*, *BDNF* in hNPCs treated with DMSO (black bars), PCI-34051 10 µM (light green bars) and PCI-34051 20 µM (dark green bars). Values are expressed as mean ± SEM $2^{-\Delta\Delta Ct}$ on the Y axis and *GAPHD* was used as reference gene. Statistical analyses were carried out using student's unpaired t-test [$p \leq 0.01$ (**), $p \leq 0.005$ (***)].

Since real-time qPCR results displayed similar effects with the two concentrations of PCI-34051, but the higher dose recapitulates better the phenotype of interest inducing cell distress/death, I decided to investigate the drug effects on hNPCs during neural differentiation using only the higher concentration of PCI-34051, 20 µM.

4.2 LiCl restores hNPC phenotype during neuronal differentiation after HDAC8 inhibition

hNPC were treated with LiCl and/or HDAC8 inhibitor during the neuronal differentiation process for 13 days. Immunostaining for Nestin and Tuj1, markers of neural precursors and

mature neurons respectively, shows that LiCl treatment increase the rate of neuronal differentiation, resulting in more Tuj1 positive cells (Fig. 14). Moreover, 20 µM of HDAC8 inhibitor affects neuronal differentiating process, but in presence of LiCl the morphology of Tuj1 positive cells in the double treatments can be partially restored.

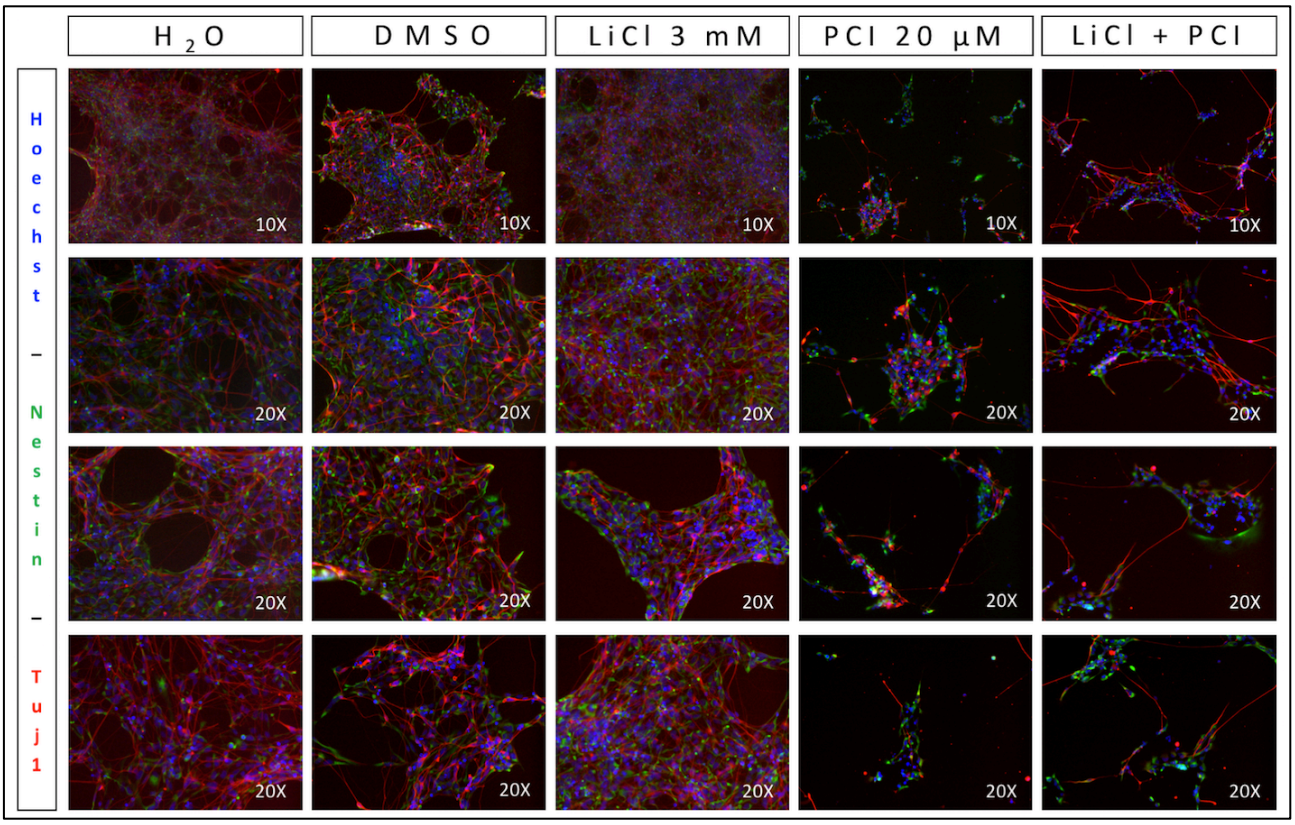

Figure 14 – Treatments of differentiating hNPC into neurons with LiCl and/or HDAC8 inhibitor (PCI-34051). Panel shows differentiating hNPCs into neuronal-like cells treated with water (as vehicle for LiCl), DMSO (as vehicle for PCI-34051), LiCl 3 mM, PCI-34051 20 µM, LiCl 3 mM + PCI-34051 20 µM at two different magnifications (10X, 20X). Images were acquired after 13 days of treatments. Hoechst-stained nuclei are shown in blue, Nestin-stained neural precursors are shown in green, and Tuj1-stained mature neurons are shown in red.

It is clear that, upon mimicking HDAC8 under-expression and activating canonical WNT pathway, cells display differences in proliferation and differentiation capabilities. All this considered, I decided to move to a more complex *in vivo* system to better characterize the molecular bases of CdLS, exploiting the *Drosophila melanogaster* model.

4.3 *Drosophila* body weight assay

Body weight is a fly feature easily measurable, and changes can be used as a read-out of a treatment or an improvement in the phenotype. CdLS flies with mutation in *Nipped-B* gene

were previously described as smaller and lighter [101], therefore I used body weight assay to test the efficacy of lithium treatment.

Male and female of adult *Drosophila melanogaster* were separated and weighted, in groups of 100 flies, based on genotype and treatments (Fig. 15, Tab. 7).

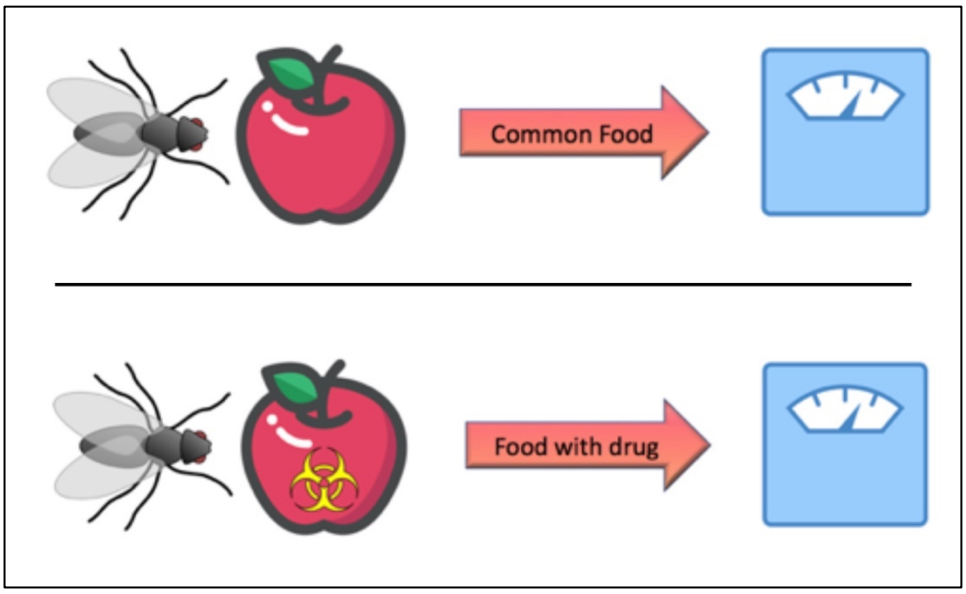

Figure 15 – Adult *Drosophila* weight assay. Schematic representation of flies' treatments before weight assay.

	Common food	Food added with LiCl
yw ♂	0,7440 mg	–
yw ♀	1,2360 mg	1,2222 mg
Nipped-B^{407} ♂	0,7141 mg	–
Nipped-B^{407} ♀	1,1780 mg	1,1571 mg

Table 7 – Data from adult *Drosophila* weight assay.

The results reported in table 7 were unexpected since mutant flies, previously described as lighter and smaller [101], do not show a significant weight phenotype, compared to the *yw* controls and in fact, I have not observed any gain of weight upon LiCl treatment. Therefore, I decided to focus on the detection of an aberrant morphological structure.

4.4 *Drosophila Nipped-B^{407}* mutants have abnormal mushroom bodies

In order to find an aberrant phenotype to better characterize the *Nipped-B^{407}* mutants, I decided to evaluate the morphology of mushroom bodies of *Drosophila melanogaster*, a well-studied specialized CNS structure involved in olfactory learning and memory in adults, by immunofluorescence staining with anti-FasII antibody. Indeed, flies heterozygous for *Nipped-B^{407}* often display mushroom bodies anomalies such as aberrant or missing lobes, when compared to control animals (Fig. 16).

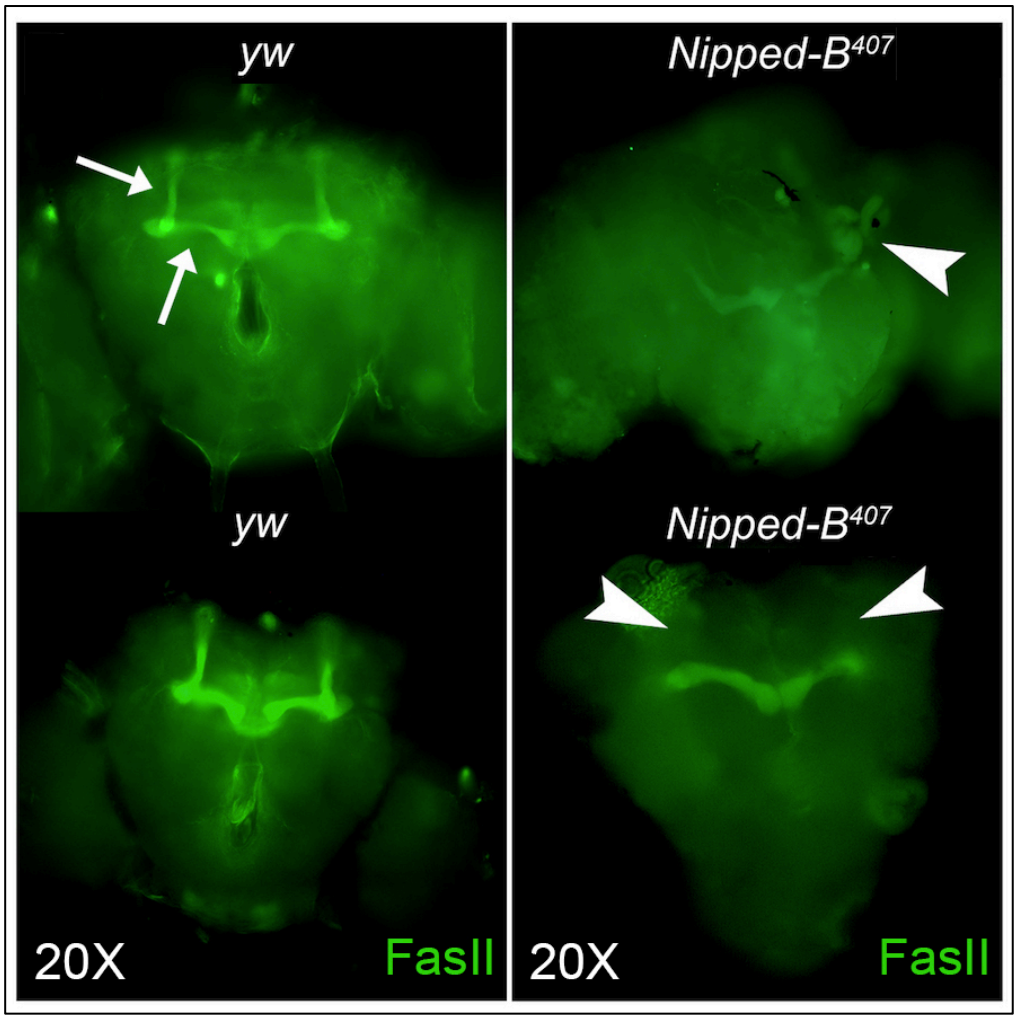

Figure 16 – *Drosophila* mushroom bodies staining. Panel show *yw* controls (on the left) and *Nipped-B^{407}* mutants (on the right) at a magnification of 20X. *yw* controls display normal mushroom bodies morphology in which α, β, γ lobes are present (white arrows). *Nipped-B^{407}* mutants display abnormal mushroom bodies morphology with twisted structure (upper arrowhead) and lacking both α lobes (lower arrowheads). Anti-FasII stained mushroom bodies in green.

Since the aberrant phenotype was clear and possible to measure, I moved to the rescuing experiment, assessing the aberrant morphology with lithium treatment.

4.5 Lithium treatment rescues adverse phenotype

Upon rearing *Nipped-B*^{407} haploinsufficient animals on food supplemented with lithium (100 mM) as an activator of canonical WNT pathway (Fig. 17), a statistically significant (Fisher exact test, p=0,0036) number of *Nippded-B*^{407} adults did not show signs of altered mushroom body development (Fig. 18). In particular, the percentage of *Nipped-B*^{407} haploinsufficient animals with abnormal mushroom body morphology decreased from 88,24% in the untreated sample to 30% in the treated animals (Tab. 8).

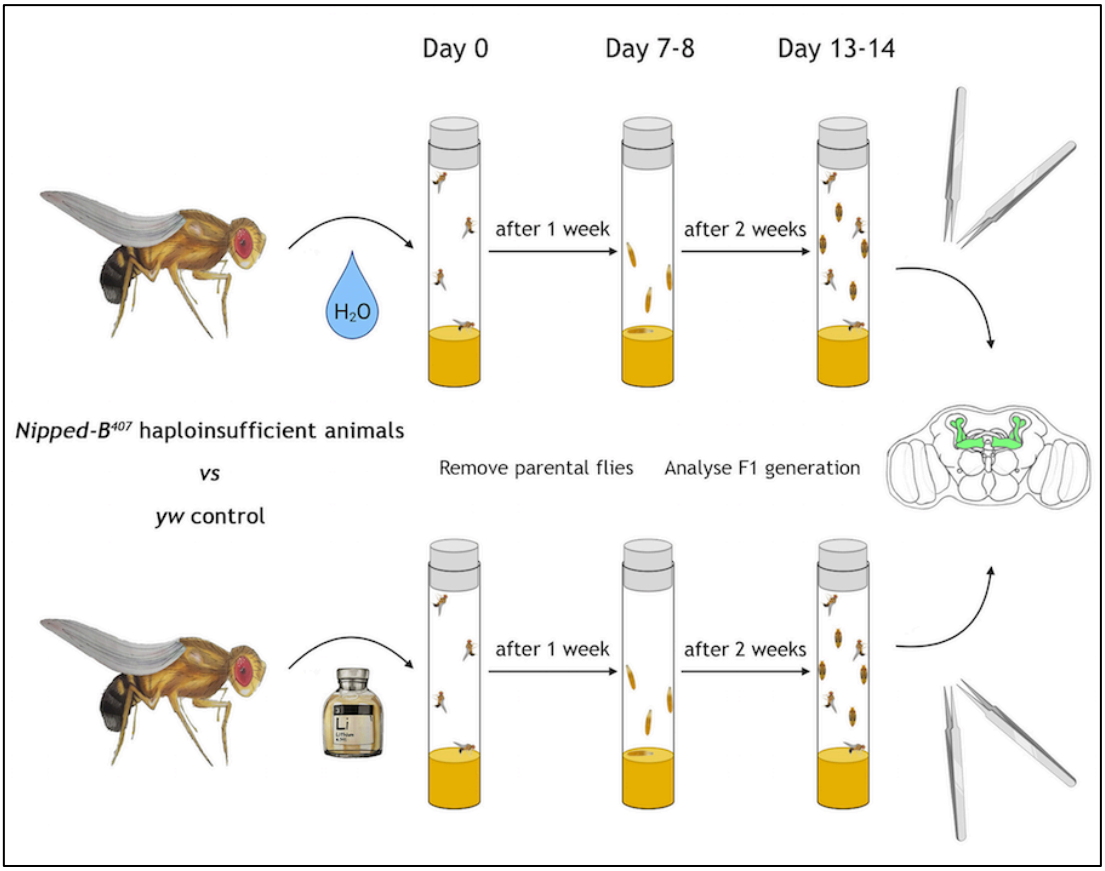

Figure 17 – Schematic representation of *Drosophila* treatments. Schematic representation of flies' treatments before brain dissection and mushroom bodies analyses in the F1 generation. Parental flies of both *yw* controls and CdLS mutants were reared on common food (upper) and food integrated with LiCl (lower) for 1 week, when they were removed allowing the larvae to grow, pupate and develop into the F1 adult flies whose brains have been dissected and analyzed.

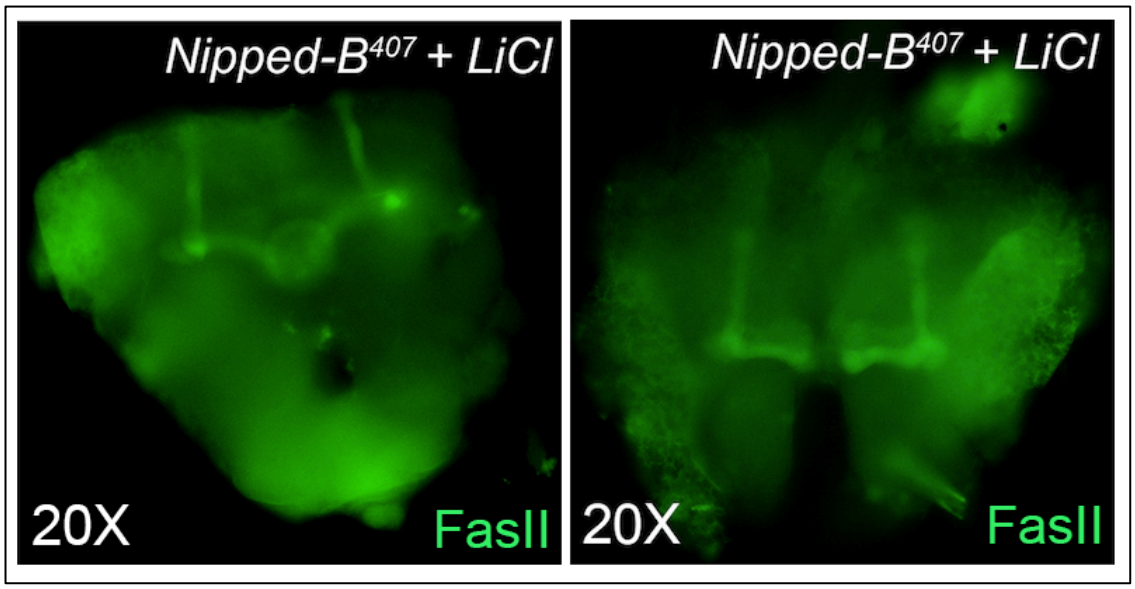

Figure 18 – Mushroom bodies rescue upon LiCl treatment. Panel show two *Nipped-B*^{407} mutants' brain after LiCl treatment at a magnification of 20X. *Nipped-B*^{407} mutants reared upon LiCl display an improved mushroom bodies morphology (compared to untreated flies in Fig. 16). Anti-FasII stained mushroom bodies are shown in green.

	Normal (%)	**Abnormal (%)**
yw	19 (95%)	1 (5%)
***Nipped-B*^{407}**	2 (11,76%)	15 (88,24%)
***Nipped-B*^{407} + LiCl 100 mM**	7 (70%)	3 (30%) ***

Table 8 – Percentage of adult *Drosophila* mushroom bodies abnormalities. Data were analyzed using Fisher's exact test (*** p=0,0036).

To assess whether such significant anatomic rescue was WNT-dependent, we analyzed gene expression in our experimental groups (n=3) (Fig. 19).

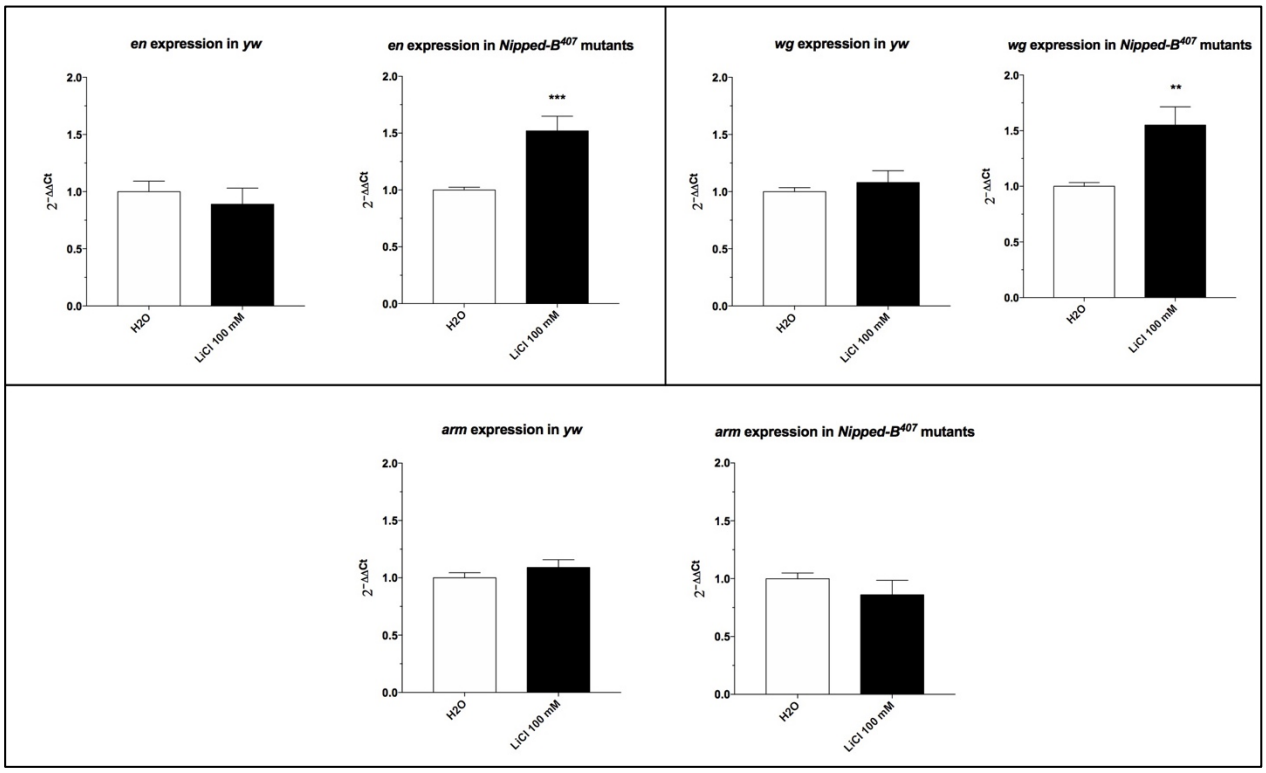

Figure 19 – *Drosophila melanogaster* qPCR analyses. Panel shows gene expression for *en*, *wg, arm* in adult *Drosophila* treated with water (as vehicle for LiCl, white bars), and LiCl 100 mM (black bars). Values are expressed as mean ± SEM $2^{-\Delta\Delta Ct}$ on the Y axis and *RpL32* was used as reference gene. Statistical analyses were carried out using student's unpaired t-test [$p \leq 0.01$ (**), $p \leq 0.005$ (***)].

Gene expression of *wg* (*WNT* ortholog) and *en* (*EN1/EN2* ortholog, target gene of canonical WNT pathway involved in embryo development) were upregulated in *Nipped-B*^{407} mutants only, while expression of *arm* (*β-catenin* ortholog) was unaffected both in *yw* controls and mutants.

The results highlighted how lithium and canonical WNT pathway activation acts on CNS structure and gene expression, but the immunostaining for the whole brain is time-consuming, therefore I decided to exploit the *Drosophila* homologous recombination to create a recombinant strain of fly that carries both the *Nipped-B*^{407} mutated allele and GFP specifically expressed in the mushroom bodies, in order to perform a direct visualization.

4.6 GFP-labelled *in vivo Drosophila* model for CdLS

I managed to create recombinant fly strains that specifically express GFP in the mushroom bodies. This result was achieved thanks to the genetic tools offered by the *Drosophila*

melanogaster model. I exploited homologous recombination to create a chromosome carrying both *Nipped-B*^{407} allele and the 201Y-Gal4/UAS-GFP system. This GAL4-UAS system allows the overexpression of a protein of interest (GFP), under the control of specific driver (*201Y*, driver for the mushroom bodies). The resulting genotype of the new strains is: *yw; Nipped-B*^{407}*, pw*$^{+}$*, 201Y-Gal4, UAS-GFP / CyO*. In the flies, mushroom bodies structure can be visualized in the living animals (Fig. 20), and directly after dissection without any staining allowing a time-saving procedure.

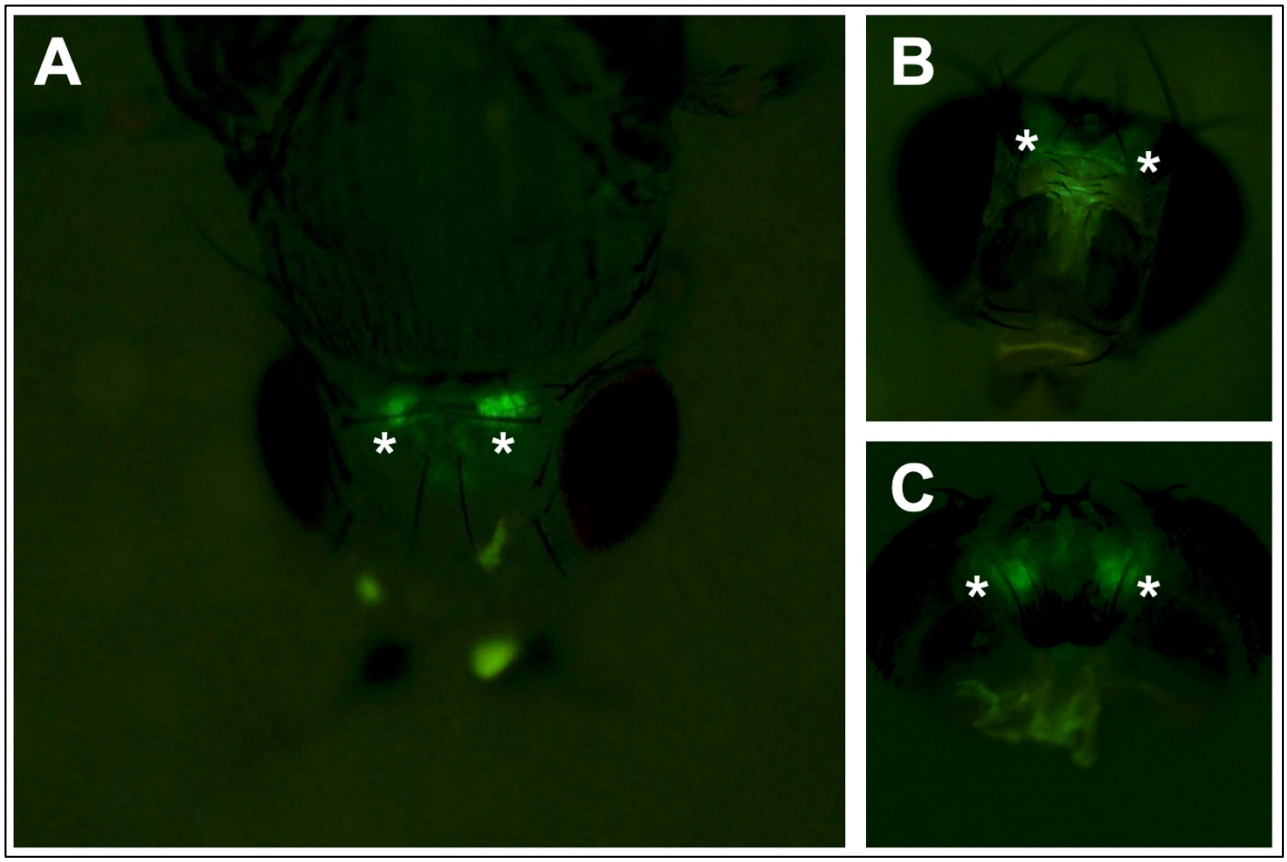

Figure 20 – *In vivo* visualization of mushroom bodies. Panel shows three different views of recombinant flies carrying both the *Nipped-B*^{407} allele and GFP protein specifically expressed in the mushroom bodies (*201Y-Gal4, UAS-GFP* construct) in adult *Drosophila* brains, highlighted by the white asterisks. (A) Head upper-rear side in the living animal. (B) Head upper side and (C) head rear side, after head removal.

5. DISCUSSION

Cornelia de Lange syndrome is a rare genetic disorder characterised by dysmorphic features, distinctive physical abnormalities and behavioral characteristics. These clinical signs can vary widely among affected individuals and range from relatively mild to severe phenotypes. 7 genes are known, so far, to be causative for this syndrome, and all of them are part, or directly interact, with the cohesin complex [6]. Diseases arising from defects in the cohesin complex, such as CdLS, still have an unclear etiopathogenesis precluding the development of targeted approaches for the young patients. In the last years many efforts have been made to unravel the molecular mechanism underlying CdLS, and some genes have been added to the list of candidates whose haploinsufficiency causes the syndrome [122]. Basic research on cohesins, cohesinopathies and the most represented CdLS, has made enormous advancements since the discovery of the “non-canonical function” of the cohesin complex in regulating gene expression [123], [124], different from the “canonical functions” implicated in regulating sister chromatids cohesion and separation during cell division and damaged DNA repair. *In vitro* and *in vivo* studies demonstrated that “non-canonical function” of the cohesin complex is the keystone to understand the molecular bases of this pathology. Many studies have been performed to analyse regulation and control of several putative target pathways that could account for the clinical heterogeneity of CdLS patients [6], [122]. Previous works in our laboratory and others, hypothesise that canonical WNT pathway, which has a pivotal role in CNS development, is altered in CdLS. In fact, this intracellular pathway controls and regulates a plethora of genes and interacts with numerous fundamental pathways during development [62], [82].

My PhD project further demonstrates the involvement of canonical WNT pathway in the pathogenesis of CdLS. First, I used an *in vitro* model of hiPSCs which is a versatile tool for the studying of neural development through its lineage differentiation. I observed the dynamics by which an impairment in the cohesin complex could reflect on a human cellular model, by treating cells with a specific inhibitor of HDAC8 [125], one of the proteins known to be implicated in the pathogenesis of CdLS. At the same time, I acted on the canonical WNT pathway through a chemical activation. Downstream analyses, showed that canonical WNT pathway activation reflects on the hiPSCs phenotype, improving the differentiation capabilities of cells in which HDAC8 was inhibited by chemical treatment. Molecular analysis, by mean of real-time qPCR on hiPSCs treated with HDAC8 inhibitor, highlighted the overexpression of *CCND1* and *BDNF*. *CCND1* is a target gene of WNT canonical pathway and *BDNF* is an important factor for growth and differentiation of new neurons.

These results represent a clear signal that cells try to compensate an upstream impairment in the cohesins mechanism that results in an impaired canonical WNT pathway signalling. After a couple of weeks of treatments, a dose-dependent cytotoxic effect of the HDAC8 inhibitor was observed. Therefore, I used the same cellular model, but I treated hiPSCs during the neuronal differentiation process for assessing whether the cells succeeded in differentiate into neurons or HDAC8 inhibition plays a role in this process. I observed that, as expected, HDAC8 inhibition interfere with the correct neuronal differentiation process and, interestingly, this impairment can be partially rescued activating canonical WNT pathway by LiCl administration. Our group achieved the same results using murine neural stem cells [125], and interestingly the LiCl treatment outcome remains consistent in human cells. In this work, I used commercial hiPSCs as a proof-of-concept experiment for testing the toxicity of the compounds (LiCl and PCI-34051) and the overall feasibility of the procedure. This approach, albeit presenting limitations (i.e., concomitant inhibition of HDAC8 and activation of canonical WNT pathway, targeting only one player of the cohesin complex machinery) is preliminary but it allows a good modelling of CdLS *in vitro*. Targeting HDAC8 is an easy way to mimic CdLS in a healthy cellular model before moving to patients derived cells. Moreover, *HDAC8* is not a common mutated gene, therefore will be difficult to collect cells from patients carrying mutations in this gene. My final goal will be switching to hiPSCs derived from patients and our collaborators, who already established and characterized hiPSCs from *NIPBL* mutated patients [111], kindly accepted to sign an MTA and send us the material for the future experiments. This material will provide a better model for precision medicine, starting with *NIPBL* mutations and, hopefully, moving to less commonly mutated gene to assess any difference in the treatment depending on the affected gene or type of mutation. Hence, I decided to move to a more complex *in vivo* system like *Drosophila melanogaster* to assess how CNS structures could be rescued upon WNT activation.

In *Drosophila melanogaster,* with this PhD project, I demonstrated how canonical WNT pathway activation through LiCl administration results in a morphological improvement in the mushroom bodies, a structure of the CNS, in the adult flies. These data point out how a molecular improvement can result in a macroscopic structural recovery. The first assay I tried was supposed to be an easy read-out of the mutations in *Nipped-B* gene. I weighted the animals, since these flies were described, in a previously published study, as smaller and lighter [101]. Unexpectedly, flies carrying the mutated allele *Nipped-B*^{407} were heavier than the controls, and this difference was also not statistically significant as opposed to the

results previously published in literature [101]. Because of these controversial results, I performed another assay that allowed me to check the presence of the mutation and to understand its role and quantify the effects of LiCl as an activator of canonical WNT pathway. I observed a structure in the CNS, that is known to be abnormal in these mutants: mushroom bodies [101]. Mushroom bodies are a symmetrical structure located in the CNS involved in insects' behaviour such as: olfactory learning and memory, courtship behaviour and elementary cognitive functions. This structure derives from the division of four mushroom body neuroblasts which are generated at an early embryonic stage and keep dividing throughout the animal's development [126]. As expected, CdLS flies carrying the *Nipped-B*^{407} allele displayed abnormal mushroom bodies morphology and upon rearing the parental flies on LiCl, I observed a rescue of the morphology of this structure in a significant percentage of mutants. For the first time ever described to my knowledge, treatment with lithium managed to improve the phenotype not only in the embryo, but also in the adult animals of the F1 generation upon treating parent flies. Mushroom bodies are considered relatable to three different regions in the mammal brain: the hippocampus, because of its involvement in learning and memory; the cerebellum, because of its involvement in learning and precisely timed motor movements; and the piriform cortex, because both piriform cortex and mushroom body are only two synapses away from the sensory layer of the olfactory system. Our group already demonstrated that cohesins are highly expressed in the cerebellum [57], and zebrafish CdLS models displayed aberrant structure in the hindbrain region [127]. Therefore, mushroom bodies can be considered a good homologous structure for studying CdLS pathogenesis in flies. Moreover, in this nervous structure, and in the human cerebellum, few cells undergo replication, this suggests that the "canonical role" of cohesin complex in the correct adhesion of sister chromatids should be marginal in this context, while the "non-canonical role" regarding gene expression should be the one mostly implicated. I then performed downstream molecular analyses using real-time qPCR to check the activation of canonical WNT pathway. I analysed three genes: *arm* (*β-catenin* ortholog), *en* (*EN1/EN2* ortholog) and *wg* (*WNT* ortholog). As expected, *arm* expression level was not affected because it does not have to be overexpressed to activate canonical WNT pathway, this protein is already present in the cytoplasm, but it needs to be activated for the nuclear translocation [128]. On the other hand, *en* and *wg* were overexpressed in the mutant flies only, due to the fact that they are both target genes of canonical WNT pathway [129], [130] but, in a physiological contest, their level are kept under control by a negative feedback of the pathway [131], [132]. When in the mutants, canonical WNT pathway is activated by other means than WNT proteins itself, these two genes are found to be highly expressed to

compensate the upstream impairment. Taken together, these data further demonstrate that cohesins has pivotal role in the development of the CNS through the activation of canonical WNT pathway.

Drosophila melanogaster turned out to be an easy and versatile model to study the molecular bases of CdLS, therefore I decided to exploit the homologues recombination to create a recombinant strain which carries both the *Nipped-B*^{407} allele and the GFP specifically expressed in the mushroom bodies. This new model, together with other existing tools, will allow time-saving procedures for future experiments and possible screening of other compounds that can activate canonical WNT pathway at different levels i.e., DCA [80], BIO [78], IQ-1 [133], CHIR-99021 [79] and many others.

In the context of human rare diseases like CdLS, it is challenging to obtain a large number of patients and most of the anomalies in the CNS are reported in the scientific literature as single case reports or with a scarce number of patients. The biggest studies that describe anomalies in the CNS of CdLS patients are from Whitehead [134] which compares neuroradiologic features of 8 CdLS patients; and from Roshan Lal [135] which collected data from 15 CdLS patients through Nuclear Magnetic Resonance (NMR). Our group, thanks to collaborators, collected the largest cohort of CdLS patients (n=155) and obtain encephalic NMR from 66 of those patients who manifested neurological symptoms (Grazioli, Parodi, Mariani *et al.,* (2021) *In press*). From the NMR data analyses emerged that a big portion of patients displayed morphological alteration in the cerebellum (52% in patients with anomalies). These alterations have been correlated with ASD behavioural problems [136], but it is challenging to diagnose ASD in CdLS patients because they have different clinical features from the ones usually present in classic ASD. CdLS patients' communication and social anxiety prevail on stereotyped and repetitive movements [137]. Our data seem to support the potential relation between cerebellum anomalies and ASD but also bolster the fact that CNS malformation in CdLS patients could arise from an aberrant activation of the canonical WNT pathway.

Correlation between cerebellum anomalies and autistic traits suggests that lithium could represents a therapeutic strategy in order to improve behavioural disabilities in CdLS patients. Canonical WNT pathway, activated by LiCl, has a pivotal role in the development of CNS and a correct reactivation of this signalling results in beneficial effects on proliferation and differentiation which could potentially improve cellular deficits. Some studies are considering lithium administration to patients with X-fragile syndrome who display similar clinical feature to CdLS [138]. Moreover, the lack of a significant relation between CNS

anomalies and single gene mutations suggests that CdLS phenotype is not caused by alteration in specific genes, but due to an impairment in intracellular pathway with which cohesins interact.

The data presented in my PhD project are in line with other experiments conducted by colleagues and other research groups using *Drosophila* [102], zebrafish [62], [127], murine cells [125], human lymphoblastoid cell lines derived from patients, organoids and mini-brains. All the data, taken together, confirm that activating canonical WNT pathway results in an improvement of neuronal differentiation capabilities, restored proliferation, and increased cell viability, making lithium a possible therapeutic approach in CdLS.

6. CONCLUSIONS

With my PhD project, I exploited different experimental *in vitro* and *in vivo* CdLS models adopting a translational approach, within the area of molecular biology and human health. I demonstrated how cohesins genes have a pivotal role, through the canonical WNT pathway, in the CNS; and that mutations in the cohesin complex interfere with the physiological differentiation of neurons from the early stages, and lead to a visible macroscopic malformation in nervous structures. My results reflect what is known about the molecular bases of CdLS and further investigate new possible players in the pathogenesis.

hiPSCs experiments and analyses should be confirmed exploiting cells derived from CdLS patients. These cells are already available, thanks to our collaborators, and they can be used to test LiCl in a known CdLS genetic background.

Furthermore, these data pave the way for a compounds screening, both *in vitro* and *in vivo* exploiting the new recombinant fly model, in order to find new therapeutic targets that can be translated into clinical practice. Moreover, I believe that this PhD project could represent an important advance for the researchers working on CdLS, providing potential implications for clinical care.

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SCIENTIFIC PRODUCTION

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- Parodi C., **Grazioli P.**, Avagliano L., George T. M., Bulfamante G. P., Finnell R. H., Massa V. *Neural tube defects: embryonic origin, cell survival equilibrium impact and clinical features.* In: Martin C., Preedy V. R., Rajendram R. "Diagnosis, Management and Modelling of Neurodevelopmental Disorders: The Neuroscience of Development". Book 1 – Elsevier *(In press)*
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- Parodi C., Di Fede E., Peron A., Viganò I., **Grazioli P.**, Finnell R. H., Gervasini C., Vignoli A., Massa V. *Chromatin imbalance as the vertex between fetal valproate syndrome and chromatinopathies.* Frontiers in Cell and Developmental Biology. *(Submitted – Under consideration)*

Oral Talks

- Congresso DiSS 2017 (Milano, IT) - Title: “Modeling Cornelia de Lange Syndrome in vitro and in vivo reveals a role for cohesin complex in neuronal survival and differentiation” **[Award: “Best oral communication” for the session “Molecular Medicine”]**
- European Society for Neurochemistry (Milano, IT); Title: “Mushroom bodies development and abnormalities in defective Wnt pathway models” **[Award: Scholarship as accepted for the Young Members' Symposium]**

Posters

- XX Congresso Nazionale SIGU (Società Italiana di Genetica Umana) (Napoli, IT) - Poster title: “Modulating the WNT pathway in Drosophila models of Cornelia de Lange Syndrome”
- European Human Genetics Conference (Milano, IT); Poster title: “Drosophila melanogaster as a model to study WNT pathway alteration in Cornelia de Lange Syndrome”

- 6th RADIZ Rare Disease Summer School (Zürich, CH); Poster title: "Cornelia de Lange Syndrome: different models and strategies to study the disease"
- 6th RADIZ Rare Disease Summer School (Zürich, CH); Poster title: "Novel diagnostic and therapeutic approaches to Rubinstein-Taybi syndrome"
- 77th Society for Developmental Biology Annual Meeting (Portland, OR, USA); Poster title: "Modulating the WNT pathway in Drosophila models of Cornelia de Lange Syndrome"
- DiSS Congress 2018 (Milano, IT); Poster title: "KMT2A mutations in patients with Rubinstein-Taybi clinical diagnosis: a shared imbalance of open and closed chromatin in overlapping syndromes?"
- DiSS Congress 2018 (Milano, IT); Poster title: "Lithium as a positive modulator of defective WNT pathway in Cornelia de Lange Syndrome models"
- MyDev 2019 (Milano, IT); Poster title: "Microbial colonization during early phases of amphibian life"
- MyDev 2019 (Milano, IT); Poster title: "In vivo and in vitro models for studying Cornelia de Lange Syndrome"
- JRC Summer School 2019 (Ispra, IT); Poster title: "In vitro assessment of HDAC inhibitors effects"
- 7th RADIZ Rare Disease Summer School (Zürich, CH); Poster title: "Wnt pathway activation in CdLS models: paving the way for future therapeutic strategies"
- 78th Society for Developmental Biology Annual Meeting (Boston, MA, USA); Poster title: "Cornelia de Lange Syndrome neurodevelopment: modulating defective Wnt pathway"
- DiSS Congress 2019 (Milano, IT); Poster title: "In vitro model for acetylation imbalance modulation in Rubinstein-Taybi syndrome"
- DiSS Congress 2019 (Milano, IT); Poster title: "Cohesin complex and canonical WNT pathway: unravelling Cornelia de Lange Syndrome molecular basis"
- XXII Congresso Nazionale SIGU (Società Italiana di Genetica Umana) (Roma, IT) - Poster title: "In vitro model for acetylation imbalance modulation in Rubinstein Taybi syndrome"
- DiSS Congress 2020 (Milano, IT); Poster title: "Studying the role of Neuroligin-3 in neurodevelopment: a link between autism and reproduction"
- DiSS Congress 2020 (Milano, IT); Poster title: "Microbial colonization and vertical transmission in Xenopus laevis"

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