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Expression Pattern for *unc5b*, an Axon Guidance Gene in Embryonic Zebrafish Development

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Branching processes such as nerves and vessels share molecular mechanisms of path determination. Our study focuses on unc5b, a member of the unc5 axon guidance gene family. Here, we have cloned the full-length zebrafish ortholog of *unc5b*, mapped its chromosome location in the zebrafish genome, and compared its expression patterns to robo4, another axon guidance family member. In situ show that *unc5b* is expressed predominantly in sensory structures such as the eye, ear, and brain. Both *unc5b* and *robo4* show robust expression in all three compartments of the embryonic brain, namely forebrain, midbrain, and hindbrain. In particular, the hindbrain rhombomere expression displays interesting patterns in that *robo4* is expressed in medial rhombomere cell clusters when compared to *unc5b* expressed in lateral rhombomere clusters. A similar medial–lateral theme is observed in other neural structures such as the neural tube. Our expression analysis provides a starting point for studying the role of axon guidance genes in embryonic hindbrain patterning.

Key words: unc5b; robo4; Hindbrain; Axon guidance; Zebrafish; Staining

INTRODUCTION

Neural and vascular patterning networks are intricate branching processes that are precisely controlled by cues from surrounding milieu (3). Four different classes of axon guidance molecules and their cognate receptors, namely the ephrins-Eph, semaphorins-plexin, netrin-unc5, and slit-roundabout (robo), are responsible for orchestrating and coordinating axon guidance to their target. *unc5b* and *robo4* are members of the unc5 and robo family, respectively, and are expressed in both neural and vascular systems. This study focuses on the neuronal expression of *unc5b*, and compares its brain expression patterns to *robo4*. unc5b, like robo4, is a single pass transmembrane receptor and contains immunoglobulin domains in the extracellular region. In the intracellular region, unc5b contains unique cytoplasmic motifs such as zonula occludens-1 (ZU-1) and death domains (1). Prior to this study, a partial *unc5b* zebrafish clone was reported (8), but the temporal and spatial expression patterns during embryonic development were not reported. Here, we have cloned the full-length ortholog of *unc5b*, characterized its genome location, performed

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in situ across developmental stages, and compared its neural expression patterns to robo4 in the developing vertebrate brain.

MATERIALS AND METHODS

Zebrafish Stocks and Reagents

Zebrafish were grown and maintained at 28.5°C (12) under MCW animal protocol number 312-06-2 guidelines. Mating was routinely carried out at 28.5°C and the embryos were staged according to established protocols (6).

RT-PCR and Antisense Probe Generation

Total RNA isolated from 24 hpf embryos was subjected to RT using oligo dT primer. Primers used to amplify were (a) CGGACTCGTTTCCATCAGCT CCT, (b) GCGTGCACATAATCCCTCAGTGC, (c) CAGGAAGTATTAGAGGTTGA, (d) GCAATCGC CATCTGTCGT, (e) GAATTCCAGAGTGAGCCC GAGGA, (f) CTGACATTCACGACTGCGCCAAT, using PCR parameters previously described (2). The PCR products were cloned into pCR4Topo vectors, sequenced from both ends, and antisense *unc5b* DIG RNA probe was made with *Spe*I linearized vector using T7 RNA polymerase. The *robo4* probe was generated as described previously (2).

Radiation Hybrid (RH) Mapping

The Zebrafish/Hamster radiation hybrid panel was obtained from University of Ottawa. PCR amplification was performed using the following primer pairs: forward: CATGTCTGCCTTCGTCTCAA, reverse: CCAGCATTCTCCAGTCACAG. The 96-well PCR samples were analyzed on 3% agarose gel and stained with ethidium bromide. The score was submitted to the Zon Lab Web site (http://134.174.23.167/zonrh-mapper/instantMapping.htm) for instant mapping information.

In Situ Hybridization and Sections

WT embryos were grown in 0.003% phenylthiourea until the desired stage, fixed overnight in 4% paraformaldehyde/PBS at 4°C, dechorionated, and stored in 100% methanol until use. Single probe whole mount in situ hybridization was carried out as described previously (15). Serial transverse robo4 and unc5b sections were generated from Spurr's epoxy resin embedded 24 hpf embryos. Embryos were washed three times in PBS, pH 7.4, dehydrated in ethanol and propylene oxide, embedded into Spurr's epoxy resin, and cured in an oven for 18 h at 68°C. Sections 1–2 μm thick were made on a Pyramitome with glass knives, floated on a drop of distilled water on a microscopic slide, and dried on a hot plate. The slides were mounted with coverslip and Cytoseal XYL medium (Richard-Allan Scientific). In situ whole mount pictures were captured with a Zeiss SVII Epi-Fluorescence stereomicroscope, and sections were captured with Carl Zeiss Axioskop2 plus upright microscope equipped with 40× or 63× differential interference contrast (DIC) objective lens and an Axiocam HRC camera. For flat mount, the yolk was removed in glycerol and the deyolked tissue was mounted on a slide containing mounting medium with a coverslip.

RESULTS AND DISCUSSION

Cloning and Mapping of unc5b

We cloned the zebrafish ortholog of human *unc5b* gene by extracting nucleotide sequences from Sanger zebrafish shotgun genome sequences that matched the amino acids of unc5b. Because members of the unc5 family are highly homologous to each other, we used synteny as another parameter of orthology determination. We used a two-prong approach where first, regions of unc5b that were least conserved between the members of the family or uniquely identifiable in unc5b were used for searching the public databases, and second, the hits were blasted to potential linkage groups. If the region was syntenic to human *unc5b*, then we proceeded with further analysis. Using the two-prong blast-mapping approach, we located two sequences that hit the extreme 5′ and 3′ end of *unc5b*, and amplified these fragments individually first (Fig. 1A, lanes 1 and 2) followed by nested PCR primer sets e and f, which confirmed the bands (Fig. 1A, lane 3). To determine linkage between the two sequences, PCR was performed with primers located at the 5′ end of the first sequence (primer a) and 3′ end of the second sequence (primer d), which generated a 2.8-kb band (Fig. 1A, lane 4). Subsequent blastn analysis of the 2.8-kb sequence mapped to linkage group 13 (data not shown), and the sequence was deposited to GenBank (accession #DQ365815). We verified the mapping data from SSAHA server by performing radiation hybrid mapping, which confirmed the location (data not shown). Linkage group 13 is syntenic to human chromosome 10 where human *unc5b* gene is located. Because *unc5b* was mapped to linkage group 13, where *cloche* mutant maps to, we checked by RT-PCR if *unc5b* was expressed in *cloche*^{m39} (11) deletion allele, and it was

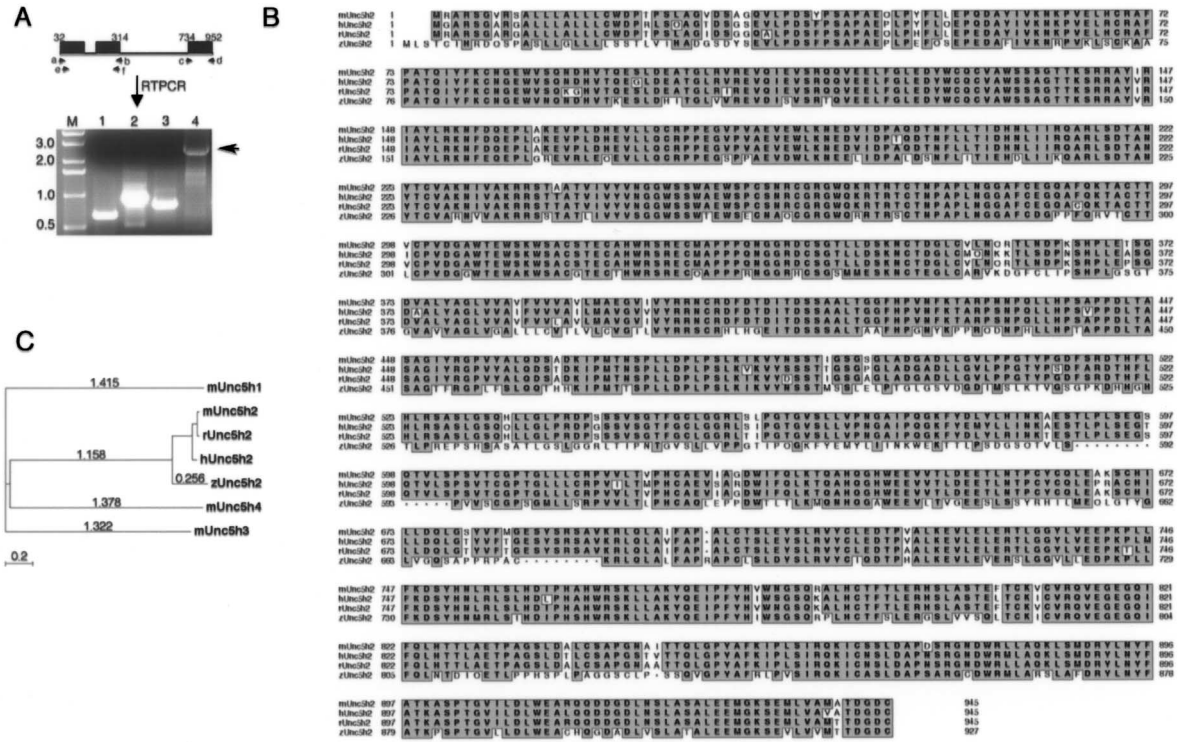

Figure 1. Cloning and phylogenetic analysis of unc5b.(A) The black boxes depicts the three exons that correspond to human *unc5b* amino acids, which hit putative zebrafish nucleotide sequence in Tblastn searches of Sanger zebrafish genome database. The six primer pairs (a to f) used for PCR are shown by a small black arrow. Lane M: marker (kb); lane 1: c and d; lane 2: a and b; lane 3: e and f: lane 4: a and d. Arrow indicates the 2.8-kb fragment of unc5b, which encompasses most of the *unc5b* gene except the first 35 amino acids. (B) Phylogenetic comparisons of zebrafish unc5b to unc5b members in other species and to mouse *unc5* family (1, 3, and 4) are shown. The CLUSTALW program, as part of the MacVector 7.0 Software package was used for generating the tree and the accession numbers for sequences used here are zUnc5b (DQ365815), mUnc5h1 (NP_694771.1), mUnc5h3 (NP_033498), mUnc5H4 (NP_694775), mUnc5b (NP_084046), rUnc5b (NP_071543), and hUnc5b (NP_734465). Evolutionary tree was built using the "Neighbor joining" method, which does not assume constant divergent rates among sequences, and evolutionary distances were calculated using "Best tree" method, which estimates the number of substitutions per site under the assumption that the distribution follows a poisson distribution. (C) Sequence homology comparisons with different unc5b family members are shown. The human, mouse, and zebrafish unc5b amino acid sequences were aligned using CLUSTALW program in MacVector 7.0. Pairwise comparisons between human and mouse sequences showed 90% homology while between human or mouse to zebrafish unc5b was 65%. Unc5h1, Unc5h2, Unc5h3, and Unc5h4 is the old nomenclature for Unc5a, Unc5b, Unc5c, and Unc5d, respectively.

present (data not shown), suggesting that *cloche* is not *unc5b*.

The zebrafish *unc5b* open reading frame encodes a protein of 929 amino acids (aa) with a single transmembrane domain and shares 65% amino acid identity to the human or mouse protein (Fig. 1B). Phylogenetic tree analysis was performed with amino acid sequences of zebrafish, mouse, human, and rat unc5b to mouse unc5 family members. The cladogram depicts that zebrafish unc5b is closer to human unc5b than mouse unc5b (Fig. 1C). Based on amino acid identity, phylogenetic and synteny relationships, we have cloned the putative full-length ortholog of *unc5b* in zebrafish. These data also confirm the partial published unc5b sequence (8), which encodes 452 amino acids.

Whole Mount unc5b *In Situ Hybridization in Embryonic Zebrafish Development*

A 2.8-kb digoxigenin (DIG) RNA probe was synthesized, and whole mount in situ was performed as described previously (2). *unc5b* expression commences postgastrulation in 12 somite embryo, and is expressed in the head, tail, and future dorsal side of the embryo (Fig. 2A, B). In the dorsal head region (Fig. 2C), *unc5b* is expressed in three distinct regions of the neural plate (asterisks), which outlines the general location of sensory and motor neurons. The *unc5b*-expressing lateral sensory neural plate cells (Fig. 2C, black asterisk) are symmetrically aligned on either side of the central motor neuron region (Fig. 2C, white asterisk) of the neural plate. In 14 somite em-

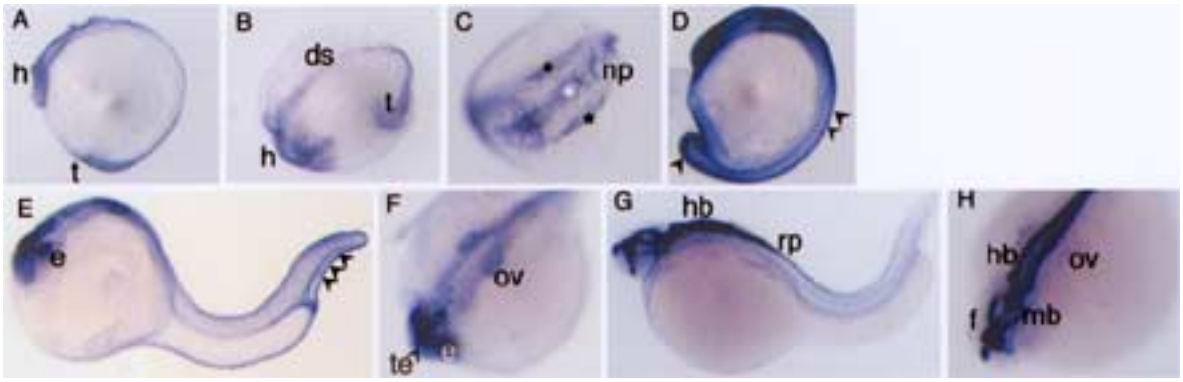

Figure 2. Expression patterns of *unc5b* in zebrafish embryonic development. A montage of *unc5b* whole mounts in situ stained embryos is shown. (A, B, C) 12 somite, (D) 14 somite, (E, F) 25 hpf, (G, H) 30 hpf. (A), (D), (E), and (G) are lateral view. (B), (C), (F), and (H) are dorsal views. The arrowheads in (D) and (E) indicate outer epidermal cells. The black asterisk and white asterisk in (C) indicate lateral sensory neural plate cells and central motor neuron region, respectively. Abbreviations used are ds: dorsal side, f: forebrain, h: head, e: eye, hb; hindbrain, mb: midbrain, np: neural plate, ov: otic vesicle, rp: roof plate, t: tail, te: telencephalon.

bryos, *unc5b* expression is ubiquitous with strong expression seen in epidermal cells that line the embryo architecture (Fig. 2D). Over the course of the next few hours in development *unc5b* expression is restricted primarily to the brain. At 25 hpf, *unc5b* expression is seen in the eye, forebrain, hindbrain, and otic vesicle (Fig. 2E). Dorsal view of the 25 hpf head region (Fig. 2F) shows prominent expression in eye, otic vesicle, and forebrain telencephalon regions. At 30 hpf (Fig. 2G), *unc5b* expression is robust in anterior regions with dorsal view (Fig. 2H) showing expression in all three regions of the brain and otic vesicle. The expression clearly crosses into dorsal hindbrain regions, and continues into the dorsal roof plate (Fig. 2G) in caudal regions of the embryo.

unc5b *Expression Comparison to robo Family Member in Embryonic Zebrafish Brain Development*

When performing whole mount *unc5b* in situ at 24 hpf we noticed that *unc5b* was expressed in lateral rhombomere cells in contrast to robo4, which we already knew was expressed in middle rhombomere cells in the same temporal window. To check whether this differential exclusivity in the gene expression patterns for unc5b and robo4 at 24 hpf is preserved in early and late embryos, we expanded our analysis to 16–18 hpf and 48 hpf time points. At 16 hpf (Fig. 3A′), *robo4* expression is observed in telencephalon, diencephalon, and mesencephalon with clear absence in the midbrain–hindbrain boundary. Further, *robo4* expression continues dorsally into the neural tube and in the notochord (Fig. 3A). *unc5b* expression is ubiquitous during this time period (Fig. 3D). At 24 hpf (Fig. 3B′, E′), a pattern of differential exclusive expression for the two genes is pronounced in the hindbrain regions anterior and posterior to the otic vesicle. In hindbrain, *robo4* expression is restricted to the medial cluster of rhombomere cells (Fig. 3B′, white asterisk) while *unc5b* expression is restricted to the lateral clusters (Fig. 3E′, black asterisk). The rhombomere boundaries in the 24 hpf robo4 hindbrain also show disproportionate staining with more expression in the middle rhombomere boundaries (Fig. 3B′) when compared to the end ones. Both genes show expression in the otic vesicle (Figs. 3B′, E′). A consolidation of the 24 hpf *unc5b* and *robo4* hindbrain rhombomere expression patterns at 24 hpf is depicted in Figure 3G. At 48 hpf, the *robo4* brain expression pattern is remarkably defined with clear demarcated regions of expression (Fig. 3C′) in the optic tectum, rhomboencephalon, mesencephalon, and diencephalon and distinct lack of *robo4* expression in the central region of the midbrain (Fig. 3C′). The telencephalon expression is also interrupted with regions of strong expression and no expression (Fig. 3C′, arrow). In the case of *unc5b*, brain expression is ubiquitous at 48 hpf and specific expression is noticed in the heart, cloaca, and posterior pronephric duct cells (Fig. 3F, black arrows). The expression patterns of netrins, ligands for unc5b, and slits, ligands for robos, have been previously reported. For netrins, only netrin-2 is observed in the fourth rhombomere during *unc5b* expression in rhombomere boundaries (14), and for slits, *slit1a* and *slit1b* are expressed in the bilateral cell clusters of hindbrain and floorplate (5) when *robo4* is expressed.

Sectional Analysis of unc5b *and* robo4 *In Situ Embryos*

To map the expression domains in greater detail, we compared serial sections of 24 hpf *unc5b* and *robo4* in situ embryos. Serial sections of 24 hpf

Figure 3. Comparisons of *robo4* and *unc5b* brain expression patterns during zebrafish embryonic brain development. Wild-type embryos probed for *robo4* and *unc5b* transcripts across three comparable developmental stages of brain development are shown. For each whole mount in situ, corresponding flat mount of the head region is depicted. (A), (B), and (C) show robo4 in situ embryos at 16, 24, and 48 hpf, respectively, along with (A′), (B′), and (C′) showing the corresponding flat mounts of the head region. Similarly, (D), (E), and (F) show unc5b in situ embryos at 18, 24, and 48 hpf along with their corresponding flat mounts of head region in (D′), (E′), and (F′). Asterisks in (B′) medial clusters of rhombomere cells and in (E′) *unc5b* expression in bilateral cluster of cells in rhombomeres. Arrows in (A) and (B) depict neural structures neural tube and notochord; arrow in (C′) shows separation of robo4 and non-robo4 expression domains in telencephalon. Short arrow in (F) shows cloaca and long arrows depict posterior pronephric duct region and heart. (G) A pictorial representation of blue regions (unc5b, E′), red regions (robo4, B′) in the rhombomeres (r1–r7) of a 24 hpf developing zebrafish hindbrain along with otic vesicle shown in purple to depict both transcript expression. (H–K) In situs for *robo4* and *unc5b* genes at 25 hpf with emphasis on vascular expression in the trunk region (I, K). Abbreviations used include a: anterior, cl: cloaca, d: diencephalon, e: eye, fb: fin bud, h: heart, hb: hindbrain, isv: intersomitic vessels, m: mesencephalon, mb: midbrain, mhb: midbrain hindbrain boundary, no: notochord, nt: neural tube, ov: otic vesicle, p: posterior, pd: pronephric duct, t: telencephalon.

unc5b in situ embryos beginning with the most anterior (Fig. 4A) and moving posteriorly (Fig. 4F) show expression in various sensory organs. In embryonic brain development, *unc5b* and *robo4* are expressed in forebrain, midbrain, and hindbrain regions. In some sections, *robo4* expression is focused in the central regions and is enveloped by *unc5b* expression in lateral position. For example, in the midbrain section, *robo4* is predominantly expressed in central midbrain regions (Fig. 4G) when compared to *unc5b* expression in more lateral regions (Fig. 4A). This theme continues into the hindbrain, where *unc5b* expression is focused on more dorsolateral rhombomere cells (Fig. 4C, D) and *robo4* in central rhombomere cells (Fig. 4I, J). In posterior sections (Fig. 4F, L), *robo4* expression is predominant in the central neural tube (Fig. 4L) when compared to *unc5b* expression, which is restricted to roof plate (Fig. 4F). The lateral–dorsal preference patterns for the two genes does not hold true throughout the embryo. For example, in forebrain telencephalon, no preference is noted for the expression patterns for both genes (Fig. 4B, H). Sensory structures such as eye and ear show prominent *unc5b* expression. In the developing eye, *unc5b* is ro-

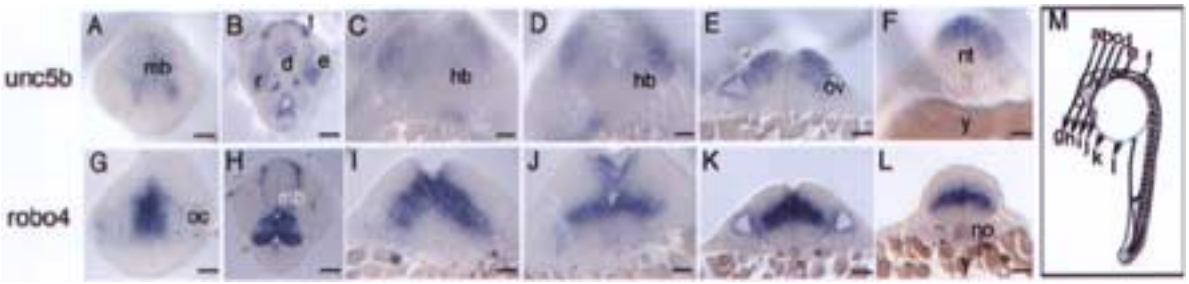

Figure 4. Comparative sectional analysis of embryonic brain regions of *unc5b* and *robo4* in situ embryos. *unc5b* and *robo4* 24 hpf whole mount embryos were embedded in Spurr's epoxy resin and sectioned as depicted in (M). All images were photographed at 40× magnification. Images were corrected for brightness, contrast, and sharpened in Adobe Photoshop. Scale bars: 50 μm. Abbreviations used include d: diencephalon, e: eye, hb: hindbrain, le: lens, mb: midbrain, no: notochord, nt: neural tube, oc: optic cup, ov: otic vesicle, r: retina, t: telencephalon, y: yolk.

bustly expressed in the ventral temporal retina (Fig. 4B), and both genes are expressed in the optic cup (Figs. 4A, G). Similarly, both genes show robust expression in the developing ear (Fig. 4E, K).

Comparative Analysis of Mouse and Zebrafish unc5b *Expression*

Previously published mouse *unc5b* expression analysis (4) helps compare and contrast the zebrafish and mouse gene expression patterns. Spatially, the mouse and zebrafish genes show a high degree of similar expression profile in sensory organs but differences in other tissues. In the developing eye, both mouse and zebrafish genes show expression in the ventral temporal retina and lower towards the central retina. Further, lens expression is observed for zebrafish *unc5b* (data not shown), but is absent for mouse *unc5b* (8). In the developing ear, *unc5b* expression is seen in both species with mouse *unc5b* expressed strongly in the semicircular canals (4) and zebrafish *unc5b* in the otic vesicles (Figs. 3E′ and 4E). In the vascular system, mouse *unc5b* is expressed in intersomitic vessels (ISVs) and CNS endothelial cells (4,8). In zebrafish, we failed to notice *unc5b* expression in developing ISVs using identical in situ conditions as that of age-matched robo4 in situ embryos (Fig. 3H, I). A recent study reported that *unc5b* knockdown embryos display ectopic ISVs sprouting defect (8), suggesting that *unc5b*$^{+}$ ISVs are guided to their target by cues from surrounding tissue. Another recent report (13) suggests that ISV sprouting defects in *unc5b* or *netrins1a* morphants are secondary to parachordal vessel defects. So it is unclear at this point whether ISV sprouting defects in unc5b morphants are primary or secondary. Interestingly, mouse *robo4* is reported to be vascular specific (9) while the zebrafish *robo4* is clearly not (2), suggesting that expression patterns for axon guidance genes differ across species.

Based on expression analysis in this study, unc5b's original name derived from "uncoordinated" phenotype mutants in *Caenorhabditis elegans* correlates well with the expression in sensory tissues. In zebrafish, *unc5b* is expressed in eye, ear, and brain, which corresponds to all relevant organ systems for coordination. Interestingly, *netrin-1* mutant mice show severe semicircular canal malformations (10). *unc5b* is the only known unc5 member expressed in the semicircular canal (4). It is not known if ear defects were observed in *unc5b* knockout mice or *unc5b* morphant zebrafish embryos (8). Our expression analysis with *unc5b* and *robo4* suggest that axon guidance genes may function during rhombomere specification in hindbrain development. Other axon guidance family members such as Ephrin-eph receptor–ligand signaling system have already been implicated in rhombomere formation by restricting cell intermingling and communication (7). Our studies not only extend these observations to other classes of axon guidance gene families such as unc5 and robos but also suggest that cooperative interactions between members of disparate axon guidance families may play a role in hindbrain development.

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