ZEBRAFISH
Volume 1, Number 3, 2004

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 *Xenopus*[9,10] and zebrafish.[11–14] Not only do these secreted BMP antagonists play an important role in the early

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Supported by NIH grants R01RR10715 and P01HD22486 (JHP), and NIH NIH/NIDCD, RO1 DC04184 and NIH/NIDCD, R01 DC05939 (KFB).
The authors thank the NIH (1-G20-RR11724), NSF (STI-9602828), M. J. Murdock Charitable Trust (96127:JVZ:02/27/97), and W. M. Keck Foundation (961582) for supporting renovation of the University of Oregon Zebrafish Facility.

stages of embryonic axis specification, they also help to specify other organ systems that depend on symmetry, including the developing vertebrate inner ear.[15] Follistatin and Chordin antagonize BMP4 activity by physically associating with BMP4, thereby blocking the binding of BMP4 to its receptor.[16–18] Genetic evidence for this interaction also exists in zebrafish: the ventralized mutant, *chordino* (*dino*),[19] lacks a functional *chordin* gene,[20] but *dino* interacts genetically with the dorsalized mutant *swirl*,[21,22] which contains a mutation in the *bmp2b* gene.[11,13]

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

To more fully understand the role of *zic2* in dorsoventral patterning and to define how *zic2* fits into the regulatory network that controls cell fate decisions in the early gastrula, we present data indicating that zebrafish *zic2a* functions in early processes specifying the dorsoventral axis and the size of the neural plate. We show that *zic2a* is expressed in the organizer region of the early gastrula, and *zic2a* transcripts persist in the presumptive axial mesendoderm until the end of gastrulation. This expression pattern suggests the hypothesis that *zic2a* may be involved in organizer function at this early stage in zebrafish development. To test this hypothesis, *zic2a* was overexpressed in zebrafish embryos, and we discovered that this treatment dorsalized wild-type zebrafish embryos, expanding the neuroectodermal domain. To place *zic2a* in the specification hierarchy of dorsoventral patterning, we expressed *zic2a* in ventralized *chordino* mutants. Results showed that *zic2a* rescued these ventralized mutant embryos. Based on the expression pattern of *zic2a*, the effects of *zic2a* overexpression in wild-type embryos, and the ability of *zic2a* message to rescue ventralized mutant embryos, we propose that *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: zic2-F: CCCCGGCGCCTTCTTYMGNTAYATG and zic2-R: GGGCAAAGATCTTCCCRCANCCNGG. The annealing temperature was 57°C, salt concentration 3mM and primer concentration 1μM. The expected product is 317 bp and spans the five zinc finger domains. Screening this cDNA library in a gridded format provided a full length *zic2a* clone. The full length *zic2a* cDNA (3132 bp long) was flanked by untranslated regions of 347 bp at the 5 prime and 1447 bp at the 3 prime side. The accession number for *zic2a* is AF151535.

Whole-Mount in Situ Hybridization

Expression analysis was performed essentially as in Oxtoby and Jowett.[33] Two-color in situ hybridizations were performed according to Jowett and Yan.[34] To make *zic2a* probe for in situ hybridizations, *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: GCGTCTAGACCTACATCGACAGAAGAAACG (nt. 1795 to 1815, with an XbaI site at the 5′), R: GCCAAGCTTCTGACAGCTCTTAGTTTTGCG

(nt. 2983 to 2963, with an XbaI site at the 5′). 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 screen[42] 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*)[22] 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 → Gly 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 clustalx[45] (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html), and the tree was constructed using the neighbor-joining method[46] 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:GCGTCTAGACAAGTGTACAATCTTTACAAC, reverse:GCCAAGCTTGATAATTCAGCGCTATCTGC). 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] http://mcbio.med.buffalo.edu/mapmgr.html).

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* gene[32] is related to *zic2a* by a gene duplication event that occurred after the divergence of tetrapod and

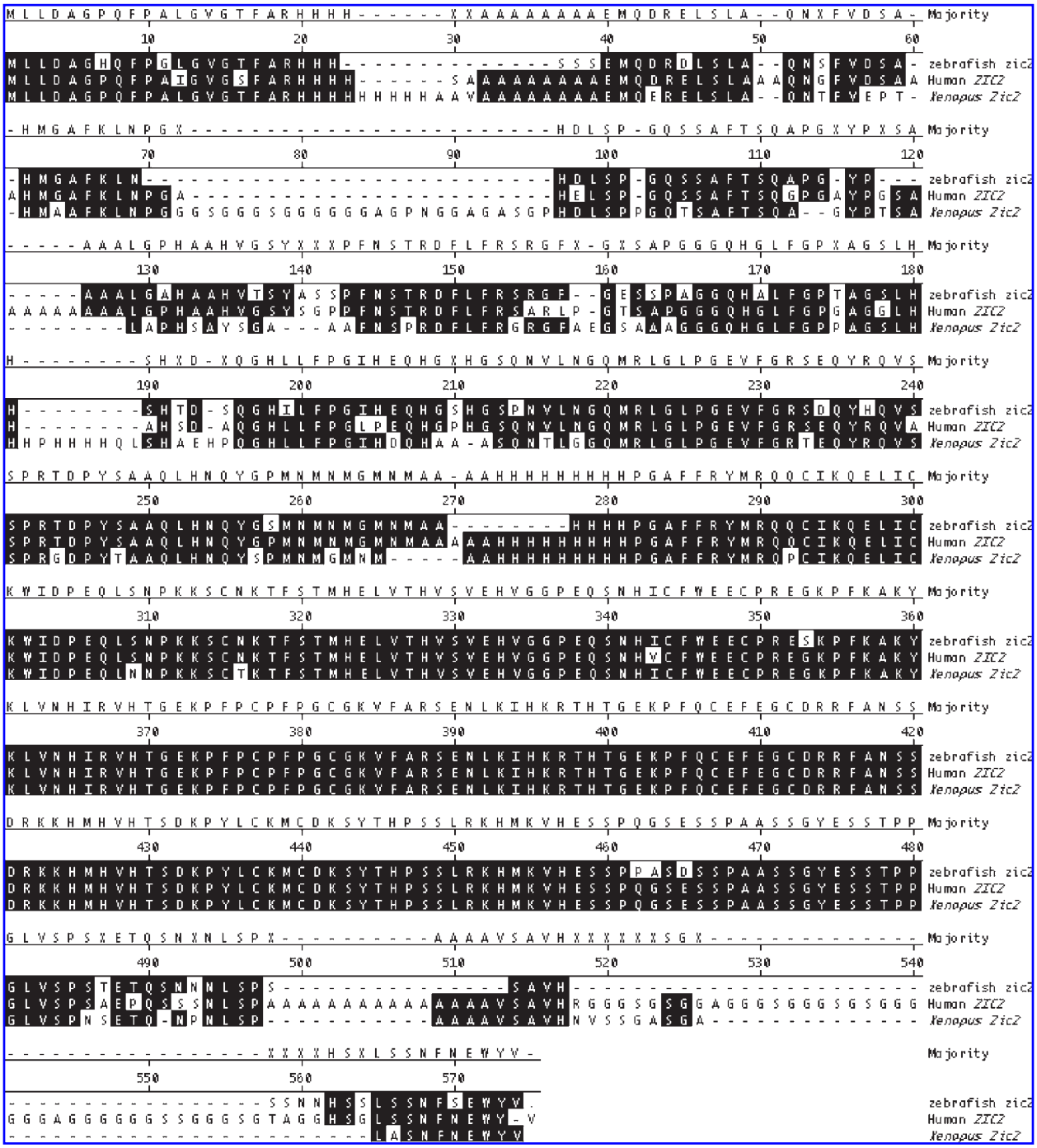

FIG. 1. Alignment of zebrafish Zic2a (Accession Number AAF73190), human ZIC2 (AF104902), and *Xenopus* Zic2 (U57453) protein sequences. The amino-acid sequence of the zebrafish Zic2a protein shows 89% and 78.9% overall identity with mouse Zic2 and *Xenopus* Zic2 proteins, respectively, with 1 and 5 changes 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.

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*

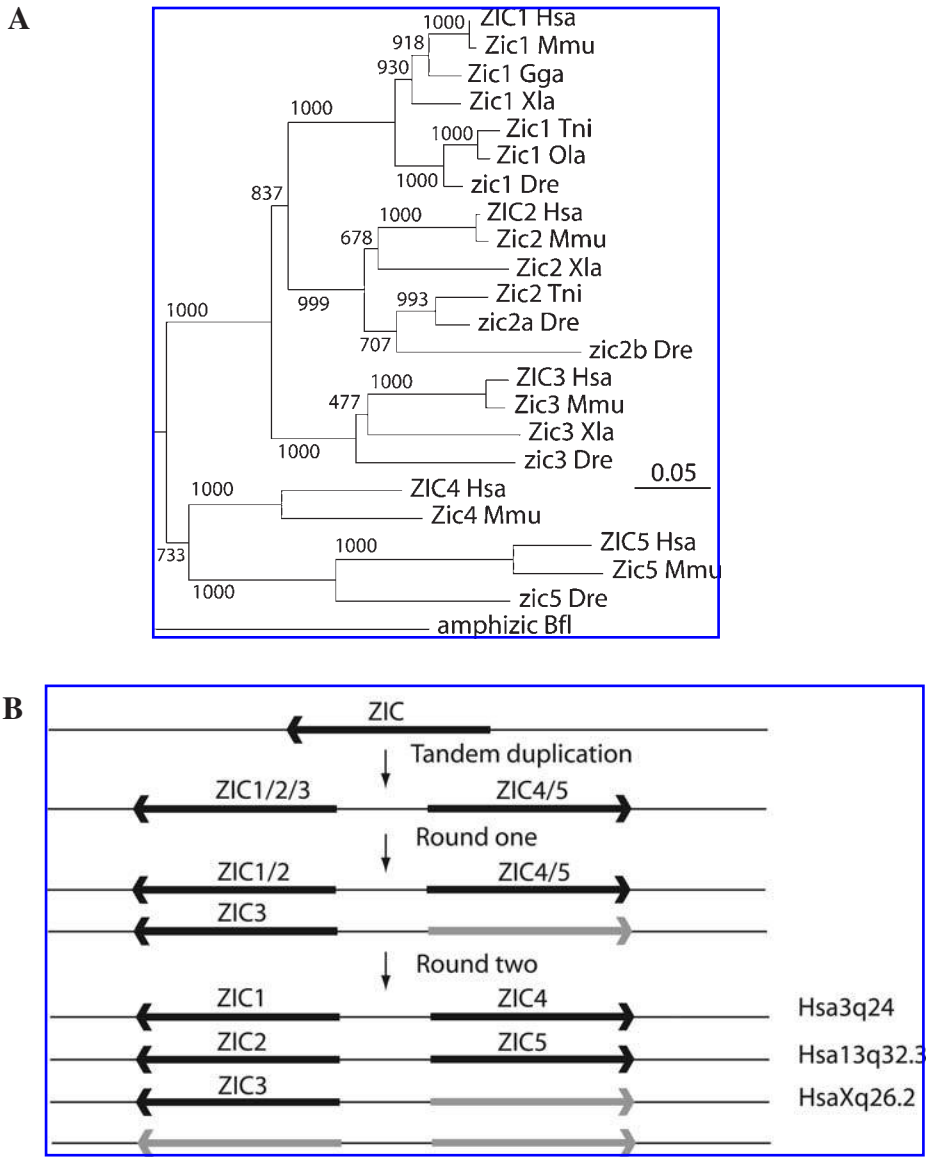

FIG. 2. The evolution of vertebrate *ZIC* genes. (**A**) A phylogenetic analysis of ZIC family proteins shows that zebrafish has two orthologs of the human *ZIC2* gene that arose in a gene duplication event before the teleost radiation. Sequence names and accession numbers: ZIC1 Hsa (human, *Homo sapiens*), NP_003403; Zic1 Mmu (mouse, *Mus musculus*), AAH50889; Zic1 Gga (chicken, *Gallus gallus*), NP_989585; Zic1 Xla (frog, *Xenopus laevis*), O73689: Zic Tni (pufferfish, *Tetraodon nigroviridis*), CAG04272; Zic1 Ola (medaka fish, *Oryzias lapides*),BAC78801; zic1 Dre (zebrafish, *Danio rerio*), NP_571008, ZIC2 Hsa, NP_009060; Zic2 Mmu, NP_033600; Zic2 Xla, BAA34264; zic2 Tni, CAG07295; zic2a Dre, NP_571633; zic2b Dre, NP_001001820; ZIC3 Hsa, NP_003404; Zic3 Mmu, NP_033601; Zic3 Xla, O57311; zic3 Dre, NP_001001950; ZIC4 Hsa, NP_115529; Zic4 Mmu, NP_033602; ZIC5 Hsa, NP_149123; Zic5 Mmu, BAC79075; zic5 Dre, NP_991290; Zic Bfl (amphioxus, *Branchiostoma floridae*), CAB96573. (**B**) A model for the evolution of vertebrate *ZIC* genes. According to the model, tandem duplication produced a pair of divergently transcribed genes that then underwent two rounds of duplication, which, after gene loss, gave the indicated human genomic distribution of *ZIC* genes.

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.[53–58] 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.[59–61] This gene experienced 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.[36]

Mapping of zic2a *and Conserved Synteny Data*

Mutations in the human ortholog of *zic2a* cause holoprosencephaly,[27] 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 (GENSCAN00000027815) 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 [(Expect = 0.0, Identities = 950/1069 (88%)]. The fugu *Zic2a* and *Zic5P* sequences are transcribed divergently as are their human orthologs. The fugu gene order on scaffold_763 is *Ebi2 – Clybl – Zic5P – Zic2a*, with order and transcript direction conserved as in the human (see Fig. 3B). Two possible explanations suggest themselves for this result. 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

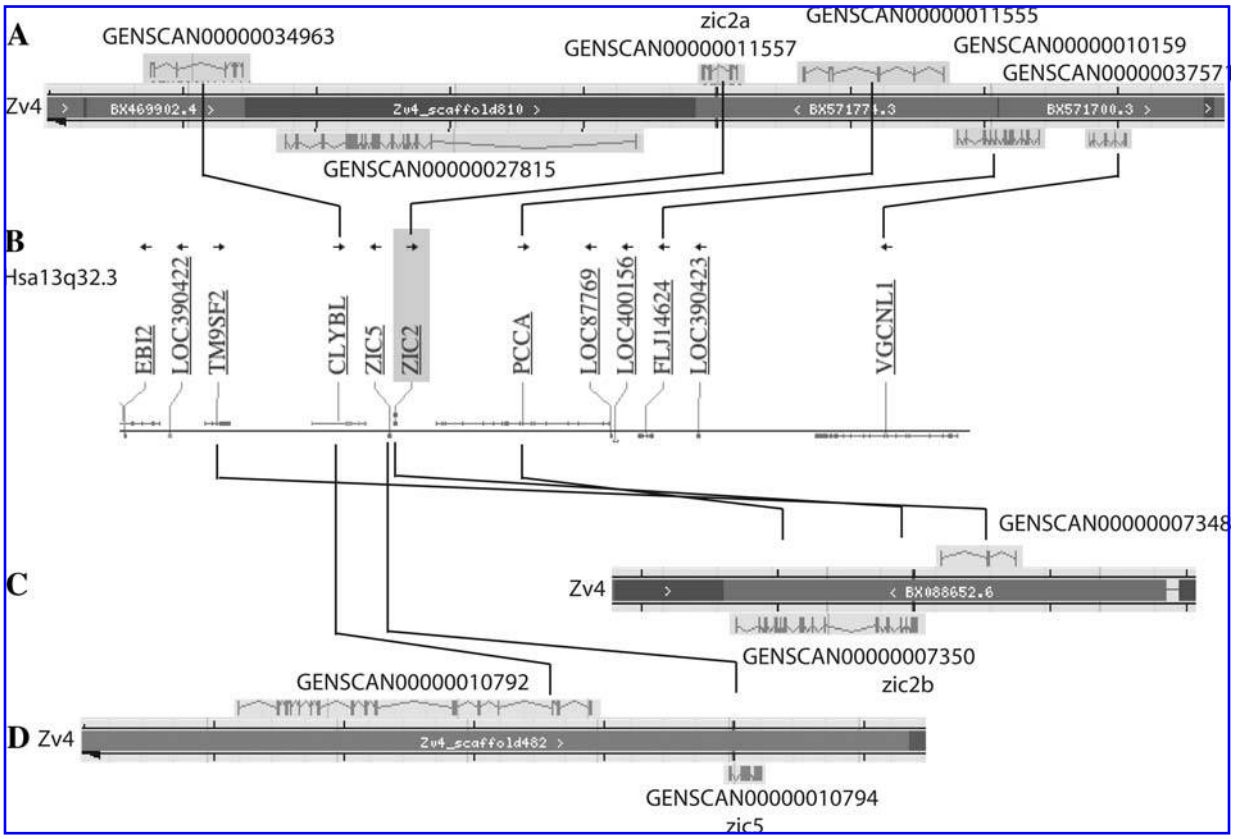

FIG. 3. The genomic context of the vertebrate *ZIC2-ZIC5* gene cluster in zebrafish. Our genetic mapping placed *zic2a* on LG3. *Top bar* shows the genomic context of *zic2a*, in the Zv4 Ensembl sequencing database segment BX571774.3 with the location of GenScan predictions shown as vertical lines representing exons and chevrons representing introns. This contig makes with its neighbors a local region of conserved synteny with a portion of human chromosome Hsa13q32.3 shown in the middle of the figure. The direction of the human transcripts is shown as *small arrows* and the extent of the genes are shown on *either side of the thin line* representing the chromosome. Zebrafish genome GenScan predictions were compared by BLAST search and lines connect them with the top hits in the searches. GenScan items without lines are putatively orthologous to other regions of the genome. Genome segment BX088652.6 contains *zic2b* flanked by two loci that are orthologous to neighbors of human *ZIC2*. The predicted locus GenScan00000007350 contains a *PCCA* ortholog at one end and the *ZIC2* co-ortholog *zic2b* at the other end. The *bottom bar* shows the genomic context of *zic5* on Zv4_scaffold482. GenScan00000010792 contains sequence at one end that has a strong best hit in a BLAST search to *CLYBL*, the nearest neighbor to *ZIC5*.

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 *zic2a* and *krox20*, which is expressed in rhombomeres 3 and 5 in the hindbrain.[33] The third more caudal region of *zic2a* expression overlaps with the lateral-most expression domain of *krox20* in rhombomere 5 (Fig. 4M). Therefore, at this stage the strongest expression of *zic2a* at the edge of the anterior neural plate extends caudally to rhombomere 5.

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

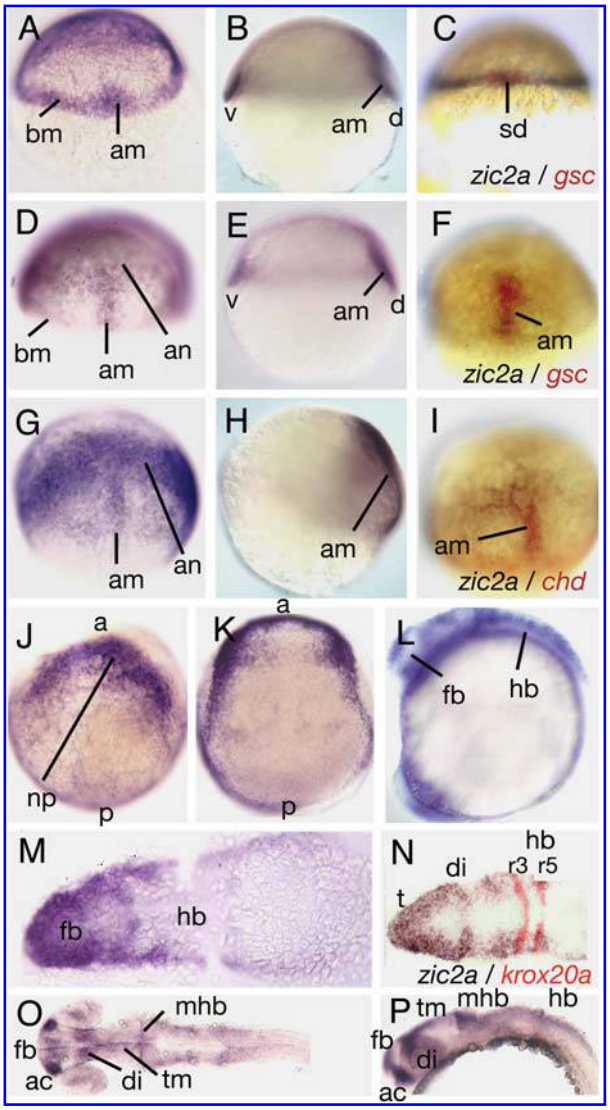

FIG. 4. Expression pattern of *zic2a*. Shield stage (**A**)–(**C**), 60% epiboly (**D**)–(**F**), 80% epiboly (**G**)–(**I**), tailbud (**J**), 1-somite (**K**), 2-somite (**L, M**), 12-somite (**N**), and 24 hpf (**O, P**). Expression of *zic2a* (blue) in the axial mesoderm overlaps that of *goosecoid* (*red*) at shield stage (**C**) and 60% epiboly (**F**), and *chordin* (*red*) at 80% epiboly (**I**). **M:** Two-color whole-mount *in situ* hybridization at the 12-somite stage with a *krox20* fluorescent probe (*red color*) and a *zic2a* digoxigenin labeled probe (*blue color*). Abbreviations: a, anterior; am, axial mesoderm; an, anterior neuroectoderm; bm, blastoderm margin; di, diencephalons; fb, forebrain; hb, hindbraind; np, neural plate; p, posterior; r3, r5; rhombomeres 3 and 5 in the hindbrain; sd, shield; t, telencephalon; tm, tectum. **A**, **C**, **D**, **F**, **G**, **I**, **J**, **K**, **M**, **N**, and **O** are dorsal views. **B**, **E**, **H**, **L**, and **P** are lateral views (in **B**, **E**, and **H**, dorsal is to the *right*; in **N** and **P**, dorsal is to the *top*, and anterior is to the *left*).

stripe of *zic2a* expression. The mesenchyme of the dorsal median fin fold, a neural crest derivative,[65] also expresses *zic2a*. At 24 hpf (Figs. 4O, 4P), *zic2a* is still strongly expressed in the forebrain, the tectum, the anterior commissure, the posterior portion of the midbrain-hindbrain boundary, and in the optic stalks and retina. We found strong expression in the dorsal spinal cord and fin mesenchyme in the dorsal tail.

The *Xenopus* ortholog of *zic2a* is expressed in neural crest precursors, which are lateral to the edge of *krox20* expression.[66] To determine whether zebrafish *zic2a* is also expressed in the neural crest, we performed two-color in situ hybridization of 2-somite stage embryos with *zic2a* and *foxd3(fkd6)*, a marker for presumptive neural crest.[35] Figures 5C and 5D show that the expression domains of the two genes have little or no overlap at the level of the hindbrain, with the *foxd3* expression domain lying lateral to that of *zic2a*. Double labeling with digoxigenin labeled *zic2a* probe and fluorescent *dlx3b*, a marker of placodes but not neural crest,[36] shows that *zic2a* and *dlx3b* partially overlap anteriorly in the forebrain and hindbrain region (Figs. 5A, 5B). Therefore, unlike its *Xenopus* ortholog, or the chick *zic2* gene,[24] the zebrafish *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.[22] 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

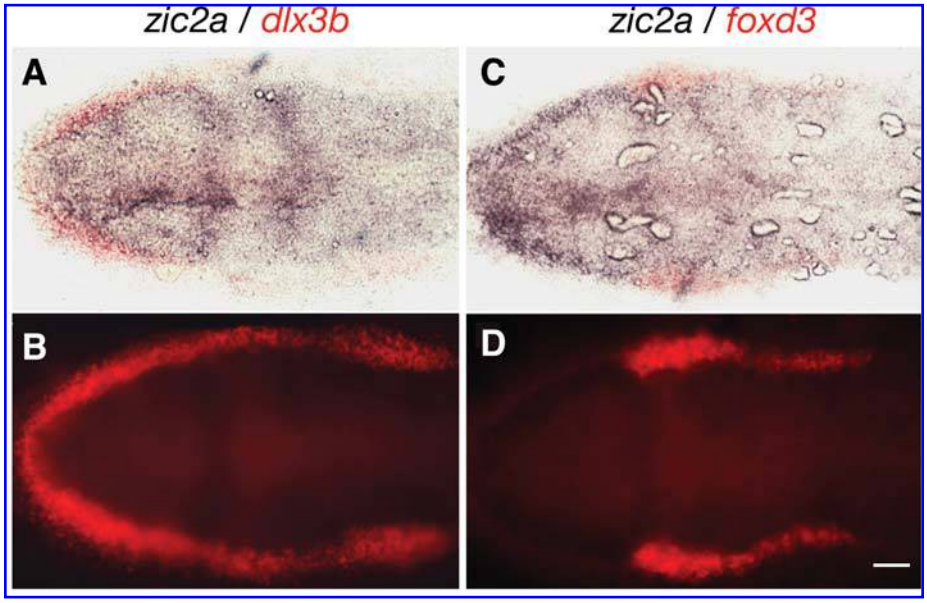

FIG. 5. Expression of *zic2a* relative to placode and neural crest markers. Two-color in situ hybridization on 4-somite stage embryos with *dlx3b*, a marker for placodes (**A**, **B**), and *foxd3*, a marker of neural crest (**C**, **D**) using fluorescent (*red*) probes, and *zic2a* using digoxigenin labeled (*blue*) probe (**A**–**D**). All are dorsal views with anterior to the *left*. Scale bar = 50 μm.

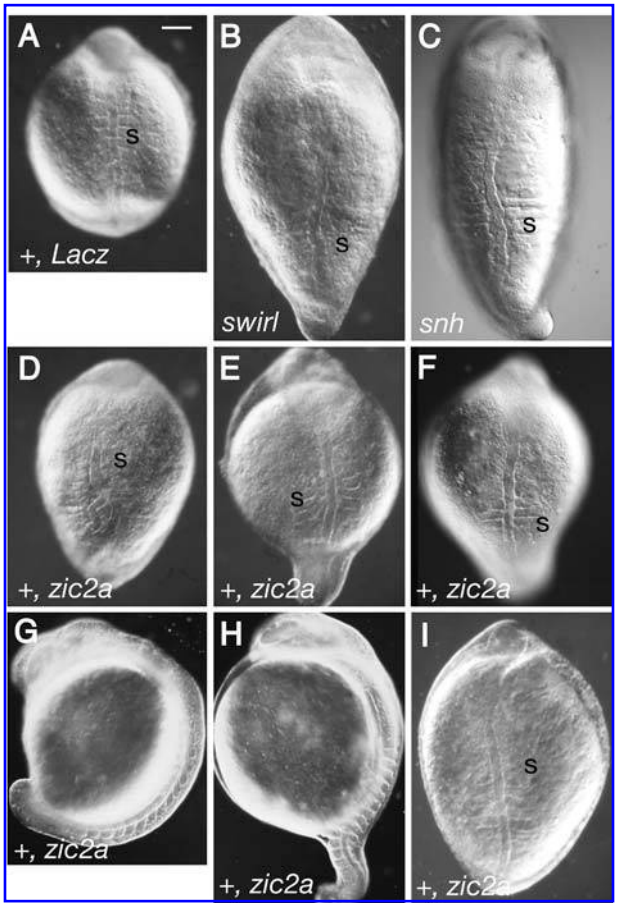

FIG. 6. Overexpression of *zic2a* in wild-type embryos resulted in dorsalized embryos. RNA for *zic2a* was injected into wild-type embryos and the embryos were observed under Nomarski optics. Wild-type embryos injected with control (*LacZ*) RNA (**A**), uninjected homozygous mutant *swirl* (**B**), and *snailhouse* (**C**) embryos, and wild-type embryos injected with *zic2a* RNA (**D**)–(**I**). Six-somite stage (**D**–**F**) or 17-somite stage (**G**–**I**). **A**–**F**, and **I** are dorsal views; **G** and **H** are lateral views. Anterior is towards the *top*. Abbreviation: s, somite.

observed in embryos homozygous for *mini fin (tolloid)*, *lost-a-fin (alk8)*, and *somitabun (smad5)* mutation phenotypes.[22] With increasingly dorsalized 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.[11,22,43,67–69]

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 dorsalized phenotype (class C5), and the yolk spilled out of the embryo before 24 hpf (hours postfertilization), as it does in some dorsalized 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 dorsalized 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 dorsalized 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*,[70] reduced domains of ventrally expressed genes like *gata1*,[37] and extended domains of neural markers like *pax2a*.[38] 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*[38] is expressed in the otic placodes, the posterior midbrain and the ventrolateral mesodermal progenitors of the pronephros (Fig. 8C). In severely dorsalized mutants, the midbrain expression domain is broadened and the pronephric expression domain is either absent or reduced and shortened in its antero-posterior length.[22] 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-

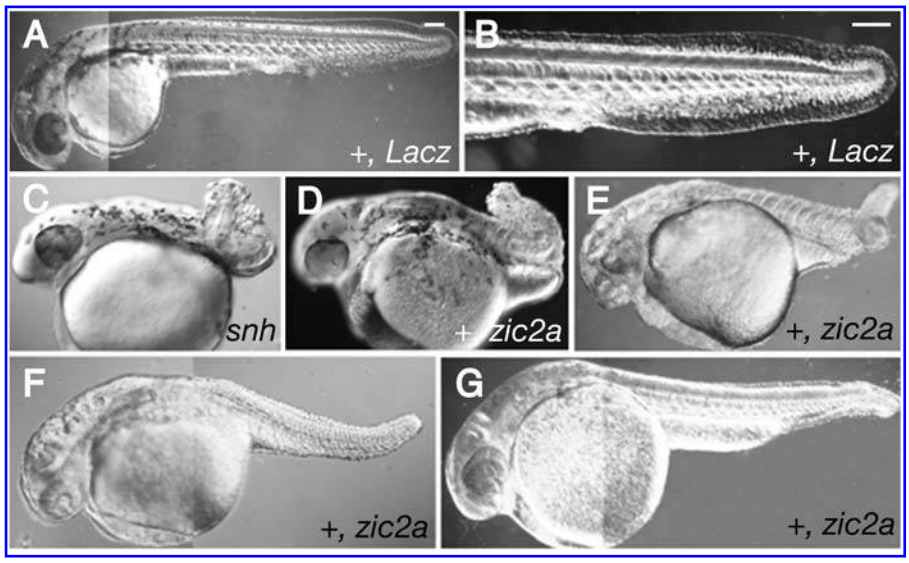

FIG. 7. Phenotypic series of dorsalization in wild-type embryos injected with *zic2a* mRNA. Side views of 24 hpf wild-type embryos injected with control (*LacZ)* RNA (**A, B**), uninjected *snailhouse* embryos (**C**), and wild-type embryos injected with *zic2a* RNA (**D–G**). **D:** C4 phenotype; **E:** C3 phenotype; **F:** C2 phenotype; **G:** C1 phenotype. (**B**) is a magnification of (**A**). Anterior is to the *left*.

tero-posterior axis (Fig. 8D) compared to wild-type embryos injected with the LacZ-containing control vector.

The *gata1* gene[37] is expressed in blood cell precursors, which are derivatives of the ventral mesoderm (Fig. 8E). In dorsalized mutant embryos, *gata1* is absent or reduced.[22] Similarly, in *zic2a*-injected wild-type embryos, *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 *zic2a* is overexpressed.

In conclusion, the *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 *zic2a* can perform the function of transducing dorsalizing signals or antagonizing ventralizing signals in zebrafish embryos.

TABLE 1. PERCENTAGE OF DORSALIZED EMBRYOS APPEARING AFTER *zic2a* OVEREXPRESSION

Injected RNA (dil.)	*No. Expts*	*n*	*% C5*	*% C4*	*% C3*	*% C2*	*% C1*	*% WT*	*% V1 (mes)*	*% V3 (din)*
Into wild-type:										
zic2 (1:2)	3	326	56	14	4	6	12	8	0	1
zic2 (1:5)	5	533	24	21	9	7	5	32	0	1
LACZ (1:5)	2	224	0	0	0	0	0	99	0	1
Into *chordino*:										
zic2 (1:2)	2	231	38	17	8	5	8	4	6	13
zic2 (1:5)	2	329	23	19	14	8	5	15	7	9
LACZ (1:5)	1	141	0	0	0	0	0	75	0	25

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.

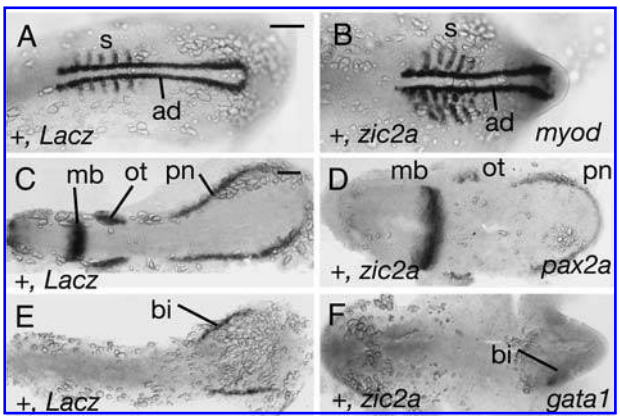

FIG. 8. Expansion of lateral mesoderm markers and reduction of ventral mesoderm markers in wild-type embryos over-expressing *zic2a*. Expression of *myod* (**A, B**), *pax2a* (**C, D**), and *gata1* (**E, F**) in wild-type embryos injected with control (*LacZ*) RNA (**A, C, E**) or wild-type embryos injected with *zic2a* RNA (**B, D, F**). Abbreviation: ad, adaxial cells; bi, blood islands; mb, midbrain; ot, otic placode; pn pronephros; s, somite.

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.[15] 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.[20,71] 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.[19] 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*).[19,72] 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-

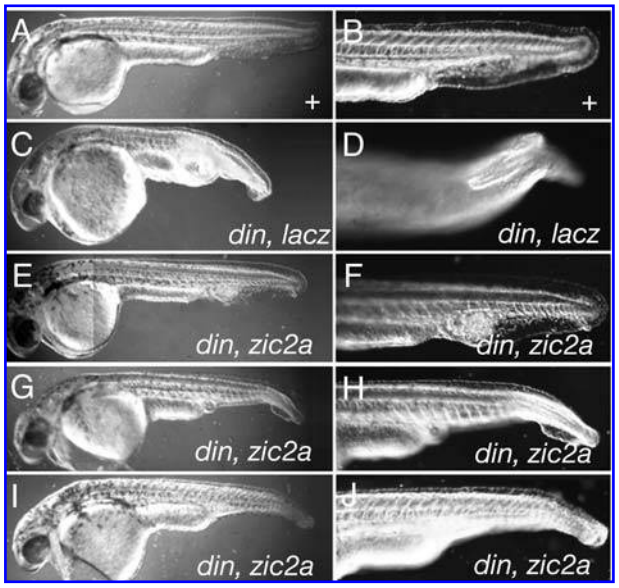

FIG. 9. Overexpression of *zic2a* rescues the ventralized phenotype of *chordino* embryos. (**A, B**) 24 hpf wild-type uninjected embryos; (**C, D**) *chordino* embryos injected with control (*LacZ*) RNA; (**E–J**) *chordino* embryos injected with *zic2a* RNA. **B, D, F, H,** and **J** are magnifications of **A, C, E, G,** and **I**, respectively. Anterior is to the *left*.

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.[31,32] 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.

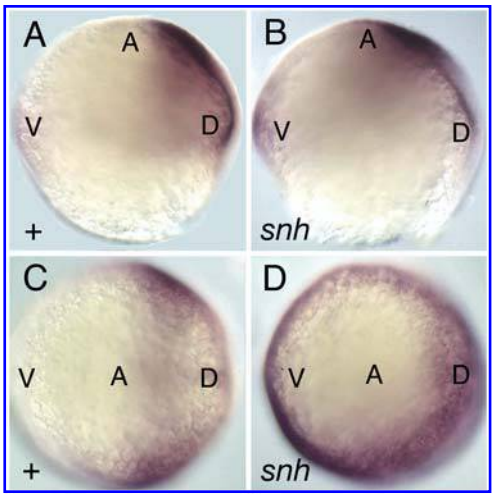

FIG. 10. Expression of *zic2a* expands ventrally in *snailhouse* (*bmp7*) mutant embryos with ventral defects. Expression of *zic2a* in wild-type (**A**, **C**) and *snailhouse* (*snh*) (**B**, **D**) embryos. **A** and **B** are lateral views; **C** and **D** are animal pole views. In all cases dorsal is to the *right*. Abbreviations: A, animal pole; D, dorsal; V, ventral.

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.[73] In zebrafish, beads soaked with BMP4 prevent *zic1* expression when implanted during gastrulation.[30] 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.[20] 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.[23] *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 [23]; 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.[74]

It is possible that *zic2a*, when overexpressed, is mimicking the effect of other *zic* genes, especially *zic2b*.[32] If that were the case, and *zic2a* is not the mediator of dorsalizing signals from *chordin*, then we expect that its expression would remain unaffected in mutations that alter the BMP pathway. The results showed, however, that mutant *snailhouse* embryos showed ectopic expression of *zic2a* in the ventral regions of the neuroectoderm. This result suggests that BMP signaling is required to restrict expression of *zic2a* to the anterior neuroectoderm at this stage of development. These findings are similar to those of Grinblat and Sive,[31] who reported the up-regulation of zebrafish *zic3* expression in ventral ectoderm of *snailhouse* mutant embryos.

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

Involvement of zic2a *in Neuroectodermal Patterning in Zebrafish*

In the present study, we have shown that expression of *zic2a* in the neuroectoderm begins at about 75% epiboly. The anterior-most domain of *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.[76] Later, *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 *zic2a* are present in the hypothalamus.

Some information about the possible late functions for *Zic2* that are unique for this *Zic* family member come from studies in humans displaying holoprosencephaly (HPE), type 5.[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[77] and a major cause for fetal loss in humans—1 in 250 induced abortions has HPE. HPE5 is associated with mutations in *ZIC2*,[27] demonstrating a requirement for *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 (*SHH*).[78,79] Because mutations in both *ZIC2* and *SHH* can cause HPE in humans, the question arises whether they act in the same or different developmental pathways. *ZIC2* expression differs from that of *SHH* in that *ZIC2* message appears predominantly in the dorsal brain whereas *SHH* is expressed in the ventral brain. In mouse models, holoprosencephaly due to mutations in *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, *Zic2* mutant mice show normal ventral neural tube formation and normal *SHH* expression; for a recent review, see Hayhurst and McConnell.[80] It is interesting to speculate that SHH induces dorsal neural fates in the anterior neural tube via a Zic2-dependent signaling mechanism.

zic2a *and Neural Crest*

Evidence from studies in the chick[24] and *Xenopus* suggest that Zic family members are involved in neural crest specification. At least four *Xenopus Zic* genes are expressed in the prospective neural plate and the neural crest.[66,73,81,82] Overexpression of *Xenopus Zic2* inhibits neurogenesis and induces neural crest differentiation.[66] A different study, however, reported that overexpression of a different *Xenopus Zic2* clone induced both neuronal and neural crest markers.[75] Similar results were obtained by overexpression of *Zic3* and *Zic1*,[74,75] whereas Zic5 specifically induced neural crest markers.[73] These studies suggest that *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 gene-specific expression patterns. The *zic2a* copy is expressed more strongly in the mesoderm of the germ ring, trigeminal ganglion, and optic stalk, while *zic2b*[32] 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.

ACKNOWLEDGMENTS

The authors thank Bruce D. Draper for use of his zebrafish cDNA library, Yi-Lin Yan for discussions and help with preparing the figures, Rob Cornell for the DN-BMPR plasmid and helpful discussions, Ruth BreMiller for tissue sectioning, Phuong Ngo-Hazelette for technical help, Amy Singer, Jocelyn McAuley, Wade Yandell, Joseph Ying and Bill Trevarrow and the staff of the Fish facility at the University of Oregon for help with fish maintenance, Yanling Wang for sequencing, Jerry Gleason for help with photography. We also thank Anandhi Jeyabalan, Erin Conlon, Bethany Barrus, and Carrie A. Rheingans for performing some of the in situ hybridization experiments and Dilip Pawar for fish culture at the University of Michigan.

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