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# Essential role of lysyl oxidases in notochord development

**John M. Gansner**[1], **Bryce A. Mendelsohn**[1], **Keith A. Hultman**[2], **Stephen L. Johnson**[2], and **Jonathan D. Gitlin**[1,2,†]

1 *Department of Pediatrics, Washington University School of Medicine St. Louis, Missouri 63110*

2 *Department of Genetics, Washington University School of Medicine St. Louis, Missouri 63110*

## Abstract

Recent studies reveal a critical role for copper in the development of the zebrafish notochord, suggesting that specific cuproenzymes are required for the structural integrity of the notochord sheath. We now demonstrate that β-aminopropionitrile, a known inhibitor of the copper-dependent lysyl oxidases, causes notochord distortion in the zebrafish embryo identical to that seen in copper deficiency. Characterization of the zebrafish lysyl oxidase genes reveals eight unique sequences, several of which are expressed in the developing notochord. Specific gene knockdown demonstrates that loss of *loxl1* results in notochord distortion, and that *loxl1* and *loxl5b* have overlapping roles in notochord formation. Interestingly, while notochord abnormalities are not observed following partial knockdown of *loxl1* or *loxl5b* alone, in each case this markedly sensitizes developing embryos to notochord distortion if copper availability is diminished. Likewise, partial knockdown of the lysyl oxidase substrate *col2a1* results in notochord distortion when combined with reduced copper availability or partial knockdown of *loxl1* or *loxl5b*. These data reveal a complex interplay of gene expression and nutrient availability critical to notochord development. They also provide insight into specific genetic and nutritional factors that may play a role in the pathogenesis of structural birth defects of the axial skeleton.

### Keywords

zebrafish; notochord; lysyl oxidase; copper; nutrition; disease model; col2a1

## Introduction

Structural birth defects are a leading cause of morbidity and mortality in humans. Despite recent advances identifying the molecular genetic basis of several such disorders, the genetic and environmental determinants of most structural birth defects remain unknown (Epstein, 1995). One of the most important environmental factors influencing the outcome of fetal development is nutrition, and epidemiologic data, twin studies, and phenotype-genotype correlations all suggest that specific nutritional influences in combination with genetic susceptibility at multiple loci have profound long-term effects on pregnancy outcome (Miles et al., 2005). While *in utero* development severely limits experimental elucidation of the mechanisms and timing of critical events affected by nutrition during early embryonic development, recent studies

†Corresponding author: Jonathan D. Gitlin, M.D., Edward Malinckrodt Department of Pediatrics, Washington University School of Medicine, 660 South Euclid Avenue, Box 8208, St. Louis, Missouri 63110, Phone: (314) 286-2846; Fax: (314) 286-2784; Email: gitlin@wustl.edu.

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suggest that the zebrafish may be an informative, genetically tractable model organism for such analysis (Jensen et al., 2006; Mendelsohn et al., 2006; Taylor et al., 2004).

The notochord is a useful structure for examining the complex interplay of genes and nutrition in early vertebrate development. This organ is readily visible throughout early zebrafish development, is the first to fully differentiate during embryogenesis, and is essential for the patterning of surrounding ectodermal, mesodermal, and endodermal tissues (Scott and Stemple, 2005). The notochord is also a critical midline structure required for locomotion in some chordates, and for axial skeletal formation in vertebrates (Scott and Stemple, 2005). Furthermore, the cell biological mechanisms underlying notochord maturation, involving cell vacuolation, matrix biosynthesis, and sheath formation, have begun to be elucidated through a series of elegant genetic experiments (Coutinho et al., 2004; Odenthal et al., 1996; Parsons et al., 2002; Stemple et al., 1996). Given the critical role of the notochord in chondrogenesis and fate determination of surrounding tissues including neural tube, heart, and skeleton (Stemple, 2005), as well as the large number of human structural birth defects arising from these tissues, elucidation of the nutritional and genetic mechanisms of notochord formation may be of direct relevance to our understanding of the etiology of congenital malformations.

During studies examining the role of copper in embryonic development, we discovered a critical role for this nutrient in late notochord formation (Mendelsohn et al., 2006). These findings suggested that specific cuproenzymes may be required for the structural integrity of the zebrafish notochord sheath, a concept supported by recent studies in *Xenopus laevis* (Geach and Dale, 2005). In this current study we have determined a role for two specific lysyl oxidase genes in notochord formation, and defined a complex biological pathway modulated by copper nutrition that underlies this process. Our data suggest that copper status during embryonic development should be interpreted in the context of environmental and genetic modulators of cuproenzyme activity. Indeed, the results raise the intriguing possibility that suboptimal copper availability, due either to nutritional or genetic variation during embryonic development, contributes to human birth defects of the axial skeleton.

## Materials and methods

### Zebrafish strains and maintenance

Zebrafish were reared under standard conditions at 28.5°C (Westerfield, 1993) and staged as described (Kimmel et al., 1995). Synchronous, *in vitro* fertilized embryos were obtained from AB or AB/WIK stocks for all experiments.

### Pharmacologic treatment

Pharmacologic compounds were purchased from Sigma (St. Louis, MO). β-aminopropionitrile (A3134) and neocuproine (N1501) were prepared as 100 mM stocks in egg water and dimethyl sulfoxide, respectively, and diluted in egg water. Embryos were incubated in compound starting at 3 hpf and examined with an Olympus SZX12 zoom stereomicroscope at 24, 30, 48, and 72 hpf. Images of representative fish mounted in 2% methylcellulose were acquired with an Olympus DP70 camera.

### Identification and annotation of zebrafish lysyl oxidase genes

Exons encoding conserved regions of the lysyl oxidase catalytic domain (Kagan and Li, 2003) were detected by TBLASTN search of the Ensembl zebrafish genome (Zv5). A clone of *lox* was commercially available (Open Biosystems #6971279); full-length cDNA sequences of all other family members were obtained using 5′ and 3′ rapid amplification of cDNA ends (RACE) and RT-PCR based on sequence homology to human, mouse, and frog lysyl oxidase sequences. Primers were as follows: *loxl1* forward primer 5′-

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CGCCTTCTTTTATTAGTCTTCTGG-3′ and reverse primer 5′-ACAGCGACGTCAGGAATTCC-3′; *loxl2a* – forward primer 5′-ATGGCGGTGTCTTCTGCATTGTGC-3′ and reverse primer 5′-CTATCTGAGGTGGTTCAGCTGGTTGC-3′; *loxl2b* – forward primer 5′-GCCATACAATCTGAGCCTCTGTC-3′ and reverse primer 5′-CTTTACCTGTGGGTCACCTGG-3′; *loxl3a* – forward primer 5′-ATGGGACAGTTTGCTAACAGC-3′ and reverse primer 5′-TTATGAGATCTTGTTGTTGAGCTGCC-3′; *loxl3b* – forward primer 5′-GTGTTTGTGTCCTTTGATGC-3′ and reverse primer 5′-GCTGTACATGAAGAGTGATCT-3′; *loxl5a* – nested 5′RACE primers 5′-CACTCGAGCTGTACGCTGAACTGGAAAGGC-3′ (first reaction) and 5′-GCACAGGGCTTGAACGCACCACATGCCC-3′ (second reaction), and 3′ PCR amplification with forward primer 5′-CATTAAGATGTGCCGCAGAGG-3′and reverse primer 5′-CTCACCCGGTGATCCTACAGTTG-3′; *loxl5b* – forward primer 5′-CCCACATCCAGAGGAGCGAA-3′ and reverse primer 5′-TCCTCACCCAGTTATGATGCAG-3′. GenBank accession numbers are: *lox* – **EF030479**; *loxl1* – **EF030480**; *loxl2a* – **EF030481**; *loxl2b* – **EF030482**; *loxl3a* – **EF030483**; *loxl3b* – **EF030484**; *loxl5a* – **EF030485**; *loxl5b* – **EF030486**. An alignment of the C-terminal portion of zebrafish and human lysyl oxidases was created in ClustalW with human CD163 as outgroup (Chenna et al., 2003), and phylogenetic analysis carried out according to the parsimony method (Felsenstein, 2005). Bootstrap values over 100 replicates are noted. Signal peptides and scavenger-receptor, cysteine rich domains were predicted using SignalP 3.0 and Motif scan (Bendtsen et al., 2004; Pagni et al., 2004). Copper binding domains, Bone Morphogenic Protein-1 (BMP-1) cleavage sites, and lysyl-tyrosyl quinone cofactor residues were identified based on homology to known lysyl oxidase sequences (Borel et al., 2001; Csiszar, 2001; Panchenko et al., 1996).

### Whole mount in situ hybridization and frozen sections

Lysyl oxidase and *col2a1* (Yan et al., 1995) probe constructs were generated as partial clones by RT-PCR and ligated into pCRII (Invitrogen). DIG-labeled antisense RNA probes were synthesized from these constructs using a DIG-labeling kit (Roche), and whole mount in situ hybridization performed as previously described (Mendelsohn et al., 2006; Thisse et al., 1993). Embryos for frozen sections were subsequently embedded in PBS containing 1.5% agarose/5% sucrose. Blocks were equilibrated in 30% sucrose at 4°C, mounted with O.C.T. (Tissue-Tek), and cut on a Leica Cryostat after freezing with liquid nitrogen. 14 μM sections were collected on Superfrost slides (Fisher) and mounted in 50% glycerol-PBS for visualization on an Olympus BX60 microscope.

### Morpholino and mRNA injections

Morpholino oligonucleotides (Nasevicius and Ekker, 2000) targeting splice sites of exons encoding the lysyl oxidase copper binding domain (Csiszar, 2001) were resuspended in Danieau buffer, diluted to include 0.05% phenol red, and injected into one- to four-cell embryos. Morpholinos targeting the start sites of *loxl1* and *loxl5b*, the 5′ splice acceptor site of an exon corresponding to exon 48 of the human *col2a1* orthologue, and standard control morpholino (Gene Tools, LLC) were likewise prepared and injected. Morpholino sequences are as follows: *loxl1* (splice) – 5′-GTGTAGATGTGGACTCACTGATGGC-3′; *loxl1* (splice) – 5′-GTAATGCCTGATGGAGACAAGAGAC-3′ (Fig. 7 and Fig. 8); *loxl1* (start) – 5′-AGTACATGCAGCATATTGAGAAGAC-3′; *loxl2b* – 5′-GATCTGGAGCAGCTAGAAAAAACAA-3′; *loxl3b* – 5′-CAGCTGCGGACATAAACAAACAAAT-3′; *loxl5b* (splice) – 5′-GCCTGTGGAATAAACACCAGCCTCA-3′; *loxl5b* (start) – 5′-TAAAGCTGTATGATTCGCTCCTCTG-3′; *col2a1* – 5′-

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CCTGAAGGTCCCTATTATAAATAAC-3′. Capped, polyadenylated mRNA for rescue experiments was generated from full-length clones of *loxl1*, *loxl5b*, and control transposase (Kawakami et al., 2004) using the mMESSAGE mMACHINE kit (Ambion), with 300 pg of mRNA injected per embryo. Rescue was calculated by taking the difference between the percentages of embryos with distorted notochords injected with lysyl oxidase and control mRNA, and then dividing this number by the percentage of embryos with distorted notochords injected with control mRNA. Statistical analysis was conducted using one-way ANOVA for independent samples based on the percentages of embryos with the distorted notochord phenotype from at least three separate experiments. In all cases, abnormal splicing was detected by RT-PCR of RNA from 15 embryos injected with control or lysyl oxidase morpholino using primers to exons flanking the putative splice site. Nucleotide sequencing of putative splice products identified on agarose or polyacrylamide gels confirmed the presence of a premature stop codon in the splice variants. The intensity of wild-type PCR products was quantified using non-saturated gel pictures and ImageJ 1.37v software (Rasband, 1997–2006), and the percentage of wild-type splice form remaining after morpholino knockdown calculated.

### Transmission Electron Microscopy

Dechorionated embryos were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate and sequentially stained with osmium tetroxide and uranyl acetate. They were then dehydrated, embedded in PolyBed 812, and thin sectioned on a Reichert-Jung Ultra-Cut. Slices were post-stained in 4% uranyl acetate and lead citrate, and viewed on a Zeiss 902 electron microscope. Photographs were recorded with Kodak EM film.

## Results

### β-aminopropionitrile recapitulates the notochord phenotype of copper deficiency

Copper deficiency, induced by either chemical (neocuproine) or genetic ($calamity^{vu69}$) means, results in a pleiotropic phenotype that includes a distorted, wavy notochord (Mendelsohn et al., 2006). Lysyl oxidases are copper-dependent enzymes that stabilize extracellular matrix by crosslinking elastin and collagens (Csiszar, 2001; Kagan and Li, 2003), a process thought to be important for maintaining notochord sheath integrity during notochord vacuolation. β-aminopropionitrile irreversibly inhibits lysyl oxidases by binding the active site of the catalytic domain (Molnar et al., 2003; Tang et al., 1983), and incubation of 3 hpf zebrafish embryos with β-aminopropionitrile resulted in striking notochord distortion and blunted somites, similar to what is observed with the copper chelator neocuproine (Fig. 1 and S. Fig. 1). Equivalent results were also obtained after incubation with semicarbazide, another known inhibitor of lysyl oxidases (data not shown), suggesting that these enzymes play a central role in notochord formation. β-aminopropionitrile treatment does not result in the pleiotropic phenotype of copper deficiency, including absence of melanin pigmentation, midbrain-hindbrain degeneration, and loss of red blood cells (Fig. 1B vs. 1C), reflecting the broader scope of cuproenzyme inhibition achieved with neocuproine. Furthermore, the effect of β-aminopropionitrile on notochord morphology was not due to copper chelation as this phenotype was not reversible with $CuCl_2$ (data not shown). Although the notochord distortion observed with β-aminopropionitrile in Figure 1 is less than that with neocuproine, the mechanisms resulting in this process are likely the same because ten-fold higher doses of β-aminopropionitrile result in a degree of distortion identical to that seen with neocuproine (S. Fig. 1).

### The zebrafish genome encodes eight lysyl oxidases

These data suggested a requirement for lysyl oxidase activity in zebrafish notochord development. The human genome encodes five lysyl oxidase family members characterized by the presence of a conserved copper binding domain and residues for a lysyl-tyrosyl quinone

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cofactor. Although experimental evidence suggests a role for two of these lysyl oxidases in elastin and collagen crosslinking (Hornstra et al., 2003; Liu et al., 2004; Maki et al., 2002; Maki et al., 2005), the precise role of each family member in extracellular matrix formation, cancer biology, and intracellular signaling remains undetermined. Using a bioinformatic approach, we identified and cloned eight unique zebrafish lysyl oxidase genes, revealing that each human lysyl oxidase has a zebrafish orthologue except LOXL4 (Fig. 2 and S. Fig. 2). While lysyl oxidase genes are well conserved between humans and zebrafish based on nucleotide sequence alignment, Loxl3a and Loxl3b lack a signal peptide and the full complement of scavenger receptor domains found in human LOXL3 (Fig. 2A and S. Fig. 2). Our analysis also revealed two novel zebrafish lysyl oxidases, Loxl5a and its paralogue Loxl5b, that are closely related to LOX and LOXL1 by phylogenetic analysis but have been not previously described in other species (Fig. 2B and S. Fig. 2A). The presence of paralogues for some zebrafish lysyl oxidases likely reflects a genome duplication event and subsequent partitioning of gene function (Lynch and Force, 2000; Woods et al., 2000).

## Four lysyl oxidases are expressed throughout the developing notochord

To elucidate the role of specific lysyl oxidases in notochord formation, we assessed lysyl oxidase mRNA expression in zebrafish embryos by whole-mount in situ hybridization. Four lysyl oxidase family members – *loxl1*, *loxl2b*, *loxl3b*, and *loxl5b* – were readily detected throughout the notochord during early zebrafish development (Fig. 3), and were specifically expressed within the vacuolar cells, as seen in frozen sections of 15 somite embryos (Fig. 4). Lysyl oxidase expression was observed as early as the 5 somite stage (Fig. 3A,M), well before notochord vacuolation begins, and was extinguished after vacuolation is complete, between 24 hpf and 48 hpf (data not shown). While *loxl1* and *loxl5b* were robustly expressed at the caudal tip (Fig. 3D,P, arrowheads), *loxl2b* and *loxl3b* were not (Fig. 3H,L), possibly reflecting a differential requirement for their activity during notochord elongation. Closer inspection revealed that *loxl5b* is expressed anterior to the notochord, in the prechordal plate region (Fig. 3O, asterix), and that *loxl1* is expressed in the hypochord (Fig. 4A, arrows), providing a new marker for this structure and suggesting a role for *loxl1* in crosslinking extracellular matrix near the developing aorta (Cleaver and Krieg, 1998;Eriksson and Lofberg, 2000). *lox*, *loxl3a*, and *loxl5a* were not expressed in or near the notochord between the 5 somite stage and 48 hpf, but could be detected by 5 dpf, while *loxl2a* was faintly and transiently expressed in a limited region of the anterior notochord before 24 hpf and was not studied further (data not shown).

## Morpholino knockdown of loxl1 or loxl1 and loxl5b together results in a distorted notochord

To determine the precise developmental functions of individual lysyl oxidases expressed throughout the notochord, we conducted knockdown experiments using morpholino antisense oligonucleotides (Fig. 5 and Table 1). Whereas control morpholino had no observable effect on developing zebrafish embryos (Fig. 5A and Table 1), a splice morpholino to *loxl1* recapitulated the notochord distortion and blunted somites observed with β-aminopropionitrile and neocuproine (Fig. 5B and Table 1), revealing that this family member is necessary for late notochord formation in zebrafish. Morpholino knockdown of *loxl2b*, *loxl3b*, and *loxl5b* induced minor changes to notochord architecture, including occasional notochord kinks (Fig. 5C–E and Table 1), and the *loxl5b* morpholino also caused caudal vein edema (Fig. 5E, arrows). In all cases, a specific effect of these splice morpholinos on lysyl oxidase gene transcription was confirmed by demonstrating abnormal splicing and generation of a premature stop codon (S. Fig. 3 and data not shown). Since very few embryos injected with morpholinos to *loxl2b*, *loxl3b*, and *loxl5b* alone developed the distorted notochord phenotype at high morpholino doses (Fig. 5C–E and Table 1), we tested all possible combinations of lysyl oxidase morpholinos. Morpholino doses of *loxl1* and *loxl5b* that did not result in notochord distortion when used alone, together recapitulated the notochord phenotype and blunted somites observed with β-aminopropionitrile and neocuproine (Fig. 5F and Table 1). This was the only morpholino

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combination resulting in a notochord phenotype (Table 1 and S. Table 1), and was similarly recapitulated with morpholinos targeting the start sites of *loxl1* and *loxl5b* (Table 1 and S. Fig. 1). Taken together, these observations indicate that these two lysyl oxidases have specific overlapping functions in notochord development. The notochord distortion elicited by combined knockdown of *loxl1* and *loxl5b* (Fig. 5F and Table 1) was specifically rescued with co-injection of either *loxl1* or *loxl5b* mRNA (Fig. 5H,I and Table 2), but not control mRNA (Fig. 5G and Table 2). Importantly, each of the lysyl oxidase splice morpholinos also resulted in head necrosis (Fig. 5B–E) that was not rescued with specific mRNA injection (data not shown), suggesting that this phenotype results from a non-specific morpholino effect. Consistent with this concept, head necrosis is not observed following treatment with either β-aminopropionitrile or neocuproine (S. Fig. 1).

## Nutrient, gene interactions in notochord distortion

Since the notochord phenotype observed with combined *loxl1* and *loxl5b* knockdown is identical to that seen with β-aminopropionitrile and neocuproine, we next examined the interaction of copper availability and lysyl oxidase gene expression in notochord formation (Fig. 6). For these experiments, doses of *loxl5b* morpholino and neocuproine were determined such that they did not cause notochord distortion when used alone (Fig. 6B,C and Table 3). In striking contrast, embryos subjected to combined treatment with these same doses of *loxl5b* morpholino and neocuproine developed distorted notochords (Fig. 6D and Table 3). Experiments using *loxl1* morpholino and neocuproine yielded similar results (Table 3), supporting the model that specific inhibition of two lysyl oxidases accounts for the notochord abnormalities observed with β-aminopropionitrile and neocuproine. Most significantly, these data also reveal that copper availability influences the phenotypic outcome of notochord development according to the genetic content of the embryo.

In this context, we also examined the role of collagen II, a structural component of the notochord sheath, in notochord formation. Collagen II is a lysyl oxidase substrate whose spatio-temporal pattern of expression corresponds with that of the notochord-expressed lysyl oxidases during early zebrafish development (Yan et al., 1995). We determined the highest dose of *col2a1* morpholino that could be injected without phenotypic abnormalities (Fig. 6E), and incubated these injected embryos in a dose of neocuproine (2 μM) that was also without effect. This combined treatment resulted in notochord distortion (data not shown), revealing the importance of copper nutrition in the context of collagen II disruption and predicting a genetic interaction between collagen II and the notochord-expressed lysyl oxidases. Consistent with this prediction, about half of the embryos injected with *loxl5b* and *col2a1* morpholinos together revealed a conspicuously distorted notochord (Fig. 6F and Table 4), demonstrating that decreased expression of *loxl5b* sensitizes embryos to *col2a1* disruption. A comparable result was also obtained using the *loxl1* and *col2a1* morpholinos (Table 4). The specificity of this effect was confirmed by detection of a splicing defect resulting from the *col2a1* morpholino that causes a premature stop codon (S. Fig. 3 and data not shown). The observation that in each case only about 50% of the embryos displayed the abnormal notochord phenotype may be due either to additional lysyl oxidase substrates that play a role in notochord formation or to submaximal disruption of *col2a1* gene expression at this morpholino dose (S. Fig. 3). Taken together, these data illustrate the complex interplay of multigenic expression and nutrition required for notochord formation in developing zebrafish embryos.

## *col2a1* expression persists in notochord vacuolar cells after lysyl oxidase inhibition

The foregoing experiments suggest that collagen II is a substrate for both Loxl1 and Loxl5b in zebrafish. As recently demonstrated (Tilton et al., 2006), *col2a1* expression persists in the vacuolar cells of the notochord at 24 hpf when embryos are treated with a dose of neocuproine that causes notochord distortion (Fig. 7B). One possible explanation for this phenomenon is

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impairment of a feedback mechanism that normally downregulates production of *col2a1* upon collagen crosslinking in the notochord sheath. Consistent with this idea, embryos treated with β-aminopropionitrile (Fig. 7C) or injected with morpholinos to *loxl1* and *loxl5b* (Fig. 7D) continued to express *col2a1* in the vacuolar cells of the notochord at 24 hpf, whereas control embryos did not (Fig. 7A). The persistent expression of *col2a1* was not due to developmental delay, as embryos added somites at the same rate under all experimental conditions (data not shown).

We next considered whether the persistent *col2a1* expression was specific to a reduction in crosslinking or was a generalized response to mechanical distortion of the notochord. To explore this idea, we incubated embryos in a dose of neocuproine (2 μM) that did not cause notochord distortion but that partially disrupted crosslinking, as revealed by the combined neocuproine and *loxl5b* studies. Persistent *col2a1* expression was observed within the notochord vacuolar cells at 24 hpf under these conditions (S. Fig. 4), similar to what is observed in neocuproine-treated embryos incubated with anesthetic to prevent notochord distortion (Tilton et al., 2006). These data are consistent with a feedback mechanism originating from the products of the crosslinking process.

The *col2a1* expression data suggest a model where lysyl oxidase inhibition affects the final stages of notochord development at a time when the extracellular matrix components, including collagen II, are already in place in the notochord sheath. The notochord sheath in zebrafish is composed of three distinct layers: an internal basal lamina, a middle fibrillar layer, and an external granular layer (Fig. 8A) (Cerda et al., 2002;Stemple, 2005). Inspection of the notochord sheath by electron microscopy revealed that inhibition of lysyl oxidase activity by neocuproine, β-aminopropionitrile, or combined *loxl1* and *loxl5b* knockdown was without effect on these layers (Fig. 8B–D). This observation is consistent with the idea that lysyl oxidase inhibition alters the final stages of notochord sheath formation.

## Discussion

These studies reveal an essential role for two specific lysyl oxidase genes, *loxl1* and *loxl5b*, in notochord formation, and demonstrate a complex interplay of gene expression and nutrient availability that is relevant to the pathogenesis of human structural birth defects (Fig. 9A,B). A role for lysyl oxidases in notochord formation was hypothesized from previous work using small molecules (Geach and Dale, 2005; Mendelsohn et al., 2006), and our current data establish a genetic basis for this pharmacologic notochord distortion. Various lysyl oxidases have been implicated in tumor metastasis, wound healing, cardiac fibrosis and elastin fiber homeostasis (Erler et al., 2006; Lau et al., 2006; Liu et al., 2004; Yu et al., 2006). However, the precise function of individual lysyl oxidase family members in development has been difficult to discern given the lack of inhibitors unique to any given enzyme. This study is the first to demonstrate that genetic deficiency of specific lysyl oxidases results in notochord distortion, and elucidates a specific and novel interaction between gene function (lysyl oxidases) and nutrition (copper) during development. While it is logical that copper-dependent enzymes such as lysyl oxidases would be inactive in the complete absence of copper, it is more difficult to predict the effect of mild copper deficiency on enzyme activity, and more importantly, to determine what components of the developing notochord would become essential in the setting of impaired – but not absent – lysyl oxidase function. The specific role for *loxl1* and *loxl5b* in notochord formation is supported by sensitization experiments using neocuproine (Fig. 6 and Table 3), and these genes may be candidates for late notochord mutants identified in previous forward genetic screens (Odenthal et al., 1996; Stemple et al., 1996) or for human disorders of the axial skeleton. Though we cannot completely rule out the possibility that the low penetrance of a distorted notochord phenotype using splice morpholinos to *loxl2b*, *loxl3b*, or *loxl5b* alone (Table 1) results from incomplete disruption of gene expression

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(S. Fig. 3), combinations involving these morpholinos did not convincingly demonstrate a role for *loxl2b* or *loxl3b* in notochord formation (Table 1 and S. Table 1).

Our data also demonstrate a genetic interaction between two lysyl oxidases (*loxl1* and *loxl5b*) and the collagen substrate *col2a1* (Fig. 6). This could reflect either direct crosslinking of this collagen substrate or crosslinking of additional extracellular matrix proteins that interact with collagen II for proper notochord formation. The latter possibility has been proposed in recent morphological studies on the notochord sheath of teleosts (Grotmol et al., 2006) and could account for observations in coatomer I protein complex mutants (Coutinho et al., 2004), where defects in the secretion of lysyl oxidases or such additional extracellular matrix components would give rise to the notochord sheath abnormalities. In all cases, reduced crosslinking of collagen fibrils from impaired lysyl oxidase activity would prevent the feedback inhibition of *col2a1* produced by notochord vacuolar cells, resulting in prolonged *col2a1* expression (Fig. 7). Alternatively, persistent *col2a1* expression could result indirectly from a general loss of notochord sheath integrity.

The observed lysyl oxidase expression patterns and phenotypes (Fig. 3, Fig. 4, Fig. 5 and data not shown) suggest that the zebrafish will be a useful model organism for elucidating the complex and specific roles of lysyl oxidases in development. In addition, the phylogenetic analysis (Fig. 2B) may prove helpful. The lack of a zebrafish orthologue to *LOXL4* is consistent with a BLAST search revealing such orthologues only in mammals (data not shown), and the markedly different expression patterns of this gene in human and mouse (Asuncion et al., 2001;Ito et al., 2001;Maki et al., 2001) may indicate that it has divergent, specialized roles in these organisms. Moreover, it is now apparent that scavenger receptor, cysteine-rich domains are a more variable feature of lysyl oxidase structure than previously anticipated. LOXL2, LOXL3, and LOXL4 are considered related because they all possess four scavenger receptor, cysteine-rich domains, which are completely absent in LOX and LOXL1. However, zebrafish *loxl3a* and *loxl3b* encode only a single scavenger receptor, cysteine-rich domain that is truncated (Fig. 2A), as does a *Xenopus* clone of *loxl3* previously assumed to be partial (S. Fig. 2C) (Geach and Dale, 2005). While the function of these domains remains unknown, a splice variant of *LOXL3* that excludes some scavenger-receptor, cysteine-rich domains has recently been described, and appears to have altered substrate specificity (Lee and Kim, 2006).

Definitive demonstration of a role for specific lysyl oxidases in notochord formation is of direct relevance to embryonic environmental exposures to nitrile compounds in plasticizers and carbamates in pesticides. Recent toxicological studies demonstrate that such pesticides induce notochord distortion in developing zebrafish embryos (Teraoka et al., 2006; Tilton et al., 2006) as well as persistent expression of *col2a1* by vacuolar cells of the notochord (Tilton et al., 2006). These findings are identical to our observations with *loxl1*, *loxl5b*, and *col2a1* morpholino knockdown combined with neocuproine treatment (Fig. 5B,F, Fig. 6 and Fig. 7), suggesting that the pesticides act through direct or indirect inhibition of these lysyl oxidase family members. Importantly, these data raise the distinct possibility that embryonic exposure to such toxins in specific genetic contexts may predispose to the later development of structural birth defects (Fig. 9B), where the inheritance of this genetic variation would not be reflected in Mendelian ratios of the observable phenotypes.

The notochord is a critical structure required for vertebral column patterning in vertebrates (Fleming et al., 2004) and its distortion in zebrafish phenotypically mimics a number of axial skeletal defects in humans including idiopathic scoliosis. Of note, while β-aminopropionitrile causes notochord distortion in zebrafish (Fig. 1B and S. Fig. 1), it is also well known to cause scoliosis in rats and other mammals (Barrow et al., 1974). Interestingly, disruptions in extracellular matrix components involved in notochord formation have been identified in clinical syndromes that include scoliosis. Two different mutations in exon 48 of the

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*COL2A1* gene cause spondyloepiphyseal dysplasia with scoliosis in humans (Tiller et al., 1995; Tiller et al., 1990). Furthermore, the kyphoscoliosis type of Ehler-Danlos syndrome (EDS VI) (Yeowell and Walker, 2000) results from loss of function mutations in the gene encoding lysyl hydroxylase type 1, which catalyzes the conversion of lysine to hydroxylysine in collagen prior to oxidization by lysyl oxidases. Taken together with the nutritional (copper) and toxicology findings noted above, these data raise the intriguing possibility that polymorphisms at multiple extracellular matrix component loci, including the lysyl oxidase genes identified here, may predispose to axial skeletal malformations in situations where nutrition or environment might otherwise be considered adequate (Fig. 9B). This concept is supported by our previous findings revealing a hierarchy of nutrient distribution to the developing embryo under limiting conditions (Mendelsohn et al., 2006) and as such is of broad relevance to our understanding of the pathogenesis of structural birth defects. The data reported here provide a testable model for these ideas with regards to common defects of the axial skeleton and reveal that zebrafish permit unique insights into the complex interplay of genes, environment and nutrition in development and disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

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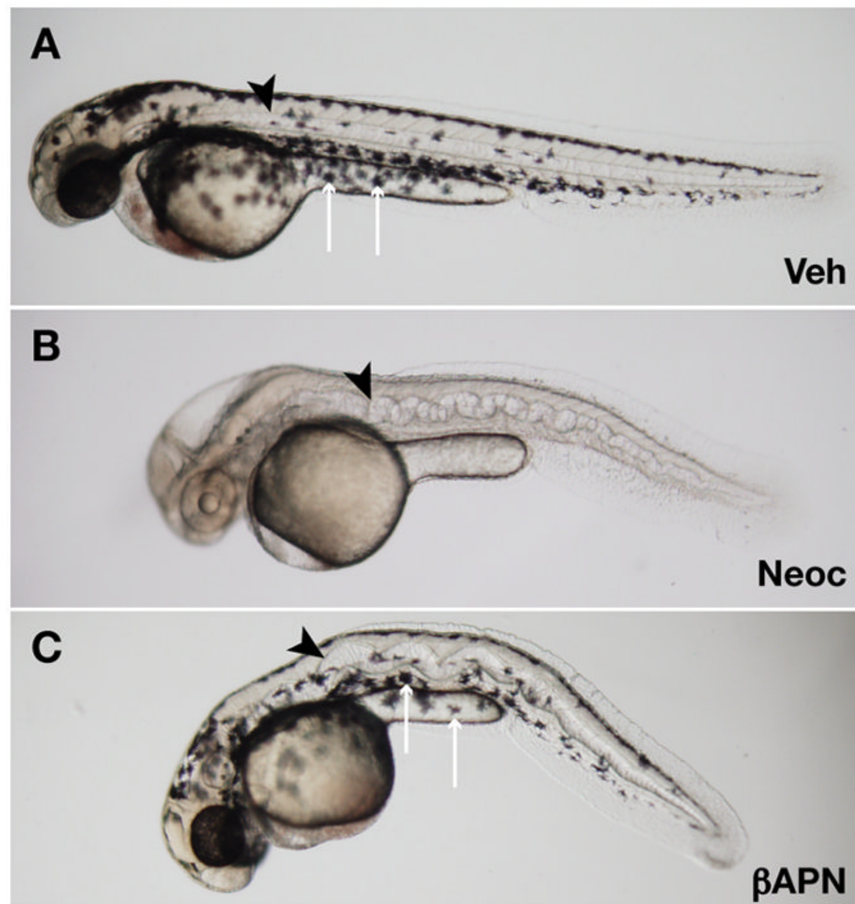


**Fig. 1.**

β-aminopropionitrile recapitulates the notochord phenotype of copper deficiency. Wild-type embryos were incubated in vehicle (A), 10 μM neocuproine (B), or 10 mM β-aminopropionitrile supplemented with 10 μM $CuCl_2$ (C). At 48 hpf, the notochord (arrowheads) is distorted in embryos treated with neocuproine (B) and β-aminopropionitrile (C); however, melanocytes (arrows) are present after β-aminopropionitrile (C) but not neocuproine treatment (B), demonstrating that β-aminopropionitrile does not act through copper chelation.

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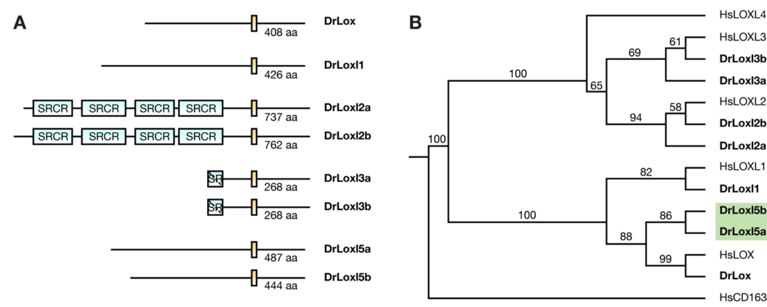


**Fig. 2.**

Lysyl oxidase family members in zebrafish. (A) The zebrafish genome encodes eight distinct lysyl oxidases, all of which contain a conserved copper binding domain depicted in yellow. Scavenger receptor, cysteine-rich domains (SRCR) of unknown function are in blue and are truncated in Loxl3a and Loxl3b (SR with strikethrough). (B) Phylogenetic tree depicting the evolutionary relationship between human and zebrafish lysyl oxidases. HsCD163 is used as an outgroup, and bootstrap values over 100 replicates are noted. Loxl5a and Loxl5b (green) represent new additions to the lysyl oxidase family that closely resemble LOX and LOXL1 in structure. Zebrafish encode orthologues to all human lysyl oxidase genes except LOXL4.

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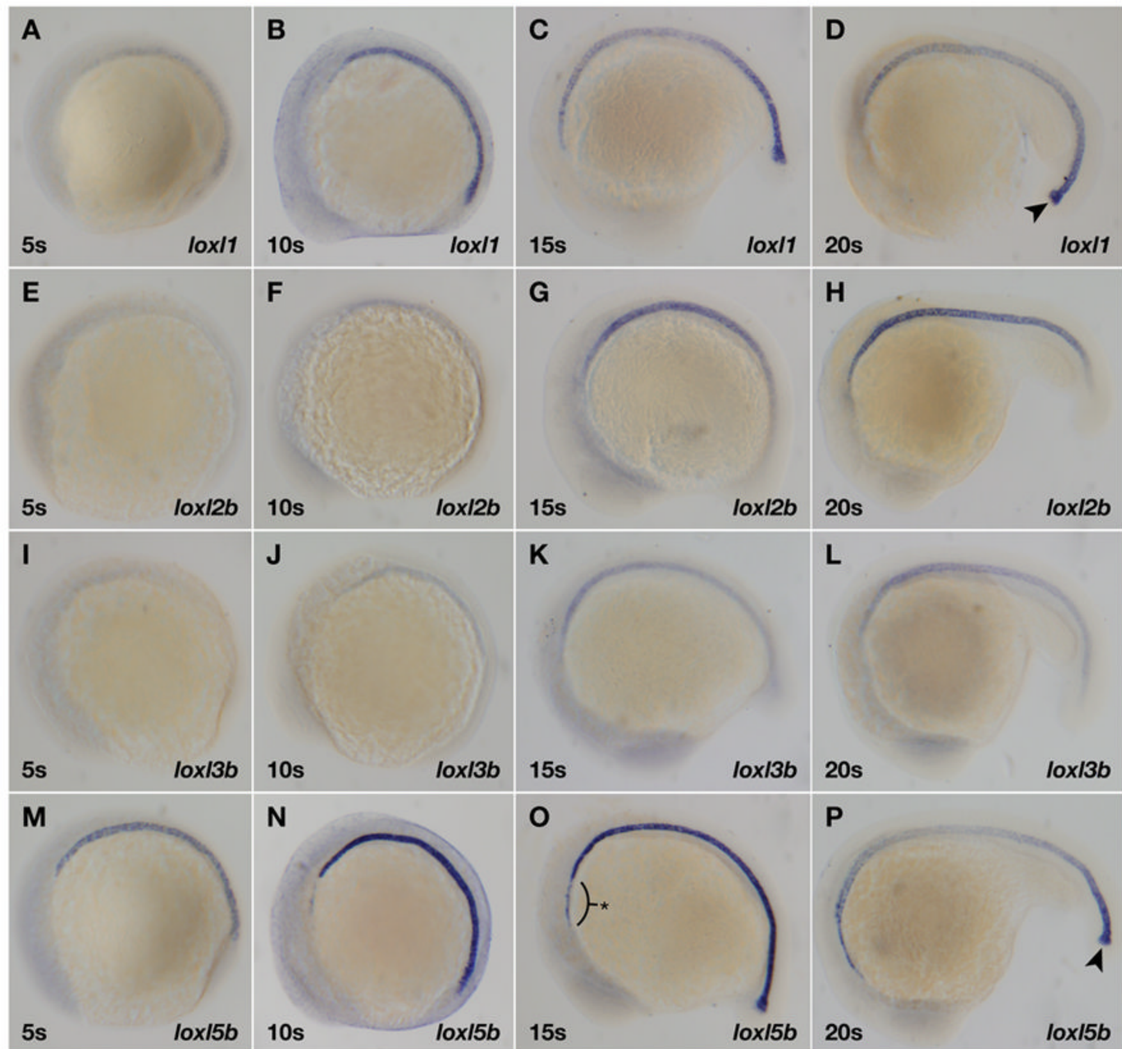


**Fig. 3.**

Four lysyl oxidases are expressed throughout the developing zebrafish notochord by in situ hybridization. (A–D) *loxl1*; (E–H) *loxl2b*; (I–L) *loxl3b*; and (M–P) *loxl5b*. Stages of development are 5 somites (A,E,I,M); 10 somites (B,F,J,N); 15 somites (C,G,K,O); and 20 somites (D,H,L,P). *loxl1* and *loxl5b* are robustly expressed at the caudal notochord tip (arrowheads) while *loxl5b* is also expressed anterior to the notochord (asterix).

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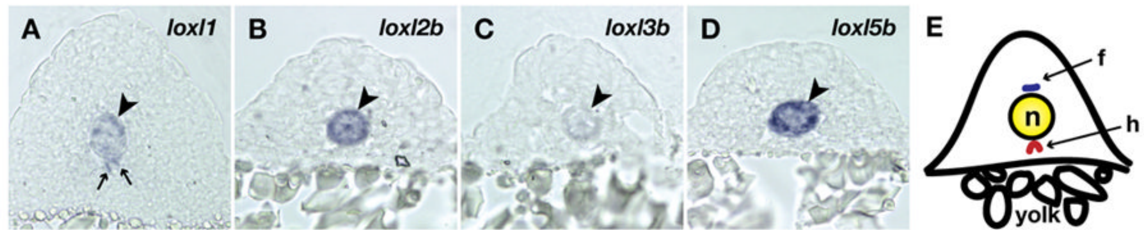


**Fig. 4.**
Lysyl oxidases are expressed by notochord vacuolar cells. (A–D) Frozen cross-sections of 15 somite embryos were obtained after in situ hybridization with the following probes: (A) *loxl1*; (B) *loxl2b*; (C) *loxl3b*; and (D) *loxl5b*. The notochord is indicated (arrowheads). Hypochord staining of *loxl1* (arrows) suggests a role for this family member in extracellular matrix crosslinking near the developing aorta. (E) Cartoon demonstrating the location of the hypochord (h) and floorplate (f), which are closely apposed to the notochord (n). Lysyl oxidases are not expressed in the floorplate.

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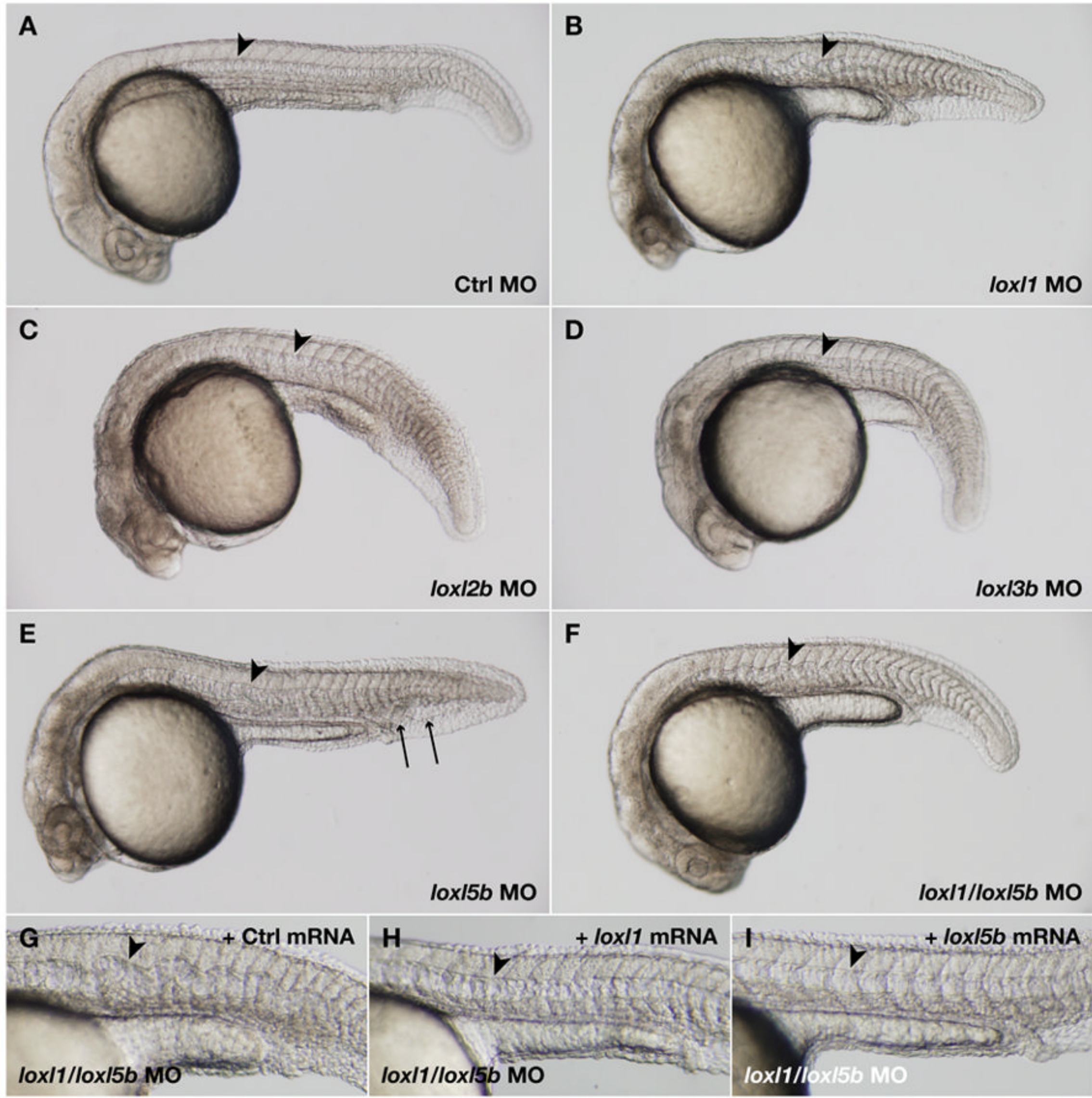


**Fig. 5.**

Morpholino knockdown of *loxl1* or *loxl1* and *loxl5b* together results in notochord distortion. Wild-type embryos were injected with morpholinos (MO) to notochord-expressed lysyl oxidases and photographed at 24 hpf. (A) 12 ng standard control; (B) 6 ng *loxl1*; (C) 3.7 ng *loxl2b*; (D) 12 ng *loxl3b*; (E) 7.4 ng *loxl5b*; (F) 2.4 ng *loxl1* and 5 ng *loxl5b*. The notochord (arrowheads) is strikingly distorted after knockdown of *loxl1* (B), and caudal vein edema develops with the *loxl5b* morpholino (E, arrows). Combining doses of *loxl1* and *loxl5b* morpholino that do not cause notochord distortion alone also recapitulates the notochord phenotype seen with neocuproine and β-aminopropionitrile treatment (F). This distortion is specifically rescued by co-injection of mRNA encoding either *loxl1* (H) or *loxl5b* (I), but not control sequence (G).

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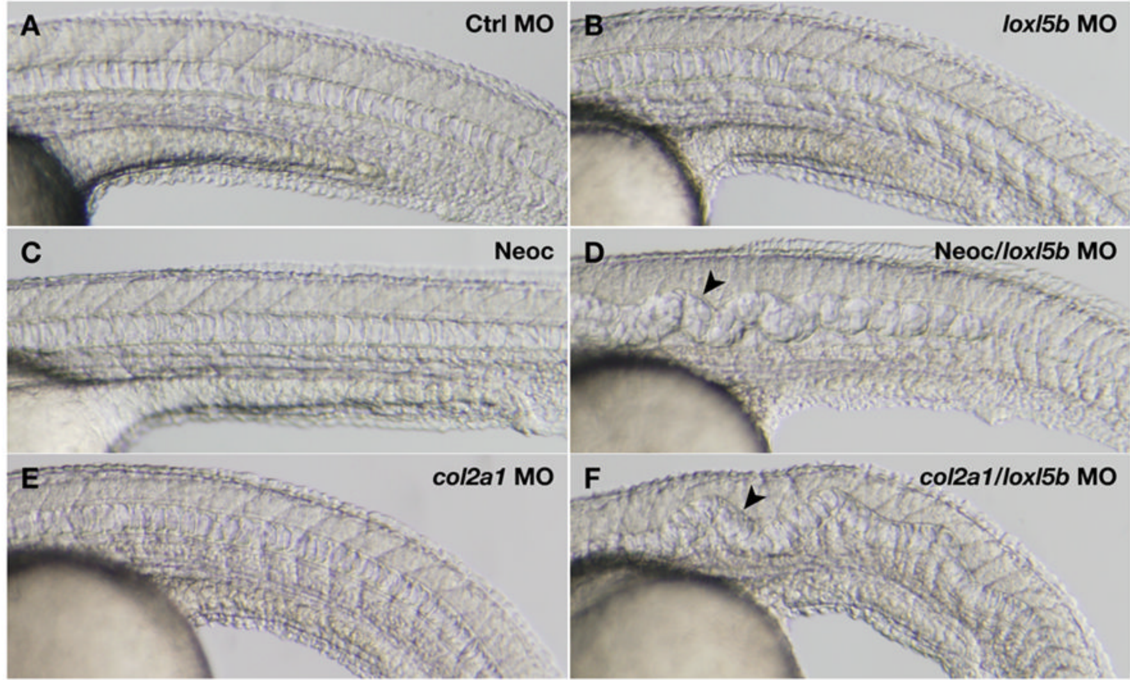


**Fig. 6.**

Partial knockdown of *loxl5b* sensitizes embryos to notochord distortion in the presence of suboptimal copper nutrition (A–D) or disruption of *col2a1* (E,F). Wild-type embryos were injected with 5 ng of control morpholino (A,C) or *loxl5b* morpholino (B,D), and incubated with (C,D) or without (A,B) 2 μM neocuproine starting at 3 hpf. Embryos injected with *loxl5b* morpholino develop notochord distortion (arrowhead) in the context of diminished copper availability (D). Embryos injected with 7.4 ng of *col2a1* morpholino (E) are sensitized to develop notochord distortion (arrowhead) upon co-injection of 5 ng of *loxl5b* morpholino (F), demonstrating a genetic interaction between *col2a1* and *loxl5b*. Photographs were obtained at 30 hpf (A–D) and 24 hpf (E,F).

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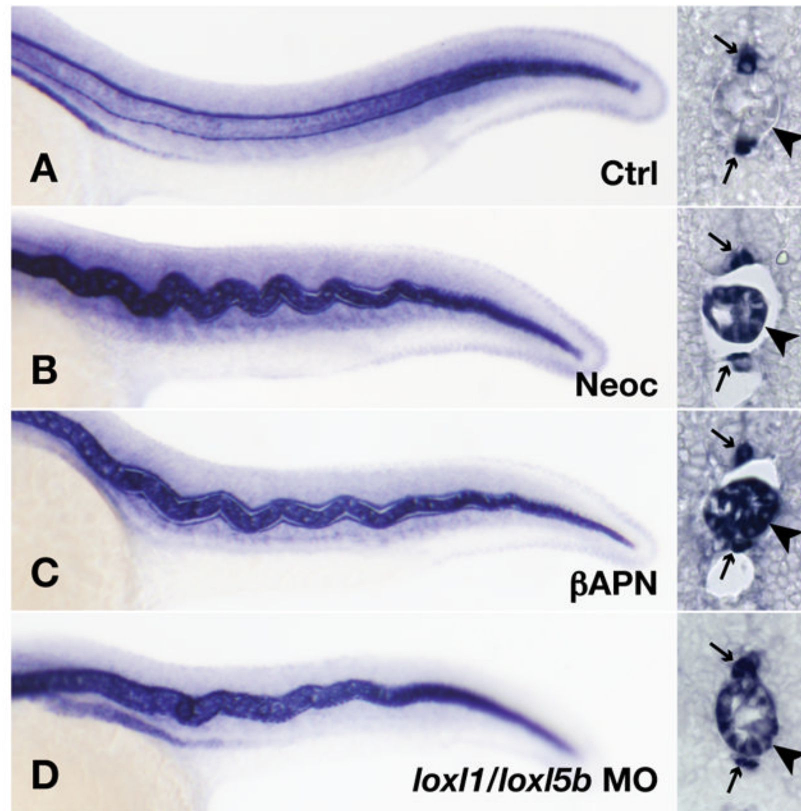


**Fig. 7.**

Expression of *col2a1* mRNA by notochord vacuolar cells persists late following lysyl oxidase inhibition. Wild-type embryos were injected with 7.4 ng control morpholino (A), incubated in 10 μM neocuproine (B), incubated in 10 mM β-aminopropionitrile supplemented with 10 μM $CuCl_2$ (C), or injected with 3.7 ng each of *loxl1* and *loxl5b* morpholino (D). In situ hybridization was carried out at 24 hpf, and frozen sections confirmed persistent *col2a1* expression following lysyl oxidase inhibition (B–D, arrowhead), as well as floorplate and hypochord staining (A–D, arrows).

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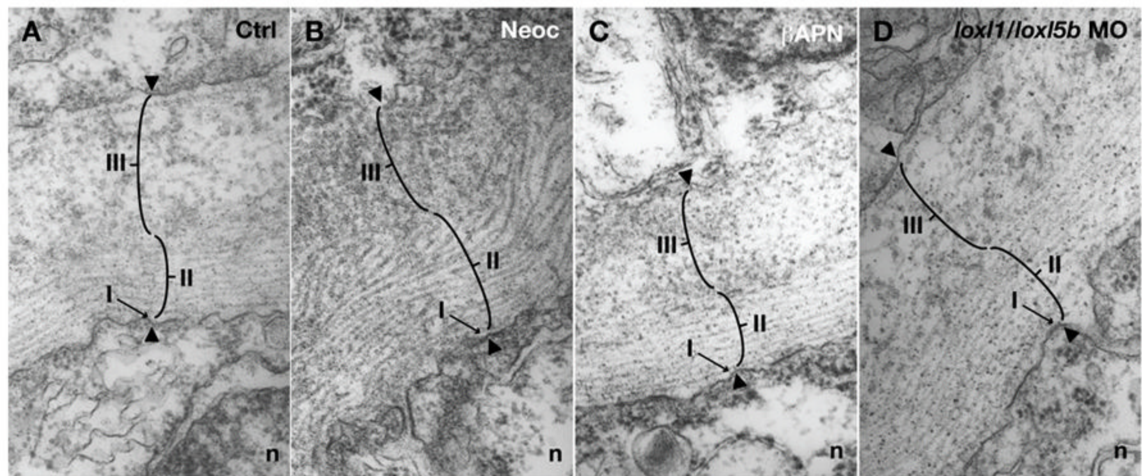


**Fig. 8.**

Electron micrographs of the notochord sheath from cross-sections of 30 hpf embryos: (A) 7.4 ng control morpholino; (B) 10 μM neocuproine; (C) 10 mM β-aminopropionitrile; (D) 3.7 ng each of *loxl1* and *loxl5b* morpholino. Sheath components include a basal lamina (I), fibrillar layer (II), and granular layer (III). All three layers are preserved after lysyl oxidase inhibition.

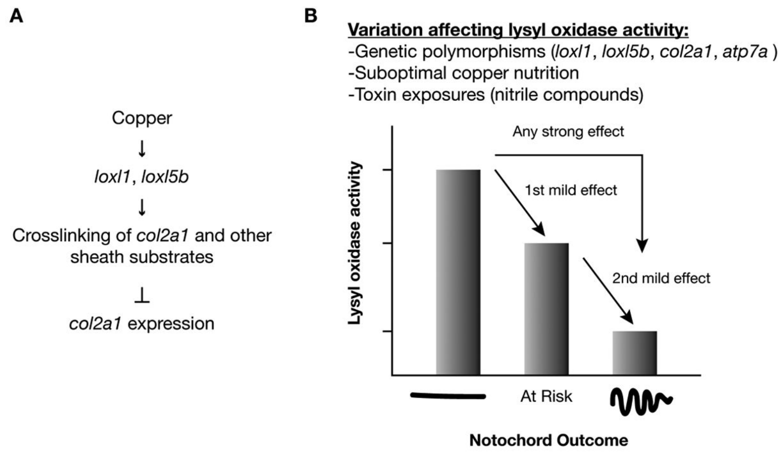


**Fig. 9.**

Model of pathways involved in notochord formation. (A) The cuproenzymes Loxl1 and Loxl5b crosslink collagen II in the notochord sheath, which suppresses vacuolar cell expression of *col2a1* as the notochord differentiates. (B) Polymorphisms at multiple genetic loci interact with environmental factors to cause disease. As a result, what is normally considered adequate nutrition may in fact be suboptimal during a specific developmental window. The gene *atp7a* encodes a transporter required for copper uptake (Mendelsohn et al., 2006).

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**Table 1**

*loxl1* is necessary for notochord formation in developing zebrafish embryos, and overlaps in function with *loxl5b*. Wild-type embryos were injected with morpholinos to notochord-expressed lysyl oxidases and examined at 24 hpf. The number and percentage of embryos with and without a distorted notochord phenotype is noted. Injection of 6 ng of *loxl1* splice morpholino causes notochord distortion in 88% of embryos, an effect not seen with morpholinos to *loxl2b*, *loxl3b*, or *loxl5b* alone. However, injection of 2.4 ng of *loxl1* morpholino and 5 ng of *loxl5b* morpholino together causes notochord distortion in 94% of embryos, demonstrating that *loxl1* and *loxl5b* have overlapping roles in notochord formation. This finding was recapitulated in separate experiments with start site (ATG) morpholinos to *loxl1* and *loxl5b*. The number of embryos examined includes dead embryos that could not be scored for notochord phenotype; the *loxl2b* morpholino causes significant embryo death at higher doses. Data shown are the pooled results of three independent experiments.

| Specific Morpholino | Dose of morpholino (ng) | # of embryos examined | Phenotype of notochord distortion | |
|---|---|---|---|---|
| | | | − | + |
| **Ctrl** | 12 | 302 | 278 (**100%**) | 0 (**0%**) |
| ***loxl1*** | 6 | 271 | 30 (**12%**) | 217 (**88%***) |
| ***loxl2b*** | 2.4 | 212 | 122 (**98%**) | 3 (**2%**) |
| ***loxl3b*** | 12 | 190 | 128 (**96%**) | 6 (**4%**) |
| ***loxl5b*** | 5 | 263 | 237 (**98%**) | 6 (**2%**) |
| ***loxl1*** | 2.4 | 297 | 272 (**99%**) | 2 (**1%**) |
| ***loxl1/loxl5b*** | 2.4/5 | 292 | 17 (**6%**) | 254 (**94%***) |
| **Ctrl** | 12 | 173 | 138 (**100%**) | 0 (**0%**) |
| ***loxl1* ATG** | 12 | 158 | 145 (**100%**) | 0 (**0%**) |
| ***loxl5b* ATG** | 12 | 159 | 140 (**92%**) | 12 (**8%**) |
| ***loxl1* ATG/*loxl5b* ATG** | 6/6 | 188 | 33 (**19%**) | 145 (**81%***) |

* $p < 0.01$ versus controls by ANOVA

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**Table 2**

The distorted notochord phenotype resulting from combined *loxl1* and *loxl5b* knockdown is specific and can be rescued by co-injection of mRNA encoding either *loxl1* or *loxl5b*. Wild-type embryos injected with control or *loxl1* and *loxl5b* morpholinos together were co-injected with control or lysyl oxidase mRNA, as indicated. The number and percentage of embryos with and without a distorted notochord phenotype at 24 hpf is noted, as well as the percentage rescue attributable to each mRNA. Exogenously-supplied *loxl1* or *loxl5b* mRNA produces a marked improvement in notochord morphology, substantially decreasing the proportion of embryos with notochord distortion. Injection of lysyl oxidase mRNA alone did not result in any overexpression phenotype. The number of embryos examined includes dead embryos that could not be scored for notochord phenotype. Data shown are the pooled results of three independent experiments.

| Specific Morpholino | Dose of morpholino (ng) | Specific mRNA | # of embryos examined | Phenotype of notochord distortion | | Rescue of notochord phenotype |
|---|---|---|---|---|---|---|
| | | | | − | + | |
| **Ctrl** | 7.4 | Ctrl | 240 | 226 (**100%**) | 0 (**0%**) | **NA** |
| ***loxl1/loxl5b*** | 2.4/5 | Ctrl | 223 | 8 (**4%**) | 195 (**96%**) | **NA** |
| ***loxl1/loxl5b*** | 2.4/5 | *loxl1* | 238 | 137 (**63%**) | 80 (**37%**[*]) | **62%** |
| ***loxl1/loxl5b*** | 2.4/5 | *loxl5b* | 251 | 197 (**86%**) | 33 (**14%**[*]) | **85%** |

[*] $p < 0.01$ versus *loxl1/loxl5b* MO with Ctrl mRNA by ANOVA

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**Table 3**

Partial knockdown of *loxl1* or *loxl5b* sensitizes embryos to notochord distortion in the presence of suboptimal copper nutrition. Wild-type embryos were injected with control or lysyl oxidase-specific morpholino, as indicated, and incubated with or without neocuproine at doses that were determined not to cause notochord distortion alone. Embryos injected with lysyl oxidase-specific morpholino and incubated in 2 μM neocuproine were sensitized to develop notochord distortion. The number of embryos examined includes dead embryos that could not be scored for notochord phenotype. Each result is pooled from three independent experiments, which were scored at 24 hpf.

| Specific Morpholino | Dose of morpholino (ng) | Pharmacologic treatment | # of embryos examined | Phenotype of notochord distortion | |
|---|---|---|---|---|---|
| | | | | − | + |
| **Ctrl** | 2.4 | None | 104 | 104 (**100%**) | 0 (**0%**) |
| **Ctrl** | 2.4 | 2 μM neocuproine | 113 | 111 (**98%**) | 2 (**2%**) |
| ***loxl1*** | 2.4 | None | 143 | 127 (**100%**) | 0 (**0%**) |
| ***loxl1*** | 2.4 | 2 μM neocuproine | 132 | 49 (**39%**) | 77 (**61%***) |
| **Ctrl** | 5 | None | 139 | 136 (**100%**) | 0 (**0%**) |
| **Ctrl** | 5 | 2 μM neocuproine | 138 | 132 (**99%**) | 2 (**1%**) |
| ***loxl5b*** | 5 | None | 147 | 126 (**95%**) | 7 (**5%**) |
| ***loxl5b*** | 5 | 2 μM neocuproine | 147 | 1 (**1%**) | 140 (**99%***) |

* $p < 0.01$ versus controls by ANOVA

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**Table 4**

Genetic interaction of *loxl1* and *loxl5b* with *col2a1*. Wild-type embryos were injected with the indicated morpholino combinations and scored for notochord distortion at 24 hpf. Partial knockdown of *loxl1* and *col2a1* together, but not alone, causes the distorted notochord phenotype. An identical result is obtained with morpholinos to *loxl5b* and *col2a1*, suggesting that diminished activity of these lysyl oxidases sensitizes embryos to notochord distortion in the presence of *col2a1* disruption. Importantly, collagen II is a lysyl oxidase substrate produced by the notochord vacuolar cells and present in the notochord sheath. The number of embryos examined includes dead embryos that could not be scored for notochord phenotype. Each result is pooled from three independent experiments.

| Specific Morpholino | Dose of morpholino (ng) | # of embryos examined | Phenotype of notochord distortion | |
|---|---|---|---|---|
| | | | − | + |
| ***loxl1*/ctrl** | 2.4/7.4 | 202 | 146 (**99%**) | 1 (**1%**) |
| ***col2a1*/ctrl** | 7.4/2.4 | 206 | 161 (**98%**) | 4 (**2%**) |
| ***col2a1*/*loxl1*** | 7.4/2.4 | 258 | 118 (**58%**) | 84 (**42%***) |
| ***loxl5b*/ctrl** | 5/7.4 | 151 | 137 (**93%**) | 10 (**7%**) |
| ***col2a1*/ctrl** | 7.4/5 | 122 | 116 (**99%**) | 1 (**1%**) |
| ***col2a1*/*loxl5b*** | 7.4/5 | 217 | 111 (**54%**) | 96 (**46%***) |

* $p < 0.01$ versus controls by ANOVA