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Distinct Tissue-Specificity of Three Zebrafish *ext1* Genes Encoding Proteoglycan Modifying Enzymes and Their Relationship to Somitic *Sonic Hedgehog* Signaling

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Proteins of the EXT (Exostosin) 1 family are known for their role in human disease. Mutations in *EXT1* cause hereditary multiple exostoses (HME), benign outgrowths of the bones, and therefore were classed as tumor suppressors. More recently, their role during embryonic development of *Drosophila* and mouse was addressed, revealing important functions of *EXT1* genes in major signaling pathways. Here, we report the isolation of three zebrafish members of the *EXT1* family, which we named *ext1a*, *ext1b*, and *ext1c*, respectively. They are expressed in restricted temporal and spatial domains during development. Both *ext1a* and *ext1b* are provided maternally and expressed during gastrulation: *ext1a* in the neurectoderm and *ext1b* in the embryonic midline and in the involuting mesendoderm of the germ ring. During somitogenesis stages, transcripts of all three *ext* genes can be found in the somitic mesoderm. Furthermore, *ext1a* is expressed in the dorsal neural tube. These expression domains become more pronounced at 24 hr postfertilization (hpf). At 48 hpf, *ext1* genes are present in the brain, while somitic expression ceases. Zebrafish have three members of the *EXT1* family, in contrast to only one EXT1 gene in mammals or *Xenopus*, consistent with the occurrence of partial genome duplications in the teleost lineage. Our expression analysis reveals that the three *ext* genes have distinct expression patterns, reflecting functional divergence after duplication. In addition, expression of *ext1a* and *ext1c* responds to elevated and reduced levels of *Sonic hedgehog* (*shh*) signaling in the somites, whereas expression of *ext1b* does not. This suggests a differential relationship between the *shh* pathway and individual *ext* gene function in zebrafish. *Developmental Dynamics 232:498–505, 2005.* © 2004 Wiley-Liss, Inc.

Key words: zebrafish; heparan sulfate proteoglycans; *EXT1*; somites; neural tube; *shh*; *smu*; *syu*

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RESULTS AND DISCUSSION

Heparan sulfate proteoglycans (HSPGs) are constituents of the extracellular matrix and play important roles during embryogenesis and cancer formation. They exert their function by regulating various signaling pathways, including Wingless/Int (Wnt), fibroblast growth factor (FGF), transforming growth factor (TGF) -β and Hedgehog (Hh; Blackhall et al., 2001; Nybakken and Perrimon, 2002; Kramer and Yost, 2003). The synthesis of HSPGs depends on the addition of a glycosaminoglycan chain to a core

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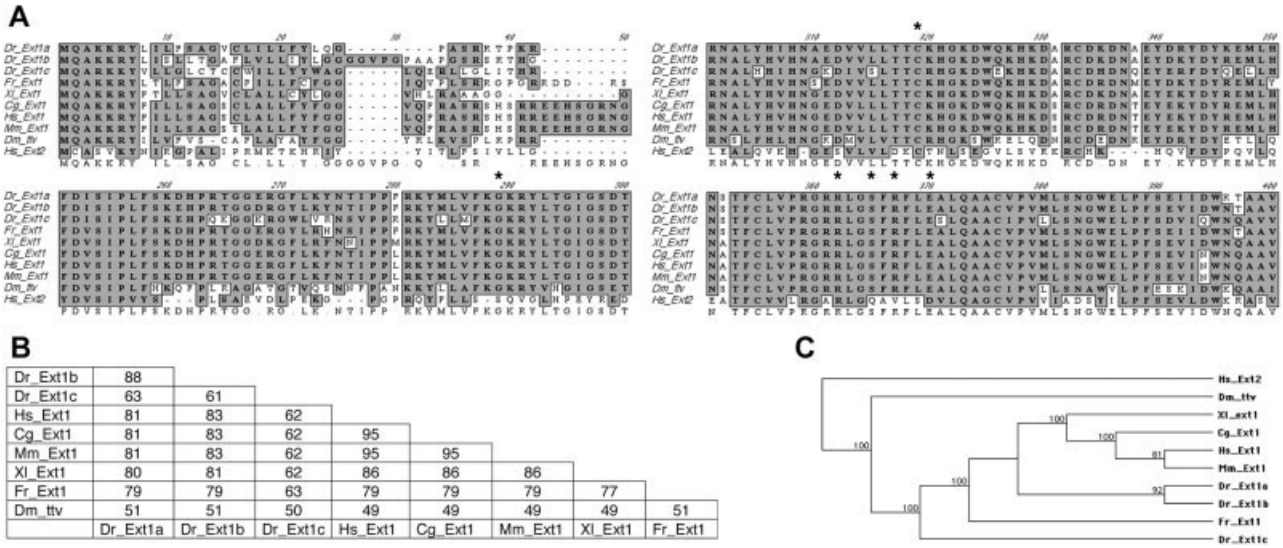


Fig. 1. Sequence comparison of EXT1 proteins. **A:** Alignment of part of EXT1 proteins of different species, including the glycosyltransferase region. After the first seven conserved amino acids, a short, less well-conserved putative stem region follows. More C-terminal regions are highly conserved. Amino acids that are critical for glycosyltransferase activity of EXT1 proteins are conserved in all three zebrafish EXT1 proteins (asterisks). **B:** Comparison of sequence identities between EXT1 proteins of different species. **C:** Phylogenetic tree of the different EXT1 proteins. All three zebrafish EXT1 proteins fall into the EXT1 clade with high bootstrap support. Cg, *Cricetulus griseus*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Fr, *Fugu rubripes*; Hs, *Homo sapiens*; Xl, *Xenopus laevis*.

protein. This reaction is catalyzed by glycosyltransferases belonging to the *EXT* family (Lind et al., 1998). In humans, mutations in these genes cause HME, benign tumors at the growths caps of the long bones (Zak et al., 2002). To date, several members of the *EXT* family have been cloned and identified in humans. These members include *EXT1*, *EXT2*, *EXT3*, and three *EXT*-like genes (Cook et al., 1993; Le Merrer et al., 1994; Hecht et al., 1995; Wise et al., 1997; Wuyts et al., 1997; Van Hul et al., 1998). In addition, orthologous genes for *EXT1* and *EXT2* were identified in mouse, *Xenopus*, *Caenorhabditis elegans*, and *Drosophila*, where the *EXT1* homolog was named *tout-velu* (*ttv*; Clines et al., 1997; Lin and Wells, 1997; Stickens and Evans, 1997; Bellaiche et al., 1998; The et al., 1999; Han et al., 2001; Katada et al., 2002). So far, only one member of each family has been identified in all species examined. In contrast to this, we describe the identification of three members of the *EXT1* family in the zebrafish genome.

Cloning of Zebrafish *ext1a*, *ext1b*, and *ext1c*

We identified zebrafish *ext1* genes by performing a BLAST search of the publicly available zebrafish databases with mouse EXT1. From this search, we retrieved three sequences, which we amplified by polymerase chain reaction (PCR) and subsequent rapid amplification of cDNA ends (RACE) PCR to generate the full-length open reading frames (ORF; see Experimental Procedures section). The predicted amino acid sequences of these proteins showed a high homology to known EXT1 proteins of other species (Fig. 1B). EXT1a shows 81% identity to mammalian EXT1 proteins, whereas EXT1b shows 83% identity. In contrast to these high values of identity, EXT1c shows only 62% identity. Zebrafish EXT1a and EXT1b show 88% identity among themselves and only 61% identity to EXT1c. Of interest, these values are lower than the respective identities within the mammalian clade. Here, EXT1 proteins show 95% identity. These values differ greatly within the protein. After the first seven amino acids, which are conserved in all species, and the putative transmembrane domain, the N-terminus up to amino acid 115 is less well-conserved. This portion is thought to form a putative stem region, which separates the signal sequence from the catalytic portion of the protein (Wei et al., 2000). Several mutations have been characterized in *EXT1* genes affecting the glycosyltransferase activity (Wei et al., 2000). The amino acids altered in these mutants are conserved in the zebrafish EXT1 paralogs (Fig. 1A, asterisks), underlining the evolutionary conservation of all three proteins. In addition, the phylogenetic tree (Fig. 1C) groups all three zebrafish EXT1 proteins in the EXT1 clade with high bootstrap support. The phylogenetic analysis furthermore suggests that *ext1a* and *ext1b* arose during a more recent duplication event in the zebrafish lineage, whereas *ext1c* is more diverged. Thus, these findings indicate that *ext1a*, *ext1b*, and *ext1c* are the orthologous zebrafish genes of other known *EXT1* genes. To analyze their expression during embryonic development, we performed in situ hybridization with riboprobes for the respective genes. As a control, we performed in situ hybridizations with sense probes of each gene that did not produce a staining (data not shown).

Expression of *ext1a*

Ext1a message is maternally provided (data not shown) and present at

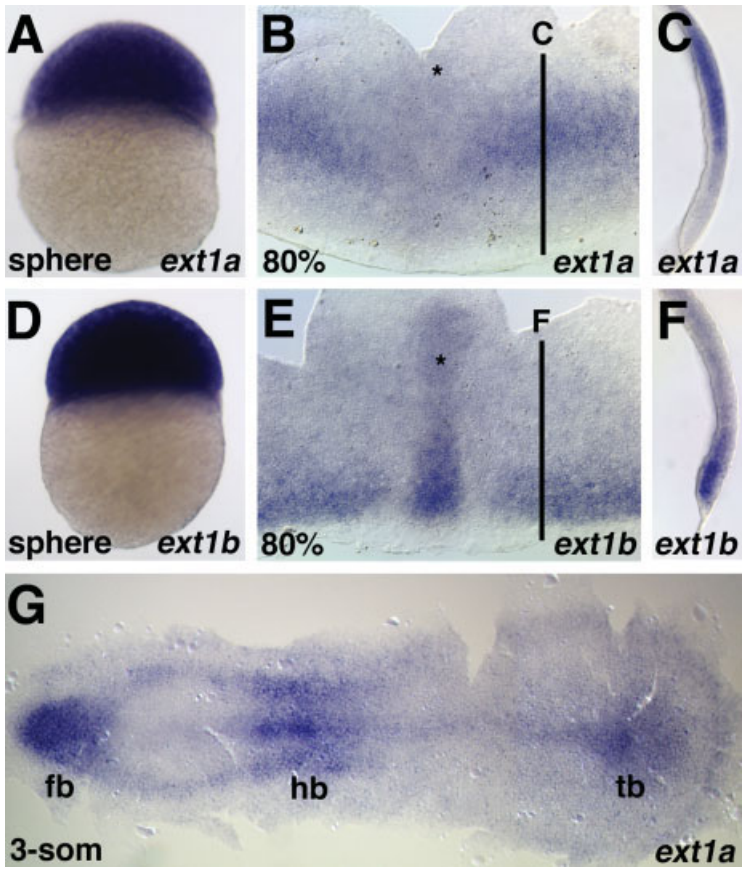


Fig. 2.

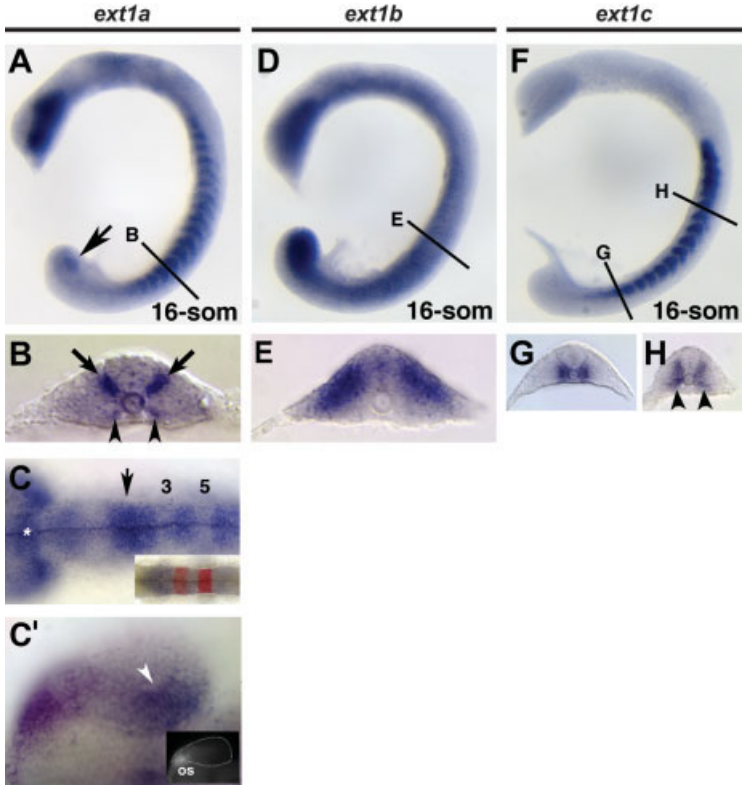


Fig. 3.

sphere stage (Fig. 2A). At approximately 80% of epiboly, *ext1a* is expressed in two ectodermal wings animal to the germ ring. These areas exclude the most dorsal structures, such as the axial mesoderm (Fig. 2B,C). At the three-somite stage, *ext1a* message is present in prospective forebrain regions, while in putative midbrain regions, it marks the outline of the neural plate (Fig. 2G). Furthermore, three stripes of *ext1a* expressing cells can be detected in the forming hindbrain. More posteriorly, *ext1a* is expressed in the tail bud and in the axial mesoderm (Fig. 2G). From the five-somite stage onward, *ext1a* RNA can be detected in the somitic mesoderm. This expression continues during somitogenesis (Fig. 3A). Cross-sections at the 16-somite stage reveal

Fig. 2. Expression of *ext1a* (A–C,G) and *ext1b* (D–F), anterior to the top (A–F), or to the left (G). **A:** Sphere stage. **B:** Eighty percent of epiboly. The embryo was opened at the ventral side and flat-mounted, dorsal side up. *Ext1a* transcripts can be detected in two wings in the prospective hindbrain region lateral to the embryonic midline (marked by asterisk). **C:** Cross-section of the embryo shown in B. **D:** Sphere stage. **E:** Eighty percent epiboly stage. Orientation of the embryo as in B. *Ext1b* RNA can be detected in the embryonic midline (marked by asterisk) and in the germ ring. **F:** Cross-section of the embryo in E. **G:** At the three-somite (3-som) stage, *ext1a* is expressed in the forebrain (fb), the hindbrain (hb), and in the tailbud (tb).

Fig. 3. Expression of *ext1a* (A–C,C′), *ext1b* (D,E), and *ext1c* (F–H) at the 16-somite stage. Anterior of the embryos is to the left. **A:** Expression of *ext1a* can be detected in the eye, the dorsal neural tube, Kupffer's vesicle (black arrow), and the somites. **B:** Cross-section at the level of the 12th somite. Expression of *ext1a* can be detected in the anterior hindbrain (arrow) and the dorsal diencephalon (asterisk). **C:** Dorsal view on the brain of the embryo in A. *Krox20* demarcates rhombomeres 3 and 5 (inset). **C′:** Expression of *ext1a* in the eye, which do not express *ext1a*. In addition, ext1a transcripts can be detected in two ventromedial (arrowheads) and two dorsomedial (arrows) domains. **D:** RNA encoding *ext1b* is expressed more ubiquitously with domains of higher expression in the tailbud region, the eye, and dorsal somites. **E:** Section at the level of the 9th somite, revealing expression of *ext1b* in the dorsal aspect of the somite. **F:** Alternating expression domains of *ext1c* can be detected in the somites. **G:** Cross-section of the embryo in F at the level of the 16th somite. **H:** Cross-section at the level of the 6th somite, revealing expression of *ext1c* in ventromedial regions of the somites (arrowheads) and adjacent to the neural tube.

staining in two ventromedial domains of the somites (Fig. 3B, black arrowheads) and in a dorsomedial compartment, adjacent to the neural tube (Fig. 3B, black arrows). In addition to the somitic expression, *ext1a* message can be detected in a segmental manner in the dorsal neural tube (Fig. 3C). Double in situ hybridization with *Krox20* reveals that rhombomeres 4 and 6 express *ext1a* (Fig. 3C, inset). Furthermore, the anterior hindbrain (Fig. 3C, black arrow) and the dorsal diencephalon (Fig. 3C, white asterisk) are positive for *ext1a* message. In the eye, expression consists of two domains: the distal retina (Fig. 3C′, white arrowhead) and the optic stalk region (Fig. 3C′), as marked by double in situ hybridization with *pax2.1* in red (Fig. 3C′, inset shows fluorescent *pax2.1* signal). Most posteriorly, Kupffer's vesicle exhibits elevated *ext1a* expression (Fig. 3A, black arrow). At 24 hpf, strong cerebellar expression is detected (Fig. 4A, black arrow, 4D, black arrow) as well as expression in the optic stalk (Fig. 4A, black arrowhead, 4F, black arrowhead) and the dorsal diencephalon (Fig. 4A, black asterisk). At this stage, the anterior part of the epiphysis shows a stronger signal compared with the posterior part (Fig. 4E, outlined with white dotted line). In general, *ext1a* expression is confined to the dorsal part of the neural tube, as revealed by a cross-section at the level of the anterior spinal cord (Fig. 4C). Furthermore, expression can be detected in the tail somites. At approximately 48 hpf, expression continues in the dorsal diencephalon and in the cerebellum. In addition, mRNA can be detected in the otic vesicle (Fig. 4B, black arrow).

Expression of *ext1b*

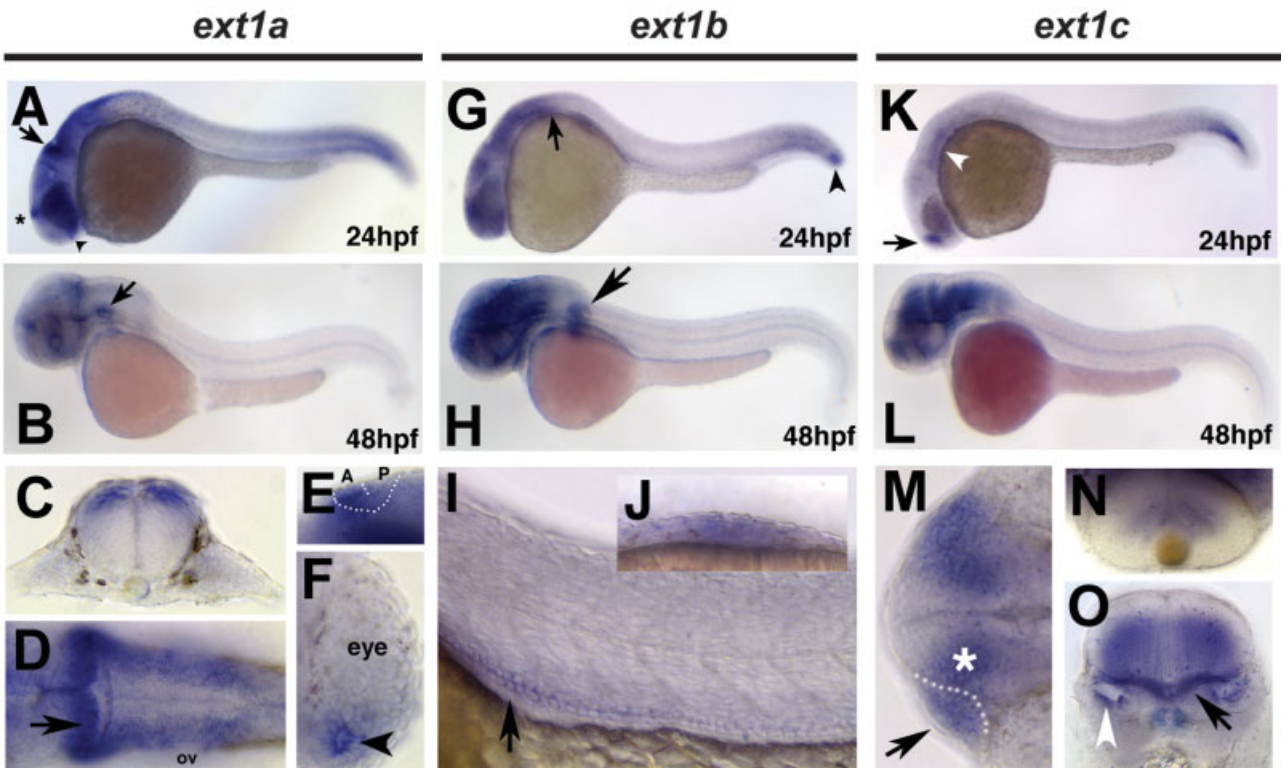
Ext1b is also provided maternally (data not shown) and transcripts can be detected at sphere stage (Fig. 2D). During gastrulation, expression consists of two domains: the germ ring and the axial mesoderm (Fig. 2E, asterisk). Cross-section at the level of the germ ring confirms mesendodermal expression (Fig. 2F). During somitogenesis stages, expression is strongest in the tail bud and the posterior somites (Fig. 3D). Cross-section reveals transcripts in the myotomal part of the somites, while no expression is detected in the sclerotomal part and in adaxial tissues (Fig. 3E). In addition, transcripts can be detected in the forming eye (Fig. 3D). At the 24 hpf stage, cells of the neural tube up to the posterior limit of the hindbrain (Fig. 4G, black arrow) are positive for *ext1b* transcripts. In addition, the posterior somites and the tail bud (Fig. 4G, black arrowhead) continue to express *ext1b*. Another mesodermal expression domain comprises cells in the region of the anterior pronephric duct (Fig. 4I, black arrow). In the forming fin buds (Fig. 4J), *ext1b* can also be detected. At approximately 48 hpf, the fin buds (Fig. 4H, black arrow) as well as the brain are positive for *ext1b* transcripts.

Expression of *ext1c*

Ext1c message can be detected first at the beginning of somitogenesis, where it is expressed in the forming somites. Later, at approximately the 16-somite stage (Fig. 3F), expression is confined to adaxial cells in the posterior part of the embryo (Fig. 3G). More anteriorly, cells adjacent to the neural tube and in ventromedial regions of the somite express *ext1c* (Fig. 3H, black arrowheads). At the end of somitogenesis, neural expression domains of *ext1c* appear, whereas the posterior somites continue to express *ext1c*. Neural domains become more elaborate at the 24 hpf stage (Fig. 4K): transcripts can be detected in the ventral rhombomeres (Fig. 4K, white arrowhead) and the telencephalon (Fig. 4K, black arrow). Furthermore, close up of the telencephalon (Fig. 4M, white asterisk) reveals that also cells of the olfactory bulbs contain *ext1c* transcripts (Fig. 4M, black arrow). At 48 hpf, transcripts can be detected in the brain (Fig. 4L). Cross-section at the level of the otic vesicle shows strong expression in the white matter (Fig. 4O, black arrow) as well as in the otic vesicles (white arrowhead) and in more dorsally located nascent neurons. At this stage, also the fin buds (data not shown) and the retina express *ext1c* (Fig. 4N).

Taken together, our results reveal the existence of at least three different members of the EXT1 family in zebrafish that most likely arose during a genome duplication event in the teleost lineage. Their expression throughout embryogenesis comprises partially overlapping and mutually exclusive domains. In addition, the expression patterns show a spatial shift over time: at somitogenesis stages, transcripts are present in the somites, while at later stages of development, the brain and the fin buds express *ext1* genes.

These expression domains partially contrast with those found in other species. For instance, the *Drosophila EXT1* homolog, *ttv* is present ubiquitously (The et al., 1999) and in *Xenopus EXT1* is expressed without tissue specificity during early embryogenesis and at adult stages (Katada et al., 2002). Of interest, the mouse *EXT1* expression pattern is more complex. In early embryonic development (5.5 to 7.5 days post coitum), *EXT1* expression is reported to be ubiquitous (Lin et al., 1998). Later, transcripts accumulate in limb buds, regions of ossification, tail, and brain (Lin et al., 1998; Stickens et al., 2000; Inatani and Yamaguchi, 2003). As we report for zebrafish *ext1a*, expression of *Ext1* was strongest in the cerebellum, which continues to express *Ext1* at postnatal stages (Rubin et al., 2002; Inatani and Yamaguchi, 2003). In addition, the mouse forebrain contains *Ext1* transcripts (Inatani and Yamaguchi, 2003). In zebrafish, we find *ext1c* present in these structures (Fig. 4K, black arrow, M), while *ext1a* expression is lost in forebrain regions (Fig. 4A). Thus, different zebrafish *ext1* genes are expressed in structures that both express the single mouse *Ext1* gene. Another site of mouse *Ext1* expression are the limb buds, homologous to fin buds in zebrafish. Here, *ext1b* and *ext1c* transcripts can be found, but no *ext1a* expression, which might have become dispensable during evolution due to the continued expression of the other two *ext1* genes. From these findings, we speculate that the diversification of *ext1* gene expression might provide another example of the duplication–degenera-


ext1a
ext1b
ext1c
A
24hpf
G
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24hpf
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48hpf
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Fig. 4.

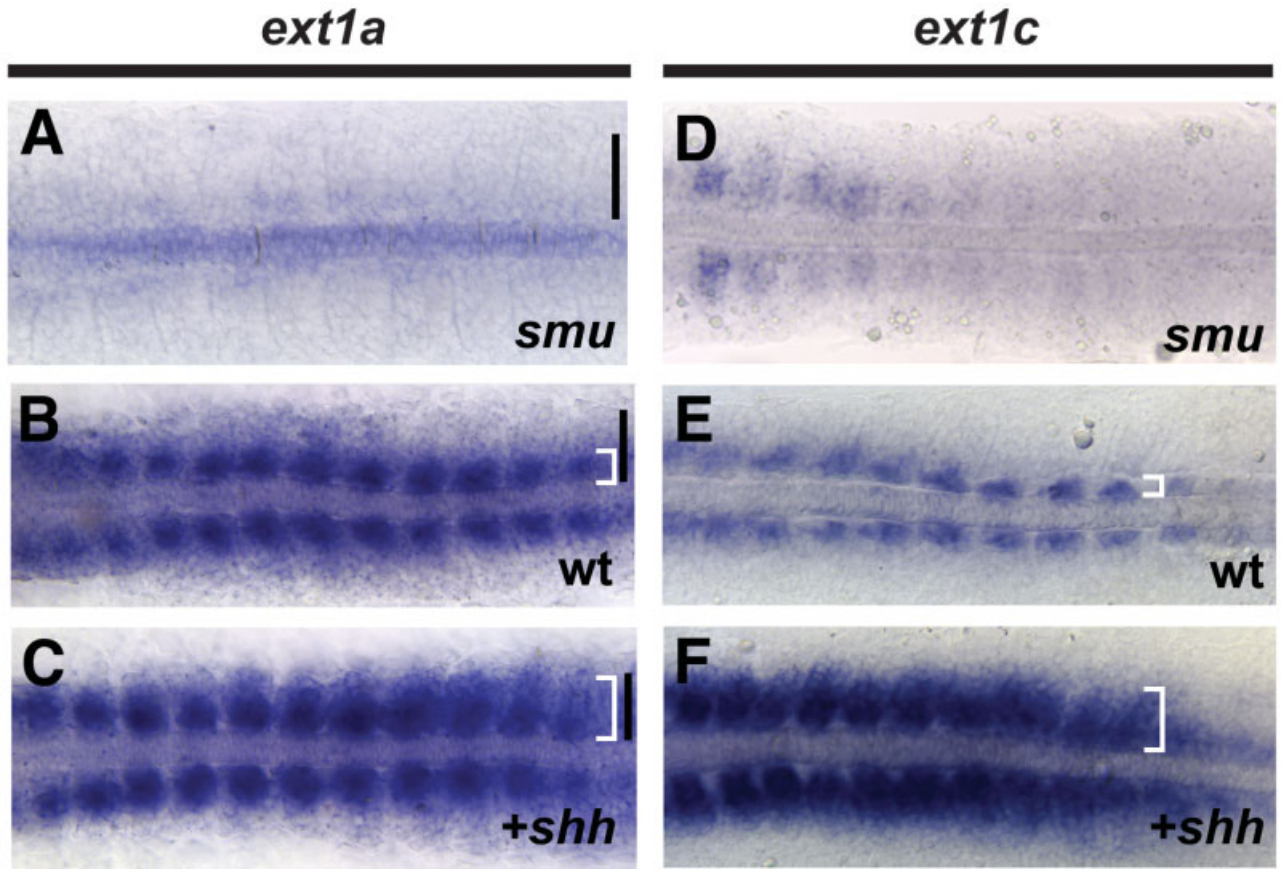
ext1a
ext1c
A
smu
D
smu
B
wt
E
wt
C
+shh
F
+shh

Fig. 5.

tion–complementation model (Force et al., 1999).

Dependence of *ext1a* and *ext1c* on Hedgehog Signaling

Previous studies in *Drosophila* have shown that EXT1 genes are necessary for the spreading of molecules belonging to the *hh* family (Bellaiche et al., 1998). Furthermore, a study in mice suggests that EXT1 proteins might negatively regulate *shh* signaling by synthesizing HSPGs, which sequester the ligand (Koziel et al., 2004). This negative regulation might suggest the existence of a feedback loop. For instance, overexpression of *shh* induces expression of the SHH receptor *patched* (Concordet et al., 1996), which also sequesters the ligand (Chen and Struhl, 1996), whereas loss of *shh* signaling leads to an absence of *patched* expression (Chen et al., 2001; Varga et al., 2001). We therefore asked whether *ext1* genes respond to *shh* in a similar way. To this end, we examined the effects of *shh* overexpression on zebrafish *ext1* genes using synthetically transcribed *shh* mRNA. We also addressed the effects of loss of hedgehog signaling by analyzing the expression of *ext1* genes in known mutants of the hedgehog pathway in fish. These mutants included *smu* (*slow muscle omitted*; Chen et al., 2001; Varga et al., 2001), which encodes for zebrafish *smoothened*, the canonical receptor for all hedgehog signals, and *syu* (*sonic you*; Schauerte et al., 1998), a deletion mutant for the zebrafish *shh* gene. The effect of altered hedgehog signaling was assessed at the 14-somite stage. In a dorsal view of this stage, *ext1a* is more strongly expressed in tissue closer to the midline (Fig. 5B). Increased hedgehog signaling led to ectopic expression of *ext1a* throughout most of the somite (Fig. 5B,C, compare white brackets). However, different expression intensities of *ext1a* could still be detected. More medially located areas of the somites showed a stronger staining, which might suggest that additional factors are required for the induction of strong *ext1a* expression. In the case of *ext1c*, the effect of *shh* overexpression was more pronounced. Upon overexpression of *shh*, a large proportion of the somitic cells expressed *ext1c* at uniformly high levels (Fig. 5E,F, compare white brackets). Of interest, expression of *ext1b* was found to be essentially normal in a gain of *shh* function situation (data not shown).

In contrast to this finding, loss of *shh* signaling (shown for *smu* mutant embryos) led to a nearly complete loss of *ext1a* expression in the somites (Fig. 5A). Similarly, *ext1c* expression could only be detected in the more anterior somites with a progressive loss of expression toward posterior regions (Fig. 5D). Again, expression of *ext1b* was not altered in *smu* mutant embryos (data not shown). Similar results were obtained when analyzing *syu* mutant fish. However, we noted a less severe reduction in gene expression of both *ext1a* and *ext1c*, probably reflecting a possible redundancy with other members of the *Hh* family (data not shown). Therefore, we conclude that expression of *ext1a* and *ext1c* in somites depends in part on functional *hh* signaling and responds to *shh* overexpression by an increase in transcription. We speculate that a putative regulatory link between zebrafish *ext1a* and *ext1c* genes and *hh* signaling exists during vertebrate somite patterning. Alternatively, the observed effects may be due to an indirect effect of slow muscle respecification, which has been shown to occur upon *shh* overexpression (Blagden et al., 1997). The finding that *ext1b* does not respond to elevated or reduced levels of *shh* signaling underscores the divergence of different members of the *ext1* family in zebrafish. Interestingly, we noted ectopic expression of *ext1a* in the notochord of *smu* mutant zebrafish at the 14-somite stage (Fig. 5A). At this stage, *shh* is produced in notochord cells (Krauss et al., 1993). In wild-type, axial mesoderm also expresses *ext1a* at the beginning of somitogenesis (Fig. 2G), but this expression is lost during later development (Fig. 3B). The prolonged expression of *ext1a* in the notochord of *smu* embryos might reflect a differential response of the *ext1a* promoter in notochord and somitic cells.

EXPERIMENTAL PROCEDURES

Cloning of Zebrafish *EXT1* Homologues

We retrieved sequences from NA6265 (*ext1a*), ctg9593 (*ext1b*), and NA205 (*ext1c*) using the ZON laboratory Blast server (http://134.174.23.160/zfBlast/Public Blast.htm). On the basis of the retrieved sequences, we designed primers to amplify parts of each gene including the 5′ end. The primers were as follows: *ext1a* fwd 5′-GCAG-

Fig. 4. Expression of *ext1a* (A–F), *ext1b* (G–J), and *ext1c* (K–O) at 24 hr postfertilization (hpf) (A–G,I–K,M) and 48 hpf (B,H,L,N,O). **A:** Neural expression of *ext1a* can be detected in the cerebellum (arrow), the dorsal diencephalon (asterisk) and the optic stalk (arrowhead). **B:** Expression in the otic vesicle (black arrow). **C:** Cross-section of the embryo in A at the level of the anterior spinal cord. **D:** Dorsal view of the embryo in A at the level of the cerebellum (black arrow) and hindbrain. Ov, otic vesicle. **E:** Dorsal diencephalon (outlined with white dots; A, anterior, P, posterior). **F:** Cross-section through the eye. Black arrowhead marks optic stalk. **G:** Expression of *ext1b* in the tailbud region (arrowhead) and in anterior neural tissue (arrow). **H:** The fins (black arrow) express *ext1b*. **I:** Close up of cells belonging to the anterior pronephric duct (black arrow). **J:** Close up of the fin bud mesenchyme. **K:** Transcripts of *ext1c* can be detected in the telencephalon (black arrow) and in the rhombomeres (white arrowhead). **L:** Expression is confined to the brain. **M:** Telencephalon (white asterisk) and the olfactory bulbs (marked by arrow and outlined by dotted white line) of the embryo in A show *ext1c* expression. **N:** Expression of *ext1c* in the retina. **O:** Cross-section of the embryo in L at the level of the otic vesicles (white arrowhead). The white matter is marked by black arrow.

Fig. 5. Dependence of *ext1a* and *ext1c* expression on Sonic hedgehog (Shh) signaling. A–F: Dorsal views of 14-somite stage embryos with anterior to the left. Expression of *ext1a* (A–C) and *ext1c* (D–F), respectively. **A,B,D,E:** In *smu* mutant embryos, expression of *ext1a* (A) and *ext1c* (D) is greatly reduced compared with wild-type (wt) embryos (B,E). **C,F:** Overexpression of *shh* results in an expansion of the somitic expression domains of *ext1a* (C) and *ext1c* (F, compare white brackets in B,C and E,F). Black lines indicate somite borders in (A–C).

GAGTTGAGACCCTTGGATTCATCAGC-3′, *ext1a* rev 5′-TGCCGTGTTTGCATGTGGTAAGGAGAACC-3′; *ext1b* fwd 5′-TGGTGGTGTTGAGGGACAAACTTCCAAACG-3′, *ext1b* rev 5′-CGTGTCCGAGCCGATGCCTGTCAA-3′; *ext1c* fwd 5′-CCTGCTGAAGCTCCCGGCGTGGC-3′, *ext1c* rev 5′-GGAAACGATGTCCTTGCCATTGTGTATGTGATG-3′. To obtain the full ORF of each gene, we performed 3′RACE reactions (GeneRacer, Invitrogen) according to the manufacturer's instructions. We used the following primers: *ext1a* 5′-GGCAAGCGCTATTTGACTGGGATTGGGTCTGAC-3′, *ext1b* 5′-TTGACAGGCATCGGCTCGGACACG-3′, *ext1c* 5′-AGATGCTCGCTGTGACAACCAGGA-3′.

Subsequently, the full-length ORF was amplified using the above-specified forward primers in combination with the following reverse primers: *ext1a* 5′-CGGAAGACAGAAATGTGCCAGCGATGG-3′, *ext1b* 5′-GTCTCTTACGGCCCCCTGTTGAACGTCCAG-3′, *ext1c* 5′-CAGCGGCTGGAATGATTGACAGGTCTGAGTG-3′. The sequences for *ext1a*, *ext1b* and *ext1c* are available under GenBank accession nos. AY34455, AY34456, and AY34457, respectively.

In Situ Hybridization and RNA Synthesis

Sense and antisense probes were produced by cloning the partial ORFs of each *ext1* gene into pCRII TOPO vectors (Invitrogen; *ext1a* and *ext1b*) or pCR4 vector (*ext1c*), followed by linearization and in vitro RNA synthesis using a digoxigenin labeling kit (Roche). In situ and double in situ hybridizations were essentially carried out as described previously (Reifers et al., 1998). *shh* mRNA was transcribed from the *shh*-T7TS Plasmid (Krauss et al., 1993) using the MessageMachineKit (Ambion). About 50 pg of *shh* were injected into zebrafish eggs at the one-cell stage.

Zebrafish Mutants

Smu mutant zebrafish were obtained from Zoltan Varga (Varga et al., 2001). *Syu* mutants were obtained from the Tübingen stock center (Schauerte et al., 1998).

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