Maternal *Xenopus Zic2* negatively regulates *Nodal*-related gene expression during anteroposterior patterning

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Summary

During the development of *Xenopus laevis*, maternal mRNAs and proteins stored in the egg direct early patterning events such as the specification of the dorsoventral axis and primary germ layers. In an expression screen to identify maternal factors important for early development, we isolated a truncated cDNA for maternal Zic2 (tZic2), encoding a zinc-finger transcription factor. The predicted tZic2 protein lacked the N-terminal region, but retained the zinc-finger domain. When expressed in embryos, tZic2 inhibited head and axial development, and blocked the ability of full-length Zic2 to induce neural crest genes. Depletion of maternal Zic2 from oocytes, using antisense oligonucleotides, caused exogastrulation, anterior truncations and axial defects. We show that loss of maternal Zic2 results in persistent and increased expression of *Xenopus nodal*-related (Xnr) genes, except for Xnr4, and overall increased Nodal signaling. Injection of a Nodal antagonist, Cerberus-short, reduced the severity of head and axial defects in Zic2-depleted embryos. Depletion of Zic2 could not restore Xnr expression to embryos additionally depleted of VegT, a T-domain transcription factor and an activator of Xnr gene transcription. Taken together, our results suggest a role for maternal Zic2 in the suppression of Xnr genes in early development. ZIC2 is mutated in human holoprosencephaly (HPE), a severe defect in brain hemisphere separation, and these results strengthen the suggestion that increased Nodal-related activity is a cause of HPE.

Key words: Zic2, Nodal, Xnr, VegT, Holoprosencephaly (HPE), Forebrain, Gastrulation, Maternal mRNA

Introduction

During early vertebrate development, proteins of the Nodal-family of TGFβ-related growth factors are responsible for the induction of the mesoderm and endoderm germ layers as well as for the subsequent patterning of the embryo along various embryonic axes (reviewed by Schier, 2003). Nodal proteins and other TGFβ proteins signal in a dose-dependent fashion and elicit different cellular responses at different times in development; thus, the regulation of Nodal expression and activity must be dynamically controlled during embryogenesis. Correspondingly, there is evidence to suggest that Nodal genes regulate their own expression in an auto-regulatory loop (Hyde and Old, 2000; Osada et al., 2000) and induce the expression of secreted Nodal antagonists of the Lefty and Cerberus families, which then limit the extent of Nodal activity (reviewed by Solnica-Krezel, 2003). During gastrulation, both Nodal signaling and Nodal inhibition are necessary for the specification of anterior mesendoderm and subsequent head formation respectively (reviewed by Robertson et al., 2003). However, the regulation of this balance between Nodal auto-induction and auto-inhibition is not well understood at the molecular level.

Experiments in *Xenopus* have demonstrated that the expression of Nodal-related genes (Xnr5/6, Sox3 and Xtcf3) is initiated and modulated by maternally inherited factors. Maternal VegT mRNA, which encodes a T-domain transcription factor, is required for the initiation of Xnr1, Xnr2, Xnr4, Xnr5 and Xnr6, and the subsequent specification of the mesoderm and endoderm germ layers (Kofron et al., 1999; Zhang et al., 1998). In vegetal cells, VegT is thought to directly initiate Xnr5 and Xnr6 expression immediately upon the onset of zygotic transcription (Hilton et al., 2003; Takahashi et al., 2000; Xanthos et al., 2002). VegT also cooperates with the maternal β-catenin pathway, which is required for dorsal axis specification, to initiate Xnr expression dorsally early in gastrulation (Agius et al., 2000; Lee et al., 2001; Xanthos et al., 2002). VegT is the only factor known to be necessary and sufficient for the induction of Xnr5/6 expression (Rex et al., 2002; Takahashi et al., 2000); however, loss-of-function experiments have demonstrated roles for several transcription factors in repressing the degree of Xnr5/6 expression. Depletion of maternal Xtcf3, FoxH1 and Sox3 mRNAs (Houston et al., 2002; Kofron et al., 2004a; Zhang et al., 2003), and morpholino oligo-induced depletion of Mixer protein (Kofron et al., 2004b) all result in increased expression of Xnr5/6. Sox3 and Xtcf3 are likely to mediate repression in the absence of stabilized β-catenin; however, the roles of FoxH1 and Mixer are surprising, as these proteins have been identified
as mediators of gene activation downstream of Nodal signaling (Chen et al., 1996b; Germain et al., 2000).

During a functional screen of maternal genes, we identified a cDNA encoding a truncated Zic2 protein that altered head formation when overexpressed in embryos. The zinc-finger proteins of the cerebellum (Zic) genes are a conserved family related to *Drosophila Odd-Paired* (Aruga et al., 1996) and encode proteins of ~500 amino acids in length. The functions of Zic proteins in early development are mostly unknown, and have been characterized as having both transcriptional activator and repressor functions (Brewster et al., 1998; Salero et al., 2001). Zic2, the focus of this work, is expressed at high levels maternally and during gastrulation in *Xenopus*. Later expression is evident in the dorsal neural tube, somites, optic vesicle and neural crest (Brewster et al., 1998; Nakata et al., 1998). Published overexpression studies in *Xenopus* found that Zic2 induced neural crest genes and inhibited neurogenesis (Brewster et al., 1998; Nakata et al., 1998), possibly by acting as a transcriptional repressor (Brewster et al., 1998). In the mouse, Zic2 is also expressed during the gastrula stages (Elms et al., 2004) and loss-of-function studies in mice indicate important roles in neural/ neural crest development (Elms et al., 2003; Nagai et al., 2000) and axon pathfinding (Herrera et al., 2003).

Mutations in human ZIC2 result in holoprosencephaly (HPE), a severe malformation of the developing brain in which the forebrain fails to form separate left and right hemispheres (Brown et al., 2001; Brown et al., 1998; Orioli et al., 2001). However, the exact developmental role of ZIC2 in brain development and in HPE remains undefined. Here we show, through maternal mRNA depletion, that *Xenopus* Zic2 has important roles in regulating anteroposterior patterning in early development through its regulation of Xnr gene expression. We show that the formation of head structures and forebrain is abnormal in embryos depleted of maternal Zic2. We also show that Zic2-depleted embryos exhibit elevated and sustained levels of Xnr gene expression and activity, and that this excess Xnr signaling has a causal role in the head defects observed. Furthermore, we show that Zic2 is required to indirectly attenuate the levels of Xnr genes induced by VegT.

**Materials and methods**

**cDNA library construction and expression screening**

A maternal cDNA expression library was constructed using mRNA isolated from two- to four-cell *Xenopus laevis* embryos according to the protocol of a cDNA synthesis kit (Stratagene Bluescript IIXR). In place of the p Bluescript vector, size-selected cDNAs were ligated into an expression vector, pCSX, a pCS2+ derived vector lacking the CMV promoter. This was removed by double digestion with HindIII and SalI, blunt-end generation with Klenow polymerase, followed by gel purification and intramolecular re-ligation of the vector backbone. The vector was then double-digested with EcoRI and XhoI and dephosphorylated to accommodate cDNA inserts. Ligation activities were transformed into XL-10 Gold competent cells (Stratagene) to yield a primary library of 1 × 10^6 clones with an average insert size of at least 1 kb. Expression screening from small pools was performed essentially as described (Tao et al., 2005), except that pools were injected vegetally at the one- to two-cell stage.

**Oocytes and embryos**

Oocytes were manually defolliculated and cultured in oocyte culture medium (OCM) at 18°C. Oocytes were injected with antisense oligos against Zic2 and cultured for 24–48 hours prior to being stimulated to mature with 2 mM progesterone. Matured oocytes were colored with vital dyes, implanted into females and fertilized using the host-transfer technique as described previously (Zuck et al., 1998). For rescue experiments, mRNAs were injected into depleted oocytes 24 hours after injection of the oligo. This allowed sufficient time for the oligos to degrade so that rescue of the Zic2 depletion could be accomplished by replacement of Zic2 and not by competition for oligo binding. Alternatively, mRNAs were injected into control or depleted embryos following recovery from the female and fertilization.

Eggs were recovered from laying females, fertilized using a sperm suspension and maintained in 0.1X MMR. For injections of mRNAs after fertilization, embryos were dejellied and transferred to 2% Ficoll/0.5X MMR at the one-cell stage. For explant assays, stage 9 embryos were dissected in 1X MMR on 2% agarose-coated dishes using sharp forceps or tungsten needles. The explants were cultured in OCM until sibling embryos reached the desired stage.

**Antisense oligos**

The antisense oligodeoxynucleotides (oligos) used were HPLC purified phosphorothioate-phosphodiester chimeric oligos (IDT) with the sequences 5’-C*T*A*CCGCTGCATGGT*G*A*T-3’ (Zic2-5MP) and 5’-T*G*T*CCGCTGCATGGTG*C*A-G-3’ (Zic2-10MP). Asterisks (*) represent phosphorothioate bonds.

A morpholino oligo (MO) against VegT was obtained from GeneTools: VegT-MO, 5’-CCCCAAGAGTTCTCTCATTCCAGC-3’ (Heasman et al., 2001).

Oligos and morpholinos were re-suspended in sterile, filtered water and injected in doses as described in the text.

**Plasmids and mRNAs**

The full-length Zic2-coding region was isolated by RT-PCR and inserted into the vector pCRII-TOPO using TOPO-TA cloning (Invitrogen). Zic2 was then subcloned into the EcoRI site of the vector pCS2+ and linearized with NotI for SP6 in vitro transcription. Zic2 was also cloned into pRN3 as a ClaI/XbaI fragment and linearized with SfiI. Capped Zic2 mRNA was synthesized using the SP6 or T3 Message Machine kits (Ambion), respectively. VegT and CerS, both in pCS2+, were digested with NotI and transcribed with SP6. RNAs were precipitated with lithium chloride, washed thoroughly in 70% ethanol and then re-suspended in sterile distilled water for injection. In vitro translation was carried out using a rabbit reticulocyte-lysate coupled transcription-translation system (TNT SP6 kit, Promega).

**Luciferase assays**

Reporter plasmids A3-luciferase, containing three tandem copies of the Mix.2 activin-response element (ARE) driving firefly luciferase, and pRLTK (25ng/embryo), containing a ubiquitous thymidine kinase promoter driving Renilla luciferase, were co-injected vegetally into either control or Zic2-depleted embryos. Embryos were frozen in triplicate during the gastrula stages and analyzed for luciferase activities according the protocol of the Luciferase Assay System (Promega).

**RT-PCR**

Analysis of gene expression was performed either by semi-quantitative, real-time RT-PCR using the LightCycler™ System (Roche) as described by Houston et al. (Houston et al., 2003), or by gel electrophoresis. For this latter method, 500 ng purified RNA was used for random hexamer-primed cDNA synthesis using MMLV (100 U/reaction; Invitrogen). cDNA reactions were diluted to 180 μl with TE [3 mM Tris (pH 8.0)/0.2 mM EDTA] and 1/20th of the diluted cDNA was used for RT-PCR. Reactions were performed on a PTC-200 (MJ Research/BioRad) with a dual 48-well Alpha block. Cycling conditions were: 94°C (2 minutes, 1 cycle), 94°C, 54°C, 72°C (10 seconds each, 27 cycles), 72°C, 10 minutes. Samples were then run on 2% agarose gels containing SYBR-Safe dye (Molecular Probes).
The primer sequences used and real-time PCR conditions are available upon request. Figures of real-time PCR presented show a representative result of each experiment, not averages of experiments, although each experiment was repeated at least twice. Real-time data were quantified against a standard curve of diluted, uninjected embryo cDNA. Undiluted control cDNA was set to 100%, thus values above 100% may not be accurately quantified as they lie outside the range of the standard curve.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed essentially as described (Sive et al., 2000). Digoxigenin-labeled antisense probes against Zic2 (CDS in pCRII-TOPO) were prepared by digestion with SpeI and transcription with T7. Xnr5 (a gift from Dr M. Asashima) was linearized with NotI and transcribed with T7. Embryos were post-fixed after staining and bleached prior to imaging.

Results

Isolation of a truncated Zic2 cDNA in a functional screen

In a functional screen of maternal cDNAs, we identified a cDNA pool that caused microcephaly and cyclopia. Isolation and identification of the active clone from this pool showed that it encoded a truncated version of the previously identified Xenopus Zic2 gene. The truncated clone (tZic2) contained the entire 3’UTR and a region of the coding region, beginning at proline 224, and containing the zinc-finger domain and the C terminus (Fig. 1A). The predicted tZic2 protein did not contain the endogenous initiating methionine; however, several in-frame methionine residues in the 5’ region of the cDNA were in good contexts for translation initiation (Fig. 1A). We translated tZic2 in vitro and detected a single band of the predicted size (Fig. 1B), suggesting that the tZic2 mRNA could produce a stable protein. Inspection of genomic DNA sequences from both mouse and Xenopus tropicalis, using online databases (data not shown), found that the start of the tZic2 sequence was within exon 1 of the Zic2 gene in both organisms. This suggested that the appearance of this truncated cDNA in the expression library occurred as a result of incomplete cDNA synthesis, and not from alternate splicing.

Embryos injected vegetally with tZic2 mRNA (500 pg) developed normally through early gastrulation, but were subsequently delayed in its completion. tZic2-injected embryos developed a shortened dorsal axis with reduced head and eye development (Fig. 1C). These results demonstrate that overexpression of tZic2 protein, lacking the N terminus, could disrupt normal axial and head development.

Nakata et al. (Nakata et al., 2000) and Kitaguchi et al. (Kitaguchi et al., 2000) showed that deletion of the N-terminal region of Xenopus Zic5 and Zic3, respectively, could generate dominant-negative proteins. Owing to the similarity of tZic2 to these constructs, we tested the hypothesis that the tZic2 cDNA could interfere with full-length Zic2 function. Co-expression of tZic2 with full-length Zic2 in animal caps showed that tZic2 efficiently blocked Zic2-mediated induction of neural crest markers, whereas tZic2 alone had no such activity (Fig. 1E).

Fig. 1. Isolation and dominant-interfering activity of a truncated Zic2 cDNA. (A) Upper panel: diagram showing the Xenopus Zic2 protein compared with the tZic2 protein. Lower panel: partial alignment of the amino acid sequences of the full-length and truncated-Zic2 proteins. Two potential starting methionines are boxed. The start of the zinc-finger domain is underlined. (B) In vitro translation of tZic2. Two preparations of tZic2 plasmid were translated in transcription-translation coupled reactions. Luciferase (Luc) ~50 kDa. (C) Injection of tZic2 causes microcephaly and cyclopia. Uninjected and tZic2-injected (500 pg) embryos at stage 40. (D) Hematoxylin and Eosin stained sections from the embryos in C showing reduced forebrains (large arrowhead) and abnormal or cyclopic eyes (small arrowhead). (E) Real-time RT-PCR analysis of neural crest (Slug, Sox9, Twist) and neural (NCAM) marker expression in animal caps cultured to stage 22. Samples and amounts injected are indicated in the key. Relative expression values; samples were normalized to ODC and expressed as a percentage of a whole stage 22 embryo sample.
These results suggest that the neural crest-inducing activity of Zic2 lies in the N terminus, and that tZic2 may inhibit endogenous Zic2 to cause microcephaly and cyclopia. However, because of the potential for cross-interference of tZic2 protein with other Zic proteins, and because tZic2 overexpression does not discriminate between maternal and zygotic roles for Zic2, we used an mRNA depletion, loss-of-function approach to specifically examine the function of maternal Zic2 in early development.

Depletion of maternal Zic2

In *Xenopus* oocytes, antisense DNA oligos bound to mRNA are recognized by an RNase-like activity, causing the degradation of the target RNA (Dash et al., 1987). mRNAs depleted in this manner are not replaced at least until the mid-blastula stage when zygotic transcription begins (Newport and Kirschner, 1982), thus oocytes fertilized following transfer into a host female produces embryos depleted of the maternal stores of specific RNAs (Torpey et al., 1992) (reviewed by Zuck et al., 1998). We designed several antisense oligonucleotides (oligos) against the 5′ region of the Zic2 mRNA and identified two that were effective in depleting maternal Zic2 to 20-40% of control levels (Fig. 2A). We confirmed that Zic2 is the only member of the family to be expressed maternally, and in addition found that the mRNA is present in both animal and vegetal regions of the oocyte (Fig. 2B), suggesting that Zic2 mRNA is not localized. Of the oligos we tested, one was more effective (10MP) and was used for the majority of experiments, although both gave similar results.

We obtained embryos from Zic2-depleted or uninjected oocytes via the host-transfer method; Zic2-depleted embryos developed normally from fertilization and initiated gastrulation at the same time as control embryos. During gastrulation, embryos lacking Zic2 were delayed in blastopore closure and ~30% of these underwent exogastrulation (Table 1). The remainder exhibited deep involution of the marginal zone and went on to develop reduced heads, wrinkled epidermis and a stunted dorsal axis (Fig. 2C,D). These embryos formed cement glands, suggesting that gross anteroposterior patterning was unaffected (Fig. 2D). In addition, depletion of maternal Zic2 did not eliminate zygotic Zic2 expression. Zygotic Zic2 mRNA was expressed correctly at the neural plate border and accumulated to normal levels by late gastrulation in maternal Zic2-depleted embryos (Fig. 2E,F). However, overall neural plate development appeared delayed (Fig. 2F) in these embryos. Histological analysis confirmed the lack of forebrain tissue in Zic2-depleted embryos (Fig. 2G, upper panel). In addition, neural tube morphology was disrupted and somite structure was abnormal. Notochords formed in Zic2-depleted embryos, but appeared larger in diameter than controls;
in some sections, a duplicated notochord was evident. Interestingly, we did not observe cyclopa in Zic2-depleted embryos as was seen in those injected with tZic2, suggesting that tZic2 may also interfere with zygotic Zic2, or possibly with other Zic proteins. Injection of Zic2 mRNA into Zic2-depleted embryos rescued normal gastrulation and head development in a majority of cases (Table 1), demonstrating that these phenotypes are due to specific depletion of endogenous Zic2.

To further characterize the head defects in Zic2-depleted embryos, we analyzed neural markers at the neural groove stage (stage 22; Fig. 2H). Consistent with the phenotype, Zic2-depleted embryos had lower levels of forebrain markers Eomes and Nkx2.1, representing both dorsal and ventral forebrain specifically. HoxB9 levels were also decreased, possibly reflecting overall neural defects. Interestingly, levels of the anterior neural marker Otx2 and the hindbrain marker Krox20 were elevated, suggesting that the depletion of maternal Zic2 results in brain defects more complex than a simple deletion of forebrain territory. Overall, these data suggest that maternal Zic2 is required for normal gastrulation movements and for proper anterior neural and axial development.

**Zic2 negatively regulates the expression of Xenopus nodal-related (Xnr) genes**

Head formation in vertebrates requires the simultaneous repression of Wnt, BMP and Nodal signaling pathways (Piccolo et al., 1999). Nodal proteins also have roles in regulating morphogenesis, and excess Nodal signaling results in brain defects such as cyclopa and exogastrulation (Piccolo et al., 1999). Nodal proteins also have roles in regulating morphogenesis, and excess Nodal signaling results in brain defects more complex than a simple deletion of forebrain territory. Overall, these data suggest that maternal Zic2 is required for normal gastrulation movements and for proper anterior neural and axial development.

**Table 1. Loss of maternal Zic2 results in exogastrulation and head defects**

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<th>Exogastrula</th>
<th>Anterior truncations</th>
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<td>Uninjected</td>
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<td>Zic2 oligo (5 ng)</td>
<td>27</td>
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<td>15/27</td>
<td>4/27</td>
<td>85</td>
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<tr>
<td>Oligo (5 ng) + Zic2 RNA (200 pg)</td>
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<td>3/27</td>
<td>4/27</td>
<td>20/27</td>
<td>26</td>
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Values represent proportion of embryos (stage 35-40), out of the total (n), that displayed the indicated phenotypes. % affected includes embryos with exogastrulation and head defects. These numbers represent the pooled data from three host-transfer experiments using oocytes from four different donor females. Zic2 mRNA was injected into oocytes.

Zic2-depleted and control embryos (data not shown). The degree of increase in Xnr expression appeared greater at the later stage, suggesting persistence, as well as upregulation, of Xnr expression.

In the mesoderm, expression of Goosecoid (Gsc), a target of Nodal-like signaling, was elevated in Zic2-depleted embryos, whereas expression of the pan-mesoderm marker Xbra was reduced, a pattern consistent with elevated Nodal-like signaling. Chordin expression was unaffected in Zic2-depleted embryos, suggesting that while the expression of some organizer genes is elevated, this may not reflect increased organizer formation. In the endoderm, we found an initial decrease in endoderm gene expression at stage 10.25, followed by elevated levels for Xsox17a and Xhex at stage 10.5, suggesting a delay in their expression. Interestingly, Cerberus expression was only slightly affected, suggesting that some, but not all, dorsoanterior markers are increased in Zic2-depleted embryos. To provide additional evidence that Zic2-depleted embryos exhibit increased Nodal-like signaling, we injected a Nodal-responsive reporter plasmid (A3-luciferse) into control embryos and siblings deficient in maternal Zic2. Analysis of luciferase activity at the mid-gastrula stage showed that activity of the reporter construct was higher in Zic2-depleted embryos (Fig. 3C). Activity at the early gastrula stage was equivalent to or slightly lower than control levels. These data show that Zic2-depleted embryos exhibit high levels of Xnr expression that is reflected by increased downstream signaling activity.

**Zic2 mRNA expression rescues Xnr expression in Zic2-depleted embryos**

In addition to phenotypic analysis, we assessed the specificity of Zic2-depletion by molecular marker analysis by real-time RT-PCR. We analyzed the levels of Xnr1, Xnr3 and Xnr5 in control uninjected embryos, Zic2-depleted embryos and Zic2-depleted embryos injected with Zic2 mRNA over three stages during gastrulation. Consistent with the phenotypic rescue, we found that injection of Zic2 mRNA restored roughly normal levels of Xnr genes by stage 11 (Fig. 4A), although the degree of rescue was less pronounced at the earlier stages. We also saw at least partially rescued levels of Nodal target genes Gsc and Xhex, whereas Chordin was unaffected by the absence of endogenous Zic2 or the presence of injected Zic2 (Fig. 4B). In a second experiment, we saw a slightly higher degree of rescue for Xnr5 (Fig. 4C). It is likely that varying penetrance of phenotype and differing rescue efficiency, as well as differences in the peak expression times of Xnr genes, contributes to variations in the amount of elevated expression or rescue that we observe. Additional sources of variability in the levels of increased Xnr expression across experiments include slight gastrulation delays in oligo-injected embryos.

**Zic2 negatively regulates the expression of Xenopus nodal-related (Xnr) genes**

Head formation in vertebrates requires the simultaneous repression of Wnt, BMP and Nodal signaling pathways (Piccolo et al., 1999). Nodal proteins also have roles in regulating morphogenesis, and excess Nodal signaling results in altered gastrulation movements (reviewed by Solnica-Krezel, 2003). Some Zic2-depleted embryos underwent exogastrulation and developed reduced head structures, we therefore tested the hypothesis that Zic2 regulates Xnr expression or signaling.

We initially examined the expression of Xnr1, Xnr3 and Xnr5 in controls and in embryos injected with a dose range of tZic2 mRNA to approximate Zic2 loss-of-function. At two stages during gastrulation, Xnr5 mRNA levels were elevated in tZic2-injected embryos in a dose-dependent manner (Fig. 3A). Similar results were also obtained for Xnr1 and Xnr3 (data not shown). In addition, overexpression of full-length Zic2 could also weakly repress Xnr5 expression, but only to ~70% of control levels (data not shown).

We next assayed the expression of Xnr genes and mesendodermal marker genes in control and Zic2-depleted embryos. Embryos were obtained by the host-transfer method, frozen at mid-gastrula stages (stage 10.25 and 10.5) and assayed for gene expression by real-time, semi-quantitative RT-PCR. We found that Zic2-depleted embryos expressed higher levels of Xnr mRNAs compared with sibling embryos (Fig. 3B). This was the case for all of the Xnr genes, with the exception of Xnr4, which was expressed at similar levels in Zic2-depleted and control embryos (data not shown). The degree of increase in Xnr expression appeared greater at the later stage, suggesting persistence, as well as upregulation, of Xnr expression.
and inherent errors in quantifying values greater than the 100% standard. Overall, these data show that injected Zic2 mRNA can reduce, to some extent, excess Xnr expression caused by Zic2-depletion and demonstrates the specificity of this depletion.

**Zic2-deficiency alters the dorsal-ventral, but not the germ layer distribution of Xnr and mesendoderm genes**

To determine to what extent loss of Zic2 results in a global derepression of Xnr transcript levels, we analyzed the expression of Xnr5 in control and Zic2-depleted embryos by in situ hybridization. Prior to gastrulation (stage 9; Fig. 5A upper panels), we detected Xnr5 in dorsal vegetal cells of both control and Zic2-depleted embryos, although Xnr5 staining was stronger in the absence of Zic2. During early-to-midgastrulation (stage 10.5; Fig. 5A lower panels), Xnr5 staining was faint or undetectable in controls but was expressed throughout the vegetal pole in Zic2-depleted embryos and enriched ventrally (Fig. 5A arrow). In no case did we observe Xnr5 expression in the equatorial or animal cells. These data suggest that Zic2 regulates Xnr5 expression in vegetal cells. Similar results were obtained for Xnr6 (data not shown).

We next examined Xnr1, Xnr3 and Xnr5 expression in isolated dorsal and ventral halves of control and Zic2-depleted embryos. In these experiments, we found that expression of Xnr1 was elevated in both dorsal and ventral halves of Zic2-depleted embryos compared with control halves, although the degree of elevation was more pronounced on the ventral side (Fig. 5B). Xnr3 and Xnr5 were also increased ventrally and either unchanged, or slightly elevated in dorsal cells. Nodal target genes Gsc and Xhex were also ectopically expressed ventrally in Zic2-depleted embryos (Fig. 5B).

To determine to what extent Zic2 regulates the expression of Xnr genes and their target genes in different germ layers, we dissected animal caps, equatorial regions and vegetal bases from control and Zic2-depleted embryos at the late blastula stage. These explants were cultured to the early gastrula stage and analyzed for expression of Xnr genes and mesendoderm markers. None of the markers analyzed showed any ectopic germ layer expression in Zic2-depleted animal caps (data not shown). Xnr5 was expressed only in vegetal explants of control embryos (Fig. 5C). In Zic2-depleted embryos, consistent with the in situ data, Xnr5 was expressed at higher levels in the bases and was not expressed in equators. Xnr1 was also elevated only in vegetal explants, as were endoderm markers Xhex and Sox17a. Gsc was elevated in both equators and bases, and Xbra was slightly decreased in equators. These results suggest that Zic2 is required to regulate the extent of Xnr expression primarily in vegetal cells.

**Nodal antagonism rescues head and axial defects in Zic2-depleted embryos**

The anterior truncations and gastrulation defect phenotypes...
Maternal Zic2 suppresses Xnr expression

of Zic2-depleted embryos correlate with the effects of excess Nodal signaling in *Xenopus* (Branford and Yost, 2002). To determine the causal role of Nodal signaling in these defects, we expressed a Nodal antagonist, Cerberus-short (CerS) (Piccolo et al., 1999), in Zic2-depleted embryos. We generated Zic2-depleted and control embryos via the host-transfer method and then injected CerS mRNA (50 pg) at the two-cell stage. Embryos were collected at the gastrula stage for RT-PCR, or reared to the tailbud stage for phenotypic analysis. At the dose used, CerS-injected control embryos developed anterior truncations (microcephaly) in a number of cases; however, this dose was sufficient to reduce the incidence of head and axial defects in Zic2-depleted embryos (Fig. 6A,C,H). CerS injection alone did not result in axial truncations. In control gastrula stage embryos, CerS injection severely reduced *Xnr1* expression, but caused an elevation of *Xnr5* and *Xnr6* (Fig. 6B), consistent with previous reports (Kofron et al., 2004a). In Zic2-depleted embryos, *Xnr1* expression was elevated, as was *Xnr5*, consistent with our previous experiments. Injection of CerS into Zic2-depleted embryos rescued *Xnr1* expression slightly but had no effect on *Xnr5* or *Xnr6* expression. Interestingly, *Xnr5* and *Xnr6* abundance was similar in embryos both depleted of Zic2 and expressing CerS, indicating lack of an additive effect (Fig. 6B). These results suggest that Zic2 acts primarily at the level of *Xnr5/6*, and only secondarily regulates *Xnr1*.

**Zic2 indirectly inhibits VegT regulation of Xnr gene expression**

VegT is both necessary and sufficient for the expression of the typical Xnr genes (Kofron et al., 1999; Takahashi et al., 2000; Xanthos et al., 2002). To identify whether Zic2 functionally interacts with VegT to regulate Xnr expression, we performed VegT loss- and gain-of-function experiments in the context of control or Zic2-depleted embryos. We first generated embryos deficient in both VegT and Zic2. For these double-depletion experiments, we used a translation-blocking-morpholino oligo
Depletion of maternal VegT reduced the expression of Xnr1 and Xnr5 to 20% of control levels but had no effect on Xnr3 expression (Fig. 7A). In embryos depleted of both Zic2 and VegT, Xnr1 and Xnr5 levels remained low. By contrast, Xnr3 was expressed at control levels in Zic2-depleted embryos (Fig. 7A, middle panel), suggesting that the elevation seen in Zic2-depleted embryos is due to excess Nodal signaling downstream of VegT, and not to dysregulation of the Wnt pathway.

Consistent with these results, loss of Zic2 also sensitized cells to ectopically expressed VegT mRNA. We injected VegT into control or Zic2-depleted embryos and assayed for Xnr5 expression at the early gastrula stage. In control embryos, VegT induced the expression of Xnr5 and Xbra in gastrula (stage 10.5) stage embryos analyzed by RT-PCR. (C,C') Examples of embryos from the experiment in B. Zic2-embryos show both microcephaly and axial truncation; the Zic2-, +CerS embryos represent a normal and a microcephalic embryo (upper and lower embryo, respectively).
Development of proteins are required for this process. It remains to be determined to what extent I-mfa proteins or other co-repressor of Zic2 in mediating repression of Xnr genes; it remains to be consistent with this idea. These results implicate the N terminus showing dominant-negative effects of the tZic2 protein are (Kitaguchi et al., 2000; Nakata et al., 2000). Our results the N terminus of Zic proteins are important for their function Zic5 generated dominant-negative constructs, suggesting that 1996a). Removal of the N-terminal regions of Xenopus myogenic bHLH proteins, such as MyoD, by retaining them in (Kitaguchi et al., 2000; Nakata et al., 2000). Our results showing dominant-negative effects of the tZic2 protein are consistent with this idea. These results implicates the N terminus of Zic2 in mediating repression of Xnr genes; it remains to be determined to what extent I-mfa proteins or other co-repressor proteins are required for this process.

We also show that Zic2 is required to regulate the expression of Xnr5. One explanation for this observation would be that Zic2 binds to the Xnr5 promoter region, causing transcriptional repression. We inspected the genomic DNA sequences for X. tropicalis Xnr5 and Xnr6, available through the genome sequencing project, and failed to find matches to three previously identified Zic2-binding elements (not shown). Elements from the human APOE promoter, which are activated by Zic2 (Salero et al., 2001), a Zic2-binding consensus sequence identical to the Gli-binding element (Mizugishi et al., 2001), and repressive Zic2 element from the human DRD1A (dopamine receptor D1a) gene (Yang et al., 2000) were not present in 3.5 kb of upstream sequence or within the first intron of Xnr5 or Xnr6 (data not shown). Additionally, these Zic2-regulated elements bear no homology to each other and Zic2 has been found to regulate transcription from basal promoters in cell line studies (Brown et al., 2005; Mizugishi et al., 2001). The Khamba allele (Elms et al., 2003) contains a point mutation that abrogates Zic2 DNA-binding activity (Brown et al., 2005), suggesting that Zic2 function requires DNA binding. Thus, Zic2 DNA-binding specificity may be dictated by complex formation with other transcription factors. The identification of Zic2-binding proteins may help to test this hypothesis.

Zic2 function in Xenopus development

Xenopus Zic2 was initially described as having both neural/neural crest-inducing activity and anti-neurogenic activity in Xenopus embryos (Brewster et al., 1998; Nakata et al., 1998). The molecular mechanisms behind these activities have not been explored in depth. Analysis of hypomorphic and potential null alleles in mice have shown that Zic2 regulates the timing and extent of neurulation and neural crest formation, as well as the patterning of the forebrain and hindbrain (Elms et al., 2003; Nagai et al., 2000). In Xenopus, Zic2 is expressed maternally and during the gastrula stages, suggesting a possible role in early developmental events. Other Zic genes are not maternally expressed (Fig. 2), thus the role of Zic2 can be studied in this context without compensation from other Zic proteins.

Depletion of maternal Zic2 results in alterations of gastrulation movements and in abnormal development of the forebrain, neural tube and notochord. These late stage effects are likely to represent embryos mildly affected (or embryos with less Zic2 depletion) as ~30% of Zic2-depleted embryos undergo exogastrulation. Elevated Nodal signaling, which is produced by the loss of Nodal antagonists, triggers exogastrulation in frog and fish embryos (Branford and Yost, 2002; Feldman et al., 2002), and abnormal gastrulation movements in the mouse (Iratni et al., 2002; Perea-Gomez et al., 2002). This same mechanism may also be responsible for exogastrulation in Zic2-depleted embryos given the increased expression and activity of Xnr genes in these embryos. The loss of Zic2 produces a milder phenotype in this regard, most likely because Nodal antagonists are still expressed in Zic2-depleted embryos (data not shown). In mouse embryos, Zic2 is also expressed prior to gastrulation (Elms et al., 2004); however, functional studies have not yet identified a role for this early phase of Zic2 expression. It will be interesting to discover if mammalian Zic2 regulates Nodal expression and gastrulation movements in a manner analogous to maternal Zic2 in frogs.

Zic2 regulation of Xnr gene expression

Several lines of evidence presented here suggest that maternal Zic2 negatively regulates the expression of Xenopus nodal-
related (Xnr) genes, primarily that of Xnr5. It is likely that maternal Zic2 serves to limit the extent of the initial wave of Xnr5 expression, which would normally be required for proper spatial and temporal regulation of Xnr1, Xnr2, Xnr3 and Nodal target genes. Among these targets are endoderm markers, which show an initial delay in expression, possibly owing to repression by high levels of Nodal signaling (Yasuo and Lemaire, 1999). It was surprising to find that Xnr4 was not upregulated in Zic2-depleted embryos, as would be expected from its expression pattern (Joseph and Melton, 1997). It is possible that Xnr4 has lost the regulatory sequences needed for autoregulation owing to functional redundancy of Xnr1 and Xnr2. Interestingly, Xnr3 which lacks typical Nodal activity, appears to have retained such regulation, as shown by our results (Fig. 7) and by the upregulation of Xnr3 in Xlefty-depleted embryos (Branford and Yost, 2002). It is unclear to what extent elevated Xnr3 is a major contributor to the Zic2-depleted embryo phenotype. These embryos do not exhibit hallmarks of Xnr3 overexpression, such as finger-like projections, and CerS, which does not inhibit Xnr3, can reduce the severity of defects.

The mechanisms by which Zic2 regulates Xnr5, and probably Xnr6, expression remain to be determined. Zic2 is dependent on VegT to regulate Xnr5; however, we found that Zic2 expression does not inhibit VegT in animal cap experiments, and Zic2 is a poor Nodal inhibitor in general. These results suggest an indirect mechanism, or the presence of other factors not present in animal caps. One likely candidate is Nodal signaling itself. We found that depletion of Zic2 and antagonism of Nodal signaling (by CerS injection) did not produce an additive effect on Xnr5 and Xnr6 expression. These results suggest a model in which Zic2 could associate with active Smad2 complexes to mediate negative feedback inhibition of Xnr5. A number of other transcription factors, including XTC3, Sox3, Mixer and FoxH1 have been recently shown to have roles in repressing Xnr5 (Houston et al., 2002; Kofron et al., 2004a; Kofron et al., 2004b; Zhang et al., 2003). Interestingly, Mixer and FoxH1 are known to associate with activated Smad2 (Chen et al., 1996b; Germain et al., 2003), but do not possess intrinsic transcriptional activation activity. Intracellular TGFβ-negative feedback loops have been described in other model systems but have not been well studied in embryos.

Zic2 in holoprosencephaly

Hemizygosity of ZIC2 underlies cases of human HPE; however, the mechanism(s) by which loss of ZIC2 causes HPE remain uncertain. In this work, we show that depletion of Zic2 results in increased Xnr expression at the gastrula stages. The majority of identified human HPE mutations are in the sonic hedgehog (SHH) pathway (Duborg et al., 2004). However, experiments in mouse and fish have also implicated Nodal loss-of-function in HPE, possibly through the induction of Shh expression in the prechordal mesoderm (Lowe et al., 2001; Rohr et al., 2001). NODAL mutations have not yet been identified in human HPE cases, although mutations in TDGF1 (CRIPTO), a NODAL co-receptor, have been found in individuals with HPE-like, but not definitive, HPE (de la Cruz et al., 2002). Mutations in human TGF, an activated-SMAD associated, homeodomain co-repressor (Wotton et al., 1999), have been reported (Gripp et al., 2000) in HPE, suggesting a role for increased NODAL activity. Our results presented here reinforce the idea that increased NODAL signaling may be a factor in HPE. Further clarification of the mechanism of Zic2 in the control of Xnr expression may be useful in the identification of additional HPE candidate genes.

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