Published in final edited form as:
Matrix Biol. 2007 January ; 26(1): 12–19.

Genomic structure and embryonic expression of zebrafish *lysyl hydroxylase 1* and *lysyl hydroxylase 2*

Valerie A. Schneider and **Michael Granato**
University of Pennsylvania School of Medicine Department of Cell and Developmental Biology Philadelphia, PA 19104-6058 215-898-2745

Abstract

Collagen biosynthesis in both invertebrates and vertebrates is critically dependent upon the activity of lysyl hydroxylase (LH) enzymes. In humans, mutations in the genes encoding LH1 and LH2 have been shown to cause two distinct connective tissue disorders, Ehlers-Danlos (Type VIA) and Bruck syndromes. While the biochemical properties of these enzymes have been intensively studied, their embryonic patterns of expression and developmental roles remain unknown. We now present the cloning and analyses of the genes encoding LH1 and LH2 in the zebrafish, Danio rerio. We find these genes to be similarly organized to other vertebrate *lh* (*plod*) genes, including the presence of an alternatively spliced exon in *lh2*. We also examine the mRNA expression patterns of *lh1* and *lh2* during embryogenesis and find them to exhibit unique and dynamic patterns of expression. These results strongly suggest that LH enzymes are not merely housekeeping enzymes, but play distinct developmental roles. The identification of these genes in the zebrafish, a genetic model organism whose development is well characterized, now provides the basis for the establishment of the first animal models for both Ehlers-Danlos (Type VIA) and Bruck syndromes.

Keywords

zebrafish; lysyl hydroxylase; lh; plod; extracellular matrix; collagen

1. Introduction

Members of the lysyl hydroxylase (LH) enzyme family catalyze the addition of hydroxyl groups to lysine residues in collagens and other proteins containing the sequence X-Lys-Gly (for review, see Myllyharju and Kivirikko, 2004). These hydroxylysine residues serve as sites of attachment for carbohydrate chains and also participate in the formation of intermolecular crosslinks (reviewed in Risteli et al., 2004). To date, three members of the LH family have been identified in vertebrates. Although the enzymes do not appear to require strict sequence specificity for substrate recognition, they do have distinct biological roles (Risteli et al., 2004; Wang et al., 2000). LH1 specifically hydroxylates lysine residues in regions of substrate proteins that form triple helices (Steinmann et al., 1995). In humans, mutations in *lh1* (also known as *plod1*) have been linked to Ehlers-Danlos (type VIA) syndrome, a recessive disorder characterized by hyperextendable skin, joint hypermobility and kyphoscoliosis (Yeowell and Walker, 2000). In contrast to LH1, LH2 also has telopeptidyl lysyl hydroxylase activity (van der Slot et al., 2003). Recently, mutations in human *lh2* (*plod2*) were linked to Bruck syndrome,

Correspondence should be addressed to M.G. (granatom@mail.med.upenn.edu)..

distinguished by fragile bones and contractures of the large joints (Ha-Vinh et al., 2004; van der Slot et al., 2003). LH3, in addition to exhibiting lysyl hydroxylase activity, is the only LH enzyme to possess additional glycosyltransferase activities that serve to further modify hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine (Heikkinen et al., 2000; Rautavuoma et al., 2002; Wang et al., 2002). Although mutations in human *lh3* (*plod3*) have not yet been identified, a mouse knockout reveals this gene has an embryonic lethal phenotype and is required for type IV collagen secretion (Rautavuoma et al., 2004; Ruotsalainen et al., 2006). More recently, we have demonstrated that *diwanka*, the zebrafish homolog of *lh3*, plays a specific role in motor axon migration (Schneider and Granato, 2006).

Although LH enzymes are recognized to play a key step in collagen biosynthesis, little is known regarding their developmental roles. While the tissue distribution of *lh* gene expression has been examined by Northern blot analysis, with the exception of *lh3*, there have been no published accounts of the in situ expression patterns of these genes during embryogenesis in any vertebrate species (Passoja et al., 1998b; Rautavuoma et al., 2004; Ruotsalainen et al., 1999; Valtavaara et al., 1997; Valtavaara et al., 1998; Yeowell et al., 1994; Yeowell and Walker, 1999). In this paper, we report the cloning of zebrafish *lh1* and *lh2* and their patterns of expression throughout the first two days of development. We find that both genes have organizational structures similar to other vertebrate *lh* genes, and that, as in other vertebrates, *lh2* exhibits alternative splicing. For the first time in any vertebrate species, we show the expression patterns of these genes during embryogenesis, and demonstrate that they display highly restricted and dynamic patterns of expression. Furthermore, we observe that *lh1* and *lh2* mRNAs are found in both unique and overlapping domains, indicative of their having distinct developmental roles. We discuss how these findings will provide the basis for future in vivo analyses of the roles of *lh* genes in development and disease.

2. Results

2.1 Cloning of zebrafish lh1 and lh2

We identified several zebrafish cDNA clones whose sequences were homologous to portions of other vertebrate *lh1* genes (see Experimental Procedures for details of cloning). Further analysis led to the identification of the full-length zebrafish *lh1* cDNA sequence (Genbank DQ020093). A subsequent BLAST analysis of the zebrafish genome assembly in Ensembl (http://www.ensembl.org/Danio_rerio/index.html) with this cDNA sequence identified the complete genomic sequence corresponding to *lh1* (Zv6_scaffold1312). SIM4 was used to predict the intron-exon boundaries of *lh1* (http://pbil.univ-lyon1.fr/sim4.php) and revealed 19 exons, whose sizes correspond closely to those of human *lh1*, as well as zebrafish *lh3* (Fig. 1A and Table 1). Consistent with the lack of Alu elements (present in several introns of human *lh1*) in the zebrafish genome, there is no conservation of intron size (Fig. 1A and Izsvak et al., 1997;Yeowell and Walker, 2000). Conceptual translation of zebrafish *lh1* predicts a 730 amino acid (aa) protein with a 20 aa signal sequence (Fig. 1B). ClustalX analysis shows this protein exhibits 67% identity to human LH1, but only 59% identity to zebrafish LH3. There are three potential N-linked glycosylation sites (NXS/T) in zebrafish LH1. Two of these sites are also conserved in other vertebrate LH1 proteins and their glycosylation has been shown to contribute to lysyl hydroxylase activity (Fig. 1B,Pirskanen et al., 1996). All amino acid residues demonstrated to be required for co-factor binding by other vertebrate LH proteins are conserved in zebrafish LH1 (Fig. 1B,Passoja et al., 1998a;Pirskanen et al., 1996).

We also identified cDNA clones whose sequences exhibited homology to portions of vertebrate *lh2* genes. Studies of *lh2* in other species show that this gene, in contrast to *lh1* and *lh3*, exhibits alternative splicing. Consistent with these studies, we identified cDNAs corresponding to the two known *lh2* splice variants in the course of cloning full-length zebrafish *lh2* (Genbank

DQ020094 (*lh2a*) and DQ020095 (*lh2b*); see Experimental Procedures for details). BLAST analysis of the zebrafish genome assembly in Ensembl revealed that the corresponding *lh2* genomic sequence is currently distributed between two contigs (Zv6_scaffold3453 and Zv6_scaffold2680). SIM4 analyses of these sequences predict that zebrafish *lh2a* contains 19 exons, whereas zebrafish *lh2b* contains an additional exon (13A) of 63 bp (Fig.1 and Table 1). Like *lh1*, exon sizes (but not intron sizes) in zebrafish *lh2* closely match those of other vertebrate *lh2* genes, as well as those of zebrafish *lh3* (Fig. 1A and Table 1). Conceptual translation predicts *lh2* to encode 733 (LH2a) and 754 aa (LH2b) proteins, which have 23 aa signal sequences and five potential N-glycosylation sites (Fig. 1B). These proteins exhibit 70% identity to human LH2a/LH2b, but only 59% identity to other zebrafish LH proteins.

2.2 Analysis of lh2 splice variants

To date, all *lh2* mutations identified in Bruck syndrome, a disorder associated with decreased levels of telopeptidyl lysyl hydroxylase (TLH) activity, are predicted to affect both splice variants of this gene (Ha-Vinh et al., 2004; van der Slot et al., 2003). However, only increased levels of *lh2b* mRNA expression appear to be associated with increased levels of TLH activity found in fibrotic lesions, suggesting that the sequence of exon 13A encodes the LH2 domain responsible for specifying TLH activity (van der Slot et al., 2003; van der Slot et al., 2004). We aligned the amino acid sequence encoded by exon 13A of zebrafish *lh2* to that of other vertebrate species (obtained from GenBank) to identify residues whose evolutionary conservation might indicate their contribution to this activity (Fig. 2A). We observed a 90% degree of conservation to human LH2b in this region, higher than the 83% conservation of the full-length proteins. We found that 11 of the 21 amino acids encoded by exon13A were invariant in the seven species examined, and an additional 7 showed only conservative changes. The high degree of conservation in these amino acids is strongly suggestive of this domain contributing to the TLH activity associated with LH2b. Further analyses will be required to determine the importance of each amino acid to this activity.

To determine whether there are differences in the temporal expression of the *lh2* splice variants, we performed RT-PCR on cDNA prepared from zebrafish embryos at various stages (Fig. 2B), using primers that distinguished these cDNAs. These studies indicate that concomitant expression of both splice variants begins during gastrulation and persists through 48 hours post fertilization (hpf), the latest stage examined. While *lh2a* mRNA levels remain relatively constant throughout this period, *lh2b* expression appears to decrease slightly during later stages of somitogenesis, increase at 24 hpf and then decline again at 48 hpf. These finding suggest that, although generally coordinately expressed, *lh2a* and *lh2b* transcripts may also be independently regulated.

2.3 Expression patterns of lh1 and lh2 during embryonic development

We performed whole mount in situ hybridization to determine the expression patterns of *lh1* and *lh2* during embryogenesis. Because there is only a 63 bp difference between the *lh2* splice variants, the *lh2* in situ probe used is unlikely to distinguish between the two transcripts. Thus, the expression patterns shown may reflect either or both *lh2* splice variants. Consistent with the RT-PCR data, expression of *lh1* and *lh2* was first detected by in situ hybridization during gastrulation. During these stages, expression of both genes was confined to the axial mesoderm, the patterns being essentially indistinguishable from one another (Fig. 3). Differences in *lh1* and *lh2* expression patterns were first detected during somitogenesis. Through out these stages, *lh1* expression was restricted to the notochord, while additional domains of *lh2* expression were observed in the hypochord, floorplate and myotome (Figs. 3 and 4).

Beginning at 24 hpf, the expression patterns of *lh1* and *lh2* shift again. As *lh1* expression in the notochord diminishes, a new domain of low-level expression appears in the sclerotomal

compartment of the ventral somite (Figs. 4C, D). Furthermore, otic vesicle expression of *lh1*, only weakly detectable at 20 somites, intensifies (Fig. 5A). A close examination of the developing ear at this stage reveals this expression is restricted to the medial surfaces of the epithelial cells lining the otic vesicle and is more concentrated in the anterior region. *lh2* mRNA expression at 24 hpf, like that of *lh1*, is downregulated in the notochord and weakly upregulated in the region of the developing sclerotome (Figs. 4G, H). Furthermore, the robust myotomal *lh2* expression that was observed throughout somitogenesis disappears, though expression in the floor plate and hypochord remains strong (Figs. 4G, H). In contrast to *lh1*, however, there is no detectable expression of *lh2* in the otic vesicle (data not shown).

As development proceeds, the domains of *lh1* and *lh2* expression remain distinct and continue to evolve. By 48 hpf, notochordal expression of *lh1* has ceased. However, a new domain of expression is observed at the posterior tip of the tail in the developing collagenous actinotrichia, which provide strength to the fin folds (Fig. 5I). *lh1* continues to be expressed in the otic vesicle, where it now can be detected in the epithelial projections of the developing semicircular canals (Fig. 5B, C). In the head of the embryo, *lh1* expression is strongly expressed in the developing neurocranium and more weakly in the mandibular and hyoid arches (Fig. 5C, G). An additional domain of *lh1* expression is observed in the pectoral fin buds, where its mRNA is present throughout the mesenchymal core of the developing fin, but absent from the overlying epithelium (Fig. 5C, E). At 48 hpf, *lh2* expression is also observed in the mesenchymal region of the pectoral fin buds, though it appears to be more concentrated in the central-most precartilagenous core than *lh1* (Fig. 5C, F). There is also robust expression of *lh2* in the developing cleithra, the bones that will connect the pelvic girdle to the skull (Fig. 5D). Within the head, high levels of *lh2* expression are found in condensing cartilage of both the mandibular and hyoid arches, in addition to the neurocranium (Fig. 5D, H). Along the length of the tail, *lh2* expression in the floor plate and hypochord persists, while a new domain of expression is observed in the developing intestine.

3. Discussion

Collagen biosynthesis is a multi-step process, requiring the coordinated activity of several different modifying enzymes (reviewed in Myllyharju and Kivirikko, 2004). Among these are the ER-resident lysyl hydroxylases, which perform co-translational modifications of lysine residues in proteins bearing the sequence X-Lys-Gly. Although the biochemical reactions they perform have been the subject of many in vitro studies, there is still much to be learned about the in vivo requirement for these enzymes, particularly during the period of embryonic development. In this paper, we have presented the cloning, sequence analysis and embryonic expression patterns of zebrafish *lh1* and *lh2*.

The zebrafish *lh1* and *lh2* genes have organizations similar to their human counterparts and, based on protein sequence similarities, are expected to exhibit comparable enzymatic activities. Though LH-mediated modifications are known to be important for collagen fibril assembly and intermolecular interactions, the critical in vivo requirement for these enzymes is underscored by the finding that, in humans, mutations in *lh1* and *lh2* result in Ehlers-Danlos (type VIA) and Bruck syndromes, respectively (Ha-Vinh et al., 2004; Risteli et al., 2004; van der Slot et al., 2003; Yeowell and Walker, 2000). Thus, our identification of the zebrafish homologs of *lh1* and *lh2* now presents the opportunity for the development of a model system for the in vivo study of these disorders. The zebrafish is an organism proven to be well suited to genetic analysis and the study of embryonic development. In the absence of identified mutations in the zebrafish *lh1* or *lh2* genes, antisense morpholino oligonucleotides can be used to produce both hypomorphic and null embryonic phenotypes (Nasevicius and Ekker, 2000). Notably, this technique may also be used to distinguish the developmental roles of the *lh2* splice variants (Draper et al., 2001). Additionally, both traditional forward genetic screens and

reverse genetic approaches (TILLING) may be used to identify fish bearing mutations in *lh1* and *lh2* (Haffter et al., 1996; Wienholds et al., 2003).

Consistent with their known roles in collagen biosynthesis, our in situ analyses of *lh1* and *lh2* demonstrate the expression of these genes in tissues shown previously to express various forms of collagen (Sprague et al., 2001). However, the dynamic patterns of *lh1* and *lh2* expression in a variety of tissues during zebrafish embryogenesis suggest that these genes do not simply play a housekeeping role, but instead participate in specific developmental events. In accordance with this hypothesis, zebrafish *lh3*, which also displays a restricted pattern of expression, plays a specific role in motor axon migration (Schneider and Granato, 2006). Importantly, our observation that *lh1* and *lh2* (and *lh3*) exhibit overlapping and distinct patterns of expression suggests that they likely modify different collagens during development, even though in vitro studies do not reveal strict substrate specificities for these enzymes (Risteli et al., 2004; Schneider and Granato, 2006; Wang et al., 2000). This view is consistent with the observation that the degree to which collagen lysine residues are underhydroxylated in individuals having Ehlers-Danlos syndrome varies with the collagen type and the tissue from which any particular collagen is isolated (Ihme et al., 1984). Comparisons of the patterns of *lh* gene expression to patterns of collagen expression could reveal which collagens are most likely to be affected in particular tissues in situations of reduced lysyl hydroxylase activity. When coupled with analyses of lysyl hydroxylase deficient embryos, these studies could identify situations in which the different LH enzymes compensate (or fail to compensate) for one another, providing insight into the in vivo differences in LH substrate specificities. In summary, our identification of the zebrafish lysyl hydroxylase genes and their expression patterns now provides the basis for the establishment of a model system that will further understanding of the critical roles these genes play in development and their contribution to disease.

4. Experimental Procedures

4.1 lh1 cloning

The zebrafish LH3 peptide sequence was used to perform a translated BLAST search of the “others” EST database in Genbank in order to identify clones encoding related proteins. ClustalX alignment of translated sequences from identified zebrafish ESTs with multiple vertebrate LH proteins (from zebrafish, rat, mouse and human) demonstrated that several clones with overlapping sequences exhibited strongest homology to LH1 (see also Schneider and Granato, 2006). Complete sequencing of three of these clones revealed that, while two clones, BI896329 and AI958709, contained only partial coding sequences, one of them, AW116639, contained the complete zebrafish *lh1* coding sequence, as well as 5′ and ′ UTR sequences.

4.2 lh2 cloning

The zebrafish LH1 and LH3 peptide sequences were used to perform translated BLAST searches of the “others” EST database in Genbank to identify clones encoding zebrafish *lh2*. A ClustalX alignment of translated sequences from zebrafish clones not encoding *lh1* and *lh3* with other vertebrate LH proteins identified two overlapping clones, CF265663 and AI585202, whose sequences exhibited homology to the C-termini of LH2 proteins. Additional analysis led to the identification of clone CB365437, whose translated sequence had homology to the N-termini of vertebrate LH2 proteins, but whose nucleotide sequence did not overlap those of CF265663 and AI585202. To identify the intervening zebrafish sequence, the PCR primers 5′ GGGACAGTCAGCCCGTTCTTGAG 3′ (forward) and 5′ TTAGGGATCTACGAAAGACACTGCT 3′(reverse) were used to amplify cDNA prepared from 24 hpf Tü wild-type zebrafish embryos. A PCR product of approximately 2 kb was cloned

into pCR-BluntII-TOPO. Sequencing of clones revealed the presence of two transcripts that were identical save for a 63 bp in-frame insertion, corresponding to the two splice variants of *lh2* identified in other vertebrate species. These constructs contained the entire *lh2* and *lh2b* coding sequences.

4.3 RT-PCR Analysis

Total mRNA was isolated (Trizol, Invitrogen) from 10 embryos at each of the indicated developmental stages and reverse transcribed to cDNA (Superscript RT II, Invitrogen). *lh2* primer pair A: 5′AGTGTGGAACATTCCTTTCCTGGC 3′ (forward), 5′ GAGAAGTGTTGTAGTTGGCAGTGG 3′ (reverse). *lh2* primer pair B: 5′ AGTGTGGAACATTCCTTTCCTGGC 3′ (forward), 5′ CTTTGGGGATCTGAGCATATCGAATG 3′ (reverse). Actin primers: 5′ AACCCTGCTCACTGAAGCCC 3′ (forward) and 5′ ATGGATGGACCTGCCTCGTC 3′ (reverse).

4.4 In situ Hybridization

Both colorimetric and fluorescent in situ hybridization reactions were performed as described in Schneider and Granato (2006). Colorimetrically labeled embryos were cleared in a 2:1 mixture of benzyl alcohol: benzyl benzoate, mounted in Canada Balsam containing 10% (v/v) methylsalicylate and imaged with a DIC filter on a Zeiss Akioskop microscope. Fluorescently labeled embryos were mounted in Vectashield (Vector Labs) and imaged on a Leica (LCS) confocal microscope.

Acknowledgments

We thank members of the Granato laboratory for helpful discussions and advice on the manuscript. This work was supported by an NRSA postdoctoral fellowship to V.S. and grants from the National Science Foundation and the National Institute of Health to M.G.

References

Draper BW, Morcos PA, Kimmel CB. Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. Genesis 2001;30:154–156. [PubMed: 11477696]

Ha-Vinh R, Alanay Y, Bank RA, Campos-Xavier AB, Zankl A, Superti-Furga A, Bonafe L. Phenotypic and molecular characterization of Bruck syndrome (osteogenesis imperfecta with contractures of the large joints) caused by a recessive mutation in PLOD2. Am J Med Genet A 2004;131:115–120. [PubMed: 15523624]

Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, et al. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development 1996;123:1–36. [PubMed: 9007226]

Heikkinen J, Risteli M, Wang C, Latvala J, Rossi M, Valtavaara M, Myllyla R. Lysyl hydroxylase 3 is a multifunctional protein possessing collagen glucosyltransferase activity. Journal of Biological Chemistry 2000;275:36158–36163. [PubMed: 10934207]

Ihme A, Krieg T, Nerlich A, Feldmann U, Rauterberg J, Glanville RW, Edel G, Muller PK. Ehlers-Danlos syndrome type VI: collagen type specificity of defective lysyl hydroxylation in various tissues. J Invest Dermatol 1984;83:161–165. [PubMed: 6432919]

Izsvak Z, Ivics Z, Hackett PB. Repetitive elements and their genetic applications in zebrafish. Biochem Cell Biol 1997;75:507–523. [PubMed: 9551176]

Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004;20:33–43. [PubMed: 14698617]

Nasevicius A, Ekker SC. Effective targeted gene ‘knockdown’ in zebrafish. Nat Genet 2000;26:216–220. [PubMed: 11017081]

Passoja K, Myllyharju J, Pirskanen A, Kivirikko KI. Identification of arginine-700 as the residue that binds the C-5 carboxyl group of 2-oxoglutarate in human lysyl hydroxylase 1. FEBS Letters 1998a; 434:145–148. [PubMed: 9738467]

Passoja K, Rautavuoma K, Ala-Kokko L, Kosonen T, Kivirikko KI. Cloning and characterization of a third human lysyl hydroxylase isoform. Proceedings of the National Academy of Sciences of the United States of America 1998b;95:10482–10486. [PubMed: 9724729]

Pirskanen A, Kaimio AM, Myllyla R, Kivirikko KI. Site-directed mutagenesis of human lysyl hydroxylase expressed in insect cells. Identification of histidine residues and an aspartic acid residue critical for catalytic activity. Journal of Biological Chemistry 1996;271:9398–9402. [PubMed: 8621606]

Rautavuoma K, Takaluoma K, Passoja K, Pirskanen A, Kvist AP, Kivirikko KI, Myllyharju J. Characterization of three fragments that constitute the monomers of the human lysyl hydroxylase isoenzymes 1-3. The 30-kDa N-terminal fragment is not required for lysyl hydroxylase activity. Journal of Biological Chemistry 2002;277:23084–23091. [PubMed: 11956192]

Rautavuoma K, Takaluoma K, Sormunen R, Myllyharju J, Kivirikko KI, Soininen R. Premature aggregation of type IV collagen and early lethality in lysyl hydroxylase 3 null mice. Proc Natl Acad Sci U S A 2004;101:14120–14125. [PubMed: 15377789]

Risteli M, Niemitalo O, Lankinen H, Juffer AH, Myllyla R. Characterization of collagenous peptides bound to lysyl hydroxylase isoforms. J Biol Chem 2004;279:37535–37543. [PubMed: 15208310]

Ruotsalainen H, Sipila L, Kerkela E, Pospiech H, Myllyla R. Characterization of cDNAs for mouse lysyl hydroxylase 1, 2 and 3, their phylogenetic analysis and tissue-specific expression in the mouse. Matrix Biology 1999;18:325–329. [PubMed: 10429951]

Ruotsalainen H, Sipila L, Vapola M, Sormunen R, Salo AM, Uitto L, Mercer DK, Robins SP, Risteli M, Aszodi A, et al. Glycosylation catalyzed by lysyl hydroxylase 3 is essential for basement membranes. J Cell Sci 2006;119:625–635. [PubMed: 16467571]

Schneider VA, Granato M. The Myotomal diwanka (lh3) Glycosyltransferase and Type XVIII Collagen Are Critical for Motor Growth Cone Migration. Neuron 2006;50:683–695. [PubMed: 16731508]

Sprague J, Doerry E, Douglas S, Westerfield M. The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research. Nucleic Acids Res 2001;29:87–90. [PubMed: 11125057]

Steinmann B, Eyre DR, Shao P. Urinary pyridinoline cross-links in Ehlers-Danlos syndrome type VI. Am J Hum Genet 1995;57:1505–1508. [PubMed: 8533783]

Suokas M, Lampela O, Juffer AH, Myllyla R, Kellokumpu S. Retrieval-independent localization of lysyl hydroxylase in the endoplasmic reticulum via a peptide fold in its iron-binding domain. Biochemical Journal 2003;370:913–920. [PubMed: 12452796]

Valtavaara M, Papponen H, Pirttila AM, Hiltunen K, Helander H, Myllyla R. Cloning and characterization of a novel human lysyl hydroxylase isoform highly expressed in pancreas and muscle. J Biol Chem 1997;272:6831–6834. [PubMed: 9054364]

Valtavaara M, Szpirer C, Szpirer J, Myllyla R. Primary structure, tissue distribution, and chromosomal localization of a novel isoform of lysyl hydroxylase (lysyl hydroxylase 3). J Biol Chem 1998;273:12881–12886. [PubMed: 9582318]

van der Slot AJ, Zuurmond AM, Bardoel AF, Wijmenga C, Pruijs HE, Sillence DO, Brinckmann J, Abraham DJ, Black CM, Verzijl N, et al. Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. J Biol Chem 2003;278:40967–40972. [PubMed: 12881513]

van der Slot AJ, Zuurmond AM, van den Bogaerdt AJ, Ulrich MM, Middelkoop E, Boers W, Karel Ronday H, DeGroot J, Huizinga TW, Bank RA. Increased formation of pyridinoline cross-links due to higher telopeptide lysyl hydroxylase levels is a general fibrotic phenomenon. Matrix Biol 2004;23:251–257. [PubMed: 15296939]

Wang C, Luosujarvi H, Heikkinen J, Risteli M, Uitto L, Myllyla R. The third activity for lysyl hydroxylase 3: galactosylation of hydroxylysyl residues in collagens in vitro. Matrix Biology 2002;21:559–566. [PubMed: 12475640]

Wang C, Valtavaara M, Myllyla R. Lack of collagen type specificity for lysyl hydroxylase isoforms. DNA & Cell Biology 2000;19:71–77. [PubMed: 10701773]

Wienholds E, van Eeden F, Kosters M, Mudde J, Plasterk RH, Cuppen E. Efficient target-selected mutagenesis in zebrafish. Genome Res 2003;13:2700–2707. [PubMed: 14613981]

Yeowell HN, Ha V, Clark WL, Marshall MK, Pinnell SR. Sequence analysis of a cDNA for lysyl hydroxylase isolated from human skin fibroblasts from a normal donor: differences from human placental lysyl hydroxylase cDNA. J Invest Dermatol 1994;102:382–384. [PubMed: 8120423]

Yeowell HN, Walker LC. Tissue specificity of a new splice form of the human lysyl hydroxylase 2 gene. Matrix Biology 1999;18:179–187. [PubMed: 10372558]

Yeowell HN, Walker LC. Mutations in the lysyl hydroxylase 1 gene that result in enzyme deficiency and the clinical phenotype of Ehlers-Danlos syndrome type VI. Mol Genet Metab 2000;71:212–224. [PubMed: 11001813]

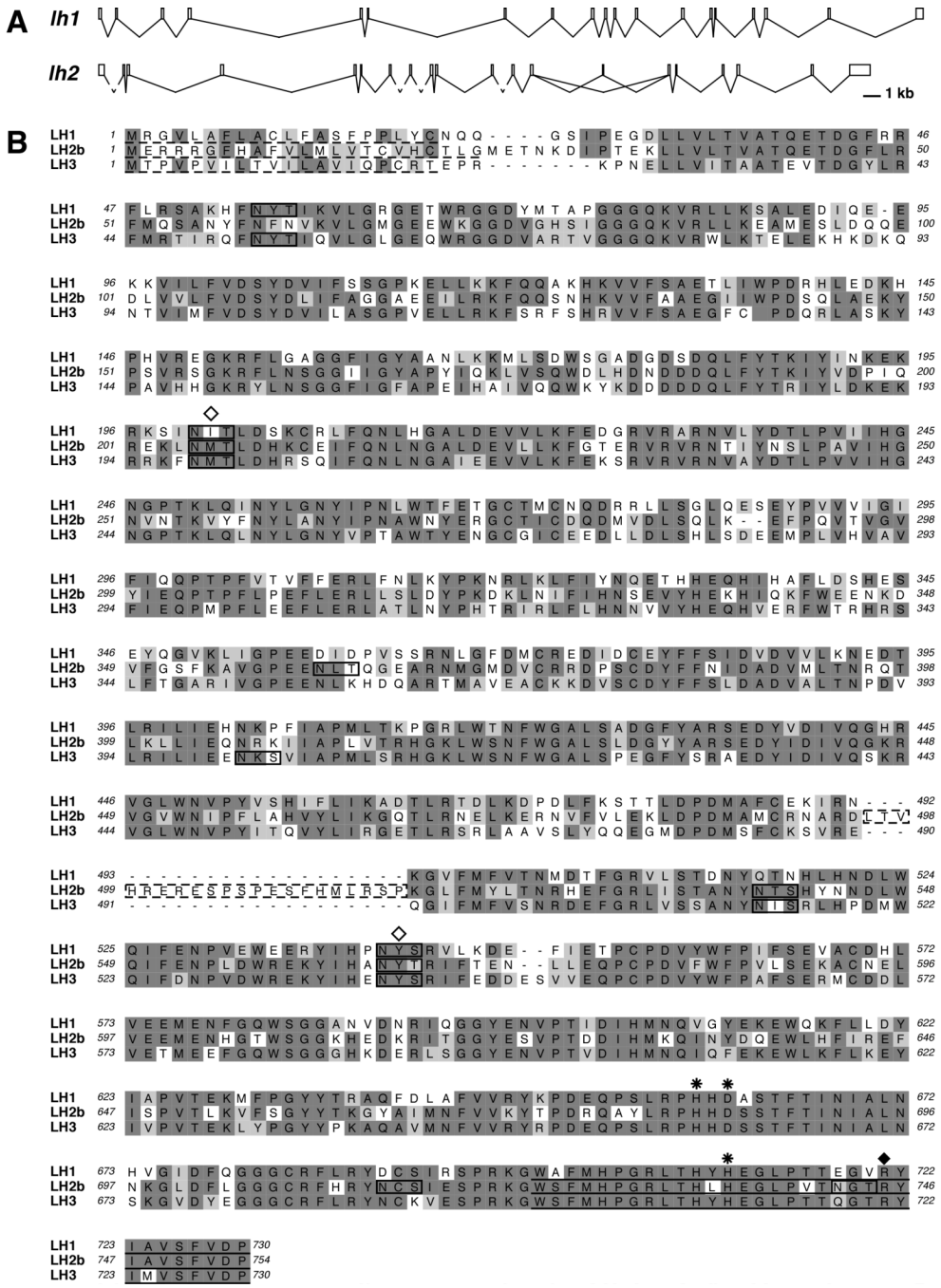

Figure 1.

Comparison of zebrafish *lh* genes. **A:** Genomic organization of zebrafish *lh1* and *lh2*. The dashed lines in *lh2* represent intron sequences whose lengths could not be determined due to the lack of available genomic sequence. **B:** ClustalW was used to align the proteins encoded by the zebrafish *lh1*, *lh2* and *lh3* genes. In LH2b, the dashed box indicates the residues encoded by the alternatively spliced exon 13A. Identical residues are shown in dark gray, similar residues in light gray. Dashed line: predicted signal sequence. Solid line: putative ER retention signal (Suokas et al., 2003). Boxes indicate potential sites for N-glycosylation. Open diamond: N-glycosylation site required for full enzymatic activity of human LH1 (Pirskanen et al.,

1996). Asterisk: Fe^{2+} binding site (Pirskanen et al., 1996). Closed diamond: 2-oxogluatarate binding site (Passoja et al., 1998a).

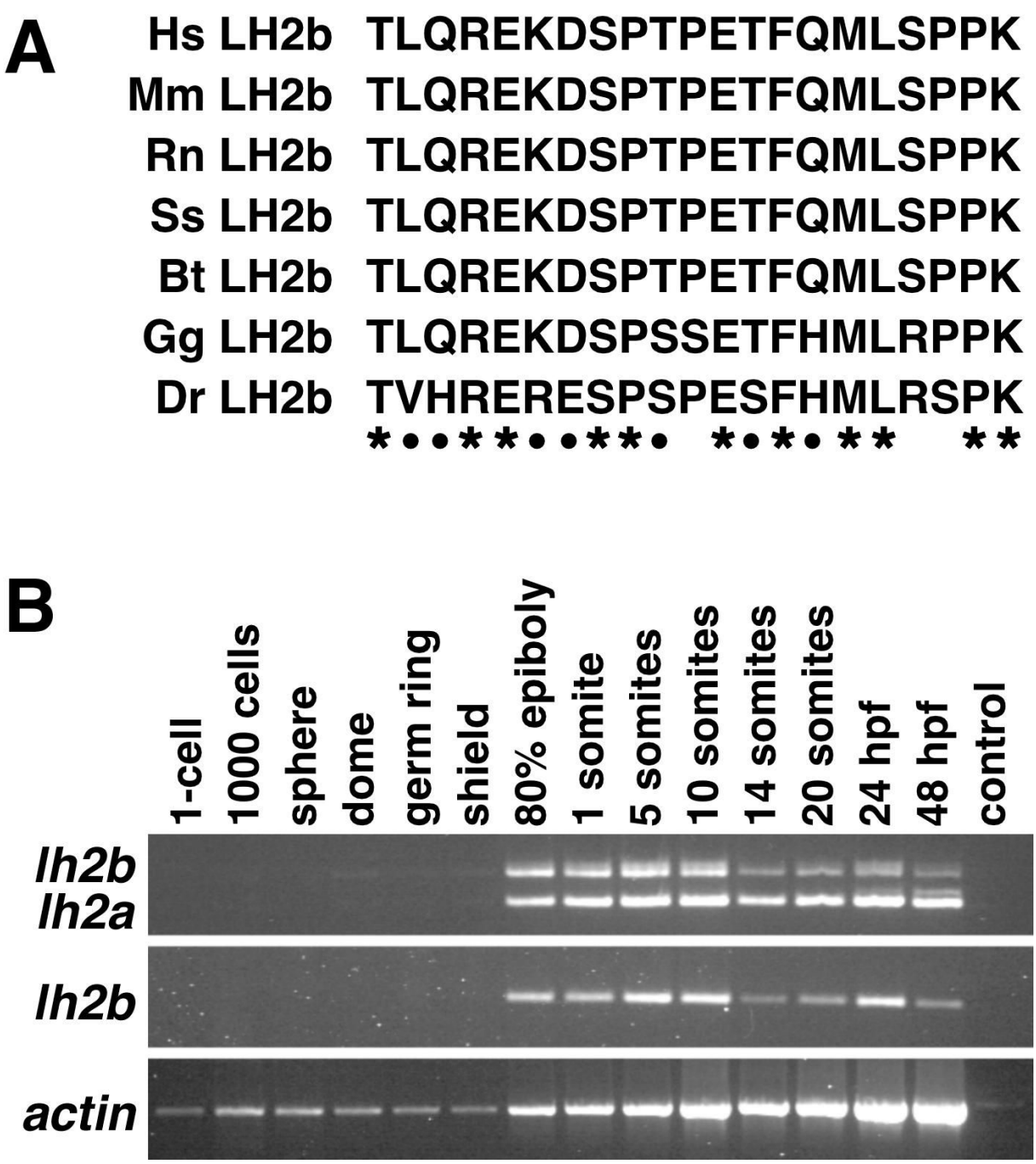

Figure 2.

Analyses of *lh2* splice variants. **A:** Alignment of the amino acid sequence encoded by exon 13A of various vertebrate *lh2* genes. Hs: Homo sapiens (NP_891988), Mm: Mus musculus (AAK20118), Rn: Rattus norvegicus (XP_579707), Ss: Sus scrofa (ABB02507), Bt: Bos taurus (XP_613110), Gg: Gallus gallus (XP_422695), Dr: Danio rerio. Asterisks indicate residues having 100% identity; closed circles show residues exhibiting conserved changes. **B:** RT-PCR analysis of zebrafish *lh2* splice variant expression at various stages of embryonic development. Top row: amplification of both *lh2b* (top) and *lh2a* (bottom) transcripts with primer pair A (see Experimental Procedures). Middle row: unique amplification of *lh2b* transcripts with primer

pair B. Note the decrease in *lh2b* expression at the 14 somite stage detected with both primer pairs. Bottom row: amplification of actin transcripts (control).

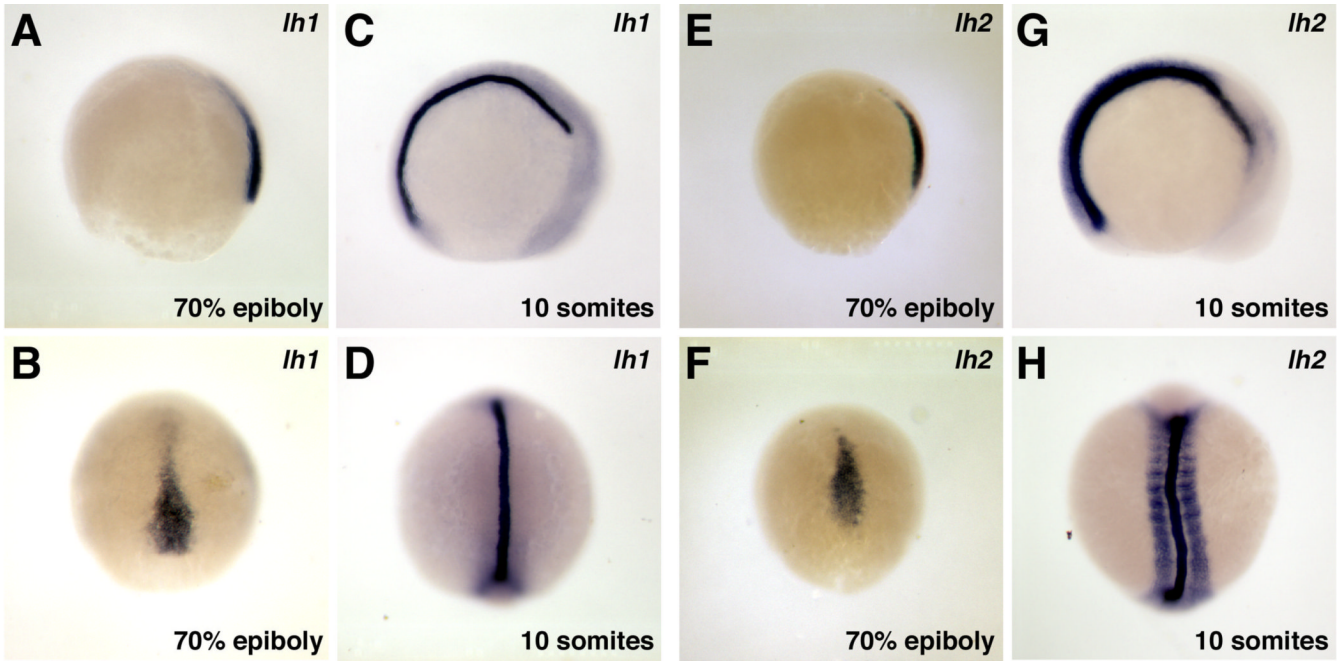

Figure 3.
Expression of *lh1* and *lh2* at early developmental stages. **A-D:** *lh1*, **E-H:** *lh2*. **A, B, E, F:** *lh1* and *lh2* are both expressed only in axial mesoderm during gastrulation (A, E: lateral views, B, F: dorsal views). **C, D, G, H:** During somitogenesis, *lh1* expression is restricted to the notochord, while an additional domain of *lh2* expression is observed in the somites and presomitic mesoderm (C, G: lateral views, D, H: dorsal views).

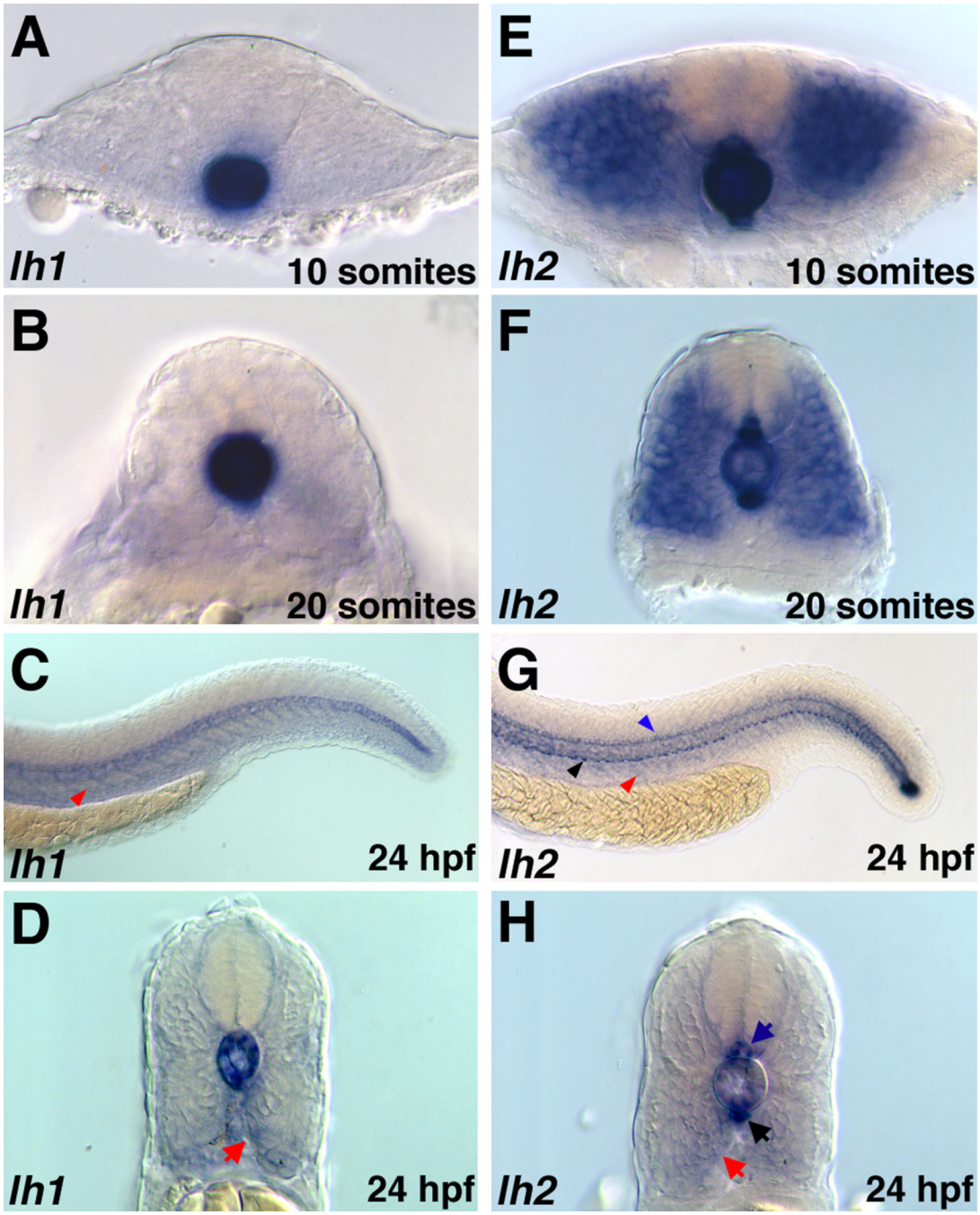

Figure 4.

Expression of *lh1* and *lh2* mRNAs in the trunk region. **A-D:** *lh1*, **E-H:** *lh2*. **AB:** Cross-sectional views showing restriction of *lh1* expression to the notochord throughout the period of somitogenesis. **E-F:** Cross-sectional views demonstrating maintenance of *lh2* in the notochord, myotome, floor plate and hypochord during somitogenesis stages. **C, D:** At 24 hpf, *lh1* expression in the notochord is reduced and low levels of expression are observed in the region of the developing sclerotome (red arrows) along the medial surface of the ventral somite (C: lateral view, D: cross sectional view) **E, F:** At 24 hpf, expression of *lh2* in the notochord and myotome is downregulated, while expression in the hypochord (black arrows) and floor plate

(blue arrows) persists. Weak expression in the region of sclerotome (red arrow) is also detected (G: lateral view, H: cross sectional view).

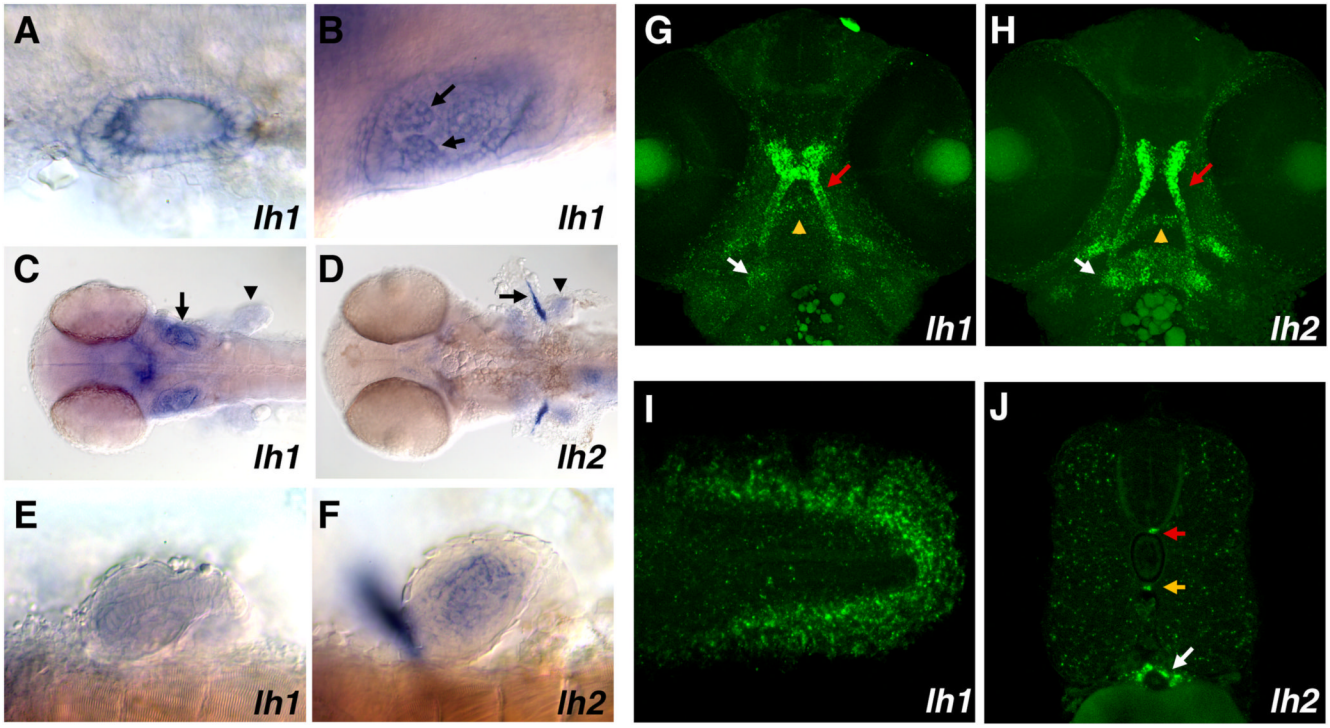

Figure 5.

Tissue specific expression of *lh1* and *lh2* mRNAs. **A, B:** Dorsal views of *lh1* expression in the otic vesicle at 24 (A) and 48 (B) hpf (anterior at right). Arrows indicate expression in the epithelial projections of the developing semicircular canals. **C, D:** dorsal views of 48 hpf embryos showing *lh1* (C) and *lh2* (D) expression in the anterior portion of the embryo. Expression of both mRNAs can be seen in the head and developing pectoral fins (arrowheads). Only *lh1* is expressed in the otic vesicles (arrow in C), whereas *lh2* is uniquely expressed in the cleithra (arrow in D). **E, F:** Higher magnification lateral views of *lh1* (E) and *lh2* (F) expression in the pectoral fins at 48 hpf. In F, the cleithrum is out of focus. Note the more intense central staining of *lh2* as compared to *lh1*. **G, H:** Confocal stacks showing *lh1* (G) and *lh2* (H) expression in head cartilages at 48 hpf (ventral views, anterior at top). Both mRNAs are strongly expressed in the neurocranium (red arrow), while *lh2* expression in the developing mandibular (yellow arrow) and hyoid arches (white arrow) is more pronounced than that of *lh1*. **I:** Lateral view of *lh1* expression in the tail at 48 hpf. Expression is restricted to the developing actinotrichia. **J:** Cross-sectional view of *lh2* mRNA expression at 48 hpf. Expression in the floor plate (red arrow) and hypochord (yellow arrow) persists at these stages. Transcripts can also be detected in the developing intestine (white arrow).

Table 1

Comparison of *lh* gene exon lengths

Exon Number	Dr *lh1* (bp)	Hs *lh1* (bp)[a]	Dr *lh2* (bp)	Mm *lh2* (bp)[b]	Dr *lh3* (bp)[c]
1	139	132	291	308	193
2	92	92	92	92	92
3	134	134	137	137	137
4	164	164	164	164	164
5	113	113	113	113	113
6	64	64	64	64	64
7	98	98	98	98	98
8	102	102	102	102	102
9	132	132	126	126	132
10	122	122	122	122	122
11	105	105	105	105	105
12	126	126	126	126	126
13	142	142	142	142	142
13A[d]	--	--	63	63	--
14	114	114	114	114	114
15	66	66	66	66	72
16	105	105	105	105	105
17	147	147	147	147	147
18	126	126	126	126	126
19	591	887	1074	1347	419

bp: base pairs, Dr: Danio rerio, Hs: Homo sapiens, Mm: Mus musculus

[a] Heikkenen et al. (1994)

[b] Ruotsalainen et al. (2001)

[c] Schneider and Granato (2006)

[d] alternatively spliced in *lh2*