Ventralized Zebrafish Embryo Rescue by Overexpression of Zic2a

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ABSTRACT

The neuroectoderm arises during gastrulation as a population of undifferentiated proliferating neuroepithelial cells. As development continues, neuroepithelial cells leave the cell cycle and differentiate into neurons and glia of the functioning central nervous system. What processes establish the spatial distribution of proliferating neuroepithelial cells? To investigate this question, zic2a was isolated from zebrafish, a homolog of the Drosophila pair-rule gene odd-paired, which is involved in nervous system patterning. At shield stage, zic2a was expressed in the zebrafish organizer and the blastoderm margin, and became restricted to the axial mesoderm in mid-gastrula. Expression of zic2a appeared in the prospective neuroectoderm during gastrulation, and later demarcated the presumptive forebrain. This expression pattern suggests that zic2a may function early in the organizer and later in the neural plate to demarcate the population of proliferating neuroectoderm. Consistent with a function for zic2a in transducing signals from the organizer, overexpression of zic2a resulted in an expansion of proliferating neuroectoderm. Furthermore, zic2a overexpression rescued the ventralized phenotype of chordino mutant embryos, which lack a functional chordin gene. Early expression of zic2 in the zebrafish organizer, and the phenotype resulting from overexpression, show a role for zic2a downstream of chordin or other secreted organizer proteins in establishing the initial size of the population of neuroectoderm cells.

INTRODUCTION

The vertebrate central nervous system contains multipotent precursor cells capable of differentiating into a variety of neuronal and glial fates.1,2 Because neural stem cells initially arise by processes that drive normal embryonic development, and these embryonic mechanisms may persist in later life to help control reserve cells that maintain homeostasis of the central nervous system after perturbation from disease or injury,3 it is important to understand the initial developmental processes that establish the extent of the embryonic central nervous system. The formation of the neuroectoderm from the dorsal ectoderm depends on inductive signals from the dorsal organizer at the early gastrula stage and secreted factors from the ventral portion of the embryo.

In Xenopus, dorsalizing signals that originate from the organizer include Noggin, Follistatin, Nodal-related-3 (Xnr3), Chordin, and Gremlin.4–8 These signals act by antagonizing the secreted Bone Morphogenetic Proteins (BMPs), which play a role in the specification of non-neural, ventral cell fates in Xenopus9,10 and zebrafish.11–14 Not only do these secreted BMP antagonists play an important role in the early
stages of embryonic axis specification, they also help to specify other organ systems that depend on symmetry, including the developing vertebrate inner ear.\textsuperscript{15} Follistatin and Chordin antagonize BMP4 activity by physically associating with BMP4, thereby blocking the binding of BMP4 to its receptor.\textsuperscript{16–18} Genetic evidence for this interaction also exists in zebrafish: the ventralized mutant, \textit{chordin (dino)},\textsuperscript{19} lacks a functional \textit{chordin} gene,\textsuperscript{20} but \textit{dino} interacts genetically with the dorsalized mutant \textit{swirl},\textsuperscript{21,22} which contains a mutation in the \textit{bmp2b} gene.\textsuperscript{11,13}

Members of the \textit{Zic} gene family are good candidates to function downstream of Chordin and BMPs. In \textit{Xenopus}, \textit{Zic1} was cloned as a downstream target of \textit{Chordin}.\textsuperscript{23} Chick \textit{Zic1, 2,} and 3 genes are widely expressed in the central nervous system, neural crest, and inner ear, where Chordin-expressing cells are found to interdigitate with longitudinal stripes of \textit{Zic1}-expressing cells in the hindbrain.\textsuperscript{24} Vertebrate \textit{Zic} genes encode zinc-finger transcription factors of the \textit{Gli} super-family, thought to be involved in patterning the neural tube.\textsuperscript{25–29} Zebrafish \textit{zic1} is strongly expressed in the presumptive forebrain at mid-gastrula.\textsuperscript{30} We reported the isolation of zebrafish \textit{zic2a} (GenBank Accession number AF151535, submitted 14-May-1999) and this gene was independently isolated by Grinblat and Sive,\textsuperscript{31} and Toyama et al.\textsuperscript{32} recently isolated an additional \textit{zic2} paralog, \textit{zic2b (zic2.2)}.

To more fully understand the role of \textit{zic2} in dorsoventral patterning and to define how \textit{zic2} fits into the regulatory network that controls cell fate decisions in the early gastrula, we present data indicating that zebrafish \textit{zic2a} functions in early processes specifying the dorsoventral axis and the size of the neural plate. We show that \textit{zic2a} is expressed in the organizer region of the early gastrula, and \textit{zic2a} transcripts persist in the presumptive axial mesendoderm until the end of gastrulation. This expression pattern suggests the hypothesis that \textit{zic2a} may be involved in organizer function at this early stage in zebrafish development. To test this hypothesis, \textit{zic2a} was overexpressed in zebrafish embryos, and we discovered that this treatment dorsalized wild-type zebrafish embryos, expanding the neuroectodermal domain. To place \textit{zic2a} in the specification hierarchy of dorsoventral patterning, we expressed \textit{zic2a} in ventralized \textit{chordino} mutants. Results showed that \textit{zic2a} rescued these ventralized mutant embryos. Based on the expression pattern of \textit{zic2a}, the effects of \textit{zic2a} overexpression in wild-type embryos, and the ability of \textit{zic2a} message to rescue ventralized mutant embryos, we propose that \textit{zic2a} functions in promoting dorsal cell fates and increasing the size of the proliferating prospective neural-plus-glial cell population.

**MATERIALS AND METHODS**

**Library Screening**

A cDNA library prepared from 6–10 hpf embryos (provided by B.W. Draper, unpublished.) was screened by degenerate PCR. The primers were designed with the application CODEHOP available in the BLOCKS web server (www.blocks.fhcrc.org) of the Fred Hutchinson Cancer Research Center in Seattle, WA. The primers were: \textit{zic2a} F: CCCGGGCCCTCCTCTTYMGN-TAYATG and \textit{zic2-R}: GGGCAAAGATCTTCC-RANCANCCNGG. The annealing temperature was 57°C, salt concentration 3mM and primer concentration 1/9262 M. The expected product is 317 bp and spans the five zinc finger transcription factors of the \textit{Gli} super-family, thought to be involved in patterning the neural tube.\textsuperscript{25–29} Zebrafish \textit{zic1} is strongly expressed in the presumptive forebrain at mid-gastrula.\textsuperscript{30} We reported the isolation of zebrafish \textit{zic2a} (GenBank Accession number AF151535, submitted 14-May-1999) and this gene was independently isolated by Grinblat and Sive,\textsuperscript{31} and Toyama et al.\textsuperscript{32} recently isolated an additional \textit{zic2} paralog, \textit{zic2b (zic2.2)}.

To more fully understand the role of \textit{zic2} in dorsoventral patterning and to define how \textit{zic2} fits into the regulatory network that controls cell fate decisions in the early gastrula, we present data indicating that zebrafish \textit{zic2a} functions in early processes specifying the dorsoventral axis and the size of the neural plate. We show that \textit{zic2a} is expressed in the organizer region of the early gastrula, and \textit{zic2a} transcripts persist in the presumptive axial mesendoderm until the end of gastrulation. This expression pattern suggests the hypothesis that \textit{zic2a} may be involved in organizer function at this early stage in zebrafish development. To test this hypothesis, \textit{zic2a} was overexpressed in zebrafish embryos, and we discovered that this treatment dorsalized wild-type zebrafish embryos, expanding the neuroectodermal domain. To place \textit{zic2a} in the specification hierarchy of dorsoventral patterning, we expressed \textit{zic2a} in ventralized \textit{chordino} mutants. Results showed that \textit{zic2a} rescued these ventralized mutant embryos. Based on the expression pattern of \textit{zic2a}, the effects of \textit{zic2a} overexpression in wild-type embryos, and the ability of \textit{zic2a} message to rescue ventralized mutant embryos, we propose that \textit{zic2a} functions in promoting dorsal cell fates and increasing the size of the proliferating prospective neural-plus-glial cell population.

**Whole-Mount in Situ Hybridization**

Expression analysis was performed essentially as in Oxtoby and Jowett.\textsuperscript{33} Two-color in situ hybridizations were performed according to Jowett and Yan.\textsuperscript{34} To make \textit{zic2a} probe for in situ hybridizations, \textit{zic2a} 3’UTR sequences (nt. 1795 to 2983), which should be gene-specific and different from other paralogs, were amplified with the polymerase chain reaction (PCR) using gene-specific primers (F: GCGTCTAG-ACCTACATCGACAGAAGAAACG (nt. 1795 to 1815, with an XbaI site at the 5’), R: GC-CAAGCTTCTGACAGCTTCTTAGTTTGC
The PCR fragment was digested with XbaI/HindIII and cloned into XbaI/HindIII-digested KS+ Bluescript vector. Antisense RNA probes were synthesized from cDNA encoding zic2a (this paper), zic1,30 krox20,33 foxd3,35 dlx3b,36 gata1,37 pax2a,38 gsc,39 and chordin.20

Zebrafish Strains

Wild-type (AB, C32, WIK, SJD) and mutant zebrafish (*Danio rerio*) were obtained from the University of Oregon Zebrafish Facility. Mutants were obtained from intercrosses of heterozygous carriers.40 Embryos were maintained at 28.5°C and staged by hours (h) or days (d) postfertilization using standard morphological criteria.41 The following ENU-induced mutations identified in the Tübingen mutagenesis screen42 were used: dino (*tt250*),19 a recessive lethal mutation caused by a small 104-bp deletion in the zebrafish ortholog of chordin;20 swirl (*ta72*) is a zygotic semidominant mutation caused by a single base-pair change in the stop codon of bmp2b;13 snailhouse (*ty68a*)22 is a hypomorphic mutation that displays a Val/L50478Gly substitution in a conserved motif of the Bmp7 prodomain.43,44

Phylogenetic and Genomic Analyses

Sequences for phylogenetic analysis were collected using the tblastn search program of NCBI BLAST (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast) to find amino acid sequences similar to zebrafish zic2a. Sequences returned by the search were then aligned by clustalx45 (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html), and the tree was constructed using the neighbor-joining method46 with bootstrapping using 1000 repeats. Genomic structures were obtained from the zebrafish Zv4 Ensembl database at http://pre.ensembl.org/Danio_rerio/. Human orthologs of zebrafish gene predictions were identified by translating BLAST analysis.

RNA Injections

The zic2a cDNA (nt. 1 to 3037) was cloned between the BamHI and XbaI sites of pCS2+ vector (D Turner, R Rupp, J Lee, and H Weintraub, unpublished results). In vitro transcription was performed with the SP6 MESSAGE MACHINE kit (Ambion, Austin, TX) according to manufacturer’s instructions. After removal of DNA template by treatment with RNase-free DNase, the mRNAs were purified with RNeasy Mini Kit (QIAGEN, Valencia, CA) and dissolved in RNase-free water. About 25 pg of capped mRNA was injected per embryo.

Mapping and Linkage Analysis

The zic2a gene was mapped by designing specific primers to amplify the 3′ untranslated region (forward:GGTCTAGACAAGTGTACAAAGTTACGCTAAGT, reverse:CGCAAGCTTGATATAGCGCTATCTG). These primers were used to amplify members of a doubled haploid mapping panel,47 and a polymorphism was detected by single strand conformation polymorphism (SSCP)48 and compared to several thousand other DNA polymorphisms previously scored on this panel.47 Map positions were determined by using Map Manager.49

RESULTS

Isolation and Phylogenetic Analysis of the Zebrafish zic2a Gene

To address the role of zic2 in ectodermal patterning, we cloned a zebrafish ortholog of the vertebrate Zic2 gene using redundant primers designed to amplify zic genes from a zebrafish embryonic cDNA library. Figure 1 shows the alignment of the zebrafish Zic2a, human ZIC2, and *Xenopus* Zic2 protein sequences, demonstrating substantial identity of structure throughout the extent of the molecule.

A phylogenetic analysis of proteins encoded by chordate Zic1, Zic2, and Zic3 genes with the nonvertebrate chordate amphioxus Zic homolog as outgroup establishes the following conclusions (Fig. 2). First, zebrafish zic2a is an ortholog of tetrapod ZIC2 because together they occupy a single clade on the ZIC family tree with very high bootstrap support (1000/1000). Second, the recently reported zic2.2 gene32 is related to zic2a by a gene duplication event that occurred after the divergence of tetrapod and
teleost lineages. Third, the Zic2 gene reported in the pufferfish Tetraodon nigroviridis is an ortholog of the zebrafish zic2a locus supported by high bootstrap values (993/1000), and is not an ortholog of zebrafish zic2b. This shows that the duplication event that gave rise to zic2a and zic2b in zebrafish occurred before the divergence of zebrafish and pufferfish lineages at the base of the teleost radiation. Thus, the duplication of teleost co-orthologs of human ZIC2

FIG. 1. Alignment of zebrafish Zic2a (Accession Number AAF73190), human ZIC2 (AF104902), and Xenopus Zic2 (U57453) protein sequences. The amino-acid sequence of the zebrafish Zic2a protein shows 89% and 78.9% overall identity with mouse Zic2 and Xenopus Zic2 proteins, respectively, with 1 and 5 changes in the zinc finger domain, respectively. It has 74.1% identity with zebrafish Zic1, with 7 changes in the zinc finger domain. Compared to Drosophila Opa, zebrafish zic2a shows 42.2% identity, with 31 changes in the zinc finger domain.
most likely occurred in the genome duplication event experienced by the ray-fin fish lineage before the teleost radiation about 300 million years ago.50–52

The phylogenetic analysis supports further conclusions regarding the origin of the vertebrate ZIC family. First, the gene duplication events that produced the several clades of ZIC genes occurred after the divergence of the cephalochordate and vertebrate lineages. This suggests that they probably occurred in the genome amplification events, which took place...
around the time of vertebrate emergence. Second, there are two ancient clades of \(ZIC\) genes, one consisting of \(ZIC1, ZIC2,\) and \(ZIC3,\) and another comprising \(ZIC4\) and \(ZIC5.\)

When the phylogenetic relationships are considered with respect to the genomic locations of human \(ZIC\) genes (http://www.ncbi.nlm.nih.gov/LocusLink/index.html), a model emerges to explain their origin (Fig. 2B). The model assumes that an ancient non-vertebrate chordate had a single \(ZIC\) gene. This zebrafish region contains BX469902.4, BX571774.3, BX571700.3 and BX571731.4; Fig. 3A). This zebrafish region is a tandem duplication event to yield two genes oriented in opposite directions. One of these two genes was the ancestor of \(ZIC1, ZIC2,\) and \(ZIC3,\) and the other was the ancestor of \(ZIC4\) and \(ZIC5.\)

In one round of genomic amplification (perhaps a genome duplication), two copies of the tandem duplicates were produced; in the leftward oriented gene, one of the copies was the ancestor of \(ZIC1\) and \(ZIC2,\) and the other was the ancestor of \(ZIC3,\) while in the rightward oriented gene, one copy was the ancestor of \(ZIC4\) and \(ZIC5,\) while the other became a pseudogene and was lost. In a second round of genomic amplification, four replicas of the tandem duplicate appeared, producing the five \(ZIC\) genes now in the human genome, and a fourth gene, the duplicate of \(ZIC3\) that was lost. This history parallels that of the \(DLX\) gene family.

**Mapping of zic2a and Conserved Synteny Data**

Mutations in the human ortholog of \(zic2a\) cause holoprosencephaly, and so \(zic2a\) might serve as a candidate for zebrafish mutations with a cyclopic phenotype. To provide a genetic map location so that \(zic2a\) can serve as a candidate gene for zebrafish mutations, we mapped the locus. The \(zic2a\) gene maps to LG3 between z8680 and z22555.

In the human genome, \(ZIC2\) and \(ZIC5\) are nearest neighbors on Hsa13q32.3. In the Zv4 assembly of the zebrafish genome (http://pre.ensembl.org/Danio_rerio/), \(zic2a\) occupies a chromosome segment (the contiguous sequences BX469902.4, BX571774.3, BX571700.3 and BX571731.4; Fig. 3A). This zebrafish region shows conserved synteny to about 2 Mb of the human genome, with the three loci PCCA, FLJ14624, and VGCNL1 on one side of \(zic2a\) and the locus CLYBL on the other side of \(zic2a\) (Fig. 3B). The zebrafish and human loci are conserved in gene order and transcription direction. The genomic contig does not have a detectable \(ZIC5\) ortholog. A sequence (GENSCAN0000027815) apparently orthologous to COL4A5 from Xq22 intrudes on the otherwise conserved synteny.

The zebrafish \(zic2b\) gene in contig BX088652.6 (Fig. 3C) also shares a conserved synteny with the human gene, and again, loci on both sides of \(zic2b\) are conserved. A zebrafish copy of the human gene PCCA, which is a nearest neighbor of \(ZIC2,\) appears in duplicate copy adjacent to both zebrafish \(ZIC2\) co-orthologs. As in the situation with \(zic2a, zic5\) is not adjacent to \(zic2b.\) Instead, the zebrafish \(zic5\) gene resides on contig Zv4_scaffold482.1 along with a second copy of a zebrafish co-ortholog of human CLYBL.

The zebrafish \(zic5\) gene is not located adjacent to \(zic2a\) or \(zic2b\) in the Zv4 assembly, despite the otherwise rather faithful conservation of this chromosome section for 450 million years of evolution. In the pufferfish Takifugu rubripes (http://www.ensembl.org/Fugu_rubripes/), the sequence SINFRUG00000151780 on scaffold_763 is orthologous to \(zic2a\) (Expect = 0.0, Identities = 365/445 (82%) in a blastx search) rather than the second best similar sequence \(zic2b\) [Expect = e-178, Identities = 309/446 (69%)]. The nearest neighbor of the fugu \(Zic2a\) gene is SINFRUG00000156741, a pseudogene we call \(Zic5P\) because its best hit is \(zic5\) from zebrafish. As in the situation with \(zic2a\) and \(zic5\) gene resides on contig Zv4_scaffold482.1 along with a second copy of a zebrafish co-ortholog of human CLYBL.

Under one explanation, the Zv4 version of the zebrafish genome database erroneously fails to place \(zic5\) next to \(zic2a\) where it belongs, a problem arising due to polymorphisms in the sequence. Alternatively, if the Zv4 data are correct, then the last common ancestor of pufferfish and zebrafish had a \(zic2a – zic5\) gene pair, but the \(zic5\) gene has become a pseudogene in fugu and zebrafish, more recently in fugu where it is easily detected, and more anciently in zebrafish, where its sequence, which should appear between the zebrafish \(zic2a\) and
clybl sequences, has degraded to a point where it is not detectable in BLAST similarity searches. Under this scenario, the current zebrafish gene zic5 must have been adjacent to zic2b, a conclusion supported by the presence of two orthologs of CLYBL in zebrafish, one adjacent to zic5 (the former neighbor of zic2b) and the other adjacent to zic2a. If this model is correct, it is important for its significance concerning the dispersal of regulatory elements. Such elements may have been disrupted if the zic2b and zic5 genes separated by chromosome rearrangement, but there would be no rearrangement associated with the loss of the zebrafish ortholog of fugu Zic5p. Thus, the regulatory elements of zic2a might be more intact than those of zic2b. Notice that the evolutionary rate of zic2b is great compared to zic2a, which might be related to relaxation of function associated with loss of regulatory elements occasioned by the chromosome rearrangement that removed zic5 from zic2b.

Zebrafish zic2a Is Expressed in the Embryonic Shield

To determine when and in which tissues zic2a is expressed during zebrafish development, we investigated its expression pattern by in situ hybridization. Transcripts of zic2a are present in the blastula as early as the 64-cell
stage (data not shown). In shield stage embryos, zic2a transcripts appear enriched in the dorsal side of the gastrula, weakly expressed in the presumptive notochord domain and the margin, including the domain that will give rise to the paraxial mesendoderm (Figs. 4A–4C). At 60% epiboly, we detect zic2a transcripts at higher levels in the axial mesendoderm and in lower levels in the anterior neuroectoderm and the blastoderm margin (Figs. 4D–4F). Double in situ hybridization analysis with a probe for goosecoid (gsc)39,62,63 shows that at shield stage (Fig. 4C) and 60% epiboly (Fig. 4F), zic2a co-localizes with gsc in its expression domain in the shield and the involuting axial mesoderm, respectively. At 75% epiboly, expression of zic2a is up-regulated in the anterior neuroectoderm, whereas in the mesodermal layer it becomes restricted to the axial mesoderm (Figs. 4G–4I). To better define the boundaries of this expression domain, we performed double in situ hybridization with chordin, which is expressed in the presumptive prechordal plate in the axial mesoderm and in paraxial regions in the neuroectoderm at 75% epiboly (Fig. 4I).64 The expression domain of zic2a in the axial mesoderm overlaps that of chordin at this stage (Fig. 4I).

In tailbud stage embryos, zic2a expression is detected at the edge of the neural plate, with highest levels in the prospective forebrain in a characteristic crescent-shaped domain (Fig. 4J). By the 1-somite stage, the zic2a expression domain at the edge of the neural tube extends further caudally (Fig. 4K). In 2-somite stage embryos, expression also appears in bilateral pairs of medial lateral stripes that subdivide the prospective head neuroectoderm into three regions (Figs. 4L, 4M). To define more precisely the boundaries of zic2a expression, we performed two-color whole-mount in situ hybridization with krox20, which is expressed in rhombomeres 3 and 5 in the hindbrain.33

In 12-somite stage embryos (Fig. 4N) zic2a is expressed in the prospective telencephalic region at high levels, and in the prospective dorsal diencephalon, the tectum, the trigeminal placode and dorsal hindbrain at lower levels. The ventral diencephalon shows only a narrow
The *zic2a* gene does not appear to be expressed in neural crest, but seems to be expressed in some placode precursors.

In conclusion, *zic2a* is transiently expressed in axial mesendoderm and in the presumptive anterior neuroectoderm and presumptive placodes, but not in the neural crest. This expression pattern suggests the hypothesis that *zic2a* may participate in the inducing activity of the organizer and play a later role in the regionalization of the neuroectoderm.

**Zebrafish zic2a Has Dorsalizing Activity**

To determine the role of *zic2a* in organizer function, we injected synthetic *zic2a* mRNA into one- or two-cell zebrafish embryos. The hypothesis that *zic2a* has a key role in organizer signaling predicts that overexpression of *zic2a* in zebrafish embryos would lead to dorsalization, an expansion of the neuroectodermal tissues derived from dorsal-lateral regions at the expense of ventrally derived tissues. To score experimental embryos, we used a standard scale for dorsalized phenotypes. Wild-type embryos (Fig. 6A) are class zero (C0). The weakest phenotypes (classes C1 to C3), which involve a reduction in the ventral tail fin, are

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**FIG. 5.** Expression of *zic2a* relative to placode and neural crest markers. Two-color in situ hybridization on 4-somite stage embryos with *dlx3b*, a marker for placodes (A, B), and *foxd3* (C, D) using fluorescent (red) probes, and *zic2a* using digoxigenin labeled (blue) probe (A–D). All are dorsal views with anterior to the left. Scale bar = 50 μm.
observed in embryos homozygous for mini fin (tolloid), lost-a-fin (alk8), and somitabun (smad5) mutation phenotypes. With increasingly dorso- nalized phenotypes, the blood and pronephric anlagen are also affected. Homozygous snailhouse (bmp7) embryos display class C4, in which the anterior somites expand laterally (Fig. 6C). The strongest phenotypes (class C5) are displayed by homozygous swirl (bmp2a) embryos (Fig. 6B), in which the somites circle around the embryo. Overexpression of zic2a in wild-type embryos shows, by the 6-somite (Figs. 6D–6F) and 17-somite stages (Figs. 6G–6I) much broader somites (Figs. 6D–F), which in the most severe cases encircled the yolk. The notochord was also broader in some severely affected embryos. More than half of the zic2a-injected embryos displayed the strongest dorsaled phenotype (class C5), and the yolk spilled out of the embryo before 24 hpf (hours postfertilization), as it does in some dorsaled mutants. Fifteen percent of the injected embryos were similar to snailhouse (snh, class C4; compare Fig. 7C (snh) to D and E), with severe truncation of the tail. Four percent of the embryos had a dorsaled phenotype of intermediate strength (class C3), in which the trunk was normal but the tail was curled over the trunk. A percentage of the embryos resembled the weaker dorsaled phenotype of class C2 (6%; Fig. 7F) and class C1 (12%) (Fig. 7G) in that the ventral fin was severely reduced or absent (Table 1). In embryos that survived to 24 hpf, the head was not affected. The zic2a-injected embryos did not appear to suffer developmental delay.

The hypothesis that zic2a activity dorsalizes embryos predicts that zic2a-injected embryos should display ventrally expanded expression domains of mesodermal genes like myod, reduced domains of ventrally expressed genes like gata1, and extended domains of neural markers like pax2a. The normal myod expression pattern includes the adaxial cells and somites (Fig. 8A), but in zic2a-injected embryos at the 6-somite stage, the anterior-posterior axis, as indicated by myod expression in adaxial cells, was shorter than normal, and the somites extended far more laterally than in embryos injected with the LacZ-containing control vector (Figs. 8A, 8B). At the 6-somite stage, pax2a is expressed in the otic placodes, the posterior midbrain and the ventrolateral mesodermal progenitors of the pronephros (Fig. 8C). In severely dorsaled mutants, the midbrain expression domain is broadened and the pronephric expression domain is either absent or reduced and shortened in its antero-posterior length. In zic2a-injected wild-type embryos, our results showed that the midbrain domain of pax2a was broadened, the otic placode expression domains were displaced latero-ventrally, and the pronephric domain was reduced and shortened in the an-
tero-posterior axis (Fig. 8D) compared to wild-type embryos injected with the LacZ-containing control vector.

The \textit{gata1} gene\textsuperscript{37} is expressed in blood cell precursors, which are derivatives of the ventral mesoderm (Fig. 8E). In dorsalized mutant embryos, \textit{gata1} is absent or reduced.\textsuperscript{22} Similarly, in \textit{zic2a}-injected wild-type embryos, \textit{gata1} expression was either missing or severely depleted (Fig. 8F) compared to controls injected with the LacZ-containing plasmid (Fig. 8E). This result is consistent with the absence of blood circulation in the most severely affected embryos at 48hpf (data not shown), and shows that ventrally-derived cells are depleted when \textit{zic2a} is overexpressed.

In conclusion, the \textit{zic2a}-injected wild-type embryos have a phenotype similar to dorsalized mutants, accompanied by a reduction in ventral cell fates (blood and ventral tail fin) and an expansion of dorsal cell fates (neural plate) and lateral cell fates (somite). These results suggest that \textit{zic2a} can perform the function of transducing dorsalizing signals or antagonizing ventralizing signals in zebrafish embryos.

\textbf{Table 1. Percentage of Dorsalized Embryos Appearing after \textit{zic2a} Overexpression}

<table>
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<th>Injected RNA (dil.)</th>
<th>No. Expts</th>
<th>(n)</th>
<th>% C5</th>
<th>% C4</th>
<th>% C3</th>
<th>% C2</th>
<th>% C1</th>
<th>% WT</th>
<th>% V1 (mes)</th>
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<td>7</td>
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<td>\textit{LACZ} (1:5)</td>
<td>1</td>
<td>141</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

The second column shows the number of independent experiments conducted. The 4th through 11th columns show the percentage of injected embryos that displayed a specific phenotype. The phenotypic class (C5 to C1 for the dorsalized embryos in decreasing phenotypic strength, V1 and V3 for the ventralized embryos in increasing phenotypic strength) is indicated at the top of the table. \(n\), total number of embryos surviving to analysis at 24 hpf.
Zebrafish zic2a Can Rescue the Phenotype of Mutants Lacking Chordin Function

What position in the hierarchy of dorsalization does zic2a occupy? The gene expression patterns suggested the hypothesis that zic2a functions downstream of chordin in the specification of dorsal fates. This hypothesis predicts that overexpression of zic2a would rescue the ventralized phenotype of chordino (tt250) mutants, which carry a null mutation in the zebrafish chordin gene. To test that prediction, we injected zic2a mRNA into one or two cell zebrafish embryos resulting from the mating of two heterozygotes, and examined their later phenotypes. Results showed that this procedure partially rescued the ventralized phenotype of chordino mutant embryos (Fig. 9 and Table 1).

Compared to wild type (Figs. 9A, 9B), ventralized chordino (tt250) embryos (Figs. 9C, 9D) normally display enlarged blood islands on the ventral tail, increased cell death on the ventral side of the yolk extension, and multiple ventral tail fins. Injection of zic2a RNA into embryos obtained from crosses between heterozygous chordino mutants resulted in dramatic reduction in the size of blood islands and in cell death in the ventral tail. Only 9% of the zic2a-injected embryos displayed the more severe ventralized phenotype of chordino (phenotype V3, Table 1), compared to 25% of ventralized class V3 phenotypes of embryos injected with LacZ RNA (Table 1). Seven percent of the zic2a-injected embryos displayed a mildly ventralized phenotype (phenotype V1, Table 1 and Figs. 9E–9H), resembling that of ogon (sizzled). Because we expected 25% homozygous chordino embryos and only 9% of embryos displayed the mutant phenotype, we concluded that overexpression of zic2a in chordino mutants rescued the mutant phenotype. Partially rescued chordino embryos were apparent in some cases due to the presence of multiple ventral tail fins (Figs. 9G, 9H). These results suggest that zebrafish zic2a can act downstream of chordin and serve as a dorsalizing signal in zebrafish.

Expression of zic2a Is Affected in Dorsalized Mutant Embryos

The hypothesis that zic2a functions downstream of dorsalizing genes like chordin predicts that zic2a expression should be affected by mutations in the BMP pathway. For this rea-
son, we studied zic2a expression in snailhouse (bmp7) dorsalized mutants at 75% epiboly.

In 12 out of 16 mid-gastrula embryos from crosses between heterozygous snailhouse carriers, zic2a expression appeared normal, in that it was restricted to the axial mesoderm and the anterior neuroectoderm (Figs. 10A, 10C). In four of the embryos, (25% of the progeny, the expected fraction of homozygous snailhouse mutants), the neuroectodermal domain of zic2a expression was expanded into ventral regions (Figs. 10B, 10D). These studies suggest that the BMP pathway is required to restrict the expression of zic2a to the anterior neuroectoderm at this stage of development in zebrafish.

**DISCUSSION**

**Involvement of zic2a in Transduction of Organizer Signals**

The results showed that zic2a is expressed in the axial mesoderm at a developmental stage in which the gene could be involved in the transduction of organizer signals. Our double in situ experiments with gsc showed that the early expression domain of zic2a in the zebrafish gastrula corresponds to the organizer (shield). In contrast, zic2b was not detected in the germ ring as was zic2a, suggesting that zic2b does not play an important role in organizer activity. The expression pattern of zic2a, however, raises the question of whether it is involved in the transduction of dorsalizing signals from the organizer. Consistent with this hypothesis, overexpression of zic2a mimicked the effects of absence or reduction of ventralizing signals as seen in the phenotypic series in which increasing severity of dorsalization is seen after injection of zic2a RNA into zebrafish embryos. Therefore, Zic2a may be involved in counter-acting the effects of ventralizing factors in the early zebrafish gastrula.

Previous reports, from both *Xenopus* and zebrafish, have shown evidence that expression of Zic genes may be regulated by opposing ventralizing and dorsalizing signals. Overexpression of either a dominant negative BMP receptor or of noggin can induce Zic3 expression in the ventromarginal zone in *Xenopus* gastrula embryos. In zebrafish, beads soaked with BMP4 prevent zic1 expression when implanted during gastrulation. These results are consistent with a sustained role for organizer-derived signals in activating opl(zic1) during gastrula stages.

In zebrafish, analysis of mutant phenotypes has identified Chordin as an essential component of the teleost organizer. We wanted to know whether zic2a is a downstream target of zebrafish chordin. Consistent with this hypothesis, overexpression of zic2a rescued the ventralized phenotype of the mutant chordino. Other BMP-antagonists, such as noggin1, gremlin or Cerberus remain to be tested. There is strong evidence from *Xenopus* that other Zic family members may be downstream targets of Chordin. Zic1 was isolated in a differential screen in *Xenopus* for genes downstream of Chordin. Zic1 is an early target of neuralizing signals, because it is first detected 30 minutes before the onset of gastrulation, 75 minutes after the initiation of Chordin expression; furthermore, Zic1 expression can be suppressed by BMP4 overexpression. Similarly, Zic3 expression in the *Xenopus* gastrula begins 30 minutes later than Chordin expression, and so is likely induced at the initial step of neural induction.
It is possible that \textit{zic2a}, when overexpressed, is mimicking the effect of other \textit{zic} genes, especially \textit{zic2b}.\textsuperscript{32} If that were the case, and \textit{zic2a} is not the mediator of dorsalizing signals from \textit{chordin}, then we expect that its expression would remain unaffected in mutations that alter the BMP pathway. The results showed, however, that mutant \textit{snailhouse} embryos showed ectopic expression of \textit{zic2a} in the ventral regions of the neuroectoderm. This result suggests that BMP signaling is required to restrict expression of \textit{zic2a} to the anterior neuroectoderm at this stage of development. These findings are similar to those of Grinblat and Sive,\textsuperscript{31} who reported the up-regulation of zebrafish \textit{zic3} expression in ventral ectoderm of \textit{snailhouse} mutant embryos.

Neither \textit{zic1} nor \textit{zic3} is expressed in the organizer,\textsuperscript{30,31} so it is unlikely that \textit{zic2a} is mimicking the function of these genes in this tissue when injected into wild-type zebrafish embryos. Overexpression of the \textit{Xenopus} ortholog of \textit{zic2a} was not reported to cause general dorsalization of embryos.\textsuperscript{66,75} It is possible, however, that the expansion of the neural tube and neural crest reported by Nakata et al.\textsuperscript{75} could be reflective of a shift from ventral to more dorsal fates.

\textbf{Involvement of \textit{zic2a} in Neuroectodermal Patterning in Zebrafish}

In the present study, we have shown that expression of \textit{zic2a} in the neuroectoderm begins at about 75\% epiboly. The anterior-most domain of \textit{zic2a} expression in the epiblast occupies a domain similar to that predicted to form the forebrain, according to the fate map of Woo and Fraser.\textsuperscript{76} Later, \textit{zic2a} is strongly expressed mainly in dorsal neural tube structures (the telencephalon, the epiphysis, the tectum, the anterior commissure, the posterior portion of the midbrain-hindbrain boundary, the dorsal hindbrain and in the optic stalks and retina). In the ventral neural tube, transcripts of \textit{zic2a} are present in the hypothalamus.

Some information about the possible late functions for \textit{Zic2} that are unique for this \textit{Zic} family member come from studies in humans displaying holoprosencephaly (HPE), type 5.\textsuperscript{27} HPE results when the embryonic forebrain, the prosencephalon fails to increase in mass and cleave to form the two lobes of the cerebral hemispheres, giving a single-lobed brain associated with defects in the skull and face. HPE is one of the most common structural anomalies of the developing forebrain\textsuperscript{77} and a major cause for fetal loss in humans—1 in 250 induced abortions has HPE. HPE5 is associated with mutations in \textit{ZIC2},\textsuperscript{27} demonstrating a requirement for \textit{ZIC2} function in the normal proliferation of cells of the central nervous system. Another type of holoprosencephaly, holoprosencephaly type 3 (HPE3) is due to mutations in sonic hedgehog (\textit{SHH}).\textsuperscript{78,79} Because mutations in both \textit{ZIC2} and \textit{SHH} can cause HPE in humans, the question arises whether they act in the same or different developmental pathways. \textit{ZIC2} expression differs from that of \textit{SHH} in that \textit{ZIC2} message appears predominantly in the dorsal brain whereas \textit{SHH} is expressed in the ventral brain. In mouse models, holoprosencephaly due to mutations in \textit{SHH} is thought to result from the loss of ventral neural tube cell fates, but the dorsal midline is also affected at the forebrain level. Unlike other mouse models for holoprosencephaly, \textit{Zic2} mutant mice show normal ventral neural tube formation and normal \textit{SHH} expression; for a recent review, see Hayhurst and McConnell.\textsuperscript{80} It is interesting to speculate that \textit{SHH} induces dorsal neural fates in the anterior neural tube via a \textit{Zic2}-dependent signaling mechanism.

\textbf{zic2a and Neural Crest}

Evidence from studies in the chick\textsuperscript{24} and \textit{Xenopus} suggest that \textit{Zic} family members are involved in neural crest specification. At least four \textit{Xenopus} \textit{Zic} genes are expressed in the prospective neural plate and the neural crest.\textsuperscript{66,73,81,82} Overexpression of \textit{Xenopus} \textit{Zic2} inhibits neurogenesis and induces neural crest differentiation.\textsuperscript{66} A different study, however, reported that overexpression of a different \textit{Xenopus} \textit{Zic2} clone induced both neuronal and neural crest markers.\textsuperscript{75} Similar results were obtained by overexpression of \textit{Zic3} and \textit{Zic1},\textsuperscript{74,75} whereas \textit{Zic5} specifically induced neural crest markers.\textsuperscript{73} These studies suggest that \textit{Zic} family members can determine ectodermal cell fate and promote the early steps in neural and
neural crest development. Both the chick Zic1 and Zic2 genes are expressed in the developing and migrating neural crest. However, the chick Zic3 gene is expressed only in the overlying superficial ectoderm and not in the neural crest.24

We examined the expression of zebrafish zic2a in neural crest. We detected little or no expression of zic2a in the foxd3-positive, prospective neural crest region in 2-somite stage embryos. Rather, zic2a expression was confined to the edge of the neural tube, as shown from double labeling experiments with krox20. We did, however, detect zic2a expression in the mesenchyme of the median fin fold, which is derived from neural crest.65 Two other gene family members, zic1 and zic3, were also reported not to be expressed in the neural crest domain.30 Therefore, it is possible that in zebrafish the involvement of Zic2 proteins in ectodermal patterning functions mainly in patterning of the neural tube. Alternatively, there may be yet unidentified Zic family members in zebrafish that are more specifically involved in neural crest development. We are currently investigating whether additional zic family members may play a role in neural crest specification in zebrafish.

The two zebrafish co-orthologs of tetrapod zic2, zic2a and zic2b, have overlapping and genespecific expression patterns. The zic2a copy is expressed more strongly in the mesoderm of the germ ring, trigeminal ganglion, and optic stalk, while zic2b32 is expressed more strongly in the posterior somites and neural retina. Thus, these two genes appear to have partitioned between them the expression domains displayed by the single Zic2 gene of frog, chicken, and mouse.24,66,74,82 This type of behavior is predicted by the hypothesis of subfunction partitioning.83–85 A search for noncoding sequences, including introns and regions flanking zebrafish ZIC2 co-orthologs that are conserved with tetrapods, coupled with the different expression patterns of zic2a and zic2b could reveal candidates for tissue-specific regulatory elements.

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