Nipbl and Mediator Cooperatively Regulate Gene Expression to Control Limb Development

Akihiko Muto1,2,3, Shingo Ikeda3, Martha E. Lopez-Burks1,2, Yutaka Kikuchi3,9, Anne L. Calof3,4,9*, Arthur D. Lander1,2,9, Thomas F. Schilling1,2,9

1 Department of Developmental & Cell Biology, University of California, Irvine, Irvine, California, United States of America, 2 Center for Complex Biological Systems, University of California, Irvine, Irvine California, 3 Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan, 4 Department of Anatomy & Neurobiology, University of California, Irvine, Irvine, California, United States of America

Abstract
Haploinsufficiency for Nipbl, a cohesin loading protein, causes Cornelia de Lange Syndrome (CdLS), the most common “cohesinopathy”. It has been proposed that the effects of Nipbl-haploinsufficiency result from disruption of long-range communication between DNA elements. Here we use zebrafish and mouse models of CdLS to examine how transcriptional changes caused by Nipbl deficiency give rise to limb defects, a common condition in individuals with CdLS. In the zebrafish pectoral fin (forelimb), knockdown of Nipbl expression led to size reductions and patterning defects that were preceded by dysregulated expression of key early limb development genes, including fgfs, shha, hand2 and multiple hox genes. In limb buds of Nipbl-haploinsufficient mice, transcriptome analysis revealed many similar gene expression changes, as well as altered expression of additional classes of genes that play roles in limb development. In both species, the pattern of dysregulation of hox-gene expression depended on genomic location within the Hox clusters. In view of studies suggesting that Nipbl colocalizes with the mediator complex, which facilitates enhancer-promoter communication, we also examined zebrafish deficient for the Med12 Mediator subunit, and found they resembled Nipbl-deficient fish in both morphology and gene expression. Moreover, combined partial reduction of both Nipbl and Med12 had a strongly synergistic effect, consistent with both molecules acting in a common pathway. In addition, three-dimensional fluorescent in situ hybridization revealed that Nipbl and Med12 are required to bring regions containing long-range enhancers into close proximity with the zebrafish hoxda cluster. These data demonstrate a crucial role for Nipbl in limb development, and support the view that its actions on multiple gene pathways result from its influence, together with Mediator, on regulation of long-range chromosomal interactions.

Introduction
Cohesin, a ring-shaped, DNA-associated protein complex, is best known for its role in tethering sister chromatids together until mitosis [1,2]. However, growing evidence indicates that cohesin, and proteins such as Nipped-B-like (Nipbl) that regulate cohesin loading onto DNA, also play critical roles in gene regulation [3–15]. In particular, it has been suggested that Nipbl and cohesin mediate interactions between promoters and distant enhancers, a process thought to involve the physical looping out of intervening DNA sequences [14–16]. For example, in Drosophila, Nipped-B (the orthologue of Nipbl) and cohesin regulate cut gene expression by controlling long-range interactions between the cut promoter and a wing-specific remote enhancer [5]. In mice, haploinsufficiency for Nipbl impairs looping that controls the selective expression of beta-globin isoforms by erythroid cells [13].

Recently, it was found that Nipbl co-localizes with the Mediator complex at promoters/enhancers of actively transcribed genes in mouse embryonic stem cells [17]. Thought to play a pivotal role in transmitting regulatory signals from gene-specific activators/ repressors to RNA polymerase II [18,19], Mediator is a large complex composed of a core that interacts with RNA polymerase II and gene-specific transcriptional regulators, and a CdK8 submodule (containing CdK8, CyclinC, Med12 and Med13) and can either negatively [20–22] or positively [23,24] regulate transcription. The reported physical interaction between Mediator and Nipbl at active genes suggests that they function together in promoter-enhancer communication, but exactly how this occurs is unknown.

Much insight into the physiological significance of cohesin’s influence on transcription has come from the study of Cornelia de Lange Syndrome (CdLS) and other “cohesinopathies”. CdLS is a congenital syndrome characterized by growth retardation, neurological dysfunction, and structural defects in multiple organs [25–30], and is caused, in most cases, by haploinsufficiency for NIPBL [31,32]. More recently it has been shown that mutations in


Author Summary

Limb malformations are a striking feature of Cornelia de Lange Syndrome (CdLS), a multi-system birth defects disorder most commonly caused by haploinsufficiency for NIPBL. In addition to its role as a cohesin-loading factor, Nipbl also regulates gene expression, but how partial Nipbl deficiency causes limb defects is unknown. Using zebrafish and mouse models, we show that expression of multiple key regulators of early limb development, including shha, hand2 and hox genes, are sensitive to Nipbl deficiency. Furthermore, we find morphological and gene expression abnormalities similar to those of Nipbl-deficient zebrafish in the limb buds of zebrafish deficient for the Med12 subunit of Mediator—a protein complex that mediates physical interactions between enhancers and promoters—and genetic interaction studies support the view that Mediator and Nipbl act together. Strikingly, depletion of either Nipbl or Med12 leads to characteristic changes in hox gene expression that reflect the locations of genes within their chromosomal clusters, as well as to disruption of large-scale chromosome organization around the hoxa cluster, consistent with impairment of long-range enhancer-promoter interaction. Together, these findings provide insights into both the etiology of limb defects in CdLS, and the mechanisms by which Nipbl and Mediator influence gene expression.

cohesin subunits SMC1A or SMC3 [33,34] or the SMC3 deacetylase, HDAC8 [35], are less common causes of CdLS. Analysis of both patient samples and animal models indicate that Nipbl haploinsufficiency causes small changes (usually less than 1.5-fold) in the expression of many hundreds of genes [3,4,11]. Analysis of both mouse and fish models of Nipbl deficiency suggests that pervasive phenotypic abnormalities result from the collective, and sometimes synergistic, effects of such small changes in gene expression [3,11].

Among the most striking abnormalities in CdLS are limb defects, which range from mild brachydactyly and clinodactyly to severe digit and limb truncations, the latter in about 1/3 of cases [26,28,36]. Limb reduction is one of the few structural defects in CdLS that is not replicated in the Nipbl-haploinsufficient mouse model, as these mice exhibit only minor changes in the shape of the ocellar process, and delays in the ossification of limb bones [3]. Hypothesizing that this difference might reflect slight differences in the threshold for triggering such defects in mouse versus man, we decided to look at development of the pectoral fin (the homologue of the mammalian forelimb) in a zebrafish model of Nipbl-deficiency, produced by injection of morpholino oligonucleotides (MO) directed against the two zebrafish nipbl [11]. Here we show that Nipbl-deficient fish display a marked reduction in pectoral fin size, which is already apparent early in fin bud development. We demonstrate that Nipbl is required for normal expression of conserved regulators of vertebrate limb growth and patterning, including fgfs in the apical ectodermal ridge (AER), shh in the zone of polarizing activity (ZPA), and several hox genes of the hoxab, hoxca and hoxd clusters. We also show that Nipbl-haploinsufficient mouse limb buds display a pattern of gene expression changes strikingly similar to those observed in Nipbl-deficient pectoral fin buds.

Pectoral fin defects have also been reported in med12-mutant zebrafish, in which Mediator function is disrupted [37]. Interestingly, we find that both the morphological and gene expression changes that occur in Nipbl-deficient fin buds are mimicked when med12 is knocked down. In particular, expression of multiple hox genes in different clusters is affected in a similar position-specific manner in both Nipbl- and Med12-deficient fish embryos, and results of experiments in which we simultaneously knock down both Nipbl and Med12 suggest that they interact genetically. Using 3-dimensional fluorescent in situ hybridization (3D-FISH) in zebrafish fin buds, we further show that Nipbls and Med12 are required for higher-order chromatin organization near the hoxa cluster. Overall, the data point to a shared, conserved role for Nipbl and the Mediator complex in the regulation of long-range enhancer-promoter interactions underlying growth and patterning of the vertebrate limb.

Results

Impaired pectoral fin development in Nipbl-deficient zebrafish

Both nipbl genes in zebrafish, nipbla and nipblb [11], are expressed in developing pectoral fin bud mesenchyme (Fig. S1A, B). To investigate their requirements in forelimb development, we generated “Nipbl-deficient” embryos in which both nipbla and nipblb were depleted by injecting either of two different sets of antisense morpholinos (MOs) designed against distinct MO target sites, as described previously [11]. Pectoral fins of Nipbl-deficient larvae were 40% shorter in length at 72 hours post fertilization (hpf) than those of control embryos (Figure 1A, B, E). This reduced size was not simply due to developmental delay, since it was much more severe than expected given the delay in development of the eye and lower jaw, which we estimated at approximately 16 hrs (Figure S2, see below). Pectoral fin defects were more severe when MOs to both nipbl mRNAs were injected, compared with either one alone (Figure S3) [11]; thus, injections of both MOs were used in subsequent experiments. Pectoral fin defects were partially rescued by co-injection of exogenous nipbla mRNA, confirming MO specificity (Figure 1C–E).

Alcian Blue staining at 5 dpf revealed that pectoral fin cartilages of the endoskeletal discs form in Nipbl-deficient larvae but are smaller (Figure 1F, G). Cell numbers in these discs were reduced by 37% and 33% along anterior-posterior (A-P) and proximo-distal (P-D) axes, respectively (Figure 1H), whereas cell size resembled controls (Figure 1F’, G’), and we found no change in cell death (Figure S4A, B), suggesting impaired growth of cartilage progenitors at earlier stages. In addition, the orderly arrangement and spacing of chondrocytes in the endoskeletal disc cells was noticeably disrupted in Nipbl-deficient fins (Figure 1F’, G’).

In zebrafish embryos, pectoral fin buds first appear at 30 hpf as shallow domes along the A-P axis, and grow and begin to fold posteriorly by 42 hpf. In Nipbl-deficient embryos, pectoral fin buds also initiate at 30 hpf but grow more slowly than controls (Figure 1I–R). TUNEL assays (Figure S4C–E) showed no increase in cell death in Nipbl-deficient fin buds (Figure S4A–E). In contrast, numbers of BrdU+ cells decreased significantly in the mesenchyme of Nipbl-deficient pectoral fin buds (Figure S4F–I). These data suggest that endoskeletal disc size reduction in Nipbl-deficient limb buds reflects cumulative effects of slower rates of cell division.

Since Nipbl is required for embryonic growth in both fish and mice [3,11], we stage-matched embryos using an independent criterion – i.e. the A-P position of the migrating posterior lateral line (p.LL) primordium labeled by in situ hybridization (ISH) for fgf10a; Figure S5A, red arrows). In controls, p.LL primordia lie just posterior to the pectoral fin buds at 22 hpf, and continue to migrate posteriorly. Based on this staging criterion the developmental delay in Nipbl-deficient embryos (summarized in Figure...
Nipbls are required for \textit{fgf} expression in the AER but not in fin bud mesenchyme

Early limb development is highly conserved from fish to mammals [38–40]. Each fin/limb bud possesses an apical ectodermal ridge (AER) and zone of polarizing activity (ZPA) [40,41], which play important roles in growth and patterning [42–44]. The AER, a thickened epithelium that rims the distal ends of the buds, is the source of Fgf signals required for P-D limb outgrowth [45–48]. The zebrafish AER expresses 4 \textit{fgf} genes: \textit{fgf4}, \textit{fgf8a}, \textit{fgf16} and \textit{fgf24} [49,50]. Of these, expression of \textit{fgf4}, \textit{fgf8a} and \textit{fgf16} was dramatically reduced in pectoral fin buds of Nipbl-deficient embryos (Figure 2A–C), which was rescued by over-expression of full-length \textit{nipbla} mRNA (Figure S6). This was not simply due to loss of AER cells since \textit{fgf24} expression was not downregulated in the Nipbl-deficient AER (Figure 2D).

Limb bud development also requires expression of \textit{fgf10a} and \textit{fgf24} in the mesenchyme [49,51,52]. However, we found no differences in \textit{fgf10a} expression between wild type and Nipbl-deficient limb buds between 22–48 hpf (Figure S7A), as well as no differences in expression of \textit{tbx5a} and \textit{fgf24}, which control the expression of \textit{fgf10a} (Figure S7B–C).

Nipbls are required for \textit{shh} expression in the ZPA and its regulation in fin bud mesenchyme

The ZPA acts as an organizing center in the posterior limb/fin bud mesenchyme in part because it produces Shh [38,40,45,50,53–55]. Shh is required for limb A-P polarity, outgrowth and Fgf expression in the AER [55]. Zebrafish Shh (\textit{shha}) and its receptor (and transcriptional target) \textit{pitrk2} are first
expressed in the ZPA at 24 hpf and expression progressively increases until 36 hpf (Figure 3A–B) [56]. In Nipbl-deficient limb buds, shha and ptc2 expression was reduced at these stages (Figure 3A, B). shha and ptc2 expression levels were also reduced in the intestine (where Nipbl is also required for development [11]; Figure 3A, B, asterisks), but unaffected in the notochord and neural tube (Figure 3A, B and unpublished data), suggesting a tissue-specific requirement for Nipbl in the expression of Shh and its receptor.

Hand2 regulates Shh expression in fin/limb buds [56–58], and we found that hand2 expression was also reduced in Nipbl-deficient fin buds (36 and 40 hpf) compared with stage-matched controls (32 and 36 hpf) (Figure 3C). In mouse limb buds, anterior expression of the transcriptional repressor, Gli3, restricts expression of Hand2 posteriorly [59]. Zebrafish pectoral fin buds also express gli3 [60] but its expression was not affected by reduction of Nipbl (Figure 3D).

Mammalian Hand2 acts together with the products of 5’-Hoxd genes [58] in the regulation of Shh expression. In pectoral fin buds of Nipbl-deficient embryos, we found that 5’-Hoxd genes, including hoxd9a-d13a (Figure 4A), were significantly downregulated (Figure 4B). Importantly, fin bud expression of hand2, hoxd10a, shha and ptc2 could all be partially rescued by exogenous nipbla mRNA (Figure S8).

Retinoic acid (RA) produced in anterior somites also regulates shha expression in pectoral fin buds (12–22 hpf [42–44,61]), as well as fin bud expression of fgf10a. However, we found no differences in expression of either the RA synthesizing enzyme aldh1a2 or the RA degradation enzyme and target gene, cyp26a1, at 13 and 19 hpf in Nipbl-deficient embryos (Figure S9).

Together, these findings indicate that Nipbls regulate the 5’-hoxd/hand2/shha signaling cascade, but do not affect the tbx5a/fgf24/fgf10a pathway that lies downstream of RA signaling, during vertebrate limb development.

Nipbls regulate expression of hox genes according to their genomic location

Hox genes belong to 13 paralog groups organized in four (mammals) or seven (zebrafish) clusters; the HoxA and D clusters are crucial for limb/fin development [56,62,63]. The most 5’-located genes (3’-Hox), such as Hoxd1, are expressed earliest in mouse limb buds, whereas expression of 5’-located genes (5’-Hox, d10-d13) begins later [64,65]. 5’-Hoxd gene expression occurs first in proximal limb buds, where it is required for Shh expression in the ZPA to establish A-P patterning [55,66], and is later restricted distally in limb buds, where it is required for proper digit formation [64,65]. Expression of hoxd genes in zebrafish fin buds is reminiscent of that in proximal mouse limb buds but appears to lack the second wave of distal expression, consistent with the lack of digits in ray-finned fish [64].

Examination of expression of multiple hox genes from the Hoxa (hoxa9, hoxa10, and hoxa13) and Hoxd (hoxd9, hoxd10, and hoxd13) clusters in the fin buds of Nipbl-deficient embryos revealed that changes in expression correlated strongly with positions of genes within clusters (Figures 4–5). Expression of five hoxd genes located at the 5’ ends of the hoxd9a-d13a cluster was severely reduced (Figure 4B), while expression of two hoxd genes located more 3’ in the cluster, hoxd3a and hoxd4a, expanded to encompass the entire bud (Figure 4C). Similarly, expression of 5’-genes in the hoxa cluster—such as hoxa9b, a10b, and a13b—was significantly
reduced in Nipbl-deficient fin buds, while a 3' gene, hoxa2b, was upregulated (Figure 5A, B). Likewise, expression of hoxe8a and hoxe9a was reduced in Nipbl-deficient fin buds while expression of hoxc1a, hoxc4a, and hoxc6a expanded posteriorly (Figure 5C, D). Thus, in all three hox clusters expressed in the pectoral fin buds, expression of genes near the 3' end of the cluster expands, whereas expression of those closer to the 5' end is reduced (Figure 5E).

Interestingly, this position-specific regulation of hox gene expression is specific to pectoral fin buds, since hox expression patterns in the neural tube were unaffected in Nipbl-deficient embryos (Figure S10).

Shh signaling from the ZPA regulates expression of several hox genes along the A-P axis of limb buds, and reduced expression of 5'-hoxa/hoxd genes as well as posterior expansion of hoxe6a expression, similar to that described above, occurs in Shh-deficient zebrafish [55]. To test if the Shh reductions resulting from Nipbl deficiency might cause the defects in hox gene expression, we treated wild-type embryos with the Shh signaling inhibitor, cyclopamine (CyA). Although CyA treatment caused some developmental delay, (~4–5 hr, based on the A-P positions of pLL primordia [compare Fig. S11A with Fig. S5A], and no more than 12 hr based on pectoral fin development), it strongly reduced expression of ptch2 as well as hoxa13b, hoxd10a and hoxd13a, while expression of hoxc4a and hoxc6a expanded posteriorly (compared with stage-matched controls, Figure S11B). These effects of CyA treatment resembled those of Nipbl depletion, but others did not - e.g. hoxd4a expression was severely reduced, and hoxe8a expression expanded posteriorly in CyA-treated embryos (Figure S11B), in contrast to Nipbl-deficient embryos (Fig. 4C, 5C). Thus, loss of Shh signaling cannot explain all of the changes in hox gene expression in Nipbl-deficient embryos, suggesting that either Nipbls regulate the expression of hox genes directly, or they do so via regulators other than (or in addition to) Shh.
Gene expression changes in limb buds of \textit{Nipbl}\textsuperscript{+/−} mutant mice fail to display obvious limb reductions, but do show some limb patterning and bone calcification defects [3]. Given the gene expression changes we found in pectoral fin buds of \textit{Nipbl}-deficient fish, we decided to investigate if \textit{Nipbl}-deficient mouse limb buds show some of the same changes. ISH for \textit{Shh} in E10.5 limb buds of \textit{Nipbl}\textsuperscript{+/−} mice revealed a marked reduction in \textit{Shh} expression in the ZPA, similar to \textit{Nipbl}-deficient fin buds (compare Figure 3A and Figure 6). This was confirmed by both Q-RT-PCR and expression microarray analysis, using RNA extracted from E10.5 limb buds harvested from stage-matched \textit{Nipbl}\textsuperscript{+/−} (n = 12) and wildtype (n = 12) littermate embryos (Table 1; also see Methods). Microarray analysis identified approximately 1000 genes as significantly over- or under-expressed in \textit{Nipbl}\textsuperscript{+/−} limb buds (Table 1 and data publicly deposited) and, similar to tissues and cells of \textit{Nipbl}\textsuperscript{+/−} mice and individuals with CdLS, most gene expression changes were typically less than 1.5-fold [3,4]. Nonetheless, statistically-significant changes in expression (mostly decreases) were observed for multiple genes in the Fgf, Bmp and \textit{Shh} pathways, as well as numerous genes in the Wnt/planar cell polarity signaling pathway. In addition, multiple genes at the 5’ and 3’ ends of the Protocadherin B cluster were downregulated (not shown), while \textit{Stag1} (which encodes a cohesin subunit) was upregulated; both of these changes are hallmarks of \textit{Nipbl} deficiency in other tissues [3].

\textbf{Figure 4.} \textit{Nipbls are required for spatial patterns of \textit{hoxd} expression in pectoral fin buds.} (A) Diagram of zebrafish \textit{hoxd} cluster. (B, C) Expression of 3’-\textit{hoxd} genes including \textit{hoxd3a} and \textit{d4a} (B) and 5’-\textit{hoxd} genes including \textit{hoxd9a-d13a} (C) was examined by ISH at 32 and 36 hpf to show both time-matched and stage-matched (\textit{nipbla/b-MO} at 36 hpf and control at 32 hpf) comparisons. Dorsal views, anterior to the top.

doi:10.1371/journal.pgen.1004671.g004

Nipbl and Mediator Regulate Limb Development

PLOS Genetics | www.plosgenetics.org 6 September 2014 | Volume 10 | Issue 9 | e1004671
Figure 5. Nipbls regulate hox gene expression according to genomic location. (A–D) Expression of genes in hoxab (A,B) and hoxca (C,D) clusters was examined by ISH. (A) 5'-hoxa, (B) 3'-hoxa, (C) 5'-hoxc, and (D) 3'-hoxc genes. Dorsal views, anterior to the top. (E) Diagram summarizing effects of Nipbl reduction on hox genes. Genes located closer to 5'-ends show reduced expression (red boxes) whereas those closer to 3'-ends become expressed across entire fin buds (green boxes).

doi:10.1371/journal.pgen.1004671.g005
Hand2 expression was expanded posteriorly following knockdown of med12, while expression of the same 5′ genes was reduced (Figure 8A).

The possibility that these similarities reflect a transcriptional relationship between Nipbl and Med12—e.g. Nipbl positively regulates Med12 expression (or vice versa)—was ruled out by direct measurements of transcript levels in the fin buds of MO-injected embryos (Figures 7E and S14). This conclusion also agrees with the mouse microarray data, which show no decrease in expression of any Mediator subunit in Nipbl+/− limb buds. Indeed, some Mediator genes (Med14, Med19, and Med12) exhibit modest increases in expression, suggesting, if anything, a negative role for Nipbl in Mediator expression (Table 1).

To test for a genetic interaction between Nipbl and Mediator, nipbla/b-MOs and med12-MOs were co-injected at subthreshold doses, and assayed for changes in pectoral fin development and gene expression. Small amounts of med12-MO (0.5 ng/embryo; low-med12-MO) caused only slight reductions in pectoral fin size and 5′-hoxa/hoxd gene expression in fin buds (Figure 8B–D). However, when combined with low doses of nipbla/b-MOs (a combination of 0.05 ng/embryo of nipbla/ MO and 0.75 ng/embryo of nipblb-MO; low-nipbla/b-MO; low-med12-MO caused reductions in 5′-hox gene expression and expansion of 3′-hox gene expression similar to those observed with higher doses of either nipbla/b- or med12-MOs alone (Figure 8D). These results suggest that Nipbl and Mediator interact functionally to regulate spatial patterning of hox gene expression in the developing limb.

Interestingly, depletion of the cohesin subunit Rad21 caused very different defects in pectoral fin development and gene expression than deficiencies for Nipbl or Med12. Rad21 depletion delayed development (by approximately 10 hrs, based on the A-P positions of pLL primordia; Figure S15), consistent with a previous report [71], but when compared with stage-matched controls all fin mesenchymal genes (including 3′-hox genes, hox6a6 and hoxd4a) were downregulated (Figure S16). Reductions in hox gene expression became more severe at later stages, although, interestingly, only in fin buds, and not in the neural tube (Figure S16).

Nipbl and Med12 regulate chromatin conformation around the hoxd cluster

Spatial- and temporal patterns of Hox gene expression are achieved through regulation of chromatin organization around Hox clusters. In mouse limb buds, for example, remote enhancers located in flanking “gene deserts” found at the telomeric (3′) and centromeric (5′) sides of the clusters regulate the proximal versus distal expression of 5′-Hox genes [15,72]; these enhancers are distinct from cis-regulatory elements within the clusters that regulate co-linear expression along the body axis [73,74]. Although these remote enhancers have been most extensively studied in mammals, some are clearly conserved and functional in teleosts [75–77]. For example, of two distinct regions in the gene desert telomeric to the mouse HoxD cluster recently shown to have proximal limb-specific enhancer activity [72], we located sequences homologous to one, CNS65, about 200 kb telomeric to the hoxa cluster in the zebrafish genome (Figure 9A).

Such results suggest that, in both fish and mice, limb bud hox gene expression depends on long-range chromosomal interactions the formation of which may be regulated by Nipbl and Mediator [17]. We tested this hypothesis by looking for changes in chromatin architecture around the hoxd cluster following Nipbl or Mediator depletion, using probes for 3D-FISH with which we can measure physical distances between the hoxd cluster and distant flanking regions on both centromeric and telomeric sides
**Table 1.** Gene expression changes in *Nipbl^-/-* mouse limb buds.

| Gene                  | Expression by microarray | Notes | Expression by Q-RT-PCR | Notes |
|-----------------------|--------------------------|-------|------------------------|-------|
|                       | FDR mut/wt               |       | FDR mut/wt             |       |
| **Shh pathway**       |                          |       |                        |       |
| *Shh*                 | 1.0% 0.82                |       | 0.48 (p<0.01)         |       |
| *Gli3*                | 9.5% 1.09                |       |                        |       |
| *Hhip*                | 2.4% 0.76                |       |                        |       |
| *Hand2*               | 2.4% 0.92                | 0.85  (p = 0.09) i, ii | 1.0% 0.90 | 0.82  (p = 0.03) |
| **FGF signaling**     |                          |       |                        |       |
| *Fgf15*               | 20.2% 0.91               |       |                        |       |
| *Fgf18*               | 1.8% 0.83                |       |                        |       |
| *Fgf9*                | 18.8% 0.88               |       |                        |       |
| *Spry2*               | 4.1% 0.89                |       |                        |       |
| **Hox Gene Expression and Function** | |       |                        |       |
| *Hoxa13*              | 22.1% 0.73               | 0.46  (p = 0.03)  |                        |       |
| *Hoxc9*               | 7.5% 0.89                |       |                        |       |
| *Hoxc13*              | 2.4% 0.85                | 0.57  (p<0.01)  |                        |       |
| *Hoxd10*              | 1.0% 1.06                | 1.27  (p<0.01)  |                        |       |
| *Hoxd11*              | 23.6% 0.93               | 0.75  (p = 0.04)  |                        |       |
| **BMP signaling**     |                          |       |                        |       |
| *Bmp2*                | 1.0% 0.79                |       |                        |       |
| *Bmp4*                | 1.0% 0.90                |       |                        |       |
| *Bmp7*                | 6.4% 1.10                |       |                        |       |
| *Bmpr1a*              | 1.0% 1.10                |       |                        |       |
| **Cohesin Function**  |                          |       |                        |       |
| *Nipbl*               | 1.0% 0.63                | 0.62  (p<0.01)  |                        |       |
| *Stry1*               | 1.0% 1.16                |       |                        |       |
| **Mediator Complex**  |                          |       |                        |       |
| *Med12l*              | 1.8% 1.11                |       |                        |       |
| *Med14*               | 4.1% 1.05                |       |                        |       |
| **Wnt/Planar Cell Polarity Pathway** | |       |                        |       |
| *Wnt11*               | 1.0% 0.87                |       |                        |       |
| *Wnt8a*               | 9.9% 1.12                |       |                        |       |
| *Fzd2*                | 2.4% 0.92                |       |                        |       |
| *Fzd8*                | 1.0% 0.86                |       |                        |       |
| *Rspo2*               | 1.8% 0.84                |       |                        |       |
| *Dshl*                | 1.0% 0.82                |       |                        |       |
| *Csnk2a1*             | 1.0% 0.89                |       |                        |       |
| *Csnk2a2*             | 7.5% 1.05                |       |                        |       |
| *Ctnk2a2*             | 7.5% 1.05                |       |                        |       |
| *Ctnk2a1*             | 1.0% 0.72                |       |                        |       |
| *Ctnk2a1*             | 1.0% 0.72                |       |                        |       |
| *Dscl*                | 8.6% 0.91                |       |                        |       |
## Table 1. Cont.

| Gene   | Expression by microarray | Expression by Q-RT-PCR | Notes | Gene   | Expression by microarray | Expression by Q-RT-PCR | Notes |
|--------|--------------------------|------------------------|-------|--------|--------------------------|------------------------|-------|
| Rspo3  | 1.0% 0.74                |                        |       | Daam2  | 1.0% 0.75                |                        |       |
| Sfrp1  | 4.6% 0.81                | iv                     |       | Nlk    | 1.0% 1.15                |                        |       |
| Sulf1  | 1.0% 0.81                |                        |       | Ppap2b | 1.0% 0.85                |                        |       |
| Fat1   | 1.0% 0.89                |                        |       | Prickle1 | 1.0% 0.91              |                        |       |
| Fat3   | 1.0% 0.78                |                        |       | Ror1   | 2.4% 0.88                |                        |       |
| Fat4   | 1.0% 0.65                |                        |       |        |                          |                        |       |

Relative gene expression levels in limb buds of stage-matched, E10.5 wildtype and Nipbl<sup>−/−</sup> mice were determined as described (see Materials and Methods), and in certain cases confirmed by Q-RT-PCR. Selected transcripts are shown.

1Involved in control of Shh expression in limb bud [101].
2Also controls Hox gene expression in the limb bud [102].
3Also controls Hox gene expression in the limb bud [69].
4Direct target of Hoxd13 in limb buds [103].
5Directs the position of the Shh expression boundary delineating the experimentally defined ZPA [104].
6Mesenchymal, involved in chondrocyte proliferation [105].
7**AER-Fgf** [106].
8**Strongly activated by HOXA13 [67].
9**HOXA13 target in limb buds [68].
10Functions as a HOX cofactor during development; complexes with HOXA9; also controls Hox and Shh expression [102].
11Non-canonical Wnt [107].
12*Canonical Wnt [108].
13**Interacts with some Wnts and Frizzleds and supports Wnt-Fz-Ror2 complex formation, and at the same reduces Wnt-Fz-LRP complex formation, thus favoring non-canonical Wnt signaling [109].
14**Wnt regulator; required for maintenance of AER and Shh signaling [110].

doi:10.1371/journal.pgen.1004671.t001
Nipbl and Mediator Regulate Limb Development

Figure 7. Med12 depletion disrupts pectoral fin morphology and gene expression similar to Nipbl depletion. (A, B) Morphology of live embryos at 52 hpf (A, lateral views) and 76 hpf (B, dorsal views). (A) Anterior halves of control and med12-MO-injected embryos (left column) and higher magnification pictures of their pectoral fin buds (right column). (B) Dorsal views of embryos at 76 hpf. (C) Whisker plots of fin lengths. Fin lengths (medians) are 430.0 μm, n = 18 (control), 275.6 μm, n = 20 (med12-MO, 2 ng), and 183.8 μm, n = 20 (med12-MO, 4 ng); *: p<10^-6. (D) Alcian blue staining of pectoral fin cartilage of control (upper) and Med12-deficient (med12-MO, 4 ng; lower) embryos at 120 hpf. Dorsal view, anterior to the top. Right column, higher magnification pictures of boxed areas of endoskeletal discs. ac, actinotrichs; ed, endoskeletal disc; sco, scapulocoracid. (E) Controls for med12-MO efficiency. RT-PCR, 36 hpf. Both pairs of med12 primers (Primer #1 and #2) show that splicing of med12 mRNA is significantly suppressed by med12-MO, with a slightly higher efficiency at 6 ng. Primer pair #1 detects both precursor and mature mRNA, whereas primer pair #2 only detects mature mRNA (see Materials and Methods). nipbl and nipblb expression was not affected by Med12 depletion. ef1α was used as a control. (F) Expression of genes involved in the 5'-hox/hand2/shha gene cassette and AER fgf genes in pectoral fin buds examined by ISH at 36 hpf. Dorsal views, anterior to the top. Similar to Nipbl-deficient embryos, shha, hand2 and 5'-hox genes in mesenchyme as well as fgf16 and fgf8b in the AER are reduced in Med12-deficient embryos (4 ng/embryo med12-MO).

doi:10.1371/journal.pgen.1004671.g007

Discussion

Multiple genes are dysregulated in fin/limb buds of Nipbl-deficient embryos

Limb reductions are among the most striking structural birth defects in CdLS [26,28,36]. Previous studies of both fish and mouse models of Nipbl deficiency, as well as of cell lines derived from human patients with CdLS, strongly suggest that such defects result from the collective and sometimes synergistic effects of numerous small changes in gene expression during development [3,4,11]. Distinct sets of gene expression changes have been found in every tissue studied thus far, providing insights into genetic pathways that underlie defects in different tissues and organs [3,4,11]. Until now, identifying gene expression changes underlying limb reductions in CdLS has not been possible, since limb reduction is one of the few structural defects in CdLS that is not obviously replicated in the Nipbl-haploinsufficient mouse model [3]. However, by combining studies of zebrafish and mice in the present study, we show that Nipbl levels are critical for limb development (Figure 1), and that Nipbl regulates expression of specific sets of genes in the embryonic limb, including many key developmental regulators that are conserved between fish and mice. Among these Fgfs, Shh, and 5'-Hox genes (Figures 2, 3, 5, and Table 1) are of particular note because of the central and conserved roles these genes play in early limb bud growth and patterning.

In the E10.5 mouse embryo, where the larger size of the limb bud (compared with zebrafish) made genome-wide transcriptional profiling feasible, levels of more than 1000 transcripts were significantly altered (Table 1 and data publicly deposited). Both the large number of affected genes and the relatively small sizes of the effects were similar to what has been observed in other tissues of Nipbl-/- mice and in cells from individuals with CdLS [3,4]. It may be noteworthy that in the mouse limb a large number of Nipbl-sensitive genes are involved in Wnt/planar cell polarity signaling. Although this finding was not further investigated here, it is possible that disruption of this pathway is related to the disorderly arrangement of endoskeletal cells that we consistently observe in developing, Nipbl-deficient fins (Figure 1F, G). It may also be noteworthy that, in Nipbl-deficient mouse limbs, several Mediator subunits are (slightly) upregulated (Table 1). As described above, upregulated Mediator function might potentially provide some compensation for Nipbl deficiency.

Interactions between Nipbl and Mediator in gene regulation

Chromatin binding studies have shown that Nipbl co-localizes with cohesin and the Mediator complex at putative regulatory elements of actively transcribed genes, suggesting that Nipbl and Mediator act together to regulate gene expression [13,17,81]. Here we provide the first in vivo evidence in support of this hypothesis: 1) Med12- and Nipbl-deficient pectoral fin buds display similar size reductions and gene expression changes—particularly within hox gene clusters; 2) subthreshold doses of nipbl- and med12-MOs synergize to reduce limb size and disrupt gene expression; and 3) both nipbl- and med12-MOs cause similar changes in chromatin conformation at the hoxa cluster.

These results support the view that Nipbl and Mediator play roles in the long-range coordination of gene expression. Moreover, the observed differential effects on expression of 3’- versus 5’-hox genes suggest an important role for Nipbl and mediator in transcriptional coordination at multi-gene loci, a result also supported by position-specific effects seen at the protocadherin beta locus in Nipbl-haploinsufficient mice [3], and by studies on the role of Nipbl in long-range control of the beta-globin locus [13].

Interestingly, instead of having position-specific effects, depletion of the cohesin subunit Rad21 led to downregulation of all 3’- and 5’-hox genes that we tested, suggesting that the gene regulatory effects of Nipbl/Mediator are not equivalent to those
of cohesin. Indeed, although cohesin has been implicated in long-range chromatin interactions [82–84], and Rad21 co-localizes at promoters and enhancers with Nipbl and Mediator [17], this co-localization only occurs at a subset of cohesin binding sites. Moreover, recent work suggests that Nipbl, but not cohesin, co-localizes with certain transcription factors [85]. Such differences may explain the markedly different results that have been observed, in both cell lines and embryos, in the changes in gene expression and chromatin organization that occur in response to depletion of cohesin versus Nipbl [11,85,86].

Figure 9. Nipbls and Med12 play roles in regulation of higher-order chromosome conformation at the Hoxd locus in pectoral fin buds. (A) Diagram of the genomic organization at the zebrafish hoxda locus. Genes in the hoxda cluster and flanking genes are shown as black boxes. Putative regulatory elements conserved between zebrafish and mouse and probes used for FISH are shown as colored ovals and lines, respectively. (B–D) Typical images of FISH. (B) Low magnification picture of a sagittal section of pectoral fin bud. Scale bar = 10 μm. (C,D) Higher magnification images of nuclei with colocalized (C) and separate signals (D). Hybridized probes are detected as green and red fluorescent dots in DAPI-stained nucleus. Scale bar = 2 μm. (E,F) Whisker plots of interprobe distances between hoxd and 3' probes (E) or hoxd and 5' probes (F) at 38 hpf. Medians, numbers of nuclei and embryos, and p-values calculated by the non-parametric Mann-Whitney U-test are shown in Table 2. Dotted lines indicate thresholds for separated (upper) and closed (lower) signals in Table 2. (G) Sizes of nuclei in pectoral fin buds (n = 30 each) were estimated at 38 hpf by measuring major and minor axes. Major axis (Ave ± S.D.): 8.58±1.63 μm (control), 8.22±1.76 μm (nipbl-a/MO, p = 0.412), and 8.14±1.43 μm (med12-MO, p = 0.280). Minor axis (Ave ± S.D.): 4.41±1.28 μm (control), 4.70±0.92 μm (nipbl-b/MO, p = 0.314), and 4.56±0.73 μm (med12-MO, p = 0.577). p-values were calculated by Student’s t-test.

doi:10.1371/journal.pgen.1004671.g009

Figure 8. Functional interactions between Nipbl and Med12 in pectoral fin development. (A) hox gene expression in pectoral fin buds of Med12-deficient embryos examined by ISH at 36 hpf. Dorsal views with anterior to the top. (B) Lateral views of pectoral fins in living larvae at 76 hpf in controls or injected with 0.5 ng med12-MO alone (low-med12-MO) or combined with low amounts of nipbl-MOs (0.05 ng nipblb-MO+0.75 ng of nipla-MO; low-nipla/b-MO). Medians: 410.1 μm, n = 16 (control), 382.2 μm, n = 24 (low-nipla/b-MOs), 385.4 μm, n = 16 (low-med12-MO alone), and 341.4 μm, n = 16 (low-med12-MO+low-nipla/b-MOs). Asterisks indicate statistical significance (p-values <0.001). (D) hox expression in larvae injected with low-med12-MO alone or combined with low nipla/b-MO. Dorsal views, anterior to the top.
doi:10.1371/journal.pgen.1004671.g008
Mediator
Regulation of chromatin conformation by Nipbl and
limb development

Direct versus indirect effects of Nipbl and Mediator in
limb development

Previous studies have proposed that limb development is controlled
by a positive feedback loop in which Shh from the ZPA and Fgfs from
the AER maintain one another’s expression [38,40,53]. Consistent
with this, we found that expression of both Shh and Fgf genes were
reduced in Nipbl-deficient limb and fin buds (Figures 2, 3 and
Table 1). As nipbla and niplbl are expressed most highly in fin bud
mesenchyme (Figure S1), it is possible that Nipbls regulate the
expression of mesenchymal genes such as shha directly, whereas
regulation of fgf expression in the AER may be indirect.

On the other hand, hox genes could be the major direct targets
of Nipbl deficiency, with effects on shha expression being
secondary. Both HoxD and Hand2 regulate Shh expression in
early limb/fin buds [57,58,87], and Hox proteins also regulate
Hand2 expression [88]. In Drosophila, Nipped-B and cohesin bind
to genes in the bithorax (Hox) complex (BX-C), specifically in cells
that express BX-C genes [81]. More recently, it has been shown
that human cohesin binds to the HOXA and HOXB clusters, and
disruption of its function reduces expression of multiple Hox
genes [83]. Our finding that three distinct hox clusters (A, C, and
D) are all affected similarly in Nipbl- and Med12-deficient
zebrafish suggests that Nipbl and Mediator play a common role in
hox locus control. Results of 3D-FISH experiments at the hoxda
cluster further suggest that Nipbl/Mediator-dependent regulation
of long-range chromatin interactions is an important part of this
role, as discussed below.

Regulation of chromatin conformation by Nipbl and
Mediator

The position-specific effects of depleting Nipbl or Med12 on hox
gene expression in the zebrafish pectoral fin bud—with 5′-genes
down-regulated and 3′ genes up-regulated—sugget a coupling of
transcriptional regulation between the two ends of hox clusters.
Our 3D-FISH results, which show that Nipbl and Med12 are
required in fin buds for long-range interactions on both sides of the
hoxa cluster, raise two possibilities for explaining the effects of
depleting Nipbl and Med12 on hox gene transcription (Figure 10).

According to one model, disruption of long-range chromosomal
interactions leads to a loss of long-range activation at the 5′ ends
and long-range repression at the 3′ ends of hox clusters (Figure 10A).
Alternatively, disruption of chromosomal conformation
may allow the 3′ remote enhancers to be replaced with other
(probably more closely located) regulatory elements, leading to
their ectopic activation (Figure 10B). These putative regulatory
elements might be fish-specific since, the orthologous 3′-Hox genes
are not upregulated in Nipbl-deficient mouse limb buds.

On the other hand, direct comparisons between mice and fish
could be misleading, due to the dynamics of hox gene expression.
In both tetrapod and zebrafish limb buds, hox gene expression
progresses through distinct stages, first being biased toward 3′
genes and later toward 5′ ones [64], as the balance of long-range
interactions shifts from telomeric to centromeric [72,77]. If E10.5
mouse hindlimb buds are not at exactly the same stage as the
pectoral fin (forelimb) buds examined here, they may not possess
the same potential to express 3′-Hox genes.

A third possibility is that some upregulation of Hox genes does
take place in the Nipbl-deficient mouse limb, similar to fish, but
the genes affected are not as close to the 3′-end of the cluster. For
example, among Hoxa genes, we observed that significant up-
regulation of Hoxd10, and possibly also Hoxd18, accompanies the
down-regulation of Hoxd11, 12 and 13 in Nipbl-deficient limbs.

Interestingly, in the zebrafish hindbrain, the effects of depletion
of Nipbl or Med12 on hox gene expression and chromosomal

Table 2. Results from 3D-FISH around the zebrafish hoxa locus.

|                | median (µm) | nuclei (embryos) | p*   | % of nuclei** |
|----------------|-------------|------------------|------|---------------|
|                |             |                  |      | closed       | separated   |
| 5′-hoxa (centromeric) |             |                  |      | closed       | separated   |
| pectoral fin buds |             |                  |      | closed       | separated   |
| control        | 0.278       | 240 (4)          | 9.58 | 5.00         |
| nipbla/b-MO    | 0.357       | 240 (4)          | 4.8×10⁻⁷ | 6.25         | 15.9        |
| med12-MO       | 0.362       | 180 (3)          | 7.9×10⁻⁹ | 5.56         | 17.2        |
| hindbrain      |             |                  |      | closed       | separated   |
| control        | 0.295       | 165 (4)          | 8.48 | 3.64         |
| nipbla/b-MO    | 0.312       | 160 (4)          | 0.092 | 8.12         | 8.12        |
| med12-MO       | 0.306       | 125 (3)          | 0.716 | 9.60         | 2.40        |
| 3′-hoxa (telomeric) |             |                  |      | closed       | separated   |
| pectoral fin buds |             |                  |      | closed       | separated   |
| control        | 0.220       | 240 (4)          | 9.58 | 1.67         |
| nipbla/b-MO    | 0.328       | 420 (7)          | 5.5×10⁻⁹ | 4.76         | 25.0        |
| med12-MO       | 0.317       | 180 (3)          | 2.2×10⁻⁴ | 6.11         | 28.3        |
| hindbrain      |             |                  |      | closed       | separated   |
| control        | 0.237       | 160 (4)          | 15.0 | 6.25         |
| nipbla/b-MO    | 0.349       | 160 (4)          | 2.2×10⁻¹¹ | 4.38         | 26.9        |
| med12-MO       | 0.337       | 160 (4)          | 1.3×10⁻⁸ | 3.13         | 21.9        |

* Evaluated by the Mann-Whitney test.
** Proportions of nuclei exhibiting interprobe distances less than half of (closed) and longer than double (separated) the control medians.
doi:10.1371/journal.pgen.1004671.t002
Interactions differ from those observed in fin buds. 3D-FISH in wild-type hindbrain cells reveals chromosomal interactions of \( \text{hoxda} \) with both 3'- and 5'-territories—despite the fact that the hindbrain expresses only 3'-\( \text{hox} \) genes. Moreover, the expression of hindbrain \( \text{hox} \) genes is unaffected in Nipbl- or Med12-deficient embryos, even though long-range interactions on the 3'-side of the \( \text{hoxda} \) cluster are markedly diminished. These results suggest: 1) that hindbrain \( \text{hox} \) gene expression is not primarily controlled by long-range enhancers (at least not on the 3'-side), and 2) that long-range interactions of \( \text{hox} \) genes are not necessarily associated with active transcription (i.e., they may sometimes represent a poised, or latent stage). Consistent with the latter idea, in mouse forebrain, where \( \text{Hox} \) genes are not expressed, the \( \text{Hoxd} \) locus still interacts with many of the same long-range elements as it does in limb bud cells [15]. Similar examples of long-range promoter-enhancer associations that do not necessarily correlate with gene expression have also been described for \( \text{Shh} \) in the mouse limb [14].

Whereas Nipbl and Med12 depletion inhibits both 3'- and 5'-chromosomal interactions of the \( \text{hoxda} \) cluster in the pectoral fin buds, in the hindbrain such depletion fails to affect 5'-interactions, suggesting a distinct underlying mechanism. In the trunk, the activation of \( \text{hox} \) gene expression is thought to reflect an anterior-to-posterior wave of chromatin decompaction, from 3' to 5', such that in anterior structures (such as the hindbrain) 5'-\( \text{hox} \) genes and adjacent sequences remain in a highly condensed state [89], associated with high levels of H3K27me3 modification [80].

Figure 10. Model of \( \text{hox} \) gene regulation by Nipbls and mediator. Along topological domains, 3'- and 5'-\( \text{hox} \) genes tend to interact with limb-specific regulatory elements in telomeric and centromeric landscapes, respectively. These interactions are required to establish proper patterns of \( \text{hox} \) gene expression in limb/fin buds and depend on Nipbl/Mediator. The long-range enhancer-promoter interactions are disrupted in the absence of Nipbl and Med12, leading to dysregulation of \( \text{hox} \) genes. (A) Expanded expression of 3'-\( \text{hox} \) genes might be allowed when released from putative remote repressors in Nipbl/Med12-deficient fin buds. (B) Alternatively, disruption of chromosomal conformation may lead to replacement of 3'-remote enhancers with (more closely located) putative ectopic enhancers that can activate 3'-\( \text{hox} \) genes strongly through long-range interactions. doi:10.1371/journal.pgen.1004671.g010
by knockdown of the Smc3 cohesin subunit, underscoring the idea, discussed earlier, that the transcriptional function of Nipbl is distinct from that of cohesin.

Understanding the variability of limb defects caused by Nipbl deficiency

Many of the mesenchymal genes (e.g. shh, hand2, 5'-hox) we find downregulated in Nipbl-deficient fin buds are essential for growth and patterning of mouse limbs. Shh+/− mice, for example, have limb truncations [90,91], and Hand2 is required for Shh expression in the ZPA [87]. Mice lacking certain genes within the HoxA or HoxD clusters have mild digit defects, while a simultaneous deletion of both HoxA and HoxD clusters causes dramatic forelimb truncations [62,63]. Our finding that expression of Shh and multiple Hox genes is reduced in the limb buds of Nipbl+/− mutant mice indicates that these genes are common targets of Nipbl in the vertebral limb, and the dysregulation of their expression is likely to be central to the etiology of limb defects in CdLS.

Nonetheless, Nipbl+/− mice display very mild limb abnormalities [3]. One likely explanation for this difference is that haploinsufficiency does not lower Nipbl levels as much as is achieved in MO-injected zebrafish embryos. Indeed, it has been observed that, due to unknown compensatory mechanisms, Nipbl+/− mice display only a 35–40% reduction in Nipbl transcripts (cf. [3], and Table 1); whereas nipbl MOs can lower nipbla and nipblb transcript levels to a much larger degree [11].

The idea that the strength of limb phenotypes is related to the degree of nipbla depletion is further supported by the observation, in zebrafish, that fin reductions are more severe when larger amounts of nipbla-MO are injected, or when both nipbla and nipblb are knocked-down, as opposed to either one alone (Figure S3). In light of this observation, it is noteworthy that only about a third of individuals with CdLS display limb abnormalities at the severe end of this spectrum [26]. A subset of this phenotypic variability likely relates to the strengths of different mutations on Nipbl protein expression (severe forelimb defects tend to correlate with nonsense or frame shift mutations [92,93]). However, it likely also reflects inter-individual variability in the functions of genes that control Nipbl expression or, like components of the Mediator complex, work together with Nipbl in the control of gene expression.

Materials and Methods

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

Fish and mouse maintenance, embryo raising and staging

Zebrafish (AB strain) were maintained and staged as described [94,95]. Embryos were stage-matched based on relative positions of posterior lateral line primordial along the A-P axis, detected by ISH with a fgf10a probe. Pectoral fin buds and the posterior end of the yolk sac extension were used as landmarks (Figure S5). Niplb+/− (Rrs strain) mice were housed, mated, and staged as described previously [3].

Microinjection of morpholino antisense oligonucleotides (MOs) and mRNA

MOs were designed to block translation (Gene Tools, Inc.), prepared at 20 mg/ml and diluted in 1× Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca (NCO3)2, 5 mM HEPES (pH 7.6)] and stored at −20°C. MO sequences are shown elsewhere (all nipbla-MOs and rad21-MO [11], and med12-MO [70]).

Full-length cDNA of nipbla was prepared by fusing partial cDNA fragments amplified by RT-PCR in pCRII-TOPO, fused with SV40 polyA sequence derived from pCS2+ and subcloned into pBS-KS+ for in vitro mRNA synthesis. Full-length capped nipbla mRNA was synthesized using mMMESSAGE mMACHINE (T3) kit (Ambion) in the presence of rGTP according to the manufacturer’s instructions. Synthesized mRNA was electrophoretically separated and a full-length mRNA was gel-isolated using RECOCHIP (TAKARA). MOs and full-length nipbla mRNA were injected into embryos at the 1–4-cell stage. A combination of nipbla-MO and niplb-MO were injected to generate Nipbl-deficient embryos, at 0.75 ng/embryo each or otherwise as indicated in figure legends.

Whole mount in situ hybridization (ISH)

Whole mount ISH of zebrafish embryos was performed using digoxigenin (DIG)-labeled antisense RNA probes as previously indicated [11]. Whole mount ISH of E10.5 mouse embryos was performed according to published protocols [96]. The 642 bp mouse Shh probe has been previously described [97]. The Hoxd12 and Hoxd13 probes were a kind gift from Denis Duboule.

Measurement of fin length

Pectoral fin lengths were measured using ImageJ from the proximal base to the distal tip in dorsal views (Whisker plots). The interquartile ranges (IQR) are shown as boxes, with the median as the horizontal lines within the boxes. The upper and lower whiskers are the highest and lowest data points within 1.5 x the IQR from the top and bottom of the box, respectively. Individual data including outliers are shown as dots. p-values are calculated by the non-parametric Mann-Whitney U test with the Bonferroni adjustment.

RNA preparation and RT-PCR

Total RNA was extracted from 20 whole zebrafish embryos for each sample, and subjected to cDNA synthesis using ProtoScript M-Mult LV First Strand cDNA Synthesis Kit (New England BioLabs). mRNA levels were examined by RT-PCR using ef1a as a control. Primers used in RT-PCR are: med12-primer #1, sense, 5'-CGCTTGGTGCTCTGCCAC-TACTC-3', antisense, 5'-CTGTGGTCTCCTGACACTTG-3'; med12-primer #1, sense, 5'-CTAAAGCTGATCTAGGAG-TAT-3', antisense, 5'-CCTTTGCGCG AACCTGTGTG-3'; nipbla, sense, 5'-GGCTACATGAGTACAGCCA-3', antisense, 5'-CATGTCAGGGGTTCCTCATA-3'; niplb, sense, 5'-CA-GACCAGAAGGAGACT-3', antisense, 5'-CTTGGTGTC-CAGGTCTTGATCTAT-3'; ef1a, sense, 5'-TCAAGCGACATCA-3', antisense, 5'-CTTGCGAGACTTTGTGAGC-CT-3'. The med12-primer #1 was designed to detect both precursor (including an intron of about 600 bases) and mature mRNA, whereas med12-primer #2 was designed at junctions of exons to detect only mature mRNA [98].

For Q-RT-PCR of mouse tissue, total RNA was isolated from somite-staged mouse hindlimbs from E10.5 embryos (WT n = 6, mutant n = 7) using the RNasey minikit (QIAGEN). cDNA was synthesized from RNA using the iScript Reverse Transcription Supermix for RT-qPCR (BioRad). cDNA was PCR amplified using the iQ SYBR green Supermix (BioRad) with a CFX96 Real-Time System (Bio-Rad). Expression changes were normalized to Hoxd13, Hoxd12, and med12 as described previously [70].
beta-2 microglobulin, and the expression of each gene was calculated using the 2-ΔΔCt method. A Student’s t test was used for statistical analysis.

Primers:

- **B2m**: atgggaagccgaacaactgt
cagctctaggggggtuattg
- **Nphl**: aagcctagatggcacacagcg
acggcaacataactagacgtg
- **Shh**: ggaacaccctatctccaagat
taatgacagatggcaacag
- **Ptc1**: gcacccagcccctaaatat
accaacaaatctctccctcg
- **Hoxd2**: cgcacacaaaccttccaaag
tgctgggtggtctgatct
- **Hoxa13**: cggacggcagaaatattgct
taatgacagatggcaacag
- **Hoxd4**: cctggcaggacacagtctc
ttcctgggtgtgagctgtt
- **Hoxd8**: gagctccggtgcttcaata
ttcctgggtgtgagctgtt
- **Hox9**: gcagggcagaggggaaacg
ttcctgggtgtgagctgtt
- **Hoxa10**: gcagggcagaggggaaagc
ttcctgggtgtgagctgtt
- **Hoxd11**: aaaggagggggcagcagat
aaagaaaaacatctcctcag
- **Hoxd12**: aaaggagggggcagcagat
aacgggcaacaataggacttg
- **Hoxd13**: ggagcagacgcaggtacgt
tggtgtaagaggccctttc

Cyclopamine (CyA) treatment

CyA was prepared at 10 mM in ethanol and stored at −20°C. Zebrafish embryos were incubated in CyA at 50 μM in embryo medium starting at 8 hpf in the dark and fixed with 4% paraformaldehyde (PFA) at indicated stages for ISH.

Proliferation and cell death

Cell proliferation was examined by bromodeoxy uridine (BrdU) incorporation assay as previously reported [98]. Incorporated BrdU was detected by staining with a rat monoclonal anti-BrdU antibody (Abcam, 1:100) and an anti-rat Alexa488-conjugated secondary antibody (Invitrogen, 1:200). Nuclei of acid-treated embryos were detected by staining whole live embryos with acridine orange (5 μg/mL for 10 min at room temperature). Cell death was examined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and acridine orange staining. For TUNEL assays, embryos were fixed at indicated stages with 2% PFA for 2 hr at room temperature and permeabilized in cold-acetone for 10 min at −20°C. Embryos were dehydrated in a graded series of methanol, at least 4 hours. Pretreated sections were loaded with probe solution prepared in hybridization buffer (50% formamide, 10% dextran sulfate in 1× SSC), covered with a cover slip, sealed with rubber cement and prehybridized for at least 2 hr at 37°C. Probes were heat-denatured by incubating the slides at 80°C for 5 min, and hybridized at 37°C for 2–3 days. After washing in 0.1× SSC at 60°C, nuclei were stained with DAPI (0.05 μg/mL) and slides were mounted for fluorescence microscopy.

Microarray analysis

Total RNA was extracted from hindlimbs (left and right) from each of 12 Nipbla−/− and 12 Nipblb−/− mouse embryos (E10.5, somite stages 35–38) [3]. The RNA was further processed by the UCI Genomics High-Throughput Facility for microarray analysis using Affymetrix Mouse Gene 1.0 ST arrays. The 24 probe cell intensity files (.Cel) were pre-processed using the Expression File Creator program of GenePattern (Broad Institute) and statistical analysis was performed using the Comparative Markers Selection module. Raw data will be made freely available to the public through Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GES60932; accession number GSE60932).

Three dimensional-fluorescence in situ hybridization (3D-FISH)

Zebrafish embryos were fixed at indicated stages in 4% PFA and sagittal cryosections cut at a thickness of 20 μm. FISH was performed on sections as described elsewhere [99]. Briefly, sections were permeabilized in 0.5% Triton X-100 in PBS and then genomic DNA was unmasked by 9 cycles of incubation at 90°C using a microwave and cooling for 2 min in 10 mM sodium citrate buffer (pH 6.0). Sections were then permeabilized in acetone for 5 min at −20°C and incubated in 50% formamide in 1× SSC for at least 4 hours. Pretreated sections were washed with 2× SSPE, and 1× SSPE for 5 min, then incubated with denatured probe overnight (4°C). Probes were prepared by nick translation (Roche) and hybridization was performed at 60°C. After hybridization, sections were washed with 2× SSPE, 1× SSPE and 0.2× SSPE, and then incubated for 10 min in 0.2× SSPE and 70% formamide. Slides were mounted with a pre-dried solution prepared in hybridization buffer (50% formamide, 15% dextran sulfate, 2× SSC), covered with a cover slip, sealed with rubber cement and prehybridized for at least 2 hr at 37°C. Probes were heat-denatured by incubating with the slides at 80°C for 5 min, and hybridized at 37°C for 2–3 days. After washing in 0.1× SSC at 60°C, nuclei were stained with DAPI (0.05 μg/mL) and slides were mounted for fluorescence microscopy.

Image analysis

Slides were examined using an Olympus confocal microscope (FV1000) and multiple optical sections along the z-axis were taken in 0.1 μm intervals. Captured images were analyzed using ImageJ. Outlines, areas, and central coordinates along x, y and z axes were measured for each fluorescent signal using the Wand tool in Image J in combination with ROI manager, and spatial distances between two closely located and differently colored signals were calculated. 60 nuclei from pectoral fin buds and 40–45 nuclei from hindbrain were analyzed for each embryo, and 3–7 embryos were used for each condition/probe set tested. Normalized inter-probe distances were plotted in probability histograms showing the mean percentage (± SD) of total nuclei from each sample displaying a given separation between fluorescent dots. Statistical significance was determined by the Mann-Whitney U test.

Supporting Information

**Figure S1** Expression of *nipbla* and *nipblb* in developing pectoral fin buds. (A) Expression of *nipbla* and *nipblb* in pectoral fin buds examined by ISH. Dorsal views, anterior to the top. (B) Transverse sections of pectoral fin buds at 36 hpf showing *nipbla* and *nipblb* expression in fin mesenchyme (me) rather than apical ectoderm (ec). Expression is also detected in endoderm (en) and neural tube (nt) but not somites (so). (EPS)

**Figure S2** Reduced pectoral fins in Nipbl-deficient embryos. Morphologies of live control (A, B, E, F) and Nipbl-deficient embryos (C, D, G, H) at 60 hpf (A–D) and 76 hpf (E–H). Dorsal (A, C, E, G) and lateral (B, D, F, H) views, anterior to the left. Pectoral fins are reduced in Nipbl-deficient embryos (arrows) and anterior ends of lower jaws are indicated (arrowheads). (EPS)

**Figure S3** Nipbla and Niplb act cooperatively in pectoral fin development. Morphologies of live control (A), Nipbl-deficient (B) and...
embryos injected with either nipbla-MO (C, D) or nipblb-MO (E, F) at indicated amounts. Dorsal views, anterior to the left. (G) Whisker plots of fin length (medians): 431.8 μm, n = 20 (control), 259.1 μm, n = 40 (nipbla/b-MO), 385.3 μm, n = 40 (nipbla-MO, 0.75 ng), 351.0 μm, n = 40 (nipbla-MO, 1.5 ng), 417.6 μm, n = 40 (nipblb-MO, 0.75 ng), and 399.7 μm, n = 20 (nipblb-MO, 1.5 ng). *: p<0.001.

Figure S4  Small fin phenotypes could be caused by reduced proliferation rather than cell death of fin mesenchymal cells. (A, B) Cell death in pectoral fins of control (A) and Nipbl-deficient larvae (B) at 120 hpf was examined by TUNEL assay. (C-E) Cell death was determined by student t-test. *: p<0.001.

Figure S5  Staging embryos by posterior lateral line primordium position. (A) Posterior lateral line (pLL) primordia (red arrows) detected by ISH for fgf10a at indicated stages move progressively posterior relative to pectoral fin buds (yellow arrows) from 22–48 hpf, indicating a 3–4 hour and 6 hour developmental delay in Nipbl-deficient embryos at 36 hpf and 48 hpf, respectively. Duplicated signals at the tail tips in some panels reflect dorsoventral bifurcation of the tail, rather than ectopic expression of fgf10a, which is a typical phenotype observed in Nipbl-deficient embryos [11]. Lateral views. (B) Summary of stage-match comparisons between control and Nipbl-deficient embryos.

Figure S6  Rescue of AER-fgf gene expression in pectoral fin buds of Nipbl-deficient embryos by exogenous nipbla mRNA. Expression of the AER fgf genes, fgf16 and fgf8a, in pectoral fin buds (arrows) of controls, embryos injected with nipbla/b-MOs, and those co-injected with nipbla/b-MO and 400 pg of nipbla mRNA was examined by ISH at 48 hpf. Dorsal views, anterior to the top.

Figure S7  Expression of genes in the fgf10a signaling pathway is unaffected in Nipbl-deficient embryos. Expression of fgf10a (A; arrows), tbx5a (B) and fgf24 (C) in control and Nipbl-deficient embryos was examined by ISH at indicated stages. fgf10a-expressing pLL primordia are marked by asterisks. Dorsal views, anterior to the top.

Figure S8  Rescue of mesenchymal gene expression in pectoral fin buds of Nipbl-deficient embryos by exogenous nipbla mRNA. Expression of genes in the shha/hand2/5′-hox gene cassette at 36 hpf in pectoral fin buds of controls, embryos injected with nipbla/b-MOs, and those co-injected with nipbla/b-MO and 400 pg of nipbla mRNA was examined by ISH. Dorsal views, anterior to the top.

Figure S9  Expression of genes of the RA signaling pathways is unaffected in Nipbl-deficient embryos. Expression of the RA synthesizing enzyme aldh1a2 and the RA-degrading enzyme cyp26a1, a target of the RA signaling, at 15 (upper) and 19 (lower) hpf in Nipbl-deficient embryos. Anterior somites are indicated by brackets. Dorsal views, anterior to the top.

Figure S10  Expression of hox genes along the anterior-posterior axis of the neural tube is unaffected in Nipbl-deficient embryos. hox gene expression at 36 hpf examined by ISH. Lateral views, anterior to the left. Pectoral fin buds are indicated by black arrows. Anterior and posterior limits of hox expression in the neural tube are indicated by red arrowheads.

Figure S11  Gene expression in pectoral fin buds of CyA-treated embryos. (A) Effects of CyA treatment on development was examined by A-P positioning of pLL primordial expressing fgf10a. Lateral views with anterior to the left. Pectoral fin buds (yellow arrows) and pLL primordial (red arrows) are pointed. Expression of fgf10a in pLL primordial and pectoral fin buds is reduced by CyA treatment and becomes undetectable in pLL primordia by 36 hpf. (B) Expression of mesenchymal genes in pectoral fin buds was examined by ISH in possible stage-match comparisons. Dorsal views, anterior to the top.

Figure S12  Analysis of expression of all mouse Hox genes in wildtype and Nipbl+/− hindlimb buds. (A-D) Expression values show hybridization intensity for probe sets representing all of genes in the four Hox clusters (although the relationship between hybridization intensity and transcript abundance is not necessarily the same for different probesets, intensity gives a rough sense of abundance). Data are graphed as mean ± SEM for the mutant (red) and wildtype (blue) samples (see Experimental Procedures). Asterisks indicate genes also shown in Table 1, for which expression changes were observed with strong or moderate statistical significance (%<FDR<7.5%; double asterisk) or weak statistical significance (FDR<25%; single asterisk); filled arrows show the directions in which expression changes were observed. Open arrows show directional changes that were also tested and confirmed by Q-RT-PCR (see Table 1), whereas “n.e.” marks genes that were tested by Q-RT-PCR and showed no detectable change in expression. The open arrow by HoxD8 is marked with a question mark because Q-RT-PCR showed a 27% elevation in expression in Nipbl+/−/− hindlimb buds, but the result was not statistically significant (P=0.14). The tight error bars on the microarray data for most transcripts, and the confirmation of all significant results by PCR, justify the ability to attach statistical significance even to these modest effect sizes. Overall, the data illustrate that expression changes in Nipbl−/− limb buds are biased toward the 5′ ends of the Hox clusters, and suggest that, as in zebrafish, effects occur at all of the expressed clusters (HoxA, C and D). In the HoxD cluster, which exhibits the highest overall hybridization intensities, the largest fold decreases are seen at the extreme 5′ end (Hoxd12, Hoxd13), and give way, as one moves in the 3′ direction, to a weak decrease (Hoxd11) and then a statistically significant increase (Hoxd10, and possibly Hoxd8). Thus, even though these effects are much smaller in magnitude than in zebrafish (where nipbl knockdown is likely much greater than in Nipbl−/− mice), they appear to show similar positional trends. (E-F) Whole mount in situ hybridization for Hoxd12 (E) and Hoxd13 (F) in E10.5 limb buds of Nipbl−/− and wildtype mice. For both Hox genes, expression is reduced significantly in both
forelimbs and hindlimbs, consistent with the results of microarray analysis and Q-RT-PCR (panels A–D, and Table 1). (EPS)

Figure S13 Expression of genes in the fgf10a pathways is unaffected in Med12-deficient embryos. Expression of fgf24 (30, 48 hpf) and fgf10a (36 hpf) in Med12-deficient embryos examined by ISH. Dorsal views, anterior to the top. (EPS)

Figure S14 Expression of med12 in Nipbl-deficient embryos. Expression of med12 in embryos injected with niplba/b-MOs was examined by RT-PCR at 6 and 36 hpf. ef1a was used as a control. (EPS)

Figure S15 Developmental delay of Rad21-deficient embryos. Effects of Rad21-deficiency (rad21-MO) at 2.5 ng/embryo) on embryonic development were examined by A-P positions of pLL primordia by ISH for fgf10a. Lateral views, anterior to the left. Pectoral fin buds (yellow arrows) and pLL primordial (red arrows) are pointed. Expression of fgf10a in pLL primordial and pectoral fin buds of Rad21-deficient embryos were detected at levels significantly lower than control and lost by 54 hpf. (EPS)

Figure S16 Expression of genes in pectoral fin buds of Rad21-deficient embryos. Effects of Rad21 reduction of expression of mesenchymal genes in pectoral fin buds were examined by ISH in possible stage-match comparisons. Dorsal views, anterior to the top. (EPS)

Figure S17 Chromosome conformation around the hoxd1 locus in hindbrain. (A, B) Effects of Nipbl- and Med12-reduction on a higher-order chromatin conformation in hindbrain was examined by 3D-FISH at 38 hpf. Interprobe distances between (A) hoxd1 and 3’ probes and (B) hoxd1 and 5’ probes shown by Whisker plots. Details of medians, numbers of nuclei and embryos, and p-values are shown in Table 2. Dotted lines indicate thresholds for separated (upper) and closed (lower) signals in Table 2. (EPS)

Acknowledgments
We are grateful to Shimako Kawauchi and Rosayela Santos for assistance in obtaining, dissecting, and staging mouse embryo limb buds for expression microarray analysis, and Marissa Macchietto for help with some ISH experiments. We thank Taifin Zhang and Ines Gehring for fish care. Microarray analysis was performed at the UCI Genomics High-Throughput Facility.

Author Contributions
Conceived and designed the experiments: AM ALC ADL TFS. Performed the experiments: AM SI MELB. Analyzed the data: AM SI MELB YK ALC ADL TFS. Contributed reagents/materials/analysis tools: YK ALC ADL TFS. Wrote the paper: AM ALC ADL TFS.

References
1. Peters JM, Tedeschi A, Schmitz J (2008) The cohesin complex and its roles in chromosome biology. Genes Dev 22: 3089–3114.
2. Renaseuro S, Losada A (2013) Cohesin, a chromatin engagement ring. Curr Opin Cell Biol 25: 63–71.
3. Kawasuchi S, Calof AL, Santos R, Lopez-Burks ME, Young CM, et al. (2009) Multiple Organ System Defects and Transcriptional Dysregulation in the Nipbl+/− Mouse, a Model of Cornelia de Lange Syndrome. PLoS Genet 5: e1000650.
4. Liu J, Zhang Z, Bando M, Itoh T, Deardorff MA, et al. (2009) Transcriptional Functions of Sister Chromatid Cohesion Proteins on cut gene expression during wing development in Drosophila. Development 132: 4743–4753.
5. Wenda KS, Yoshihara K, Itoh T, Bando M, Koch B, et al. (2008) Cohesin mediates transcriptional insulation by CTCF/binding factor. Nature 451: 796–801.
6. Parello V, Hadjur S, Spivakov M, Leleu M, Sauer S, et al. (2008) Cohesins Functionally Associate with CTCF on Mammalian Chromosome Arms. Cell 132: 422–433.
7. Rhodes JM, Bentley FK, Print CG, Dorsett D, Misulovin Z, et al. (2010) Chromatin regulatory archipelago controls Hox genes transcription in digits. Cell 147: 1132–1145.
8. Amano T, Sagai T, Tanabe H, Mizushina Y, Nakazawa H, et al. (2009) Chromosomal dynamics at the Shh locus: limb buds-specific differential regulation of competence and active transcription. Dev Cell 16: 47–57.
9. Montavon T, Noshikoyna N, Mascrez B, Joye E, Thevenet L, et al. (2011) A regulatory archipelago controls Hox genes transcription in digits. Cell 147: 1132–1145.
10. Ferrai C, Pombo A (2009) 3D chromatin regulation of Sonic hedgehog in the limb buds. Dev Cell 16: 9–11.
11. Muto A, Calof AL, Lander AD, Schilling TF (2011) Multifactorial Origins of Lange Syndrome. PLoS Biol 9: e1001181.
12. Dorsett D, Strom L (2012) The ancient and evolving roles of cohesin in gene transcriptional activation. Mol Cell 43: 422–433.
13. Chien R, Zeng W, Kawauchi S, Bender MA, Santos R, et al. (2009) Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell 22: 43–53.
14. Akoulitchev S, Chiaulev S, Reinberg D (2000) TFIIH is negatively regulated by cdk7-containing mediator complexes. Nature 407: 102–106.
15. Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422: 198–207.
16. Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422: 198–207.
17. Kagey MH, Newman JJ, Blodeau S, Zhan Y, Orlando DA, et al. (2010) Mediator and cohesin connect gene expression and chromatin architecture. Nature 467: 430–435.
18. Potts J, Sawyer AM (2006) Controlling Mediator function. Curr Opin Genet Dev 16: 199–206.
19. Amano T, Sagai T, Tanabe H, Mizushina Y, Nakazawa H, et al. (2009) Chromosomal dynamics at the Shh locus: limb buds-specific differential regulation of competence and active transcription. Dev Cell 16: 47–57.
20. Amano T, Sagai T, Tanabe H, Mizushina Y, Nakazawa H, et al. (2009) Chromosomal dynamics at the Shh locus: limb buds-specific differential regulation of competence and active transcription. Dev Cell 16: 47–57.
21. Amano T, Sagai T, Tanabe H, Mizushina Y, Nakazawa H, et al. (2009) Chromosomal dynamics at the Shh locus: limb buds-specific differential regulation of competence and active transcription. Dev Cell 16: 47–57.
22. Amano T, Sagai T, Tanabe H, Mizushina Y, Nakazawa H, et al. (2009) Chromosomal dynamics at the Shh locus: limb buds-specific differential regulation of competence and active transcription. Dev Cell 16: 47–57.
Mercader N, Fischer S, Neumann CJ (2006) Prdm1 acts downstream of a
57. Yelon D, Ticho B, Halpern ME, Ruvinsky I, Ho RK, et al. (2000) The bHLH
56. Sakamoto K, Onimaru K, Munakata K, Suda N, Tamura M, et al. (2009)
52. Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, et al. (1997) The
47. Grandel H, Schulte-Merker S (1998) The development of the paired fins in the
46. Grandel H, Draper BW, Schulte-Merker S (2000) dackel acts in the ectoderm
45. Prykhozhij SV, Neumann CJ (2008) Distinct roles of Shh and Fgf signaling in
44. Niederreither K, Vermot J, Schuhbaur B, Chambon P, Dolle P (2002)
38. Mercader N (2007) Early steps of paired fin development in zebrafish
36. Dorsett D, Krantz ID (2009) On the Molecular Etiology of Cornelia de Lange
35. Deardorff MA, Bando M, Nakato R, Watrin E, Itoh T, et al. (2012) HDAC8
34. Deardorff MA, Kaur M, Yaeger D, Rampuria A, Korolev S, et al. (2007)
33. whistler L, Selfridge EH, Kimmel CB (1988) Early steps of paired fin development in zebrafish and mouse. Development 129: 973–984.
32. Sasaki S, Sato H, Okada Y, Dahiya S, Kato K, et al. (2006) Transient engagement of the cohesin complex is essential for mouse pectoral fin development. Dev Biol 298: 9–18.
31. Misulovin Z, Schwartz YB, Li X-Y, Kahn TG, Gause M, et al. (2008) Mutations in Cohesin Complex Members SMC3 and SMC1A Cause a Mild Developmental Retardation. Am J Hum Genet 80: 485–494.
30. Niederreither K, Melki R, Bienz M (1997) Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development. Mech Dev 62: 175–182.
29. Williams TM, Williams ME, Kuick R, Misek D, Mitiku K, et al. (2005) Candidate downstream regulated genes of Hox group 13 transcription factors with and without monosomic DNA binding capability. Dev Biol 279: 462–480.
28. Shiozaki H, van der Reijden MI, Ye Z, Kolovos P, et al. (2014) Cohesin associates with transcriptionally active regions of the Drosophila melanogaster genome. Chromosoma 117: 89–102.
27. De Marre LE, Jeng J, Cotney J, Reilly SK, Yin J, et al. (2013) The genomic landscape of cohesin-associated chromatin interactions. Genome Res 23: 1224–1234.
26. Ahn D, Ho RK (2008) Tri-phasic expression of posterior Hox genes during the onset of mouse limb bud development. PLoS Genet 4: e1000901.
25. Kopan R, Ilagan MX, Hui CC, Liu J, Young RA (2000) The Hippo pathway regulates organ size and induces apoptosis. Nature 408: 202–207.
24. Shin CH, Chung WS, Hong SK, Ober EA, Verkade H, et al. (2009) Multiple roles for Med12 in vertebrate endoderm development. Dev Biol 331: 467–479.
23. Woltering JM, Noordermeer D, Leleu M, Duboule D (2014) Conservation and divergence of regulatory strategies at hox Loci and the origin of tetrapod digits. PLoS Biol 12: e1001773.
22. Wieland T, Essery F, Frasch M, Hertweck C, et al. (2010) Comment on: "Mutations in Cohesin Complex Members SMC3 and SMC1A Cause a Mild Developmental Retardation" [Am J Hum Genet 80: 485-494]. Am J Hum Genet 87: 180-181.
21. Visel A, van Oudenaarden A, Li J, Grun D, et al. (2010) Whole-genome binding of the cohesin complex. Nature 464: 363-367.
20. De Inverno A, Ubeda M, Ullrich A, Yancopoulos GD, et al. (2003) A new activator for Sonic hedgehog signaling. Nature 426: 383-389.
19. Verheugen P, Vriend G, van der Sluijs C, van der Geer P (2011) Structure similarity searching without sequences. Nat Rev Genet 12: 664-676.
18. Wehrs T, MacArthur PC, Kustanovich B, Kim J, et al. (2010) Cohesin associates with transcriptionally active regions of the Drosophila melanogaster genome. Chromosoma 117: 89–102.
17. Cano DA, del Rio C, Aguado D, Perez-Alonso F, et al. (2006) A switch between topological domains underlies HoxD gene collinearity in mouse limbs. Science 340: 1241617.
16. Schubert CA, Pongor S, Hebrok M, Beachy PA, et al. (2001) Conservation of chromatin organization in the interphase nucleus. Proc Natl Acad Sci U S A 108: 12782–12786.
15. Schneider I, Aneas I, Gehre AR, Dahn KD, Nogeria MA, et al. (2011) Appendage expression driven by the Hoxd13 global control region is an ancient gnathostome feature. Proc Natl Acad Sci U S A 108: 12782–12786.
14. Putnam NW, Waterston RH, Venter JC, et al. (2002) The genome sequence of Drosophila melanogaster. Science 298: 1304-1351.
13. Wolf E, Hofer S, Wildbichler P, Deininger P, et al. (2010) Human limb abnormalities caused by disruption of hedgehog signaling. Trends Genet 26: 364-373.
12. de Ligt J, Hoeijmakers JHJ, van Oers JM, et al. (2009) Spatially confined folding of chromatin in the interphase nucleus. Proc Natl Acad Sci U S A 106: 3812–3817.
11. Schneider I, Aneas I, Gehre AR, Dahn KD, Nogeria MA, et al. (2011)Appendage expression driven by the Hoxd13 global control region is an ancient gnathostome feature. Proc Natl Acad Sci U S A 108: 12782–12786.
10. Visel A, van Oudenaarden A, Li J, Grun D, et al. (2010) Whole-genome binding of the cohesin complex. Nature 464: 363-367.
9. Wietzerbin J, Hadjantonakis AK, Le Meur E, et al. (2003) The role of mesenchymal cells in the expression of the Hoxd13 boundary. Development 130: 1013–1023.
8. Winter J, Jabaudon-Dubuisson D, Monfils JD, et al. (2006) Cohesin regulates Hox gene expression in the mouse forelimb by recruiting Mediator. Dev Cell 11: 613-625.
7. Jones RA, Sefton BM, Hannon GJ, Weinberg RA (2000) Hippo, a conserved guardian of organ size. Cell 103: 857-868.
6. Yang Z, Li X-Y, Hacia JG, et al. (2010) The cohesin complex associates with transcriptionally active regions of the Drosophila melanogaster genome. Chromosoma 117: 89–102.
5. Fradis M, Sellitto M, Sciutti D, et al. (2001) Human limb abnormalities caused by disruption of hedgehog signaling. Trends Genet 26: 364-373.
4. Wulfkuhle JS, Poirier P, Obenchain S, et al. (2004) The cohesin complex associates with transcriptionally active regions of the Drosophila melanogaster genome. Chromosoma 117: 89–102.
3. search for enhancer sequences in the context of tissue-specific gene expression. Nat Rev Genet 12: 283–293.
2. Wieland T, Essery F, Frasch M, Hertweck C, et al. (2010) Comment on: "Mutations in Cohesin Complex Members SMC3 and SMC1A Cause a Mild Developmental Retardation" [Am J Hum Genet 80: 485-494]. Am J Hum Genet 87: 180-181.
1. search for enhancer sequences in the context of tissue-specific gene expression. Nat Rev Genet 12: 283–293.
genotype-phenotype correlations in Cornelia de Lange Syndrome. Hum Mutat 31: 1216–1222.

94. Westerfield M (1995) The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). Eugene, OR: University of Oregon Press.

95. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310.

96. Kawachi S, Takahashi S, Nakajima O, Ogino H, Morita M, et al. (1999) Regulation of lens fiber cell differentiation by transcription factor c-Maf. J Biol Chem 274: 19254–19260.

97. Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, et al. (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75: 1417–1430.

98. Shepard JL, Stern HM, Pfaff KL, Amatruda JF (2004) Analysis of the cell cycle in zebrafish embryos. Methods Cell Biol 76: 109–125.

99. Solovei I, Grasser F, Lancet C (2007) FISH on Histological Sections. CSH Protoc 2007: pdb prot4729.

100. Muller S, Neuser M, Kohler D, Cremer M (2007) Preparation of Complex DNA Probe Sets for 3D FISH with up to Six Different Fluorochromes. CSH Protoc 2007: pdb prot4730.

101. Itou J, Taniguchi N, Oishi I, Kawakami H, Lotz M, et al. (2011) HMGB factors are required for posterior digit development through integrating signaling pathway activities. Dev Dyn 240: 1151–1162.

102. Capellini TD, Di Giacomo G, Sahi V, Brendolan A, Ferretti E, et al. (2006) Phox1/Phox2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression. Development 133: 2263–2273.

103. Sahi V, Viganò MA, Cocchiarella F, Mantovani R, Zappavigna V (2008) Hoxd13 binds in vivo and regulates the expression of genes acting in key pathways for early limb and skeletal patterning. Dev Biol 317: 497–507.

104. Lettice LA, Williamson I, Wiltshire JH, Pelsue S, Devenney PS, et al. (2012) Opposing functions of the ETS factor family define Shh spatial expression in limb buds and underlie polydactaly. Dev Cell 22: 459–467.

105. Liu Z, Lavine KJ, Hung IH, Ornitz DM (2007) FGF18 is required for early chondrocyte proliferation, hypertrophy and vascular invasion of the growth plate. Dev Biol 302: 90–91.

106. Hung IH, Yu K, Lavine KJ, Ornitz DM (2007) FGF9 regulates early hypertrophic chondrocyte differentiation and skeletal vascularization in the developing stylopod. Dev Biol 307: 300–313.

107. Pandur P, Lasche M, Eisenberg LM, Kuhl M (2002) Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 418: 636–641.

108. Darken RS, Wilson PA (2001) Axis induction by wnt signaling: Target promoter responsiveness regulates competence. Dev Biol 234: 42–54.

109. Yamamoto S, Nishimura O, Misaki K, Nishita M, Minami Y, et al. (2006) Cthr1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt-receptor complex. Dev Cell 15: 23–36.

110. Nam JS, Park E, Turcotte TJ, Palencia S, Zhan X, et al. (2007) Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. Dev Biol 311: 124–135.