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Zebrafish *atonal* Homologue *zath3* Is Expressed During Neurogenesis in Embryonic Development

Xukun Wang,[1] Alexander Emelyanov,[2] Vladimir Korzh,[1,2] and Zhiyuan Gong[1]*

Basic helix-loop-helix (bHLH) transcriptional activators function in development of various cell lineages, including the central nervous system. One of the bHLH proteins, Math3/Xath3/NeuroM, was suggested to act as a late proneural gene in the mouse, *Xenopus*, and chick. Here, we isolated a zebrafish homologue, named *zath3*, and analyzed its expression pattern in zebrafish embryos. In the neural plate, *zath3* is expressed first in the primordia of the tegmentum and trigeminal ganglia and three classes of primary neurons: sensory neurons, interneurons, and motor neurons. During later development, *zath3* transcripts were localized along the boundaries of the optic tectum in the midbrain and rhombomeres of the hindbrain. Analyses of *zath3* expression in three mid-hindbrain boundary mutants, *acerebellar*, *no isthmus*, and *spiel-ohne-grensen*, indicated that distribution of *zath3* mRNAs in the midbrain and hindbrain was dramatically disturbed. In addition, these mutants also affect expression of *zath3* in the neuroretina. *Developmental Dynamics 227:587–592, 2003.* © 2003 Wiley-Liss, Inc.

Key words: neuroM; MATH3; neuroD; basic helix-loop-helix (bHLH); *acerebellar*; *no isthmus*; *spiel ohne grensen*

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INTRODUCTION

The central nervous system (CNS) consists of two classes of cells: neurons and glia. In both invertebrates and vertebrates, neurons and glia are generated from common progenitor cells (Walsh and Cepko, 1992; Condron and Zinn, 1994; Levison and Goldman, 1997), and both cell types contribute to formation of boundaries of different regions within the CNS (Trevarrow et al., 1990). A specific class of basic helix-loop-helix (bHLH) transcription factors plays crucial roles in neural fate determination. In vertebrates, it has been suggested that the early proneural bHLH factors (e.g., Neurogenin or Ngn) act in the proliferation zone before migration of neuroblasts and the late bHLH factors (e.g., NeuroD) act during their migration at the initiation of differentiation (Lee, 1997). Recently, it has been found in chick that a novel bHLH *atonal*-related gene, *neuroM,* acts at the transition between undifferentiated, premigratory, and differentiating, migratory neural precursors (Roztocil et al., 1997). In *Xenopus,* the homologue of *neuroM, Xath3* converts ectoderm into a neural fate in a manner similar to *neuroD* (Takebayashi et al., 1997).

So far, most known bHLH genes involved in neurogenesis of vertebrates have been described in the zebrafish, e.g., *ngn1, ngn3, neuroD, ndr1a, ndr1b, ndr2, zash1a, zash1b, zath1, zath5,* and *olig2* (Allende and Weinberg, 1994; Blader et al., 1997; Korzh et al., 1998; Liao et al., 1999; Sawai and Campos-Ortega, 1999; Itoh and Chitnis, 2001; Wang et al., 2001; Park et al., 2003). However, the zebrafish homologue of *ath3/neuroM* has not been reported. In the present study, we isolated an *ath3* cDNA clone and named it *zath3*. As a first step in understanding its roles in development of the CNS, its expression pattern was investigated in both wild-type embryonic zebrafish and several mutants that are defective in formation of boundaries within the CNS.

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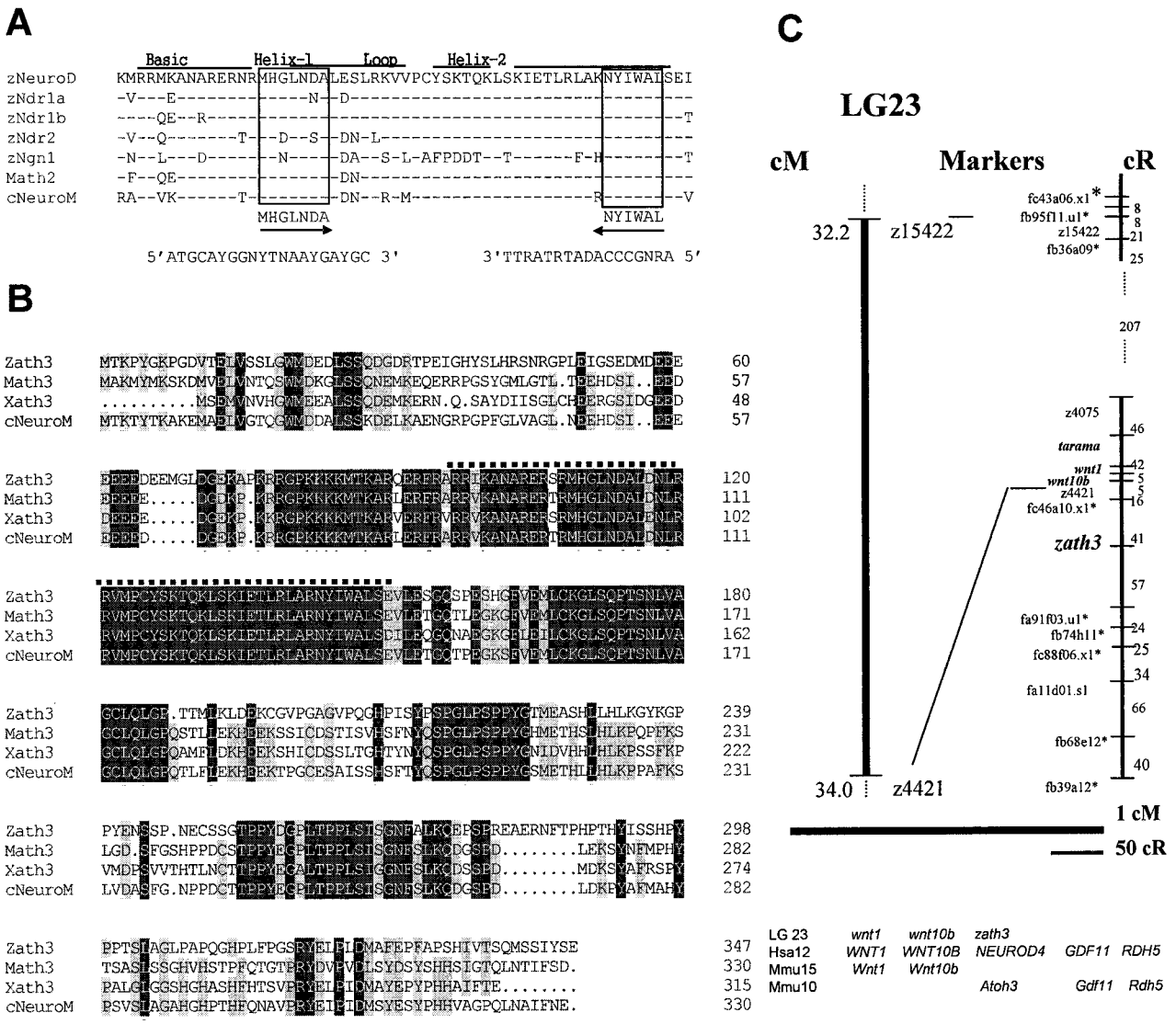

Fig. 1. Cloning, sequencing, and genome mapping of *zath3* cDNA clone. **A:** Alignment of the basic helix-loop-helix (bHLH) domain from selected proneural bHLH proteins, including chick NeuroM. The two boxed regions were used to design degenerate polymerase chain reaction primers, and the primer sequences are shown underneath the boxes: N = A, C, G, and T; D = A, G, and T; R = G and C; Y = C and T. **B:** Amino acid alignment of zebrafish Zath3, mouse Math3, *Xenopus* Xath3, and chick NeuroM. Identical amino acid residues are dark-highlighted, and similar residues light-highlighted. Dots represent gaps inserted for maximal alignment. Dotted line demarcates the bHLH domain. **C:** Partial linkage map indicating the location of *zath3* in relation to different markers. The distance is indicated in centimorgans or centirays. Genes mapped are in bold font, other markers with prefixes z and f are adapted from http://134.174.23.167/zonrhmapper/RHLg/. At the bottom of the map is shown a conserved synteny of zebrafish, mouse, and human chromosomes containing *ath3*.

RESULTS AND DISCUSSION

As described in the Experimental Procedures section and Figure 1A, a full-length zebrafish *zath3* cDNA clone was isolated by polymerase chain reaction (PCR) using degenerate primers and by subsequent cDNA library screening. Sequencing analysis indicated that the cDNA clone contained an insert of 1969 bp and had an open reading frame of 347 amino acids. Sequence alignment of Zath3 with MATH3, Xath3, and NeuroM are shown in Figure 1B. Zath3 shares ~96% sequence identity with these ATH3 proteins in the bHLH domain and also exhibits significant sequence homology outside of this domain. Phylogenetic analysis of Zath3 and all available members of vertebrate Atonal-like bHLH proteins, including Neurogenin and NeuroD families, confirmed that Zath3 is in the same cluster as MATH3, XATH3, and NeuroM (data not shown).

By using the T51 zebrafish-hamster somatic cell radiation hybrid (RH) panel (Kwok et al., 1998), *zath3* was mapped to ~34 cM from the top of the linkage group (LG) 23 (Fig. 1C). A comparison of *zath3* and other genes mapped to the same area of LG23 with their homologues on human and mouse chromosomes revealed a conserved synteny in these regions among zebrafish, mouse, and human genomes. According to Woods et al. (2000), zebrafish LG23

has the closest relationship with human chromosome 12 and mouse chromosomes 10/15. Consistent with this, we noticed that, in the region containing *zath3*, at least three other genes (*tarama*, *wnt1*, and *wnt10b*) have been mapped. The homologous human genes *NEUROD4*, *TARAMA*, *WNT4*, and *WNT10B* were also found in a small region of chromosome 12 (Fig. 1C). In the mouse, *wnt1* and *wnt10b* were located on chromosome 15, whereas *athod3* (*MATH3*) was on chromosome 10. By comparing mouse and human genomes, two other mouse genes near *athod3*, *gdf11*, and *rdh5*, were found to have homologs (*GDF11* and *RDF5*) on human chromosome 12 (Fig. 1C).

The spatial and temporal expression of *zath3* was examined during zebrafish embryogenesis by whole-mount in situ hybridization. *zath3* mRNA was first detected at the beginning of neurulation (10 hours postfertilization (hpf); not shown). At 11 hpf, the *zath3* expression domain includes a central chevron-shaped cluster and two lateral clusters in the anterior neural plate, and bilateral triple stripes in the posterior neural plate (Fig. 2A). The chevron-shape cluster of *zath3*-positive cells at the midline is reminiscent of *ngn1*-positive cells, which develop into the tegmentum (Korzh et al., 1998). Similarly, the two lateral clusters are in position of primordia of the trigeminal ganglia. The posterior stripes from the medial to lateral probably represent three populations of primary neurons of the spinal cord: motoneurons, interneurons, and Rohon-Beard sensory cells (Korzh et al., 1993, 1998; Blader et al., 1997). By 16 hpf, in the anterior CNS, *zath3* is expressed similar to *ngn1*, *zash1b*, and *neuroD* (Allende and Weinberg, 1994, Blader et al., 1997; Korzh et al., 1998) in two main domains: the olfactory bulbs and trigeminal ganglia (Fig. 2B). In addition, a small cluster of *zath3*-positive cells was detected on one side near the midline at the level of dorsal diencephalon (Fig. 2B). A majority of embryos analyzed at this stage (72 of 105 or 68% of embryos) have this cluster on the right side. This observation suggested that asymmetry of the dorsal diencephalon might appear in development as early as 16 hpf, several hours earlier than that previously suggested (Sampath et al., 1998). From 18 hpf, its expression was mapped to several neuronal clusters in the anterior brain, most prominently in the telencephalon (Fig. 2C). At 18 hpf, *zath3* expression was observed in the ventral neural tube and, in the tail bud, weak *zath3* expression was also detected in the precursors of the medial floor plate (Fig. 2D). In summary, it seems that expression of *zath3* during the period of early neurogenesis is mostly confined to primary neurons.

When secondary neurogenesis starts around 22 hpf (Kimmel and Westerfield, 1990), the pattern of *zath3* expression becomes more complicated. Posteriorly, the expression was limited to the ventral neural tube (Fig. 2G). Cross-sections revealed that the expression in the dorsal clusters probably correspond to interneurons and the ventral ones adjacent to the floor plate may represent secondary motoneurons (Fig. 2E,F). At 30 hpf, *zath3* expression in the brain became even more complicated, occurring in several clusters in the ventral-most part of the midbrain and in seven double segmental clusters in the hindbrain (Fig. 2H,I). These double segmental clusters are reminiscent of radial glial cells that formed curtains along segmental boundaries (Trevarrow et al., 1990). They consist of two components: the midline clusters that are more intensively stained were previously defined by expression of *zash1b*, *hlx1*, and *zp-50* (Allende and Weinberg, 1994; Fjose et al., 1994; Hauptmann and Gerster, 1996); the less-stained lateral clusters form seven loops that are likely lateral extensions of medial clusters (Fig. 2H–J).

In the zebrafish retina, neurogenesis starts in the ventrorostral portion at ~24 hpf (Korzh et al., 1998) and is expanded clockwise with the morphogenetic wave (Neumann and Nusslein-Volhard, 2000). To compare *zath3* expression with expression of other bHLH genes, in situ hybridization was also performed by using a *neuroD* antisense probe. At 48 hpf, *zath3* expression appears in most of the retinal neuroepithelium except for the outmost layer (Fig. 3A). In contrast, *neuroD* expression is strongly expressed in the outermost layer but weakly in the inner layers (Fig. 3B). In addition, the two genes also have distinct but overlapping expression domains in optic tectum, tegmentum, and hypothalamus and may represent different stages of neuron differentiation. At 72 hpf, an intensive *zath3* expression is present in the inner nuclear layer (INL) of the retina (Fig. 3C), whereas *neuroD* is expressed in the ganglion cell layer (GCL) and outer nuclear layer (ONL; Fig. 3D). This finding is reminiscent of the situation in differentiated chick retina, where *neuroM* is expressed in INL and *neuroD* in GCL and ONL (Roztocil et al., 1997).

To illustrate the roles of *zath3* in the zebrafish development, we also examined *zath3* expression in two mutants defective in formation of the mid-hindbrain boundary (MHB): *acerebellar* (*ace*$^{-/-}$) and *no isthmus* (*noi*$^{-/-}$). In *ace*$^{-/-}$, mutant of *fgf8*, the MHB and cerebellum are absent, whereas in *noi*$^{-/-}$, mutant of *pax2.1*, not only these structures, but also the optic tectum and optic stalk are affected (Krauss et al., 1991; Brand et al., 1996; Fürthauer et al., 1997; Macdonald et al., 1997; Reifers et al., 1998). In the wild-type, *zath3* is expressed along boundaries of the optic tectum (Fig. 4A,B) and *zath3*-positive cells are absent in the medial part of the optic tectum (Fig. 4B,J). Thus, in the midbrain as well as in the hindbrain, *zath3*-positive cells defined boundaries of the brain regions. In *ace*$^{-/-}$, the pattern of *zath3* expression in the optic tectum and hindbrain is disorganized and ectopic *zath3*-positive cells appear in the posterior optic tectum (Fig. 4D,E). In the retina, the expression is reduced in the posterior retina (Fig. 4F, compared with the wild-type retina in Fig. 4C). In *noi*$^{-/-}$ embryos, the expression pattern is even more disorganized in the midbrain and hindbrain. The ectopic *zath3* cells appear in the optic tectum en masse (Fig. 4G,H). Interestingly, in the eyes, expression of *zath3* is almost absent except for the ectopic cluster of cells located outside the eye and dorsal to the choroid fissure (Fig. 4I). As the ventral retina forms as a result

of migration of cells from the optic stalk (Holt, 1980), our observation may suggest that *zath3*-positive cells of the retina originate outside the eye and migrate into the eye later. Probably in *noi*$^{-/-}$, where the optic stalk is affected, these cells are unable to migrate into the eye and differentiate in an ectopic position.

The *spiel ohne grensen* (*spg*$^{-/-}$) mutant is defective in *pou2* and has no MHB (Hauptmann et al., 2002). In this mutant, the expression pattern of *zath3* revealed a different set of deficiencies. Compared with wild-type embryos, not only the MHB but also boundaries between other domains of expression in the ventral midbrain and diencephalon are absent in the *spg*$^{-/-}$ mutant, leading to appearance of continuous domains of *zath3* expression (Fig. 4K). Also, there is a general shortening of the anteroposterior neuroaxis, especially in the optic tectum. Based on these observations, it seems that *pou2* plays a role not only in the MHB but also widely in formation of boundaries between segments of the anterior neural tube.

The expression pattern of *zath3* in the hindbrain suggests that this gene may play a role during formation of segmental boundaries. Here, the curtain-like rows of the radial glial cells align segmental boundaries on both sides (Trevarrow et al., 1990). Several molecular markers, including *hlx-1*, *zp50*, and *zash1b*, display similar expression patterns in these regions, consistent with their role in early differentiation of cells adjacent to the boundary (Allende and Weinberg, 1994; Fjose et al., 1994; Hauptman and Gerster, 1996). *zath3* is expressed in a similar manner and may play a similar role. Of interest, in *ace*$^{-/-}$ and *noi*$^{-/-}$ mutants, the organized pattern of *zath3* expression in the hindbrain has been affected, indicating that *fgf8* and *pax2.1* genes may function in maintaining segmentation in the hindbrain. Consistent with this role, both genes are expressed in the hindbrain at particular stages during early development (Krauss et al., 1991; Reifers et al., 1998). For example, *pax2.1* is expressed in segmentally distributed commissural neurons (Mikkola et al., 1992).

EXPERIMENTAL PROCEDURES

Isolation of Zebrafish *zath3* cDNA Clone and DNA Sequencing

The initial PCR was carried out against genomic DNA with degenerate primers designed based on the conserved bHLH domain of several proneuronal bHLH transcription factors, including NeuroM (Fig. 1A). Multiple fragments were amplified from genomic DNA, but only the predicted 100-bp fragment was recovered for cloning. Of 28 random clones sequenced, one displayed an amino acid sequence 100% identical to the bHLH domain of cNeuroM (Roztocil et al., 1997). Subsequently, two gene-specific primers were designed based on the 100-bp sequence to extend this clone by rapid amplification of cDNA end (RACE) -PCR using a zebrafish embryonic cDNA library (Gong et al., 1997) as a template. A 700-bp 5′ RACE fragment was amplified and confirmed by DNA sequencing. The fragment was then used as a probe to screen the same embryonic cDNA library. Among nine positive clones obtained, the clone with the longest insert (~2 kb) was characterized and it contains a complete open reading frame of Zath3 protein. DNA sequencing was performed by using an automated sequencer with the ABI prism BigDye Termination, Cycle sequencing Ready Reaction Kit (Perkin Elmer).

Genome Mapping

T51 radiation hybrid panel (zebrafish-hamster hybrid cell lines; Research Genetics; Kwok et al., 1998; Geisler et al., 1999) was used to map *zath3*. The following two primers were used (M1: 5′ TCTGTATACTCTTGATGTCC 3′; M2: 5′ ACTTTATGAGAATCGGTAGC 3′). The pair of primers amplified a 243-bp genomic DNA fragment located ~250 bp downstream of the stop codon. PCR was performed for 35 cycles with an annealing temperature of 52°C. The PCR products were analyzed by agarose gel electrophoresis. The chromosome position was determined by linkage analysis of a PCR length variant between the gene and other markers using software from the Max-Planck-Institut für Entwicklungsbiologic (http://wwwmap.tuebingen.mpg.de:8082/rh/). The genome map data of human and mouse were obtained from the two Web sites: human, http://www.ncbi.nlm.nih.gov/genome/guide/human; and mouse, http://www.ncbi.nlm.nih.gov/genome/guide/M_musculus.html

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization using probes labeled with digoxigenin was performed according to Korzh et al. (1998). Some of the hybridized

Fig. 2. Expression of *zath3* during zebrafish embryonic development. All embryos were hybridized with *zath3* antisense riboprobe except for those in E and F, which were hybridized with both *zath3* and *shh* probes. **A:** Eleven hours postfertilization (hpf), dorsal view with the anterior toward the left. The left side of the neural plate (shown partially) is a mirror image of the right side, and the midline is shown by a dash-dot line. **B:** At 16 hpf, novel domains of expression included the telencephalon (t) and the asymmetric *zath3*-expressing cluster (arrow), which may correspond to the epiphysis. The dash-dot line defines midline. **C:** At 18 hpf, lateral view of the anterior brain to reveal expression in the anterior diencephalons, thalamus (tl), telencephalon (t), and epiphysis (ep). **D:** At 18 hpf, in the tail bud, weak *zath3* expression was detected in the precursors of the medial floor plate (arrowhead), and more anteriorly, in the ventral neural tube. The dotted line outlines the notochord. **E,F:** At 24 hpf, transverse section at the level of the hindbrain and spinal cord, respectively. Here, *zath3* expression maps to the motoneurons (mn) and interneurons (in). **G:** At 28 hpf, side view of the spinal cord revealed the segmental distribution of the *zath3*-positive cells in the ventral neural tube (arrows). **H,I:** Clusters of *zath3*-positive cells in the forebrain, midbrain, and hindbrain. The double clusters in the hindbrain are reminiscent of the curtains of radial glia on both sides of the segmental boundary. The boxed area is magnified in J. The *zath3*-positive cluster consists of two components: the thick medial cluster resides in the neural tube and more disperse semi-oval cluster in the lateral mesoderm. Black arrows, the segmental clusters of *zath3*-positive cells in the midbrain and ventral diencephalons; blue arrows, segmental clusters in the ventral hindbrain; straight lines, segmental boundaries. fp, floor plate; ht, hypothalamus; m, midbrain; mhb, mid–hindbrain boundary; n, notochord; rb, Rohon-Beard neurons; tg, trigeminal ganglion; wm, whole-mount. Scale bars = 100 μm in A–D,G,H,I and 25 μm in E,F,J.

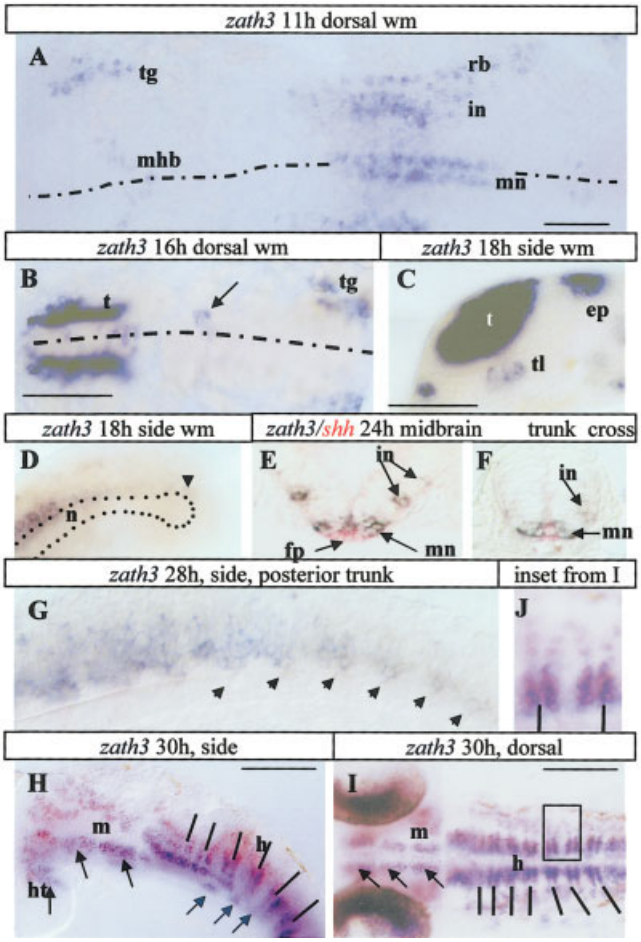

Fig. 2

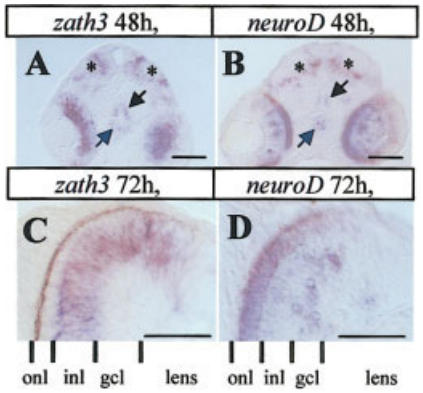

Fig. 3. *zath3* is expressed in the zebrafish retina. **A,B:** At 48 hours postfertilization (hpf), transverse sections at the level of eyes. Embryos were stained for mRNA expression of *zath3* (A) and *neuroD* (B), respectively. Asterisks, optic tectum; black arrows, tegmentum; blue arrows, hypothalamus. **C,D:** At 72 hpf, a magnified image of zebrafish retina stained for *zath3* (C) and *neuroD* (D) expression. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; wm, whole-mount. Scale bars = 50 μm in A–D.

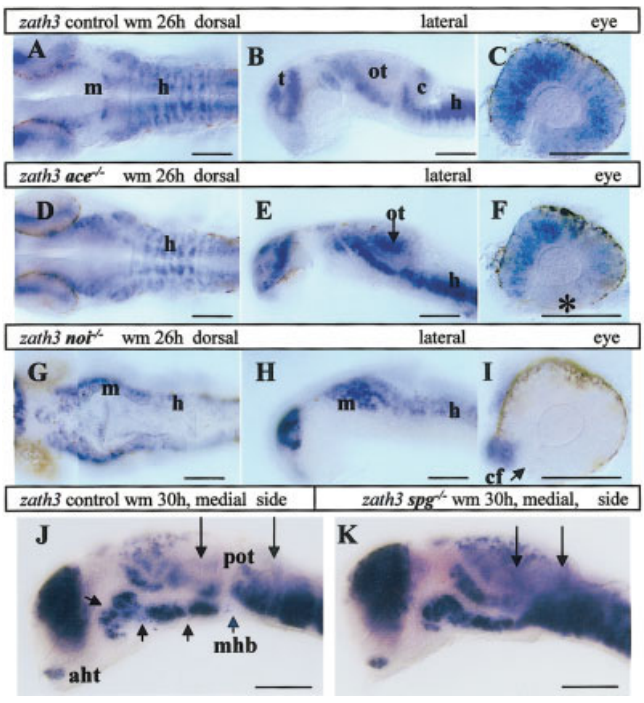

Fig. 4. *zath3* expression in wild-type embryos (**A–C**) and selected mutants: *ace*$^{-/-}$ (**D–F**), *noi*$^{-/-}$ (**G–I**), and *spg*$^{-/-}$ (**J,K**). All embryos were hybridized with *zath3* probe, and stages are indicated. A,D,G: Dorsal view; B,E,F: lateral view; C,F,I: eye. The asterisk in (F) indicates the lack of *zath3* expression in the ventral retina. J: In the 30 hpf wild-type embryos, *zath3* expression domains close to the midline clearly divided at the mid-hindbrain boundary (blue arrow). Other gaps separate domains in the midbrain and diencephalon are indicated by short black arrows. The anteroposterior (A-P) extent of the optic tectum is defined by two long arrows. K: In the *spg*$^{-/-}$ embryo, all these gaps are absent and the optic tectum shortened, demonstrating general shortening of the A-P neuroaxis. aht, anterior hypothalamus; c, cerebellum; cf, choroid fissure; h, hindbrain; m, midbrain; mhb, mid–hindbrain boundary; ot, optic tectum; pot, posterior optic tectum; wm, whole-mount. Scale bars = 100 μm in A–K.

embryos after staining were also embedded in 1.5% agar–sucrose and sectioned by a cryostat. Photographs were taken by using an AX70 compound microscope (Olympus, Japan) or a Leica dissecting microscope (Leica, Switzerland).

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