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 (Xnrs) 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 pBluescript 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 *Hin*dIII and *Sal*I, 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 *Eco*RI and *Xho*I and dephosphorylated to accommodate cDNA inserts. Ligations 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 0.1×MMR. For injections of mRNAs after fertilization, embryos were dejellied and transferred to 2% Ficoll/0.5×MMR at the one-cell stage. For explant assays, stage 9 embryos were dissected in 1×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*CCGTGCGACTGTGC*C*C*A-3′ (Zic2-10MP). Asterisks (*) represent phosphorothioate bonds.

A morpholino oligo (MO) against *VegT* was obtained from Gene-Tools: VegT-MO, 5′-CCCGACAGCAGTTTCTCATTCCAGC-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 *Eco*RI site of the vector pCS2+ and linearized with *Not*I for SP6 in vitro transcription. *Zic2* was also cloned into pRN3 as a *Cla*I/*Xba*I fragment and linearized with *Sfi*I. Capped *Zic2* mRNA was synthesized using the SP6 or T3 mMessage mMachine kits (Ambion), respectively. *VegT* and *CerS*, both in pCS2+, were digested with *Not*I 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 *Spe*I and transcription with T7. *Xnr5* (a gift from Dr M. Asashima) was linearized with *Not*I 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). Analysis of histological sections showed that *tZic2*-injected embryos had severely reduced forebrains and either cyclopic, reduced or absent eyes (Fig. 1D). 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).

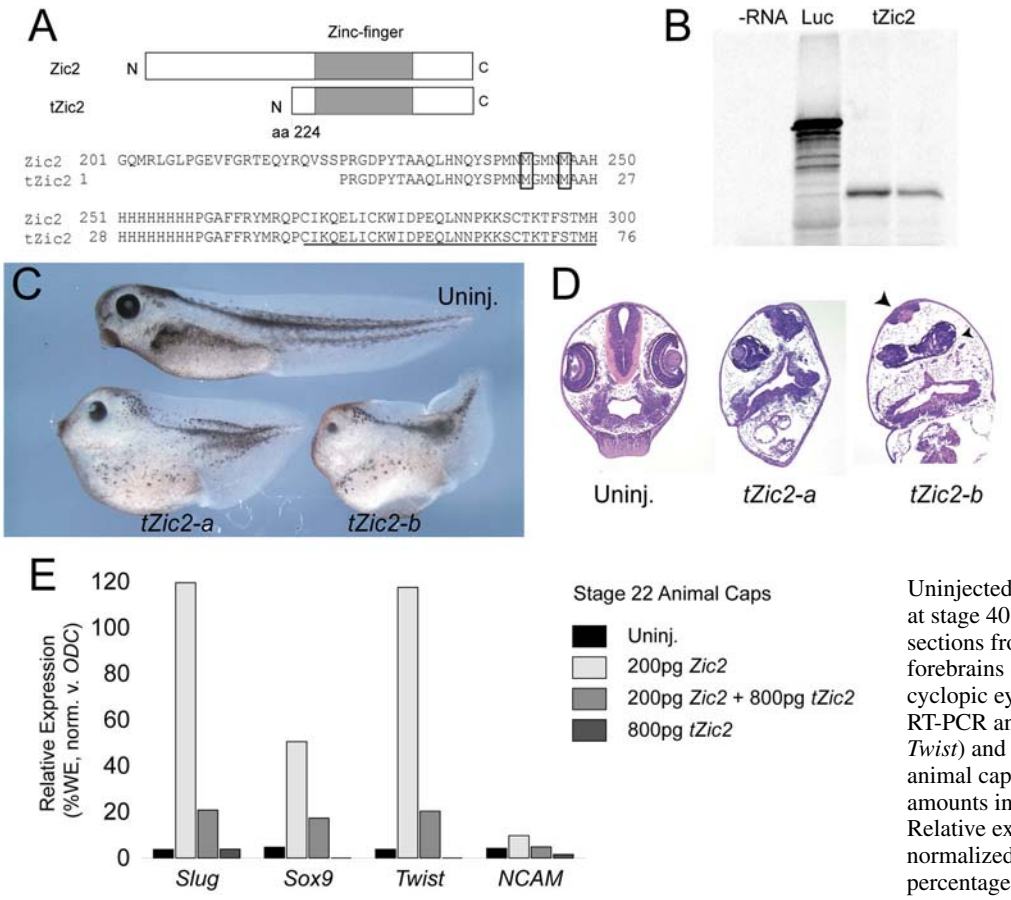

Fig. 1. Isolation and dominant-interfering activity of a truncated *Zic2* cDNA. (A) Upper panel: diagram showing the *Xenopus* Zic2 protein compared with the tZic2 protein. Lower panel: partial alignment of the amino acid sequences of the full-length and truncated-Zic2 proteins. Two potential starting 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;

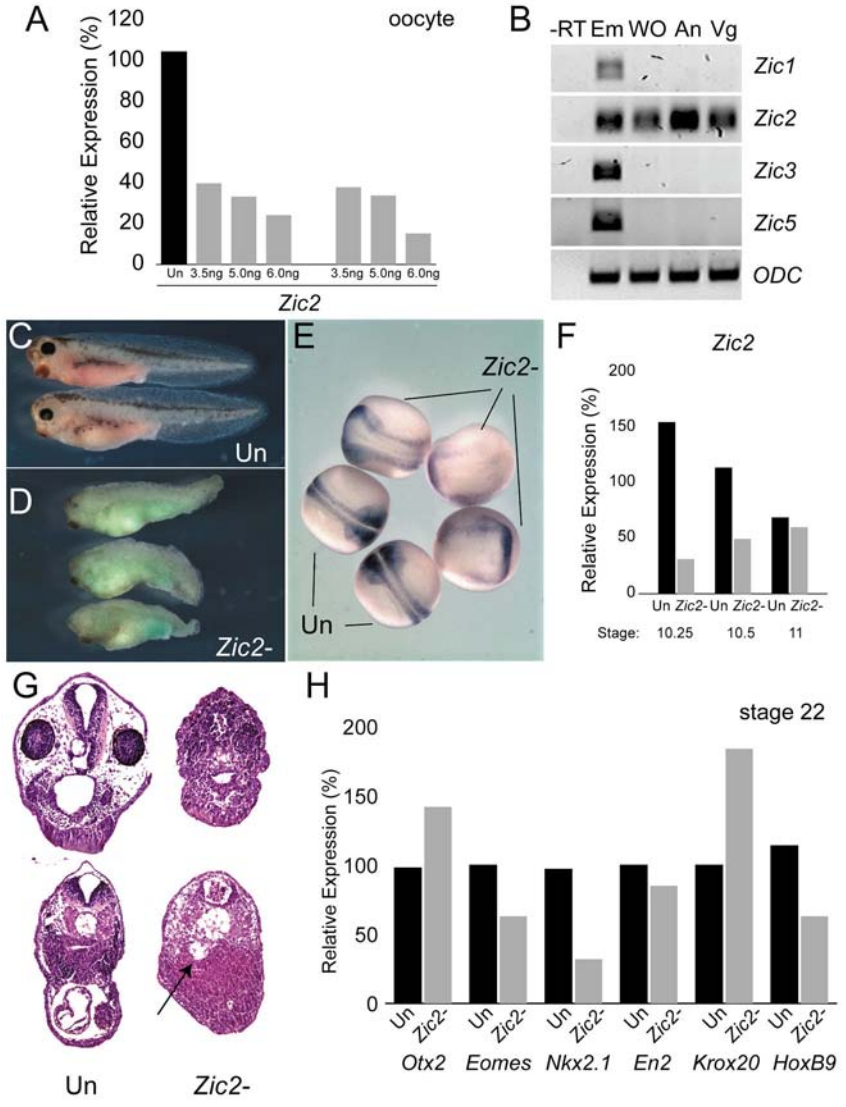

Fig. 2. Depletion of maternal *Zic2*. (A) Dose-dependent depletion of *Zic2* from oocytes of two different donor females. Oligo 10MP was injected at the indicated doses and oocytes were cultured for 24 hours before analysis by RT-PCR. Samples were quantitated relative to uninjected oocytes (Un) from female 1 (black bar). (B) *Zic2* is the only Zic gene expressed maternally. RT-PCR of *Zic1*, *Zic2*, *Zic3* and *Zic5* expression in stage 10.5 embryos (Em; stage 14 for *Zic5*), stage VI oocytes (WO) or animal and vegetal halves of oocytes (An and Vg). (C,D) Representative phenotypes of uninjected (C) and *Zic2*-depleted (D) embryos at stage 38/39. (E,F) Zygotic *Zic2* is expressed in maternal *Zic2*-depleted embryos. (E) Whole-mount in situ hybridization of *Zic2* expression in stage 18 uninjected (two lower left embryos) and maternal *Zic2*-depleted embryos (three upper right). Although neural tube formation is delayed, *Zic2* is expressed anteriorly and at the neural plate border. (F) Gastrula stage series of *Zic2* expression in uninjected and *Zic2*-depleted embryos. (G) Histological sections of embryos from C,D taken through the head (upper panel) or just behind the level of the otic vesicle (lower panel). In each panel, the uninjected embryo is on the left and the *Zic2*-depleted embryo is on the right. The arrow indicates a duplicated notochord. (H) Real-time RT-PCR analysis of neural markers in control and *Zic2*-embryos. Relative expression values were normalized to *ODC* and expressed as a percentage of a stage 22 embryo.

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

	n	Exogastrula	Anterior truncations	Normal	% Affected
Uninjected	36	0/36	0/36	36/36	0
Zic2 oligo (5 ng)	27	8/27	15/27	4/27	85
Oligo (5 ng) + *Zic2* RNA (200 pg)	27	3/27	4/27	20/27	26

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.

in some sections, a duplicated notochord was evident. Interestingly, we did not observe cyclopia 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 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.

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 *Xsox17α* and *Xhex* at stage 10.5, suggesting a delay in their expression. Interestingly, *Cerberus* expression was only slightly affected, suggesting again 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 restored 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

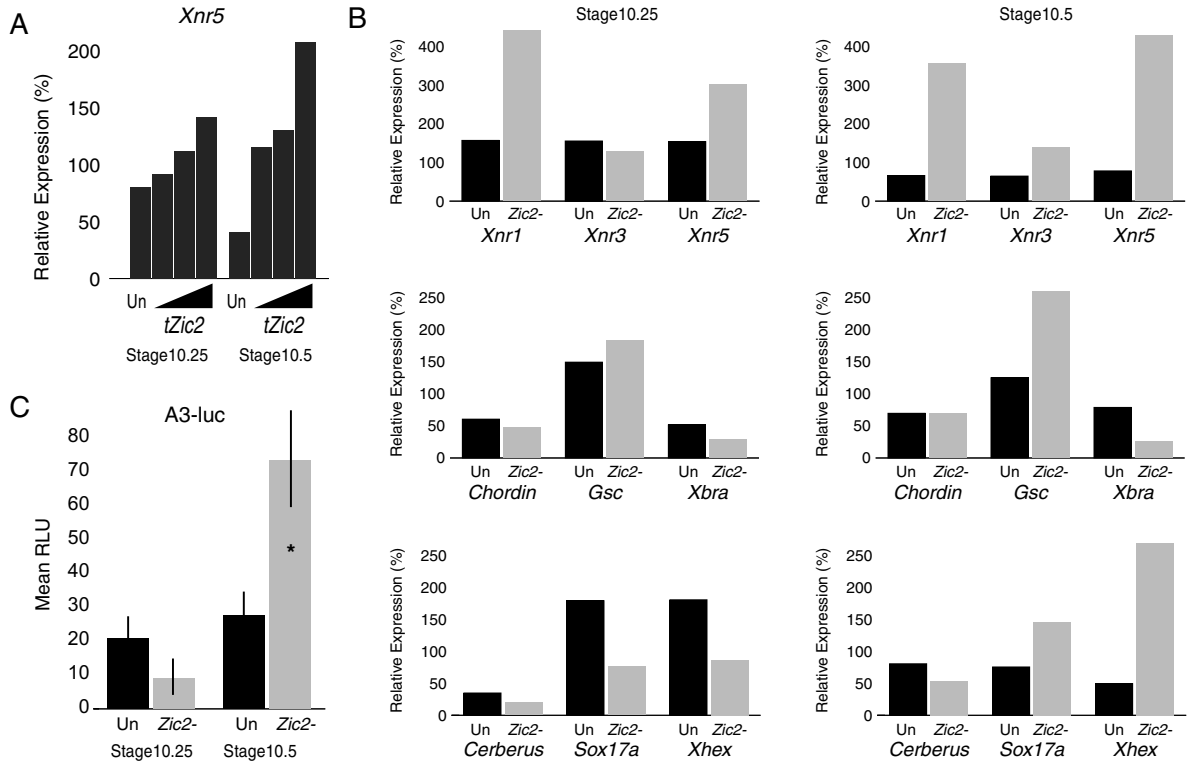

Fig. 3. *Zic2* regulates Xnr expression during gastrulation. (A) Dose-dependent induction of *Xnr5* by injected *tZic2* RNA (330 pg, 660 pg, 990 pg) in whole embryos assayed by real-time RT-PCR. Un, uninjected embryo. (B) Expression of Xnrs, mesoderm and endoderm markers in control (Un) and *Zic2*-depleted (*Zic2*-; 5 ng oligo 10MP) embryos assayed by real-time RT-PCR at stage 10.25 (left panel) and stage 10.5 (right panel). (C) Increased activin-like activity in *Zic2*-depleted embryos. Mean relative luciferase units (RLU)=A3-luciferase activity (3× activin response element)/RLTK (*Renilla* luciferase, thymidine kinase promoter) activity. Error bars show s.d. in a representative experiment; the asterisk indicates statistical significance (P<0.05).

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 de-repression 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 mid-gastrulation (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 *Xsox17α*. *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

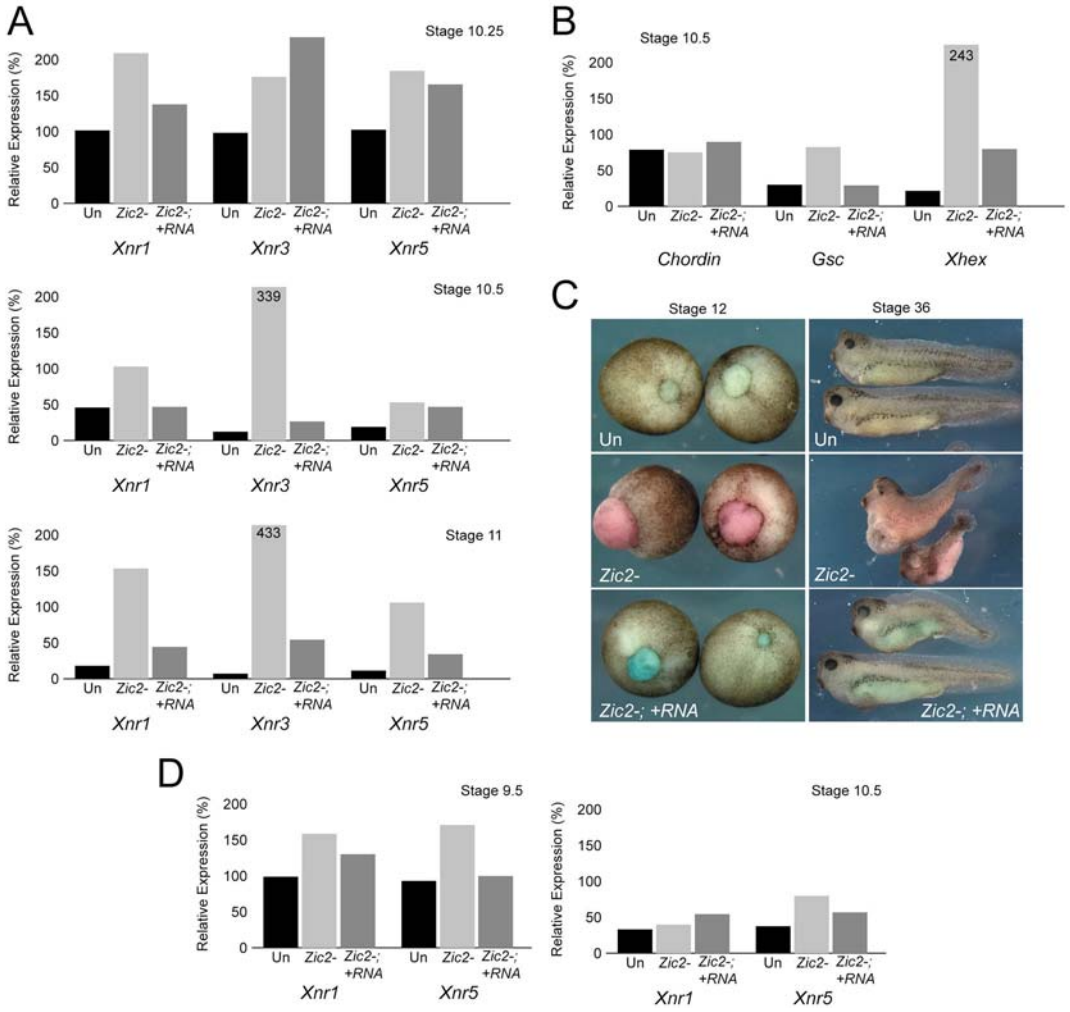

Fig. 4. Rescue of Xnr expression and normal development by injected *Zic2* mRNA. (A) Expression of *Xnr1*, *Xnr3* and *Xnr5* in uninjected controls (Un), *Zic2*-depleted embryos and *Zic2*-depleted embryos (5 ng 10MP) injected with *Zic2* mRNA (75 pg into oocytes prior to host transfer). Expression values are relative to the uninjected stage 10.25 sample. (B) Expression of *Chordin*, *Gsc* and *Xhex* in the same samples as A. (C) Examples of embryos from the experiment in A,B shown at two stages, late gastrula (stage 12, left panels) and tailbud (stage 36, right panels). (D) Expression of *Xnr1* and *Xnr5* in a second rescue experiment, as in A, except that 200 pg of *Zic2* mRNA was used.

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,C′). *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

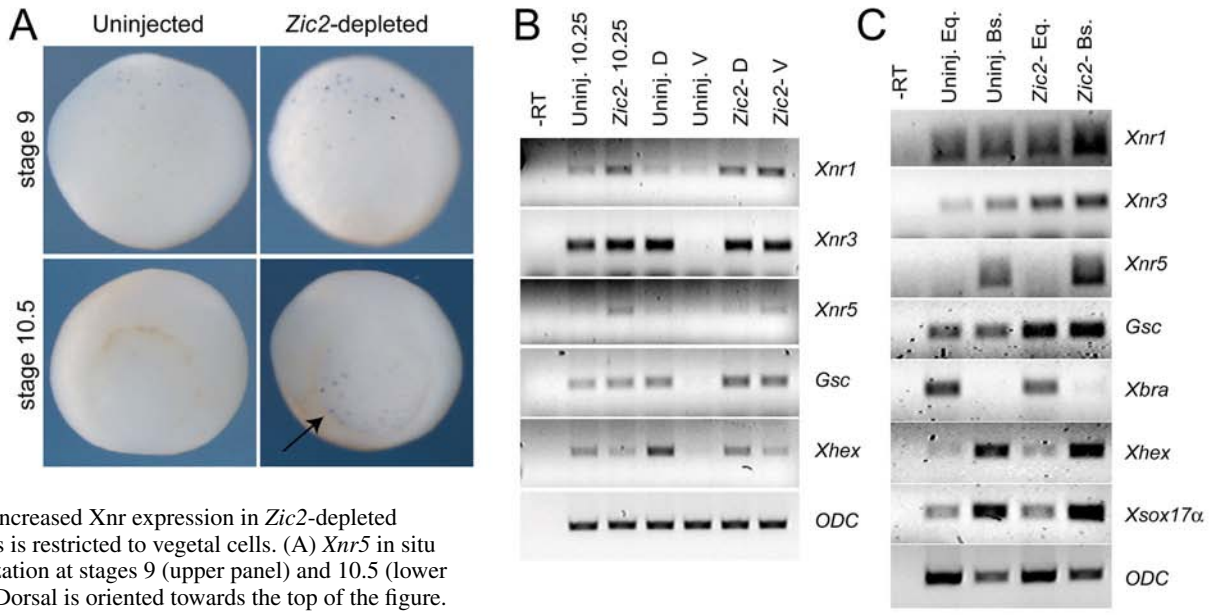

Fig. 5. Increased Xnr expression in *Zic2*-depleted embryos is restricted to vegetal cells. (A) *Xnr5* in situ hybridization at stages 9 (upper panel) and 10.5 (lower panel). Dorsal is oriented towards the top of the figure. *Xnr5* is expressed in dorsal vegetal cells of uninjected and *Zic2*-depleted embryos. *Xnr5* expression at stage 10.5 is undetectable in control embryos (0/11) but is present ventrally in *Zic2*-depleted embryos (8/13; arrow). (B) Expression of Xnr genes and Xnr target genes in dorsal (D) and ventral (V) halves at stage 10.25. Uninjected (Uninj.) 10.25 and *Zic2*-depleted (*Zic2*-) 10.25 are intact embryos. –RT, stage 10.25 sample processed in the absence of reverse transcriptase. All genes shown are expressed ventrally. (C) Expression of Xnr genes and Xnr target genes in equatorial (Eq) and vegetal base (Bs) explants.

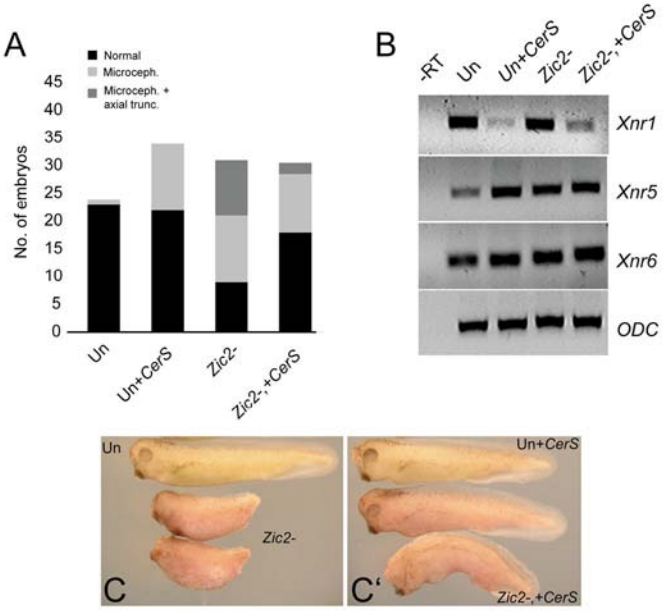

Fig. 6. Rescue of *Zic2*-depletion by injection of CerS. (A) Analysis of microcephaly (microceph.) and axial truncations (axial trunc.) in control stage 33 embryos (Un), controls injected with 50 pg CerS mRNA (Un+*CerS*), Zic2-depleted embryos (*Zic2*-) or *Zic2*- embryos injected with 50 pg *CerS* mRNA (*Zic2*-, +*CerS*). Data were pooled from two host-transfer experiments. Bars represent the number of embryos with the indicated phenotype. Axial truncations were present in a subset of embryos with microcephaly. (B) Expression of *Xnr1*, *Xnr5* and *Xnr6* 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).

(MO) against *VegT* (Heasman et al., 2001) in order to reduce the possibility of non-specific effects caused by the injection of additional phosphorothioate 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*/VegT double-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 a three- to fourfold increase in *Xnr5* levels (Fig. 7B), whereas *VegT* overexpression in *Zic2*-depleted embryos resulted in a further elevation of *Xnr5* expression. These results suggested that *Zic2* antagonizes Xnr induction by VegT. To test if Zic2 could act as a direct antagonist of VegT, we expressed *VegT* and *Zic2* mRNAs in animal caps assays (Fig. 7C). Injection of *VegT* induced the expression of *Xnr5* and *Xbra* in animal caps; however, co-injection of *Zic2* had no effect on this induction. *Zic2* alone did not induce any of the genes we tested; however, we noted that embryos left intact accumulated pigment cells in anterior regions (not shown). Because this is an outcome of *Zic2* overexpression (Nakata et al., 1998) (our observations), we conclude that the *Zic2* mRNA used in this experiment was indeed active. These data suggest that Zic2 can repress the extent of Xnr induction by VegT, and that this repression is indirect or involves proteins or signals not present in animal cells.

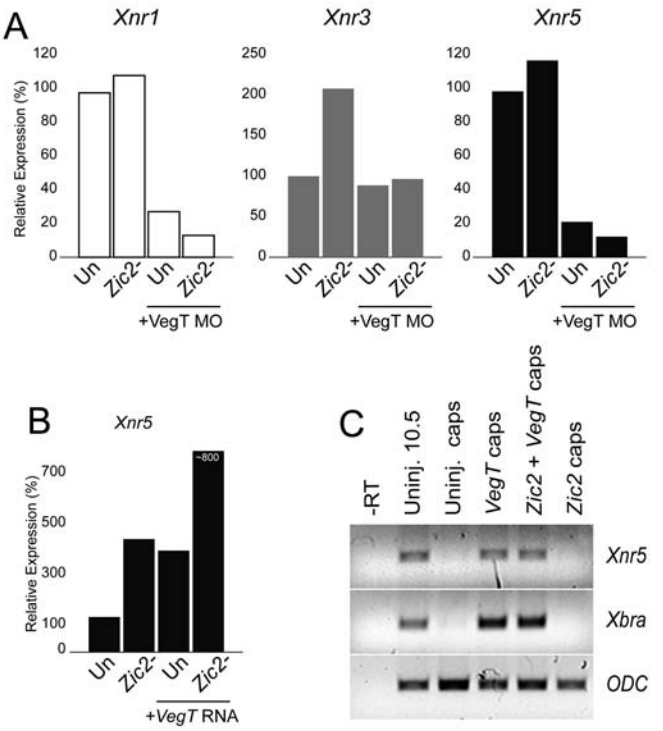

Fig. 7. *Zic2* antagonizes VegT regulation of Xnr expression. (A) Expression of *Xnr1*, *Xnr3* and *Xnr5* in whole embryos at stage 10.5. Un, uninjected; *Zic2*-, 5 ng 10MP; *VegT* MO, 18 ng morpholino oligo. (B) Expression of *Xnr5* in uninjected and *Zic2*-depleted embryos in the absence or presence of *VegT* mRNA (200 pg). Oligos were injected vegetally in A and *Zic2* oligo and *VegT* RNA were injected animally in B. (C) Zic2 does not inhibit VegT in animal caps. *Zic2* mRNA (500 pg), *VegT* mRNA (250 pg) or a combination of the two, were injected at the two-cell stage. Animal caps were dissected at the early gastrula stage and cultured for 2 hours.

Discussion

Function of the Zic2 protein

Previous structure-function studies of Zic proteins have identified the C-terminal half (including the zinc-finger domain) as having DNA-binding and transcriptional activation properties (Mizugishi et al., 2001). By contrast, the N terminus was shown to have a repressive function in cultured cells, mediated by interactions with the co-repressor I-mfa (Mizugishi et al., 2004). I-mfa proteins are thought to act as repressors of myogenic bHLH proteins, such as MyoD, by retaining them in the cytoplasm or blocking their DNA-binding (Chen et al., 1996a). Removal of the N-terminal regions of *Xenopus* Zic3 and Zic5 generated dominant-negative constructs, suggesting that the N terminus of Zic proteins are important for their function (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 implicate 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 Xtnr5* and *Xtnr6*, 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 *Xtnr5* or *Xtnr6* (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 *Khumba* 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 is which Zic2 could associate with active Smad2 complexes to mediate negative feedback inhibition of *Xnr5*. A number of other transcription factors, including XTcf3, 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., 2000), but do not posses 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 *TGIF*, 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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