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Zic3z Defines the Dorsal and Vegetal Neuroectoderm in the Zebrafish Embryonic Development

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Abstract: The *Zic* family is a group of genes encoding zinc finger proteins that are highly expressed in the mammalian cerebellum. *Zic* genes are the vertebrate homologue of *Drosophila* pair-rule gene, *odd-paired* (*opa*), which plays important roles in the parasegmental subdivision as well as in the visceral mesoderm development of *Drosophila* embryos. Recent studies on human, mouse, frog, fish and ascidian *Zic* homologues support that *Zic* genes are involved in a variety of developmental processes, including neurogenesis, myogenesis, skeletal patterning, and left-right axis establishment. In an effort to explore possible functions of Zic proteins during vertebrate embryogenesis, we initially examined more detailed expression pattern of zebrafish homologue of *zic3* (*zic3z*). *zic3z* transcripts are detected in the neuroectoderm, neural plate, dorsal neural tube, and brain regions including eye field during early embryonic development. Marker DNA studies found that *zic3z* transcription is modulated by BMP, Wnt, and Nodal signals particularly in the dorsal and vegetal neuroectoderm at gastrula. Interfering with *zic3z* translation with *zic3z*-specific morpholino causes abnormal brain formation and expansion of the optic stalk cells. Retinal ganglion cells (RGCs) undergo abnormal neuronal differentiation. These findings suggest that *zic3z* defines the dorsal and vegetal neuroectoderm to specify brain formation and retinal neurogenesis during early embryonic development.

Key words: zebrafish, *zic3z*, Wnt, Nodal, BMP, neuroectoderm, optic stalk, retina, and retinal neurogenesis.

The *Zic* genes are vertebrate homologues of *odd-paired*, the Drosophila pair-rule gene. The five known mammalian and four *Xenopus* Zic proteins contain five tandem C_2H_2 zinc fingers that are highly conserved across species (Herman et al 2002). These *zic* genes are expressed in overlapping, but distinct patterns and have distinct roles in vertebrate development. *Zic1* appears to play an important role in skeletal patterning (Aruga et al., 1999) and cerebellar development in mouse (Aruga et al., 1994, 1998, 2002a). Individuals with *zic2* mutations show a number of neural defects including holoprosencephaly (Brown et al., 1998 Nagai et al., 2000), spina bifida, and neurulation delay and delay in neural crest development (Nakata et al., 2000) in human and mouse. These results support that *Zic2* is involved in formation of the neural tube and neural crest. In addition, targeted deletion of the *Zic3* gene in the mouse and mutations in the DNA binding domains of human *Zic3* result in neural tube defect (NTD) (Ferrero et al., 1997; Gebbia et al., 1997 Carrel et al., 2000; Klootwijk et al., 2000; Inoue et al., 2007) and X-linked heterotaxy (HTX-1, OMIM 306955) or situs ambigus (Purandare et al., 2002; Ware et al., 2004), suggesting that *Zic3* plays an important role in neural tube formation (Ferrero et al., 1997; Gebbia et al., 1997 Carrel et al., 2000; Klootwijk et al., 2000; Inoue et al., 2007) and left-right axis establishment (Purandare et al., 2002 Ware et al., 2004; Gebbia et al., 1997; Purandare et al., 2000). *Xenopus Zic5* [orthologous to murine and human *Zic4*] is also important for neural crest development in *Xenopus* embryos (Nakata et al., 2000).

Gain-of-function and loss-of-function analyses of the Zic genes support that Zic proteins regulate gene expression (Aruga, 2004). Zic proteins in fact bind Gli-binding DNA sequences in a sequence-specific manner, but with lower affinity than Gli proteins (Mizugishi et al., 2001; Koyabu et al., 2001). It is of interest that Zic physically interacts with Gli via their zinc-finger domains, raising the possibility that Zic proteins act as transcriptional cofactors to modulate the hedgehog-signaling pathway (Koyabu et al., 2001). Zic proteins activates transcription from several promoters of

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genes such as *ApoE* (Salero et al., 2001), *Math1* (Ebert et al., 2003), *dopamine receptor* (Yang et al., 2000). Zic proteins thus appear to affect gene expression via transcriptional regulation.

In this report, we demonstrate that *zic3z* is expressed in the eye primordial cells during somitogenesis as well as in the retina proliferate zone at pharyngula stage. We further show that concurrent abrogation of *zic3z* transcripts using morpholino oligonucleotides induces several defects in the brain, optic stalk, and retina. We further discuss the possible function of Zic3z in formation of brain, opitic stalk, and retina.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish were raised, maintained, and staged as described in *The Zebrafish Book* (Westerfield, 1995). Embryos were obtained by spontaneous spawning and appropriate stages of the embryos were fixed in 4% paraformaldehyde in PBS.

Constructs

cDNA encoding the full-length *zic3z* was cloned by RT-PCR from total RNA extracted from five-somite stage and 24hpf embryos. *Antivin, sqt, β-catenin, boz,* and *bmp4* cDNAs encoding full-length proteins were subcloned into the pcGlobin vector (Roet al., 2004). Expression vector for *zic3z*-GFP was constructed by inserting the PCR fragment containing the 5' UTR and coding region of *zic3z* into the *Hind*III and *Nco*I of the pCS2+GFP plasmid.

RNA and morpholino injection

Capped RNAs were transcribed with SP6, T7 RNA polymerase using the mMessage mMachine Kit (Ambion). Embryos were injected at the 1-4-cell stage with *sqt* (5 pg), *atv* (5-20 pg), *β-catenin* (100 pg) and *boz* (5 pg), *bmp4* (50 pg) synthetic mRNAs. Capped mRNAs were diluted in 0.1 M KCl solution containing 0.5% Phenol Red. An antisense morpholino (Gene-Tools, Inc., Oregon, USA) was designed to target *zic3z*-5'UTR(*zic3z*-MO): 5' GCTCAATCGAGAA AAACAAA 3', *chordin* (*din*-MO): 5' ATCCACAGCAGC CCCTC CATCATCC 3' as well as a standard negative control morpholino (control-MO): 5' CTTCAATGTAGCA AAGACC 3'. Morpholinos were dissolved in 1× Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5 mM HEPES, pH 7.6), and then further diluted with distilled water and 0.5% Phenol Red. Morpholinos were injected into the yolk at the 1- to 4-cell stage at a concentration of 500 μg-1ng. In some RNA injections, *GFP* (100 ng/l) RNAs were added as control.

In situ hybridization

In situ hybridization was carried out as described (Hauptmann and Gerster, 1994) using the following mRNA: *pax2.1* (Macdonald et al., 1997), *vax1 and vax2* (Masaya et al., 1992), *ngn1* (Blader et al., 1997 Kim et al., 1997), *fgf8* (Crossley and Martin, 1995), *lim1* (Toyama and Dawid, 1997), *pax6* (Krauss et al., 1991); *krox20* (Wilkinson et al., 1989), *ath5* (Matsuda et al., 2004) *islet1* (Appel et al., 1995), *wnt1* (Liu and Joyner, 2001), and rx1 (Chuang et al., 2001). Two color in situ hybridization was performed as described by Hauptmann and Gerster (1994). Embryos were photographed using a CoolSNAP-Pro camera system attached to a Leica M stereomicroscope.

RESULTS

Zebrafish *zic3* expression pattern

The expression pattern of *zic3* has been reported in mouse (Suzuki et al., 1997 Ekker et al., 1997), *Xenopus* (Nakata el al., 1997; Kitaguchi et al., 2002), zebrafish (Grinblat and Sive, 2001), and chick (McMahon et al., 2007) embryos. We further determined more detailed characteristics of *zic3* expression domains during embryogenesis using whole mount in situ hybridization. *zic3z* transcripts are initially accumulated at late-blastula stage (sphere stage, 4hpf), soon after midblastula transition, and restricted in the dorsal half of the blastoderm, which gives rise to prospective organizer (Fig. 1A). In particular, we found that *zic3z* transcripts are present in the prospective dorsal organizer region of zebrafish earlier than in the mouse (Suzuki et al., 1997; Ekker et al., 1997; Elms et al., 2004), *Xenopus* (Nakata et al., 1997; Kitaguchi et al., 2002), and zebrafish *zic3* (Grinblat and Sive, 2001). As gastrulation proceeds, *zic3z* is expressed in the entire prospective dorsal neuroectoderm as a *zic2* gene (Grinblat and Sive, 2001; Kudoh et al., 2004) (Fig. 1 B, C, D) and blastoderm marginal cells (Fig.1 B, C). At the tail bud stage (10 hpf), *zic3z* transcripts are predominantly present in the forebrain, diencephalon, hindbrain, and segment plate. *Zic3z* transcripts in the hindbrain overlap with the expression domain of *fgf8*, a prospective forebrain and hindbrain marker (Fig. 1E, F). In the embryos of *Zoep*mutant, which lacks mesendoderm and displays cyclopic eye, the expression domain is significantly reduced in comparison to the wild type. It is worth noting that diencephalon primodium cells are fused in *Zoep* mutant embryos compared with wild type embryos (Fig. 1G). During somitogenesis (5-10 somite), *zic3z* is transcribed in the eye primordial cells as well as in the neural plate, forebrain, diencephalon, hindbrain, and segmental plate (Fig. 1H, I). *Zic3z* expression domain in the eye primordial cells continues until pharyngula stages at 24 hpf and 48 hpf (Fig. 1K, L). At pharyngula stage, *zic3z* expression in the neural retina becomes restricted to the retina proliferate zone (Fig. 1K, L) (Herman et al., 2002). *zic3z* transcripts also appear in the dorsal neural tube, and tail bud region

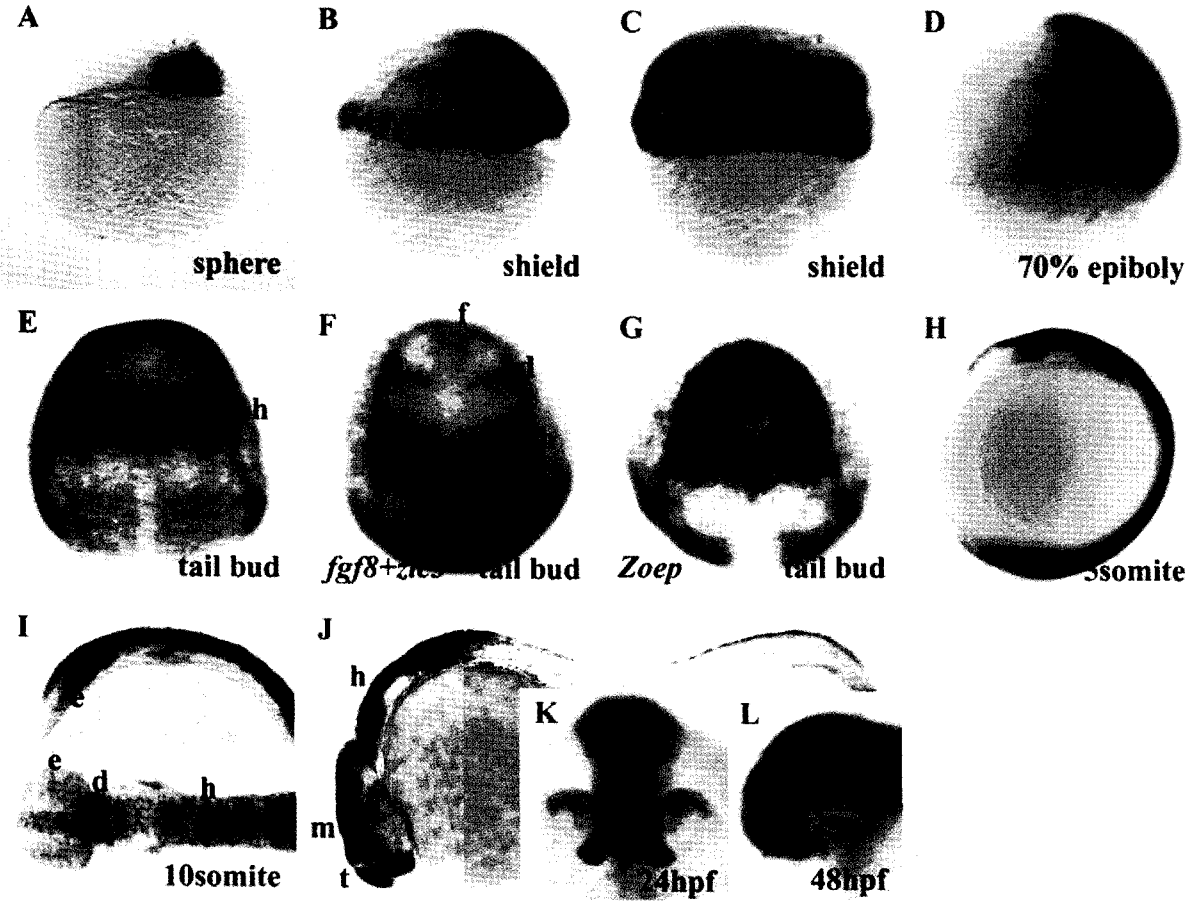

Fig. 1. Expression pattern of *zic3z* at the early embryonic stages. *Zic3z* expression was detected by whole mount in situ hybridization. (A) The transcripts of *zic3z* are initially accumulated at the sphere stage (4 hpf) and (B-D) restricted in cells at the dorsal half of the blastoderm, blastoderm margin, and posterior dorsal quadrant of the prospective ectoderm at the 50% and 70% epiboly stage. (E-G) At tail bud stage (10 hpf), *zic3z* is expressed in the neural plate including forebrain, diencephalon, and hindbrain primodium cells. (G) In *Zoep* mutant, *zic3z* expressing diencephalon primodium cells are fused compared with WT embryo. (H, I) After segmentation stage, *zic3z* is expressed in the anterior telencephalon, posterior diencephalon, midbrain, hindbrain, paraxial mesoderm and dorsal neural tube. Also, *zic3z* begins to appear in the eye primordial cells at 10 somite stage (14 hpf), and after 24 hpf (J, K), 48 hpf (L), *zic3z* expression is specified in retina cells. t: telencephalon, f: forebrain, d: diencephalon, m: midbrain, h: hindbrain, e: eye.

(Fig. 1J). Recent report suggests that Zic3 together with Zic2 synergistically controls neurulation and segmentation of paraxial mesoderm in mouse embryo (Inoue et al., 2007). Taken together, the zic3z expression pattern suggests that its expression in the prospective dorsal neuroectoderm at the early developmental stage, ie, the shield stage, plays an important role in formation of the brain pattern at tail bud.

Nodal, BMP, and Wnt govern *zic3z* expression pattern in the neuroectoderm

Because *zic* genes are regulated by BMP (Nakata et al., 1997; Grinblat et al., 1998; Rohr et al., 1999), Nodal, and Wnt signals in the neuroectoderm (Weber and Sokol, 2003), we tested if Nodal, BMP, and Wnt modulate the expression pattern of *zic3z* using whole-mount in situ hybridization.

We initially examined the effects of Nodal on *zic3z* transcription by injecting a small (5 pg) or large (20 pg) amounts of RNA for the Nodal/Activin inhibitor, Atv/Lefty1 (Thisse and Thisse, 1999). The Nodal inhibitor largely eliminates *zic3z* transcripts from the blastoderm marginal cells and presumptive dorsoanterior neuroectoderm, but not in the dorsal organizer region (Fig. 2D-I). In contrast, overexpression of the Nodal-related protein Squint ectopically stimulates the *zic3z* transcription and extends its expression domain toward the ventral side of the embryos injected with *squint (5 pg)* RNA (Fig. 2J-L). We next examined the effects of BMP signals on *zic3z* expression pattern by injecting *bmp4* RNA or *chordin* morpholinos. Knock-down of *chordin* expression largely reduces the *zic3z* expression in the dorso-anterior neuroectoderm but not in the blastoderm marginal cells at the mid-gastrula stage (Fig. 2P, R), while overexpression of *bmp4* completely eliminates the zic3z transcripts from the blastoderm

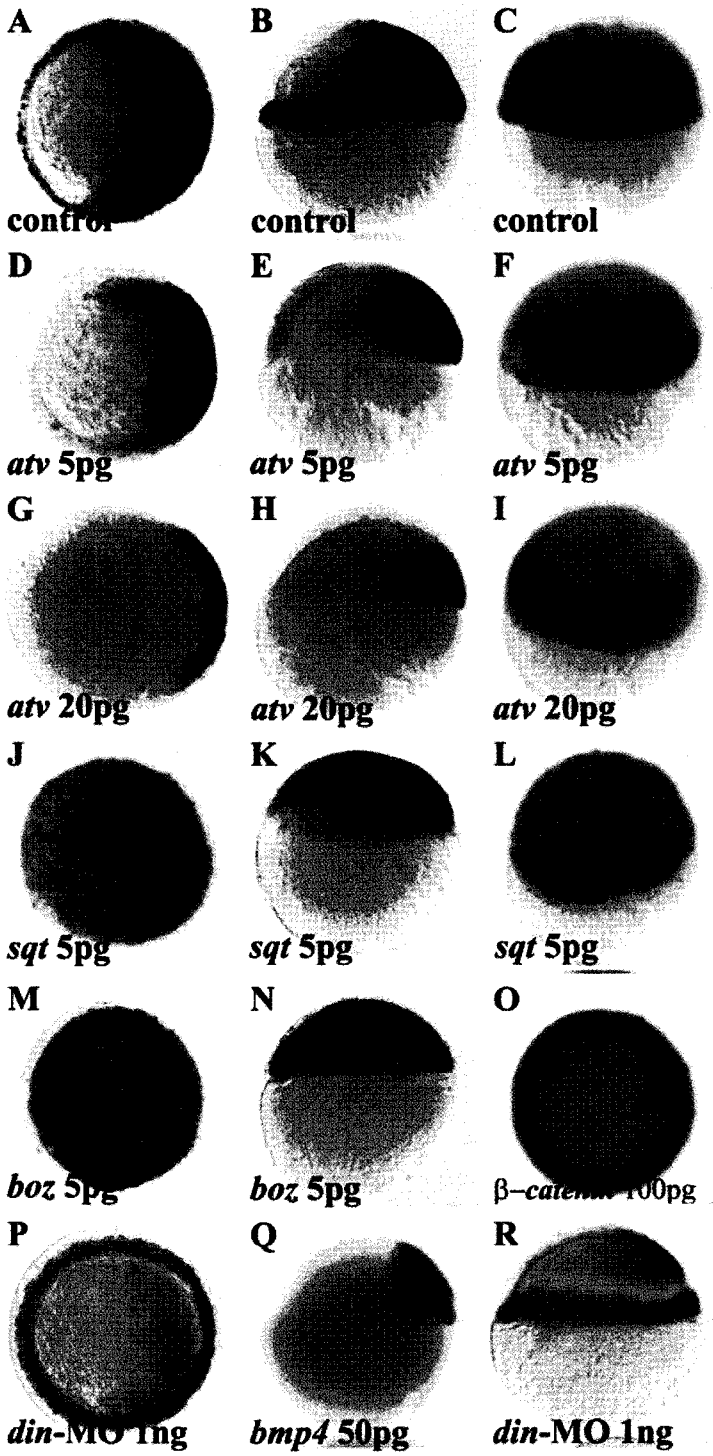

Fig. 2. Regulation of *zic3z* expression during gastrulation. *Zic3z* expression is regulated by Nodal signaling. (A-C) Expression of *zic3z* in wild type. (D-L) *zic3z* expression is regulated by Nodal signaling in a dose-dependent manner. *Zic3z* expression domain is reduced (D-I) or ectopically induced (J-L) at the mid-late gastrula stage in the embryos injected with 5 pg of *atv RNA* (D-F), 20 pg of *atv* RNA (G-I), 5 pg of *sqt* RNA (J-L). (M-O) *Zic3z* expression is ectopically induced in embryos injected with 5pg of *boz* RNA (M, N) and 100 pg of β-catenin (O). (P-R) Both in *chordin* morpholino (1ng) and *bmp4* RNA (50 pg) injected embryos, *zic3z* expression is reduced in the anterior-neuroectoderm, respectively. (A, D, G, J, M, O, P) animal pole views, (B, E, H, K, N, Q, R) lateral views, (C, F, I, L) dorsal pole views. All embryos are at 50% epiboly stage.

marginal cells and largely reduces them in the anterior neuroectoderm (Fig. 2Q). Taken all together, these results confirm that proper expression of *zic3z* both in the blastoderm marginal cells and in the dorsoanterior neuroectoderm is under the control of Nodal and BMP signals in the zebrafish embryos.

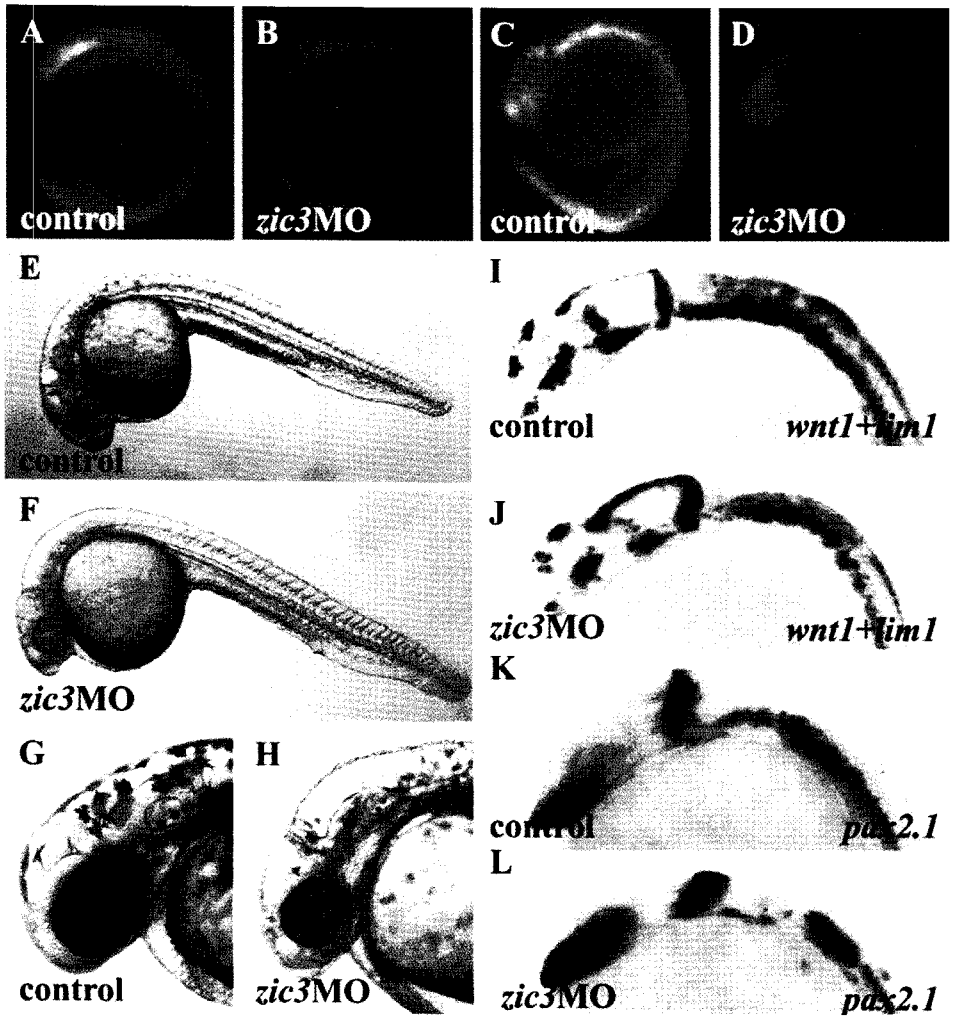

Fig. 3. The size of the MHB, hindbrain domain is narrowed by abrogation of *zic3z*. (A, B) Expression of *fez-like* in the forebrain is unaffected in *zic3z* morphant embryos at the tail bud stage. (C, D) Hindbrain neurons marked by *Ngn1* expression at the three-somite stage are reduced in *zic3z* morphant embryos. (E-H) Expression of *hoxb1b* in posterior-neuroectoderm is enlarged (E, F), MHB and hindbrain domain is narrowed by *zic3z*-MO at the 10 somite stage. (G-J).

Because induction of neural tissue in zebrafish is initiated in early blastoderm by stabilization and nuclear transcription of the transcriptional activator β-catenin in the presumptive ectoderm on the future dorsal side (Schier, 2001), we examined if the β-catenin-mediated Wnt signal modulates the *zic3z* expression. Overexpression of β-catenin (100 pg) ectopically induces *zic3z* expression in the neuroectoderm of gastrula (Fig. 2O) as overexpression of *boz does* (Fig. 2M, N). These results strongly indicate that expression of *zic3z* in the dorsoanterior neuroectoderm involves Wnt signal as well as Nodal and BMP signals.

Zic3z is required for the brain patterning

In order to define the original function of Zic3z in processing embryonic neural induction, we assayed biological function of *zic3z* by conducting *zic3z* knock-down experiments. Injection of antisense morpholino against the 5' untranslated regions of *zic3z* into the yolk of one to four cell stage embryos (0.8 ng) efficiently inhibits translation from the *zic3z: GFP* RNA containing the 5'UTR plus coding sequence of *zic3z* (Fig. 3B, D) while control morpholino does not (Fig. 3A, C). *zi3z* morphants display head deformities such as thinning of the cerebellum and enlargement of the ventricles in the hindbrain (Fig. 3E-H) approximately by 80%, suggesting that *zic3z* specifies brain patterning.

We further analyzed various molecular markers of brain patterning to determine molecular genetic elements associated with the defects caused by the zic3z knock-down. Knock-down of *zic3z* does not alter *fez-like* (*fezl*) expression at tail bud stage (Fig. 4A, B), but causes extension of the *hoxb1b* expression domain toward the posterior neuroectoderm (Fig. 4E, F). Knock-down of *zic3z* significantly closes *Pax2.1* and *fgf8* expression area in the midbrain-hindbrain boundary (MHB), and hindbrain (Fig. 4I, J) and perturbs the *ngn1* expression particularly in the hindbrain at the 3-

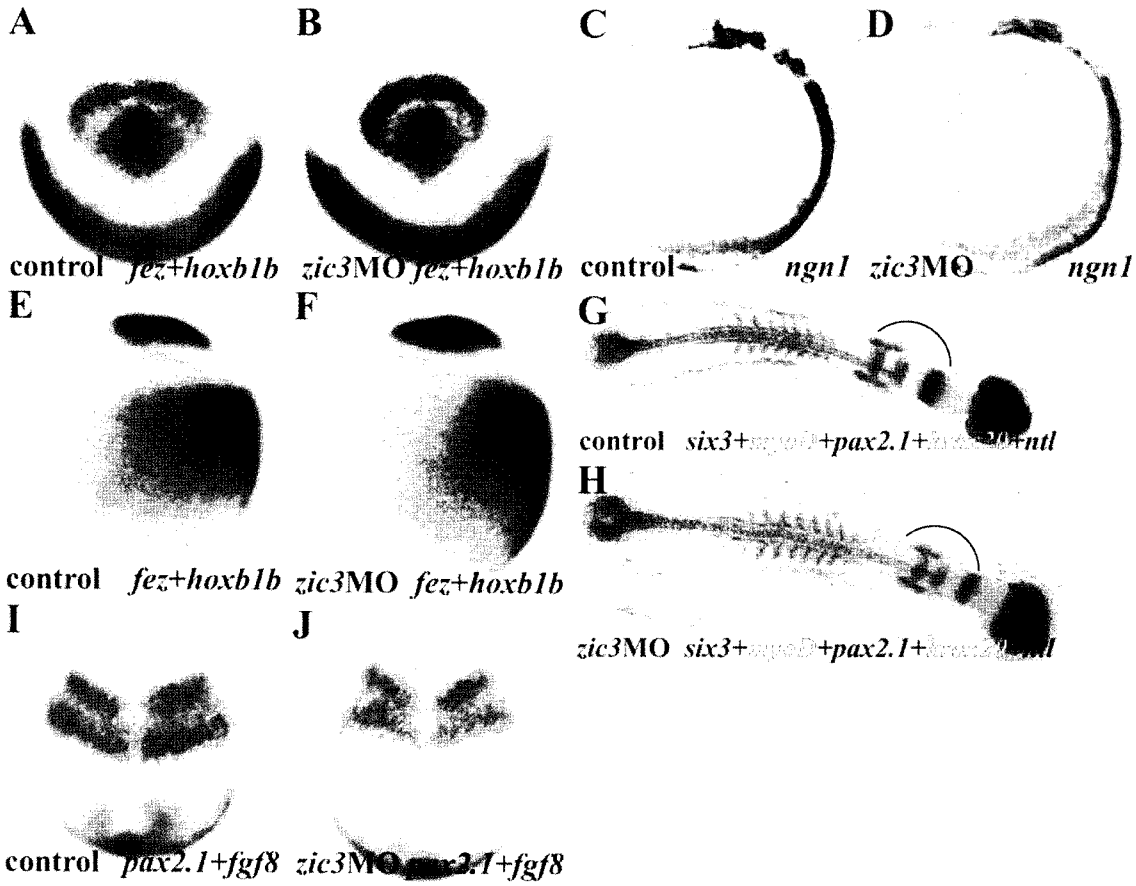

Fig. 4. Blocking *zic3z* translation with *zic3z*-MO leads to a cerebellar hypoplasia. (A-D) Injection of *zic3z*-MO inhibits translation from the *zic3z: GFP* RNA containing the 5'UTR and coding sequence of *zic3z* at the 10 somite, 24 hpf. (E-H) Loss-of-function phenotype after *zic3z*-MO mediates interference with *Zic3z* activity at 24 hpf (E, F) and 32 hpf (G, H). *Zic3z* morphants show abnormal development of in the midbrain, hindbrain and eye size and shape. (I-L) Expression of *lim1, wnt1* (I, J) and *pax2.1* (K, L) is reduced in the *zic3z* morphants of cerebellar region at the 24 hpf (indicated by arrowhead). (A-L) All embryos are shown in lateral views.

somite stage (Fig. 4C, D). Knock-down of *zic3z* abrogates *pax2.1* expression in the hindbrain but not in the MHB region of the injected embryos (Fig. 3K, L). Co-staining with *wnt1* and *lim1* probes show that knock-down of *zic3z* suppresses the *lim1* expression in the cerebellum, and rhombomeres 1 and 2 without changing the *wnt1* expression pattern (Fig. 3I, J). These results support that *zic3z* is required for the formation of brain pattering. In addition, these results are consistent with the observation that hypoplastic change occurs in the cerebellar anterior lobe of Zic1/Zic2 trans-heterozygotic and Zic3-deficient mice (Aruga et al., 1998, 2002a) and homozygous Bent tail (Bn/Y, Zic3-dificient) mice (Aruga et al., 2004).

Abrogation of *zic3z* expression expands optic stalk cells toward retina and undifferentiated retinal ganglion cells (RGC)

Because *zic3z* is expressed in the neural retina (Fig. 1I-L), and knock-down of *zic3z* expression induces abnormal eye size and shape (Fig. 4E-H), we studied changes in gene expression associated with the eye defects in the *zic3z* morphants using markers for the optic stalk and retina cells. Expression domains of *fgf8* (Fig. 5C, E), *pax2.1* (Fig. 5A, B, D, F, I, J), *vax1 and vax2* (data not shown) are largely expanded to the dorsal retina in the *zic3z* morphants. It has been shown that *Shh* overexpression causes a failure of separation of the eye primodium from the diencephalon, resulting in fusion between the eye and the diencephalon over a large region (Ekker et al., 1995; Hallonet et al., 1999; Macdonald et al., 1995). This phenotype appears to be caused by hypertrophy of the optic stalks at the expense of pigment epithelium and neural retina (Wilson et al., 1995). We examined whether the expansion of optic stalk toward the retina is either due to degeneration of retinal neurons coupled with over proliferation of optic stalk or optic nerve cells, or due to retinal cells changing fate and differentiating as optic stalk or optic nerve as in *shh* overexpression. We assessed the expression of *pax6* and *rx1* genes that are initially expressed throughout the retina and later in proliferate cells of the cilliary marginal zone (Macdonald

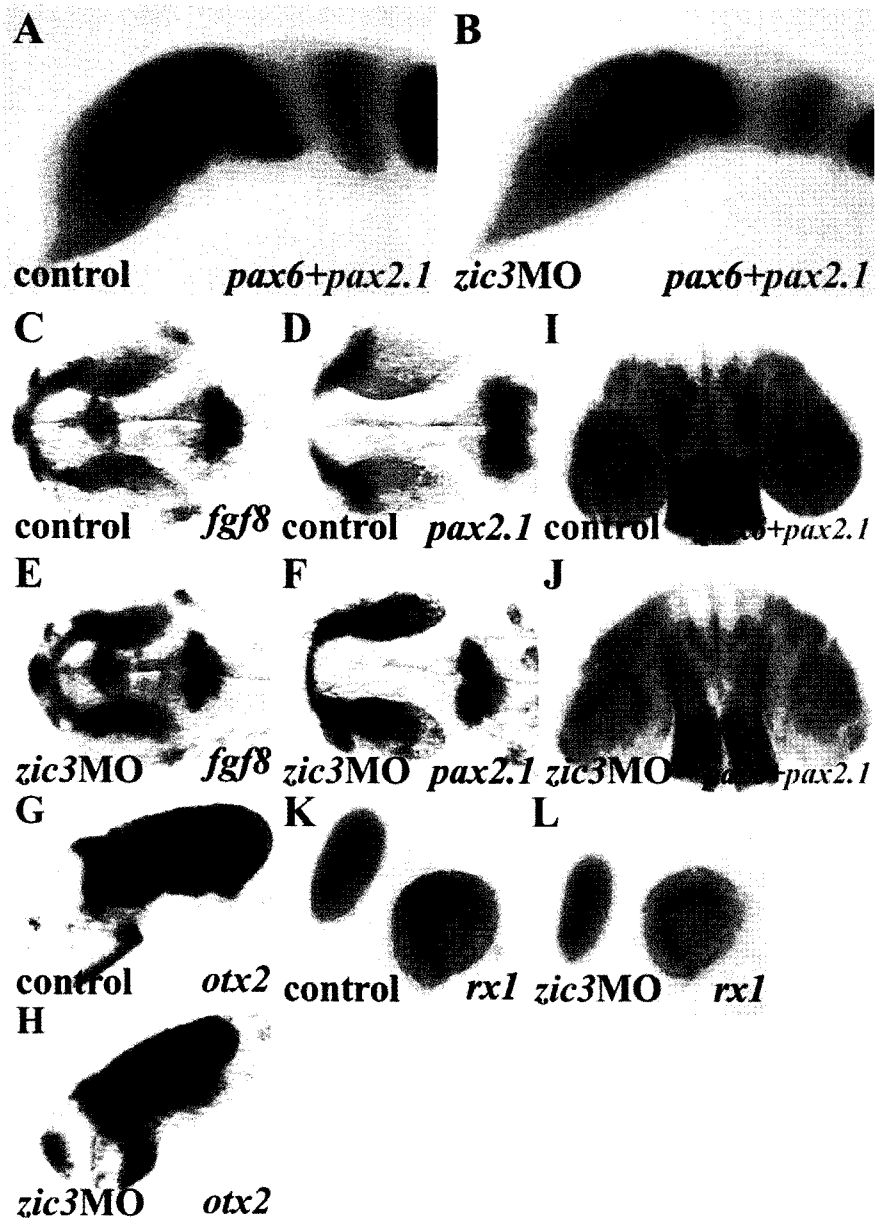

Fig. 5. Blocking *zic3z* translation with *zic3z*-MO leads to expansion of optic stalk toward the retinal region. Injection of *zic3z* morpholino causes strong expansion of *pax2.1* (A, B, I, J: red color, D, F: blue color) and *fgf8* (C, E) expression into retinal region at the 10 somite, 28hpf. However, *rx1* (K, L) and *pax6* (A, B, I, J: blue color) expression is unaffected in the retina at the 20 somite stage. In *zic3z*-MO injected embryos, *otx2* expression domain in midbrain is smaller than control embryos. (A, B, G, H, K, L), lateral views, (C, D, E, F, I, J), flat mount views.

and Wilson, 1997; Marther et al., 1997; Chuang et al., 1999). The expression of *pax6* (Fig. 5A, B, I, J) and *rx1* normally persists throughout the retina of *zic3z* morphant embryos at the 10 and 20 somite stages (Fig. 5K, L). *pax2.1* expression expands to dorsal retina whereas *pax6* expression does not change in the *zic3z* morphant embryos (Fig. 5A, B, I, J). These data indicate that expansion of optic stalk to the retina is due to degeneration of retinal neurons coupled with over-proliferation of optic stalk or optic nerve cells.

We further determined the molecular elements associated with the fate changes in *zic3z* morphants by analyzing the expression patterns of neuronal makers *ath5* for retinoblasts and postmitotic neurons prior to full differentiation (Ichiro and Wilson, 2000) and *islet1* (Korzh et al., 1993 Inoue et al., 1994) for retinal ganglion and inner nuclear layer cells. While the retinal cell markers *pax6* and *rx1* are not affected (Fig. 5A, B, I, J, K, L) in *zic3z* morphant embryos, the transcripts of both *ath5* (Fig. 6A-D) and *islet1* (Fig. 6E, F) are severely reduced or completely eliminated in the retinal ganglion cells (RGC) at 30 hpf and 48 hpf. These data suggest that proper expression of *zic3z* is needed not only for differentiation but also for maintenance of retina

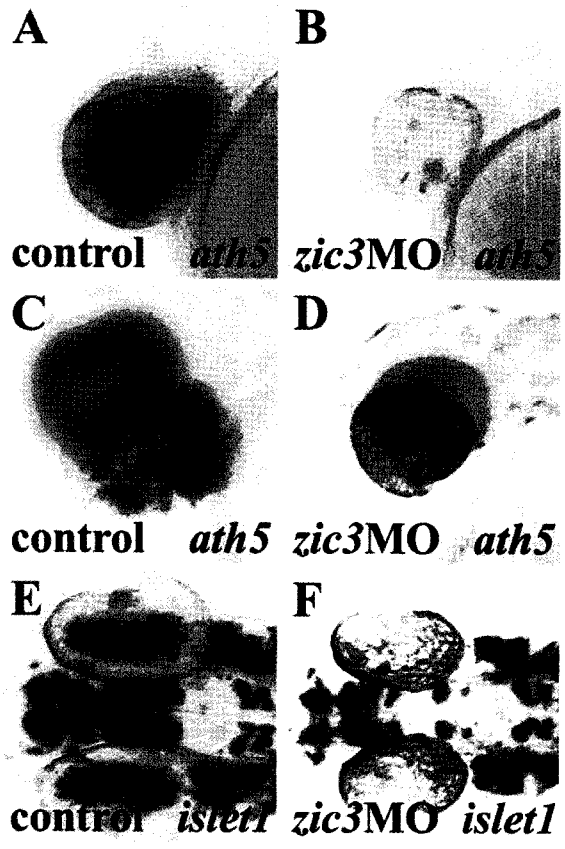

Fig. 6. Knockdown of *zic3z* expression affects retinal neurogenesis. (A-F) The zebrafish atonal homologue *ath5* (A, C) and *islet1* (E) expression appears in the central region of the differentiating retina at the 30 hpf (A), 35 hpf (E) and 48 hpf (C). In *zic3z*-MO injected embryos, *ath5* (B, D) and *islet1* (F) expression is strongly reduced. (A-D), Lateral views; (E, F), flat mount views.

DISCUSSION

Regulation of *zic3z* expression in the blastoderm marginal cells, dorsal organizer region, and neuroectoderm

Studies of *zic3z* expression pattern and overexpression (Fig. 1 and 2D-I) suggest that *zic3z* expression is under the control of the neuroectoderm inducing signals such as BMP, Nodal, and Wnt signals during early embryogenesis. This notion is consistent with the previous findings that *zic3* expression is activated or regulated by Nodal (Weber et al., 2003), BMP (Nakata et al., 1997; Grinblat et al., 2001; Weber et al., 2003)-related molecules, a member of the TGFβ superfamily and Wnt signals (Weber et al., 2003).

Overexpression of *sqt* (Fig. 1J, K, L), β-catenin (Fig. 1O) and *boz* (Fig. 1M, N) ectopically induces *zic3z* expression in the dorsal neuroectoderm. On the other hand, knock-down of *antivin* (Fig. 2D-I), *bmp4*-RNA (Fig. 1Q) or *chordin* (Fig. 1P, R) downregulates or abolishes *zic3z* expression in the dorsal neuroectoderm and blastoderm marginal cells, but fail to modulate *zic3z* expression in the dorsal organizer. It is thus conceivable that *zic3z* expression in the blastoderm marginal cells, dorsal organizer region, and neuroectoderm is under differential combination of BMP, Nodal and Wnt signals or other unknown signals. It remains to be further elucidated how *zic3z* expression is differentially regulated in those areas.

Zic3z specifies cerebellum and hindbrain pattern

Although the *Zic3z* transcripts are predominantly present in the forebrain, diencephalon and hindbrainregion, knock-down of *zic3z* does not affect its expression pattern only in the forebrain domain (Fig. 3A-F, G, H). At the same time, the knock-down shifts posterior neuroectoderm to anterior neuroectoderm (Fig. 3E, F) while it closes MHB and hindbrain domain at the late gastrula stage and somite stage in *zic3z* morphant embryos (Fig. 3G-J). Even at 24 hpf, and 32 hpf, the *zic3z* morphants develop very thin cerebellum and enlarged hindbrain ventricle space together with normal development of the forebrain, and MHB (Fig. 4E-H). At the same time, *lim1* and *pax2.1* transcripts are decreased only in the cerebellum and hindbrain neurons (Fig. 4I-L). This observation is consistent with that of a targeted null allele for murine zic3 and mutations in zic3 of human patients that display congenital defects, such as X-linked heterotaxy, neural tube defect (Ferrero et al., 1997; Gebbia et al., 1997 Carrel et al., 2000; Klootwijk et al., 2000), and cerebellar hypoplasia (Aruga et al., 1998, 2002). We thus postulate that *zic3z* is likely to function for cerebellum and hindbrain patterning rather than for forebrain patterning.

Zic3z and eye development

Expression studies of *zic* family genes in mouse (Nagai et al., 1997; Nakata et al., 1998), chick (Zhang et al., 2004; McMahon et al., 2007), *Xenopus* (Herman et al., 2002), and zebrafish (Thisse et al., 2001) found that *zic* genes are expressed in the developing eye. *Zic2* is a determinant directing ipsilateral RGC projection in mouse (Herrera et al., 2003) while *zic3* helps to pattern the chick retina for intra-retinal axon guidance (Zhang et al., 2003). In the case of *zic3z*, its transcripts appear in the eye primordial cells at 13-14 hpf (Fig. 1I), and in the neural retinal region, and become restricted in the retina proliferate zone at the 25 hpf afterward (Fig. 1K, L). Our loss-of-function experiment showed that *zic3z* controls the formation of optic stalk cells and that its loss induces the expansion of *pax2.1, fgf8*, and *vax* gene expressed in domains (Fig. 5A-H) without altering the fate of retinal cells (Fig. 5A,B, G, H). It has been shown that *pax2.1* expressing optic stalk primordial cells initially appear at the 12 hpf, and are differentiated as reticular astrocytes of the optic nerve in zebrafish embryos (MacDonald et al., 1997). It is possible that *zic3z* functions as a negative regulator in the formation of optic stalk cells

indirectly since the *pax2.1* transcripts appear in the optic stalk cells at 12 hpf. Considering that *zic3z* encodes a zinc-finger transcription factor, it has been proposed that *zic3z* regulates the expression of secreted or membrane-bound molecules (Zhang et al., 2003). Likewise, zic3z is to regulate *pax2.1* transcription for the development of optic stalk. On the other hand, Jeremy and his colleagues (2005) reported that cell-intrinsic factors are sufficient to activate neurogenesis in the zebrafish retina, but also that cell-cell signals may act earlier in development to establish these cell-intrinsic factors or to modulate their activity in order to bring about retinotopic differences at the time of neurogenesis. Development of the zebrafish retina is under control of Hh signaling. One of the earliest functions of Hh signaling is to induce optic stalk tissue at the expense of neural retina (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). Studies on molecular network among *zic3z*, *shh*, and other elements will elucidate more detailed biological function of *zic3z* during optic stalk development.

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REFERENCES

Altshuler DM, Turner DL, and Cepko CL (1991) Specification of cell type in the vertebrate retina. In Cell Lineage and Cell Fate in Visual System Development. Cambridge, MA: MIT Press. pp. 37-58.

Appel B and Eisen JS (1998) Regulation of neuronal specification in the zebrafish spinal cord by Delta function. *Development* 125: 371-380.

Aruga J (2004) The role of *Zic* genes in neural development. *Mol Cell Neurosci* 26: 205-221.

Aruga J, Inoue T, Hoshino J, and Mikoshiba K (2002) Zic2 controls cerebellar development in cooperation with Zic1. *J Neurosci* 22: 218-225.

Aruga J, Mizugishi K, Koseki H, Imai K, Balling R, Noda T, and Mikoshiba K (1999) Zic1 regulate the pattering of vertebral arches in cooperation with Gli3. *Mech Dev* 89: 141-50.

Aruga J, Minowa O,Yanginuma H, Kuno J, Nakai T, Noda T, and Mikoshiba K (1998) Mouse Zic1 is involved in cerebellar development. *J Neurosci* 18: 284-293.

Aruga J, Nagai T, Tokuyama T, Hayashizaki Y, Okazaki Y, Chapman VM, and Mikoshiba K (1996). The mouse *Zic* gene family: homologues of the *Drosophila* pair-rule gene *odd-paired*. *J Biol Chem* 271: 1043-1047.

Aruga J, Yokota N, Hashimoto M, Furuichi T, Fukuda M, and Mikoshiva K (1994) A novel zinc finger protein, *Zic* is involved in neurogenesis, especially in the cell lineage of cerebella granule cells. *J Neurochem* 63: 1880-1890.

Benedyk MJ, Mullen JR, and DiNardo S (1994) Odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos. *Gene Dev* 8: 105-117.

Bernardos RL, Lentz SI, Wolfe MS, and Raymond PA (2005) Notch-Delta signaling is required for spatial pattering and Muller glia differentiation in the zebrafish retina. *Dev Biol* 278: 381-95.

Birgbauer E, Cowan CA, Sretavan DW, and Henkemeyer M (2000) Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. *Development* 127: 1231-1241.

Blader P, Fischer N, Gradwohl G, Guillemont F, and Strahle U (1997) The activity of neurogenin1 is controlled by local cues in the zebrafish embryo. *Development* 124: 4557-4569.

Brown SA, Warburton D, Brown LY, Yu CY, Roeder ER, Stengel-Rutkowski S, Hennekam RC, and Muenke M (1998) Holoprosencephaly due to mutaions in ZIC2, a homologue of Drosophila odd-paired. *Nat Genet* 20: 180-183.

Brittis P and Silver J (1994) Exogenous glycosamingoglycans induce complete inversion of retinal ganglion cell bodies and their axons within the retinal neuroepithelium. *Proc Natl Acad Sci* USA 91: 7539-7542.

Carrel T, Purandure S, Harrison W, Elder F, Casey B, and Herman GE (2000) The X-linked mouse mutation bent tail is associated with a deletion of the *Zic3* locus. *Hum Mol Genet* 9: 1937-1942.

Chuang JC and Raymond PA (2001) Zebrafish genes *rx1* and *rx2*help define the region of forebrain that give rise to retina. *Dev Biol* 231: 13-30.

Crossely PH and Martin GR (1995) The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* 121, 439-451.

Dakubo GD, Wang Y, Mazerolle C, Campsall K, McMahon AP, and Wallace VA (2003) Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. *Development* 130, 2967-2980.

Deiner MS, Kennedy TE, Fazeli A, Serafini T, Tessier-Lavigne M, and Sretavan DW (1997) Netrin-1 and DCC mediate axon guidance locally at the optic disc : loss of function leads to optic nerve hypoplasia. *Neuron* 19: 575-589.

Dickinson ME, Krumlauf R, and McMahon AP (1994) Evidence for a mitogenic effect of *wnt-1* in the developing mammalian central nervous system. *Development* 120, 1453-1471.

Dornseifer P, Takke C, and Campos-Ortega JA (1997) Overexpression of a zebrafish homologue of the Drosophila neurogenic gene Delta perturbs differentiation of primary neurons and somite development. *Mech Dev* 63: 159-171.

Ebert PJ, Timmer JR, Nakada Y, Helms AW, Parab PB, Liu Y, Hunsaker TL, and Johnson JE 2003 *Zic1* represses *Math1* expression via interactions with the *Math1* enhancer and modulation of *Math1* autoregulation. *Development* 13: 1949-1959.

Ekker SC, Ungar AR, Greenstein P, von Kessler DP, Porter JA, Moon RT, and Beachy PA (1995) Patterning activities of vertebrate *hedgehog* proteins in the developing eye and brain. *Curr Biol* 5: 944-955.

Ferrero GB, Gebbia M, Pilia G, Witte D, Peier A, Hopkin RJ, Craigen WG, Shaffer LG, Schlessinger D, Ballabio A, and Casey B (1997) A microscopic deletion in Xq26 associated

with familial situs ambigus. *Am J Hum Genet* 61: 395-401.

Gebbia M, Ferrero GB, Pilia G, Bassi MT, Aylsworth A, Penman-Splitt M, Bird LM, Bamforth JS, Burn J, Schlessinger D, Nelson DL, and Casey B (1997) X-linked situs abnormalities result from mutations in *ZIC3*. *Nat Genet* 17: 305-308.

Grinblat Y and Sive H (2001) *Zic*-gene expression marks anero-posterior pattern in the presumprive neuroecotderm of the zebrafish gastrula. *Dev Dynam* 222: 688-693.

Haddon C, Smithers L, Schneider-Maunoury S, Coche T, Henrique D, and Lewis J (1998) Multiple Delta genes and lateral inhibition in zebrafish primary neurogenesis. *Development* 125: 359-370.

Hallonet M, Hollemann T, Pieler T, and Gruss P (1999) *Vax1*, a novel homeobox-containing gene, directs development of the basal forebrain and visual system. *Genes Dev* 13: 3106-3114.

Hauptmann G and Gerster T (1994) Two-color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. *Trends Genet* 10, 266.

Herman G E and El-Hodiri HM (2002) The role of Zic3 in vertebrate development. *Cytogenet Genome Res* 99 :229-235.

Inoue A, Takahashi M, Hatta K, Hotta Y, and Okamoto H (1994) Developmental regulation of *islet-1* mRNA expression duting neuronal differentiation in embryonic zebrafish. *Dev Dyn* 199: 1-11.

Inoue T, Hatayama M, Tohmonda T, Itohara S, Aruga J, and Mikoshiba K (2004) Mouse Zic5 deficiency results in neural tube defects and hypoplasia of cephalic neural crest derivatives. *Dev Biol* 270: 146-62.

Inoue T, Ota M, Mikoshiba K, and Aruga J (2007) Zic2 and Zic3 synergistically control neurulation and segmentation iof paraxialmesoderm in mouse embryo. *Dev Biol* 306: 669-684.

Jeremy NK, Brian AL, and Herwig B (2005) Staggered cell-intrinsic timing of *ath5* expression underlies the wave of ganglion cell neurogenesis in the zebrafish retina. *Development* 132, 2573-2585.

Joseph RW and Sergei YS (2003) Identification of a phylogenetically conserved activin-responsive enhancer in the *Zic3* gene. *Mech Dev* 120: 955-965.

Kim CH, Bae YK, Yamanaka Y, Yamashita S, Shimizu T, Fujii R, Park HC, Yeo SY, and Huh TL (1997). Overexpression of neurogenin induces ectopic expression of *HuC* in zebrafish. *Neurosci Lett* 239: 113-116.

Kitaguchi T, Mizugishi K, Hatayama M, Aruga J, and Mikoshiba K (2000) *Xenopus brachyury* regulates mesodermal expression of *Zic3*, a gene controlling left-right asymmetry. *Dev Growth Differ* 44: 55-61.

Kitaguchi T, Nagai T, Nakata K, Aruga J, and Mikoshiba K (2000) *Zic3* is involved in the left-right specification of the *Xenopus* embryo. *Development* 127: 4787-4795.

Klootwijk R, Franke B, van der Zee CE, de Boer RT, Wilms W, Hol FA, and Mariman EC (2000) A deletion encompassing *Zic3* in bent tail, a mouse model for X-linked neural tube defects. *Hum Mol Genet* 9: 1615-1622.

Koyabu Y, Nakata K, Mizugishi K, Aruga J, and Mikoshiba K (2001) Physical and functional interaction between Zic and Gli proteins. *J Biol Chem* 276: 6889-6892.

Krauss S, Johansen T, Korzh V, Moens U, Ericson JU, and Fjose A (1991) Zebrafish *pax* (*zf-a*) : a paired box-containing gene expressed in the neural tube. *EMBO J* 10: 3609-3619.

Kuo JS, Patel M, Gamse J, Merzdorf. C, Liu X, Apekia V, and Sive H (1998) *Opl*: a zinc finger protein that regulates neural determination and patterning in *Xenopus*. *Development* 125: 2867-2882.

MacDonald R, Barth KA, Xu Q, Holder N, and Wilson SW (1995) Middle signaling is required for *Pax* gene regulation and patterning of the eyes. *Development* 212: 3267-3278.

MacDonald R, Scholes J, Strahle U, Brennan C, Holder N, Brand M, and Wilson S (1997). The Pax protein is required for commissural axon pathway formation in the rostral forebrain. *Development* 124: 2397-2408.

Masai I, Stemple DL, Okamoto H, and Wilson S (2000) Midline signals regulate retinal neurogenesis in zebrafish. *Neuron* 27: 251-263.

Matsuda N and Mishina M (2004) Identification of chaperonin CCT gamma subunit as a determinant of retinotectal development by whole-genome subtraction cloning from zebrafish no tectal neuron mutant. *Development* 131: 1913-25.

McMahon A, Junette K, and Merzdorf C (2007) The expression of *zic1*, *zic2*, *zic3*, and *zic4* in early chick embryos. *Dev Biol* 306: 355-366.

Mizugishi K, Aruga J, Nakata K, and Mikoshiba K (2001) Molecular properties of Zic proteins as transcriptional regulators and their relationship to GLI proteins. *J Biol Chem* 276: 2180-2188.

Nadean LB, Sima P, Joseph B, and Tom G (2001) *Math5* is required for retinal ganglion cell and optic nerve formation. *Development* 128: 2497-2508.

Nagai T, Aruga J, Takada S, Gunther T, Sporle R, Schughari K, and Mikoshiba K (1997) The expression of the mouse *Zic1, Zic2* and *Zic3* genes suggests an essential role for *Zic* genes in body pattern formation. *Dev Biol* 182: 299-313.

Nakata K, Nagai T, Aruga J, and Mikoshiba K (1997) *Xenopus Zic3*, a primary regulator both in neural and neural crest development. *Proc Natl Acad Sci* USA 94: 11980-11985.

Nakata K, Nagai T, Aruga J, and Mikoshiba K (2002) *Xenopus Zic* family and its role in neural and neural crest development. *Mech Dev* 75: 43-51.

Niehrs C (1999) Head in the WNT : the molecular nature of Spemann's head organizer. *Trends Genet* 15: 314-319.

Oxtoby E and Jowett T (1993) Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res* 21: 1087-1095.

Purandare SM, Ware SM, Kwar KM, Gebbia M, Bassi MT, Deng JM, Vogel H, Behringer RR, Belmont JW, and Casey B (2002) A complex syndrome of left-right axis, central nervous system and axial skeleton defects in *Zic3* mutant mice. *Development* 129: 2293-2302.

Ro H, Soun K, Kim EJ, and Rhee M (2004) Novel vector systems optimized for injecting in vitro-synthesized mRNA into zebrafishembryos. *Mol Cells* 17: 373-376.

Rohr KB, Schulte-Merker S, and Tautz D (1999) Zebrafish *zic1* expression in brain and somites is affected by BMP and hedgehog signaling. *Mech Dev* 85: 147-159.

Salero E, Perez-Sen R, Aruga J, Gimenez C, and Zafra F (2001) Transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter. *J Biol Chem* 276: 1881-1888.

Take-uchi M, Clarke JDW, and Wilson S (2003) Hedgehog signaling maintains the optic stalk-retinal interface through

the regulation of *Vax* gene activity. *Development* 130: 955-968.

Thisse C and Thisse B (1999) Antivin, a novel and divergent member of the TGF b superfamily, negatively regulates mesoderm induction. *Development* 126 :229-240.

Toyama R and Dawid IB (1997) *Lim6*, a novel LIM homeobox gene in the zebrafish: comparison of its expression pattern with *lim1*. *Dev Dyn* 209: 406-417.

Yang Y, Hwang CK, Junn E, Lee G, and Mouradian MM (2000) ZIC2 and Sp3 repress Sp1-induced activation of the human D1A dopamine receptor gene. *J Biol Chem* 275: 38863-38869.

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