Cornelia de Lange Syndrome: *NIPBL* haploinsufficiency downregulates canonical Wnt pathway in zebrafish embryos and patients fibroblasts

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Cornelia de Lange Syndrome is a severe genetic disorder characterized by malformations affecting multiple systems, with a common feature of severe mental retardation. Genetic variants within four genes (*NIPBL* (Nipped-B-like), *SMC1A*, *SMC3*, and *HDAC8*) are believed to be responsible for the majority of cases; all these genes encode proteins that are part of the ‘cohesin complex’. Cohesins exhibit two temporally separated major roles in cells: one controlling the cell cycle and the other involved in regulating the gene expression. The present study focuses on the role of the zebrafish *nipbl* paralog during neural development, examining its expression in the central nervous system, and analyzing the consequences of *nipbl* loss of function. Neural development was impaired by the knockdown of *nipbl* in zebrafish. *nipbl*-loss-of-function embryos presented with increased apoptosis in the developing neural tissues, downregulation of canonical Wnt pathway genes, and subsequent decreased Cyclin D1 (*Ccn1d*) levels. Importantly, the same pattern of canonical Wnt pathway and CCND1 downregulation was observed in *NIPBL*-mutated patient-specific fibroblasts. Finally, chemical activation of the pathway in *nipbl*-loss-of-function embryos rescued the adverse phenotype and restored the physiological levels of cell death. 

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Cornelia de Lange Syndrome (CdLS) is a multiple malformation syndrome typically involving developmental delay, specific facial features, behavioral abnormalities, and major congenital malformations. Almost any organ can be affected, but the most commonly observed defects are the ones that affect the neurodevelopmental, gastrointestinal, and musculoskeletal systems. There is a broad spectrum of clinical involvement, with increasing recognition of a much milder phenotype than previously appreciated. Clinically, a common feature of CdLS is mental retardation that is often associated with enlarged brain ventricles, thinning or atrophy of white matter, brainstem hypoplasia, and cerebellar vermal hypoplasia or agenesis. During development, the central nervous system (CNS) arises from the neural plate that folds up on its anterior–posterior (AP) axis to form a tube divided in three main units: the anlagen of the prosencephalon (forebrain), the mesencephalon (midbrain), and the rhombencephalon (hindbrain). Caudally, the tube will develop into the spinal cord. These coordinated events are achieved through a finely tuned balance between cell proliferation and programmed cell death. Within each region, a large diversity of neuronal cell types is generated, each with distinct identities in terms of their morphology, axonal trajectory, synaptic specificities, and neurotransmitters. This fundamental specification is controlled by dorsoventral (DV) and AP gradients of molecules. For example, components of the Wnt family are known to induce anterior structures (such as forebrain), whereas bone morphogenetic proteins are responsible for neurogenesis in the dorsal neural tube.

Genetically, CdLS is caused by both autosomal-dominant and X-linked mutations. Approximately, 60% of the cases are due to genetic defects in one of the four genes: *NIPBL* (Nipped-B-like), *SMC1A*, *SMC3*, and the recently identified *HDAC8*. *NIPBL* gene was the first to be found associated with CdLS. *SMC1A* was subsequently identified as an additional gene whose disruption produces the phenotypic findings of the CdLS, whereas one CdLS case was reported having a mutation in *SMC3*. *HDAC8* was found to be mutated in CdLS patients, consistent with its role in controlling the acetylation status of *SMC3*. Most recently, mutations in *RAD21*, a structural protein in the cohesin complex, have been found in children with a spectrum of disabilities overlapping with the CdLS syndrome, presenting with milder cognitive impairments consistent with a so-called

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**Abbreviations:** CdLS, Cornelia de Lange Syndrome; Cond1, Cyclin D1; Cond2, Cyclin D2; CNS, central nervous system; AP, anterior–posterior; DV, dorsoventral; WISH, whole-mount in situ hybridization; RPMI, Roswell Park Memorial Institute serum; q-PCR, quantitative RT–PCR; TBST, tris-buffered saline/Tween 20 buffer; ABC, active form of beta-catenin; Fmi/Stain, Flamingo/Starry

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The cohesin complex is evolutionarily conserved from fungi to human cells, and its canonical role is the regulation of chromatid separation during cell division. In particular, the cohesin complex controls sister chromatid cohesion during S phase. In the somatic cells of vertebrates, four core subunits forming the complex have been identified: SMC1 (SMC1A or SMC1B), SMC3, SCC1, and either SA1 or SA2. Associated with the core complex, other proteins assist during cell division, for example, NIPBL, which appears to facilitate the uploading of the complex, or WAPL, which enables complex dissociation. A non-canonical role has recently been observed, whereby the cohesin complex also represents a key regulator of gene expression. In Drosophila, NIPBL ortholog (i.e., Nipped-B) has been shown to participate in the long-distance activation of cut and Ultrabithorax gene expression. In zebrafish (Danio rerio), cohesins regulate runx transcription factors and hematopoiesis. Interestingly, it seems that functionally, the cell cycle control and gene expression regulation are temporally separated. For example, Drosophila mutants for orthologs of cohesin complex genes show a defect in axon retraction that physiologically occurs in post-mitotic neurons, hence in cells that have exited the cell cycle. In zebrafish, cohesins are expressed in both proliferating and non-proliferating cells, while mice that are heterozygous for Nipbl have severe developmental defects and generally altered gene expression in the absence of cell cycle or sister chromatid impairment.

A nipbl-deficiency model in zebrafish shows altered expression of genes involved in endodermal differentiation along with a spectrum of heart, gut, and visceral organ defects that bear a striking similarity to those observed in CdLS patients. In the present study, we have used two combined approaches, zebrafish embryos and patient-derived cells, in order to dissect molecular mechanisms underlying the neurodevelopmental disorders. First, the effects of nipbl loss of function in the developing CNS of zebrafish, in particular at the hindbrain level, were characterized. The results obtained from functional studies in zebrafish embryos were then confirmed in fibroblasts of CdLS patients with known mutation in NIPBL.

**Results**

**Zebrafish nipblb is expressed in the CNS.** In accordance with the literature, nipblb was ubiquitously expressed during early somitogenesis in zebrafish embryos. The expression progressively decreased in the trunk and appeared specifically localized in the CNS at 24 hpf (hours post fertilization) (Figure 1a). As seen in this dorsal view, expression of nipblb (Figure 1b) was detected in the diencephalon and mesencephalon, and highlighted the progression of hindbrain ventricle opening. Histological longitudinal and transverse sections of 24 hpf embryos clearly showed that nipblb is expressed throughout the entire dorsal–ventral (DV) and AP axis of the CNS (Figures 1c and d). Colocalization with krox20, a marker of rhombomeres III and V, showed that nipblb expression extended caudally as far as rhombomere V at this developmental stage (Figure 1e).

**Inhibition of Nipblb function in zebrafish embryos affect CNS development.** Embryos injected with nipblb-morpholino (MO) began to exhibit defects at 24 hpf, including brain defects (50% of nipblb-MO injected, total n = 200, Figures 2a and b). In particular, nipblb-loss-of-function embryos were microphthalmic and microcephalic. In addition, nipblb-MO-injected embryos were presented with a short, curved tail (40% of nipblb-MO injected, total n = 180, Figure 2b), a phenotype previously described by Muto et al. following nipblb loss of function. nipblb-MO efficiency and specificity were then confirmed in fibroblasts of CdLS patients with known mutation in NIPBL.
were verified by western blot experiments in which Nipbl protein levels were reduced by 39% in nipblb-MO-injected embryos at 24 hpf, compared with stage-matched control embryos (Figures 2c and d).

**nipblb downregulation affects cell survival but not cell proliferation.** When compared with controls, nipblb-MO-injected embryos showed increased programmed cell death in the CNS at 24 hpf, whereas apoptosis was less pronounced in CNS of control embryos (Figures 3a–d). Interestingly, the TUNEL staining showed that the apoptotic cells were most evident in the tissues expressing the nipblb gene (compare Figure 1a and Figures 3b–d). Previous studies in zebrafish described p53-dependent apoptosis following cohesin smc3 knockout, and p53 upregulation in mutants for the cohesin subunit rad21. It is well known that MO molecules could elicit undesirable off-target effects such as activation of the p53 protein. Importantly, reversal of p53-dependent cell death by p53 knockdown did not affect cell death caused by specific gene loss of function.18 To verify that apoptosis in nipblb-MO-injected embryos was due to the specific loss of function of this gene and not a result of p53 activation, we co-injected p53-MO with nipblb-MO. In p53-MO + nipblb-MO-injected embryos, the amount of apoptotic cells were comparable to nipblb-loss-of-function embryos, which verified that neural apoptosis was specifically caused by knocking down nipblb (n = 42) (Supplementary Figure S1). These results suggest that nipblb is required for cell survival during neurulation in zebrafish embryos.

To determine whether nipblb loss of function also alters the proliferation rate, embryos were stained with the proliferative cell nuclear antigen antibody. As expected from previously published studies on cohesin loss of function,10,19 we found that entry into the S phase was not compromised, as the number of proliferative cell nuclear antigen-positive cells in nipblb-MO-injected embryos at 24 hpf was not significantly increased in comparison with controls (Supplementary Figure S2).

**nipblb loss of function alters Wnt signaling pathway expression.** Owing to the expression of nipblb throughout the CNS, we further investigated whether CNS AP formation was altered in nipblb-loss-of-function embryos at 24 hpf. The expression of AP identity markers, such as hoxb2a, pax2a, and krox20, was not altered in nipblb-MO-injected embryos (Supplementary Figure S3), suggesting that the CNS is patterned correctly. However, we found that wnt1 expression was severely altered in the hindbrain of nipblb-MO-injected embryos at 24 hpf (80%, total analyzed embryos n=120; Figures 4a and b). It was possible to observe nipblb knockdown phenotypes with different degrees of fusion of the wnt1 signal in the hindbrain between rhombomere II and the spinal cord: the most severe phenotype included a complete fusion of the wnt1 hindbrain signals (20%, total n=120), the intermediate phenotype expressed multiple points of wnt1 fusion along the AP hindbrain axis (40%, total n=120), and the least severe phenotype presented either as a single point of wnt1 fusion or as an incomplete opening of the ventricle (20%, total n=120, Figures 4a and b).

To investigate whether the observed defects were specific for the mis-regulation of wnt1 expression following nipblb loss of function or were caused by morphological defects in hindbrain formation, we analyzed the expression of atoh1, a marker of hindbrain dorsal progenitors and ventricle opening.23 atoh1 expression was not altered in nipblb-MO-injected embryos at 24 hpf (90%, total n=50; Supplementary Figures S4A and B), showing that anatomical patterning of the developing brain is not affected by nipblb loss of function.
As defects in neural tube patterning might disrupt neuronal differentiation, we examined the dopaminergic population (thyrosin-hydroxilase-positive neurons) at 24 hpf following nipblb loss of function. It is well known that formation of mesencephalic dopaminergic neurons is directly not only by diffusible signals from the notochord, floor plate, and isthmic organizer such as Shh and Fgf, but also by Wnt1 and other extrinsic factors. In nipblb-loss-of-function embryos, we found that neurodifferentiation of this population was not adversely affected (Supplementary Figures S4C and D).

Importantly, comparable alterations in gene distribution observed in wnt1 were also observed in axin2 expression pattern, a key downstream component of the pathway typically used as a readout of the cascade (Figures 4c and d).

Expression profiling of the WNT/beta-catenin pathway target genes in patients fibroblasts and nipblb-MO-injected embryos. In zebrafish embryos, nipblb loss of function resulted in alterations of the Wnt/beta-catenin pathway, modeling the molecular defects underlying NIPBL-mutated CdLS patients. Hence, we sought to analyze the expression profile of this signaling pathway in fibroblasts of patients, comparing it with that of cells obtained from age-, ethnicity- and gender-matched healthy controls. Fibroblasts were believed to represent a valid model for cohesin deficiency in humans as in studies on gene expression, consistent altered profiles were observed in primary cultures of fibroblasts and lymphoblastoid cell lines.

It was possible to exclude involvement of the non-canonical pathway, as both DVL1 and DVL2 are comparable in patients and control group (Supplementary Figures S5A and B).
Analysis of the expression of direct targets of the canonical pathway, such as Cyclins, by quantitative PCR (q-PCR) showed a trend toward the reduction of Cyclin D1 (CCND1) in NIPBL-mutated patients compared with healthy controls (Figure 5a). As it is known that Cyclins can compensate for the deficiency of other isoforms in mammals, we also analyzed Cyclin D2 (CCND2) and found it to be significantly upregulated (P < 0.01) (Supplementary Figure S5C).

Consistently, a mean reduction of CCND1 levels of almost 50% was observed in patients samples when compared with healthy controls (Figure 5c, Supplementary Figure 5D). 

Ccnd1 was also found to be significantly downregulated in nipblb-MO-injected embryos by q-PCR (Figure 5b), western blot analysis with a decrease of Ccnd1 expression by 42% (Figure 5d), and whole-mount in situ hybridization (WISH) analyses (Figures 5e and f). The latter showed that the
expression of ccnd1 was altered in nipblb-MO-injected embryos at 24 hpf in comparison with controls (40%, total n = 30).

In addition, we found an average of 18% reduction of the active form of beta-catenin (ABC) in patients compared with controls after normalization (Figure 5g, Supplementary Figure 5D). The same pattern was observed in nipblb-MO-injected embryos with a statistically significant reduction of ABC levels of 80% (Figure 5h).

Rescue of nipblb deficiency phenotype by chemical induction of canonical Wnt pathway. As our data suggested that the adverse phenotype caused by knocking down of nipblb in zebrafish embryos and in patients fibroblasts was mediated by attenuation of the canonical Wnt pathway with a subsequent decreased in Ccnd1 expression, we sought to rescue the phenotype in nipblb-loss-of-function embryos by chemical induction of the Wnt pathway by lithium chloride (LiCl) treatment. The concentration used in three sets of experiments failed to produce visible effects in control embryos (minimum 30 per experiment), but was able to strikingly reverse the defects induced by nipblb-MO injection (minimum 30 per experiment). In particular, the cephalic structures showed a restored morphology, and the wnt1 distribution pattern is similar to that of control embryos (Figures 6a–c). Apoptosis levels determined by TUNEL staining were reduced after LiCl treatment in nipblb-MO-injected embryos to levels comparable to control embryos (Figures 6d–f). Western blot analysis of nipblb-MO-injected embryos treated with LiCl showed that the canonical Wnt pathway was induced, as ABC levels were increased by fourfold compared with controls and more than 10-fold compared with nipblb-MO-injected embryos (Figures 6g–i). Both differences were statistically significant. Notably, Ccnd1 protein levels following LiCl treatment were induced more than 3.7-fold compared with nipblb-MO-injected embryos and 2.4-fold increase compared with controls. (Figures 6h–j).
Discussion

In this study, two different modeling strategies were used to analyze abnormalities underlying CdLS, focusing on NIPBL, which is the most commonly mutated gene in patients affected by the disease. A developmental study in vivo using zebrafish embryos was initially used to identify gene pathways that might contribute to the phenotype, followed by a validation study in which primary cultures of CdLS patients fibroblasts were subsequently tested.

First, the expression of nipblb in the CNS of the zebrafish embryos at 24 hpf, the time when the neurulation process is almost completed, was evaluated. This alleviates an existing data gap in the literature, as the expression pattern of zebrafish nipblb at different developmental stages had been previously reported. The results showed a dynamic pattern of expression regulated spatio-temporally, with specific localization at the level of the cephalic neural tissues. The other zebrafish paralog nipbla had been reported with a similar expression pattern and function in the developing visceral organs.

Using MO technology, a model of haploinsufficiency in zebrafish embryos was generated. This model mimics the genetic haploinsufficiency of CdLS patients with mutation in NIPBL. The nipblb-loss-of-function embryos showed severe alterations of the head morphology, with various degrees of microcephaly, microphthalmia, and incomplete development of these cephalic structures.

This adverse phenotype correlated with increased apoptosis in the areas where nipblb is normally expressed, suggesting that the cohesins have an important functional role in cell survival of specific tissues during vertebrate development. In our system, the proliferation rate seemed unaffected. Interestingly, the expression distribution of a number of markers of AP development appeared comparable to control embryos, wnt1 distribution, on the other hand, was significantly impaired at the hindbrain level, the prospect pons, medulla oblongata, and cerebellum, in mammals. We ruled out that wnt1 expression alterations were caused by abnormalities in anatomy of developing structures as atoh1, which expression covers the entire dorsal and lateral aspect of the cerebellar anlage, was properly distributed in nipblb-MO-injected embryos. The wnt1 localization, however, failed to be restricted to the lateral tissues in these embryos. This is consistent with previous data indicating the presence of underlying antero-posterior pattern in the absence of Wnt signals.

The canonical Wnt pathway was found to be altered in nipblb-MO-injected embryos and in patients fibroblasts. In both cases, the ABC was downregulated. Ccdn1, a known direct target of the pathway, was consequently downregulated in both models. Noticeably, the abnormal cephalic phenotype observed in nipblb-MO-injected embryos is strongly reminiscent of that observed after Ccdn1 knockdown.

Using LiCl as a well-known chemical activator of the Wnt pathway, which negatively regulates GSK3β inhibition of beta-catenin, it was possible to rescue the adverse phenotype in nipblb-MO-injected embryos at all levels: anatomical cephalic structures appeared morphologically normal, the programmed cell death levels were restored to physiological levels seen in controls, and Ccdn1 protein levels were increased. Interestingly, wnt1 pattern of expression appeared rescued in zebrafish nipblb-MO-injected embryos by LiCl treatment, suggesting that in our model, the primary adverse event is at the Gsk3β/beta-catenin complex level, and that chemical compensation triggers a negative feedback on wnt1 expression control. In fact, it has been reported a negative feedback through upregulation of Axin2 in a cancer model.

Our CdLS zebrafish model suggests a role for the canonical Wnt pathway perturbation in CNS abnormalities. In a murine model of autosomic recessive microcephaly, the brain alterations are caused by decrement in expression levels of Wnt1-regulated genes, and overexpression of beta-catenin in neural cells could rescue the defects. Several animal models with alterations in Wnt-related gene expression show partially overlapping phenotype: the well-known masterblind (mbl) mutant, which develops reduction of the telencephalon, optic vesicles, and an abnormal AP patterning, was found to be a spontaneous axin1 mutant. Since then several mouse (for example, Hesx1, Six3, and Tcd3) and zebrafish mutants have been described with comparable phenotype at the AP patterning of the CNS during embryonic development. In humans, HESX1 and SIX3 have been found causative of hypopituitarism and septo-optic dysplasia, a developmental disorder characterized by severe defects in the brain and eye. In 1996, a case report described a 5-year-old CdLS patient autopsy that revealed hypoplasia of the optic systems, hypothalamic nuclei, corpus callosum, and cerebellar vermis. Moreover, the septum pellucidi, fornix, and anterior commissure were rudimentary. This case showed features of septo-optic dysplasia combined with commissural dysplasia and cerebellar vermic hypoplasia.

It is known that decreased levels of Wnt1 signaling results in the downregulation of Ccdn1 that leads to apoptosis in several animal models. It is important to note that in mammals, there is partial compensation by the upregulation of Ccdn2 as it was observed in our cell system. It is also known that GSK3β overexpression induces increased apoptosis in vivo perturbing proliferation and maturation, resulting in the loss of immature neurons. The reduction of Wnt ligand levels or the removal of the canonical pathway component beta-catenin results in fewer neuroepithelial/radial glial stem cells and in precocious neuronal differentiation in mouse embryos.

Further, it has been shown that in murine models of autism-like disorders, the most conspicuous phenotype is an increase in programmed cell death in the developing cerebellum. In CdLS patients, alterations in the development of cerebellar structures at anatomical levels are known, but more robust analyses with magnetic resonance imaging could help unraveling the link between autism features, seizures, and altered CNS development in these patients.

Increased apoptosis in our in vivo system led us to hypothesize that, at least to some extent, the mental retardation and autism-like behavior typically associated with CdLS patients could have their roots in this abnormality. The reported autopsy showed reduction of Purkinje cells number in the cerebellum, and our data could implicate an imbalance of cell death control during development as causative.

Several pieces of evidence suggest a possible regulation of the Wnt pathway by cohesins in non-dividing cells in different models. In skin fibroblasts expressing a mutated form of HDAC8, WNT3A, WNT2, WISP2, and FZD8 were found to be downregulated compared with wild-type cells. In Drosophila, SMC3 appears important to control expression of Flamingo/
Starry night (Fmi/Stan) the orthologs of vertebrate Celsr, which is known to transduce the Wnt signaling cascade in the non-canonical pathway to control cell polarity. It is interesting to note that this regulation might be two-way, as in colorectal cancer, the known upregulation of beta-catenin appears to control expression of SMG3.

In conclusion, this paper shows for the first time, to our knowledge, a regulation of the Wnt-canonical pathway by cohesins during development in zebrafish embryos and in fibroblasts from CdLS patients. This suggests a potential underlying mechanism for the developmental abnormalities observed in the CNS of CdLS patients.

Materials and Methods

Animals. Breeding wild-type fish of the AB strain were maintained at 28 °C on a 14-h light/10-h dark cycle. Embryos were collected by natural spawning, staged according to Kimmel et al. and raised at 28 °C in fish water (Instant Ocean, United Pet Group, Blacksburg, VA, USA; 0.1% methylene blue) in Petri dishes, according to established techniques, approved by the veterinarian (OVSC) and the animal use committee (IACUC) at the University of Oregon, in agreement with local and national sanitary regulations. Embryonic ages are expressed in somites (s), or hours post fertilization (hpf).

To activate Wnt/beta-catenin signaling, LiCl was added to fish water for 30 min at 10–12 s stage at a concentration of 0.3 M at 28 °C. Treated embryos were then washed three times with water and allowed to develop to 24 hpf.

In situ hybridization, histological analysis, and immunohistochemistry. WISH experiments were carried out as described by Thiese et al. Immunohistochemistry analyses were performed according to the method described by Panzer et al. For each experiment, a minimum of 30 controls and MO-injected embryos were analyzed. To clone nphpb probe, a PCR was used (primers in Supplementary Table S1); and hox20 probe was synthesized as described in Oxtoby and Jowett. Wnt probe was synthesized as described in Mollen et al. axin2 probe was synthesized as described in Shimizu et al.; atoh1 probe was synthesized as described in Millimaki et al.; pax2a probe was synthesized as described in Krauss et al.; hox2a probe was synthesized as described in Prince and colleagues. For histological sections, stained embryos were re-fixed in 4% paraformaldehyde, dehydrated and stored in methanol, and wax-embedded and sectioned (5 μm). For immunohistochemistry experiments, embryos were exposed to specific primary antibodies (Supplementary Table S3), followed by appropriate biotinylated secondary antibodies (Vector Laboratories, Burlington, CA, USA). Images of embryos and sections were acquired using a microscope equipped with digital camera with LAS Leica Imaging software (Leica, Wetzlar, Germany). Images were processed using the Adobe Photoshop software (Adobe System Incorporated, San José, CA, USA), and, when necessary, different focal planes of the same image have been taken separately and later merged in a single image.

TUNEL staining. For TUNEL assay, a minimum of 24 embryos (per experimental group) were fixed with 4% paraformaldehyde for 2 h at room temperature. Embryos were washed with methanol at −20 °C and then twice with PBC (0.001% Triton X-100, 0.1% sodium citrate in PBS) for 10 min. Staining for apoptotic cells was performed using the AP-PBC (0.001% Triton X-100, 0.1% sodium citrate in PBS) for 10 min. Staining for TUNEL assay, a minimum of 24 embryos (per experimental group) were fixed with 4% paraformaldehyde for 2 h at room temperature. Embryos were incubated at 37 °C for 1 h, washed, stained, and mounted for microscopic imaging.

Injections. To repress nphpb mRNA translations, an ATG-targeting MO (nphpb-MO) was synthesized (Gene Tools LLC, Philomath, OR, USA); nphpb-MO: 5′-GTCCCCCATCTTGGCGAAGAAGAGGGA-3′, and used at the concentration of 1.pmol/embryo in 1 × Danieau buffer (pH 7.6) as previously reported. As a control, a standard control MO oligonucleotide (ctrl-MO) was injected. In all experiments, nphpb-MO-injected embryos were compared with embryos injected with the same amount of ctrl-MO at the same developmental stage. p53-MO was designed (Gene Tools) and used as described in Robu et al.

Cell cultures. Biopsies from four CdLS patients with known mutation in NIPBL and three healthy controls (Supplementary Table S4) were used in this study. Samples were obtained after signed informed consent. Healthy donors, two male and one female Italian pediatric patients, were undergoing surgical procedures for dermatological testing. Skin biopsies were collected from CdLS patients and pediatric controls, and then finely shredded using a scalpel in sterile conditions. Skin fragments were cultured in RPMI medium supplemented with 20% fetal bovine serum. After 7–10 days, fibroblasts started to grow, and as soon as cell confluence was reached, cells were collected, following standard trypsination procedure, suspended in Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) or radio immunoprecipitation assay buffer, and stored at −80 °C. For any experiments, same passaged (maximum P = 3) cells were compared.

Reverse transcription-PCR and real-time q-PCR assays. RNA extraction was performed following the manufacturer’s protocol. Superscript II enzyme (Life Technologies) was used for cDNA synthesis. Quantitative-reverse transcription (RT)-PCR amplification of zebrafish embryos (minimum 30 per experimental group) were carried out in a total volume of 25 μ containing half volume of SYBR Green (Life Technologies). For normalization purposes, eif-α RNA level was tested in parallel with the gene of interest. Primers are reported in Supplementary Table S1. Melting curve derivatives were assessed for each run.

q-RT-PCR experiments on human samples were performed with Universal Probe Master system (Roche Diagnostics). For this set of experiments, a LightCycler 480II (Roche Diagnostics) was used.

Primers and probes were selected according to the Software Probe Finder (Roche Diagnostics) and are reported in Supplementary Table S2. ABL gene was used as reference and healthy patients cells as standard control.

Western blot. Fish embryos (minimum 30 per experimental group) and human fibroblasts were lysed in radio immunoprecipitation assay buffer and homogenized. Samples were boiled for 10 min at 95 °C with 20 × reducing agent (Bio-Rad, Hercules, CA, USA). Thirty micrograms of protein samples were size-fractionated by pre-cast gel (4–15%, Bio-Rad) and transferred with following standard protocol. The polyyvinylidene fluoride membranes (Bio-Rad) were blocked with 10% nonfat dry milk in TBST (TBS, pH 7.5, containing 0.1% Tween 20) for 1 h at room temperature and subsequently incubated with the primary antibody. Primary antibodies were used at appropriate working dilutions (Supplementary Table S3), in 5% milk/TBST overnight at 4 °C. Horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO, USA) were used for 1 h at room temperature. The antigen signal was detected with the immune-star chemiluminescence detection system (Bio-Rad) as specified by the manufacturer. A StrpAblot Stipping Buffer (Euroclone S.p.A., Milan, Italy) was used to recover membranes. Densitometry analyses were performed using Alliance instrument and Uviband software (Uvitec, Cambridge, UK). Beta-actin was used as reference for protein concentrations and then data was expressed normalized against control levels. For zebrafish embryos, experiments were run as three independent experiments and for human fibroblasts in duplicates.

Statistical analysis. For q-RT-PCR experiments, data were statistically analyzed applying a two-tailed t-test setting P < 0.05 as significant. Data were analyzed using the comparative ΔΔCT method, both t-test and S.D. values refer to samples triplicates. For western blot experiments, raw data were analyzed applying a two-tailed paired t-test. Significance was set as P < 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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1. Kline AD, Krantz ID, Sommer A, Kiewer M, Jackson LD, FitzPatrick DR et al. Cornelia de Lange syndrome: clinical review, diagnostic and scoring systems, and anticipatory guidance. Am J Med Genet A 2007; 143A: 1287–1296.

2. Krantz ID, McCaulum J, DeScipio C, Kaur M, Gillis LA, Yaeger D et al. Cornelia de Lange syndrome is caused by mutations in NIPBL, the human homolog of Drosophila melanogaster Nipped-B. Nat Genet 2004; 36: 631–635.
3. Dearordaff MA, Wilde JJ, Albrecht M, Dickinson E, Tennstedt S, Braunschütz D et al. RAD21 mutations cause a human cohesinopathy. Am J Hum Genet 2012; 90: 1014–1027.
4. Lumen A, Krumlauf R. Patterning the vertebrate neuraxis. Science 1996; 274: 1109–1115.
5. Altmann CR, Brivanlou AH. Neural patterning in the vertebrate embryo. Int Rev Cytol 2001; 199: 147–182.
6. Deardorff MA, Bando M, Nakato R, Watrin E, Itoh T, Minamino M et al. HDAC8 mutations in Cornelia de Lange syndrome affect the cohesin acetylation cycle. Nature 2012; 489: 313–317.
7. Bose T, Gerton JL. Cohesinopathies, gene expression, and chromatin organization. J Cell Biol 2010; 189: 201–210.
8. Dorsett D, Strom L. The ancient and evolving roles of cohesin in gene expression and DNA repair. Curr Biol 2012; 22: R240–R250.
9. Rollins RA, Morpillo C, Dorsett D. Nipped-B, a Drosophila homolog of chromosomal adherins, participates in activation by remote enhancers in the cut and Ultrabithorax genes. Genetics 1999; 152: 577–593.
10. Horsfield JA, Anagnostou SH, Hu JK, Cho KH, Geisler R, Lieschke G. Running rings around chromosomes to trim axons and target dendrites. Dev Cell 2008; 14: 156–158.
11. Dorsett D. Running rings around chromosomes to trim axons and target dendrites. Dev Cell 2008; 14: 156–158.
12. Altmann CR, Brivanlou AH. Neural patterning in the vertebrate embryo. Int Rev Cytol 2001; 199: 147–182.
13. Kawauchi S, Calof AL, Santos R, Lopez-Burks ME, Young CM, Hoang MP et al. Cooperative roles of p53 activation and pRb-dependent regulation of Runx genes. Nature Genet 2010; 42: 897–905.
14. Roue G, Pichereau V, Lincet H, Colomer D, Sola B. Cyclin D1 mediates resistance to Wnt signal through upregulation of conductin/αxin2 in colorectal and liver tumors. Mol Cell Biol 2002; 22: 1184–1193.
15. Bhujwalla JM, Durak O, Tsai LH. ASPP regulates Wnt signaling pathway activity in the effecting organ. Genes Dev 2011; 25: 1593–1598.
16. Rhodes JM, Bentley FK, Print CG, Dorsett D, Misulovin Z, Dickinson EJ. Genetic interaction between the homeobox transcription factors HESX1 and SI3X is required for normal pituitary development. Dev Biol 2008; 324: 322–333.
17. Martinez-Barbera JP, Rodriguez TA, Beddington RS. The homeobox gene Hes1 is required in the anterior neural ectoderm for normal forebrain formation. Dev Biol 2000; 223: 422–430.
18. Altmann CR, Brivanlou AH. Neural patterning in the vertebrate embryo. Int Rev Cytol 2001; 199: 147–182.
19. Prince VE, Moens CB, Kimmel CB, Ho RK. Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. Dev Biol 1993; 152: 293–303.
20. Krauss S, Johansen T, Korzh V, Fjose A. Expression pattern of zebrafish pax genes governing cell fate and cell polarity. Development 2003; 130: 4421–4436.
21. Staal FJ, Noort MvM, Strous GJ, Dattani M, Martinez-Barbera JP. Absence of SIX3 mutations induces apoptosis in human cancer cells. Neoplasia 2004; 6: 7–14.
22. Gissi G. SM3C knockdown triggers genomic instability and p53-dependent apoptosis in human and zebrafish cells. Mol Cancer 2006; 5: 52.
23. Roue G, Pichereau V, Lincet H, Colomer D, Sola B. Cyclin D1 mediates resistance to Wnt signal through upregulation of conductin/αxin2 in colorectal and liver tumors. Mol Cell Biol 2002; 22: 1184–1193.
24. Rouessa E, Krieglstein K. Induction and specification of midbrain dopaminergic cells: focus on Hesx1. Development 2003; 130: 4421–4436.
25. Shimizu T, Yamanaka Y, Ryu SL, Hashimoto H, Yabe T, Hirata T. The development of mice expressing a single D-type cyclin. EMBO J 1999; 18: 393–406.
26. Roue G, Pichereau V, Lincet H, Colomer D, Sola B. Cyclin D1 mediates resistance to Wnt signal through upregulation of conductin/αxin2 in colorectal and liver tumors. Mol Cell Biol 2002; 22: 1184–1193.
27. Horsfield JA, Anagnostou SH, Hu JK, Cho KH, Geisler R, Lieschke G. Running rings around chromosomes to trim axons and target dendrites. Dev Cell 2008; 14: 156–158.
28. Horsfield JA, Anagnostou SH, Hu JK, Cho KH, Geisler R, Lieschke G. Running rings around chromosomes to trim axons and target dendrites. Dev Cell 2008; 14: 156–158.
29. Joly JS, Joly C, Schulte-Merker S, Braunschütz D et al. RAD21 mutations cause a human cohesinopathy. Am J Hum Genet 2012; 90: 1014–1027.
30. Lumen A, Krumlauf R. Patterning the vertebrate neuraxis. Science 1996; 274: 1109–1115.
31. Altmann CR, Brivanlou AH. Neural patterning in the vertebrate embryo. Int Rev Cytol 2001; 199: 147–182.
32. Deardorff MA, Bando M, Nakato R, Watrin E, Itoh T, Minamino M et al. HDAC8 mutations in Cornelia de Lange syndrome affect the cohesin acetylation cycle. Nature 2012; 489: 313–317.
33. Bose T, Gerton JL. Cohesinopathies, gene expression, and chromatin organization. J Cell Biol 2010; 189: 201–210.
34. van de Water S, van der Wettering M, Joore J, Esseling J, Bink R, Clevers H et al. Ectopic Wnt signal determines the eyelens phenotype of zebrafish masterblind mutant. Development 2001; 128: 3877–3888.
35. Heiseberg CP, Houart C, Take-Uchi M, Rauch GJ, Young N, Coutinho P et al. A mutation in the Gsk3β-binding domain of zebrafish Masterblind/αxin1 leads to a fate transformation of telencephalon and eyes to diencephalon. Genes Dev 2001; 15: 1427–1434.
36. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 1996; 93: 8455–8459.
37. Wu D, Pan W. GSK3: a multifaceted kinase in Wnt signaling. Trends Biochem Sci 2010; 35: 181–188.
38. Lusty B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U et al. Negative feedback loop of Wnt signaling through upregulation of conductin/αxin2 in colorectal and liver tumors. Mol Cell Biol 2002; 22: 1184–1193.
39. Buchman JM, Durak O, Tsai LH. ASPP regulates Wnt signaling pathway activity in the effecting organ. Genes Dev 2011; 25: 1593–1598.

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