Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 273 (2004) 23–37

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Repression of nodal expression by maternal B1-type SOXs regulates germ layer formation in *Xenopus* and zebrafish

Chi Zhang,[a] Tamara Basta,[a] Laura Hernandez-Lagunas,[b] Peter Simpson,[b] Derek L. Stemple,[c] Kristin B. Artinger,[b] and Michael W. Klymkowsky[a,*]

[a] *Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Boulder, Colorado 80309-0347, USA*
[b] *Department of Craniofacial Biology, University of Colorado Health Sciences Center, Denver, CO 80262, USA*
[c] *Wellcome Trust Sanger Institute, Genome Campus, Hinxton, Cambridge, CB10 1SA, UK*

Received for publication 27 February 2004, revised 24 May 2004, accepted 26 May 2004
Available online 14 July 2004

Abstract

B1-type SOXs (SOXs 1, 2, and 3) are the most evolutionarily conserved subgroup of the SOX transcription factor family. To study their maternal functions, we used the affinity-purified antibody antiSOX3c, which inhibits the binding of *Xenopus* SOX3 to target DNA sequences [Development. 130(2003)5609]. The antibody also cross-reacts with zebrafish embryos. When injected into fertilized *Xenopus* or zebrafish eggs, antiSOX3c caused a profound gastrulation defect; this defect could be rescued by the injection of RNA encoding SOX3ΔC-EnR, a SOX3-engrailed repression domain chimera. In antiSOX3c-injected *Xenopus* embryos, normal animal–vegetal patterning of mesodermal and endodermal markers was disrupted, expression domains were shifted toward the animal pole, and the levels of the endodermal markers SOX17 and endodermin increased. In *Xenopus*, SOX3 acts as a negative regulator of *Xnr5*, which encodes a nodal-related TGFβ-family protein. Two nodal-related proteins are expressed in the early zebrafish embryo, *squint* and *cyclops*; antiSOX3c-injection leads to an increase in the level of *cyclops* expression. In both *Xenopus* and zebrafish, the antiSOX3c phenotype was rescued by the injection of RNA encoding the nodal inhibitor Cerberus-short (CerS). In *Xenopus*, antiSOX3c's effects on endodermin expression were suppressed by injection of RNA encoding a dominant negative version of Mixer or a morpholino against SOX17α2, both of which act downstream of nodal signaling in the endoderm specification pathway. Based on these data, it appears that maternal B1-type SOX functions together with the VegT/β-catenin system to regulate nodal expression and to establish the normal pattern of germ layer formation in *Xenopus*. A mechanistically conserved system appears to act in a similar manner in the zebrafish.

Keywords: SOX3; B1-SOXs; Nodals; Germ layer specification; Mesoendoderm; Embryonic axis specification; *Xenopus*; Zebrafish

Introduction

The patterning of the metazoan embryo depends upon the interplay between cellular asymmetries and inductive interactions. In many nonamniotic organisms, cellular asymmetry is initially predominant; these asymmetries are established during oogenesis, modified during meiotic maturation, and activated by fertilization (Slack, 1991). In placental mammals, the initial asymmetries are subtler and their relationship to the embryonic axes less direct; that said, there is clear evidence for asymmetries early in mouse and human development (Zernicka-Goetz, 2002). The clawed frog *Xenopus laevis* has been a particularly fruitful model system for studying the role of maternal components in axis determination and cellular differentiation. As laid, the egg has an obvious animal–vegetal axis defined overtly by the distribution of pigment and yolk platelets; that axis is modified by sperm entry to define the dorsal/ventral and anterior/posterior axes of the later embryo (Kumano and Smith, 2002; Lane and Sheets, 2002; Pandur et al., 2002). A number of maternal RNAs are asymmetrically distributed in the oocyte and mature egg (Chan and Etkin, 2001). Of these, the vegetally localized mRNA encoding the T-box containing transcription factor VegT plays a critical role in subsequent embryonic patterning. Depletion of maternal VegT mRNA by antisense oligonucleotide injection into late stage oocytes disrupts the localization of other maternal RNAs (Heasman et al., 2001) and leads to defects

* Corresponding author. Fax: +1-303-492-7744.
E-mail address: klym@spot.Colorado.edu (M.W. Klymkowsky).

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.05.019

in endomesodermal differentiation (Houston et al., 2002; Xanthos et al., 2001; Zhang et al., 1998).

Zygotic gene expression begins in earnest in *Xenopus* at the midblastula transition (MBT). Recent studies, however, indicate that developmentally critical zygotic gene expression begins earlier. Yang et al. (2002) found that zygotic expression of *Xnr5* and *Xnr6* can be detected at the 256-cell stage. *Xnr5* and *Xnr6* encode nodal-related proteins, members of the TGFβ family of secreted proteins (Chang et al., 2002; Schier, 2003). They have been implicated in both endodermal differentiation and in the establishment of the Nieuwkoop center (Takahashi et al., 2000), a dorsal–vegetal group of cells specified by the 32-cell stage (Gimlich, 1986). *Xnr5* expression is regulated by VegT and the β-catenin/TCF system (Hilton et al., 2003; Rex et al., 2002; Yang et al., 2002). Maternal VegT binds directly to sites within the *Xnr5* promoter and acts as a transcriptional activator while TCFs bind to and suppresses *Xnr5* expression. Activation of cytoplasmic β-catenin following sperm entry and cortical rotation relieves TCF-mediated repression and allows *Xnr5/6* transcription.

Another maternal factor, SOX3, regulates *Xnr5* expression and binds directly to sites within the *Xnr5* promoter (Zhang et al., 2003). SOX3 is one of a subset of SOX proteins that antagonize the β-catenin/TCF system of the early *Xenopus* embryo (Zorn et al., 1999a). SOX3 is a member of the B group of SOX proteins, defined by the presence of a HMG box with approximately 50% sequence identity to the SRY (*s*ex-*r*elated on the *Y*) protein. The B-type SOXs are the most phylogenetically conserved of the SOX proteins (Bowles et al., 2000). B-type SOXs are also conserved with respect to embryonic expression. In ascidians (Miya and Nishida, 2003), hemichordates (Lowe et al., 2003), echinoderms (Kenny et al., 1999), and vertebrates (Avilion et al., 2003; Girard et al., 2001; Penzel et al., 1997; Zhang et al., 2003), B-type SOXs are supplied maternally, distributed asymmetrically, and later expressed within the developing nervous system (Sasai, 2001; St Amand and Klymkowsky, 2001).

SOX proteins bind to DNA in a sequence-specific manner through interactions with the minor groove. Binding leads to the introduction of a sharp bend in the DNA (Bewley et al., 1998; Love et al., 1995) that can facilitate or suppress interactions between other regulatory factors (Scaffidi and Bianchi, 2001). The SOXs examined to date bind to variants of a common core consensus sequence AACAAT, although their optimal binding sites are likely to be longer (Mertin et al., 1999; van Beest et al., 2000). The β-catenin-binding LEF/TCF proteins are also HMG-box transcription factors, and the SOX binding consensus sequence defines a subset of Wnt/β-catenin-regulated LEF/TCF sites (Klymkowsky, 2004). B-type SOXs have been divided into transcriptional activator B1 and transcriptional repressor B2 subgroups (Uchikawa et al., 1999). This is clearly an oversimplification, however, given recent evidence that the B1-type SOX, SOX3, acts as transcriptional repressor in the early *Xenopus* embryo (Zhang et al., 2003; see below).

In the context of early developmental events, zygotic expression of B1-type SOXs plays a critical role. In the sea urchin, down-regulation of B1-type SOX proteins by a β-catenin-dependent process is essential for normal endodermal differentiation and morphogenesis (Kenny et al., 2003). In the mouse, the B1-type SOX, SOX2, collaborates with Oct3/4 to regulate FGF4 expression (Yuan et al., 1995). FGF4 acts in an autocrine–paracrine manner to maintain the pluripotency of embryonic and extraembryonic cells (Avilion et al., 2003; Goldin and Papaioannou, 2003). All of these studies, however, focus on zygotic B1 SOX functions. The role, if any, of maternally expressed genes is more difficult to study given the perdurance of maternally supplied proteins. In the mouse, such studies require the generation of conditional mutations, typically via a cre-lox strategy. The gene must be mutated in the oocyte lineage without disrupting oogenesis. One obvious way to circumvent this problem is to introduce a reagent that directly inactivates or inhibits the maternal protein. In the case of SOX3, we have generated such a reagent, an affinity-purified antibody directed against the C-terminal domain of the *Xenopus* SOX3 polypeptide. The antibody blocks SOX3 binding to DNA (Zhang et al., 2003) and cross-reacts with zebrafish. Using this antibody, we examined the functions of maternal B1 type SOXs in *Xenopus* and zebrafish. The results indicate a phylogenetically conserved role for B1-type SOX proteins in establishing the primary animal–vegetal axis of the early embryo through the regulation of nodallike protein expression.

Materials and methods

Embryos and their manipulation

Xenopus embryos were prepared, injected, and maintained according to standard laboratory methods (Dent et al., 1989; Sive et al., 2000). Zebrafish embryos were maintained according to standard protocols (Westerfield, 1994) and pressure injected at the 1- to 8-cell stage with antiSOX3c antibody together with fluorescein dextran (LFD) as a lineage tracer. For rescue experiments, RNA encoding SOX3ΔC-EnR was injected in combination with rhodamine dextran (LRD) as a lineage tracer. The antiSOX3c and antiXTCF3c (antiTCF3c) antibodies have been described previously (Zhang et al., 2003). Antibody concentrations were determined by Lowry assay, and all dilutions were performed using antibody injection buffer. Zebrafish embryonic extracts (approximately 16–20 postfertilization) were prepared following the Zfin web protocol (http://zfin.org/zf_info/zfbook/cont.html); immunoblot analysis was carried out as described previously (Zhang et al., 2003). For RT–PCR analyses, RNA was extracted from manipulated embryos at the 50% epiboly stage using Trizol.

Immunocytochemistry

Whole-mount immunocytochemistry of *Xenopus* was carried out as described in Dent et al. (1989) with minor modifications. Zebrafish embryos were fixed in 4% paraformaldehyde for 1–2 h at room temperature, followed by 100% methanol. Embryos were incubated in primary antibody overnight, washed, and incubated with secondary antibodies overnight. The antiSOX3c antibody was used, diluted 1:2000. Antibody staining was visualized using alkaline phosphatase-conjugated secondary antibodies (Bio-Rad) and the BCIP/NBT reaction.

Plasmids and morpholinos

pCS2 plasmids encoding *Xenopus* SOXD-V5H, SOX2-V5H, SOX3-V5H, SOX3ΔC-EnR-myc, and SOX3ΔC-VP16-myc are described in Zhang et al. (2003). The pCS2.CerS plasmid was supplied by E. DeRobertis (UCLA) (Piccolo et al., 1999); mixer and dominant negative mixer-EnR plasmids (Henry and Melton, 1998) were supplied by D. Melton (Harvard). The ability of various RNAs to rescue the effects of antiSOX3c was determined by injecting RNA into fertilized eggs followed by antibody injection. A morpholino that specifically blocks translation of the SOX17α2 RNA (Hudson et al., 1997) was generously supplied by Aaron Zorn (U. Cincinnati). A control morpholino was purchased from GeneTools, Inc.

In situ hybridization and quantitative RT–PCR analyses

Whole-mount in situ hybridization of *Xenopus* was carried following standard methods (Sive et al., 2000) using digoxigenin-labeled probes against *brachyury* (J.C. Smith, Cambridge, UK, and Wolfgang Driever, U. Frieberg, Germany), *wnt11* (K. Mowry, Brown U., USA), *endodermin* (supplied by Aaron Zorn, U. Cincinnati), *goosecoid* (Eddy DeRobertis, UCLA), *squint* and *cyclops* (David Kimmelman, U. Washington). Quantitative RT–PCR was carried as described in detail in Zhang et al. (2003). The primers for *endodermin* were [upstream: 5′-AGC AGA AAA TGG CAA ACA CAC-3′—downstream: 5′-GGT CTT TTA ATG GCA ACA GCT-3′] (Sasai et al., 1996) and those for *XSOX17*β were [upstream: 5′-CAG GTG AAG AGG ATG AAG AG-3′—downstream: 5′-CAT TGA GTT GTG GCC CTC AA-3′(Hudson et al., 1997). The primers for *Xnr5* and *Xnr5* are as described in Zhang et al. (2003). Primers for zebrafish *EF1α* were [upstream 5′-GGC CAC GTC GAC TCC GGA AAG TTC-3′—downstream 5′ CTC AAA ACG AGC CTG GCT GTA AGG-3’]; *squint* [upstream 5′-CCC ACA CCA GTA GAT GAA ACC TTC-3′—downstream 5′-CCA GGT GCC TCA GTG CAG GAA ACC′3′]; and *cyclops* [upstream 5′-GCT GCT GTT TTC AGA GCA GCA GGA C-3′—downstream 5′-CGG TAT GCA TTG TAC TTC TTA GGG-3′].

Zebrafish EST isolation and analysis

Predicted zebrafish B1-type SOX protein sequences, with the exception of DrSOX19 (Vris and Lovell-Badge, 1995), DrSOX31 (Girard et al., 2001), and DrSOX21 (Rimini et al., 1999), were taken from the Ensembl *Danio rerio* whole genome assembly Version 15.2.1 (http://www.ensembl.org/Danio_rerio/). The protein names and corresponding Ensembl/NCBI identifiers are listed in Table 3. Protein sequence alignment was performed using the Web-based ClustalW1.8 program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html), and its graphical representation was generated using Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

Results

To study the role of maternal SOX3 in *Xenopus laevis*, we first tested the effects of injecting a morpholino directed against the 5′ UTR and coding sequence of SOX3 RNA. While the morpholino induced a decrease in the level of SOX3 polypeptide and some changes in gene expression (Zhang et al., 2003), it produced no overt embryonic phenotype (data not shown). While disappointing, this result was not completely unexpected given the high level of maternal SOX3 polypeptide present (Fig. 1C) (Zhang et al., 2003) and initiation of SOX2 expression in the late blastula (Kishi et al., 2000). SOX2 has been found to be functionally similar to SOX3 in the mouse (Avilion et al., 2003) and in *Xenopus* (Kishi et al., 2000; see below). Given the lack of an overt SOX3 morpholino phenotype, we turned to injection of the antiSOX3c antibody. antiSOX3c, generated against the C-terminal 20-amino acids of the *Xenopus* SOX3 polypeptide, blocks the binding of endogenous *Xenopus* SOX3 to specific DNA target sequences (Zhang et al., 2003). When RNAs encoding epitope-tagged forms of *Xenopus* SOX2 and SOX3 were injected into fertilized eggs, it was apparent that antiSOX3c was more efficient at immunoprecipitating SOX3 than SOX2 (Fig. 1A). The C-terminal region of SOX3 detected by antiSOX3c is conserved between B1-type SOX proteins; it differs at 7 out of 20 positions from *Xenopus* SOX2 (Fig. 1B).

Injection of fertilized *Xenopus* eggs with 50 ng per embryo of antiSOX3c led to a profound gastrulation defect (Figs. 2B, D; Table 1). At lower amounts of antibody, gastrulation was more often complete, but subsequent development was often aberrant (Fig. 2C). Often a distinctive ‘taillike’ protrusion was observed (Figs. 2B, C). Four controls were used to confirm the specificity of the antiSOX3c phenotype. Incubation of the antiSOX3c antibody with the KLH-peptide antigen against which it was raised completely abolished all effects of the antibody on development (data not shown). Fertilized eggs injected with equal or higher concentrations of a similarly generated, affinity-

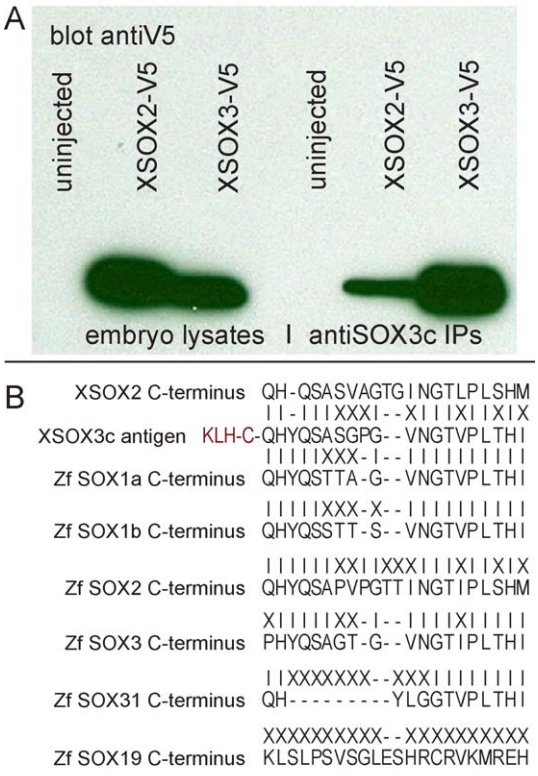

Fig. 1. AntiSOX3c selectivity. (A) To assess the degree of antiSOX3c's cross-reaction with SOX2, fertilized eggs were injected with RNA encoding epitope-tagged forms of the two polypeptides. At ~stage 8 embryonic lysates were prepared, immunoprecipitated with antiSOX3c, separated by SDS–PAGE, and analyzed by immunoblot using a mouse antiV5 antibody. While SOX2V5 was expressed at higher levels than SOX3V5, the antiSOX3c antibody preferentially precipitated the SOX3V5 polypeptide. (B) The antiSOX3c antibody was generated using the C-terminal 20 amino acids of *Xenopus* SOX3 fused to keyhole limpet hemocyanin (KLH). This region of the SOX3 polypeptide differs at 7 positions and requires one insertion and one deletion to be aligned with *Xenopus* SOX2. The various B1 type SOXs found in zebrafish (Table 4), with the exception of ZfSOX19, all share sequence similarity with the antiSOX3c epitope. (C) To assess the specificity of the antiSOX3c antibody in zebrafish, extracts of stage 8 *Xenopus* ("Xen") and 16–20 h postfertilization zebrafish ("Zeb") embryos were analyzed by immunoblot (left panel—immunoblot, right panel—Ponceau stained blot before antibody incubation). In both *Xenopus* and zebrafish extracts, the antiSOX3c antibody stains a prominent band of approximately 35 kDa (a minor band of approximately 90 kDa is occasionally seen in *Xenopus* extracts).

purified rabbit antibody directed against the C-terminal 20 amino acids of XTCF3 developed completely normally (Fig. 2A; Table 1). Immunostaining revealed the persistence of both antiSOX3c and antiTCF3c antibodies through stage 30 of development (see below). Finally, and most importantly, we were able to rescue the phenotypic effect of antiSOX3c injection in a significant percentage of embryos by injecting either SOX3ΔC-EnR or SOX2V5H RNAs. SOX3ΔC-EnR is a chimeric version of *Xenopus* SOX3 in which the C-terminal epitope recognized by antiSOX3c has been replaced by the engrailed transcriptional repressor domain. SOX3ΔC-EnR mimics the activity of wild-type SOX3 in the early *Xenopus* embryo (Zhang et al., 2003). XSOX2V5H is an epitope-tagged form of *Xenopus* SOX2 which, like SOX3, inhibits β-catenin-dependent axis formation in the early embryo (data not shown). When both blastomeres of 2-cell embryos, derived from antiSOX3c-injected eggs, were injected with RNAs encoding either SOX3ΔC-EnR or SOX2V5H, we observed rescue of the antiSOX3c phenotype in approximately 30% of embryos (Fig. 2E). When fertilized eggs were injected first with SOX3ΔC-EnR or SOX2V5H RNAs and then with antiSOX3c, we observed an increase in the extent of phenotypic rescue to approximately 40% (Fig. 2F; Table 1). No rescue was observed when antiSOX3c-injected embryos were injected with RNAs encoding SOX3ΔC-VP16, an activating version of SOX3 or the divergent, I-type SOX, SOXD (Fig. 2F; Table 1). Injection of SOX3ΔC-VP16 produces a gastrulation defect on its own (data not shown).

Characterization of the antiSOX3c phenotype

Dorsal injection of SOX3 RNA ventralizes *Xenopus* embryos (Zhang et al., 2003; Zorn et al., 1999a). In embryos that complete gastrulation, such as those shown in Fig. 2C, antiSOX3c injection appears to produce a limited dorsalization, with a dorsal–anterior index (Kao and Elinson, 1988) of between 7 and 8 (with 5 being normal). Given that a known target of SOX3 regulation, *Xnr5* (Zhang et al., 2003), is expressed very early during development (Yang et al., 2002), the late stage defects found in antiSOX3c-injected embryos are likely to be indirect. We therefore concentrated our characterization of antiSOX3c's effect on markers of differentiation expressed in late blastula/gastrula stage embryos (stages 9–12).

The T-box transcription factor *brachyury*, an immediate-early marker of mesodermal differentiation (Smith et al., 1991), is regulated in part by the Wnt–β-catenin/TCF system (Vonica and Gumbiner, 2002) and plays key roles in both mesodermal differentiation and the morphogenic movements of gastrulation (Cunliffe and Smith, 1992; Kwan and Kirschner, 2003). In uninjected and antiTCF3c-injected embryos, *brachyury* expression occurs in a uniform ring around the blastopore and within the nascent notochord (Figs. 3A, C). In antiSOX3c-injected embryos staining for *brachyury*, RNA was highly irregular—stronger in some

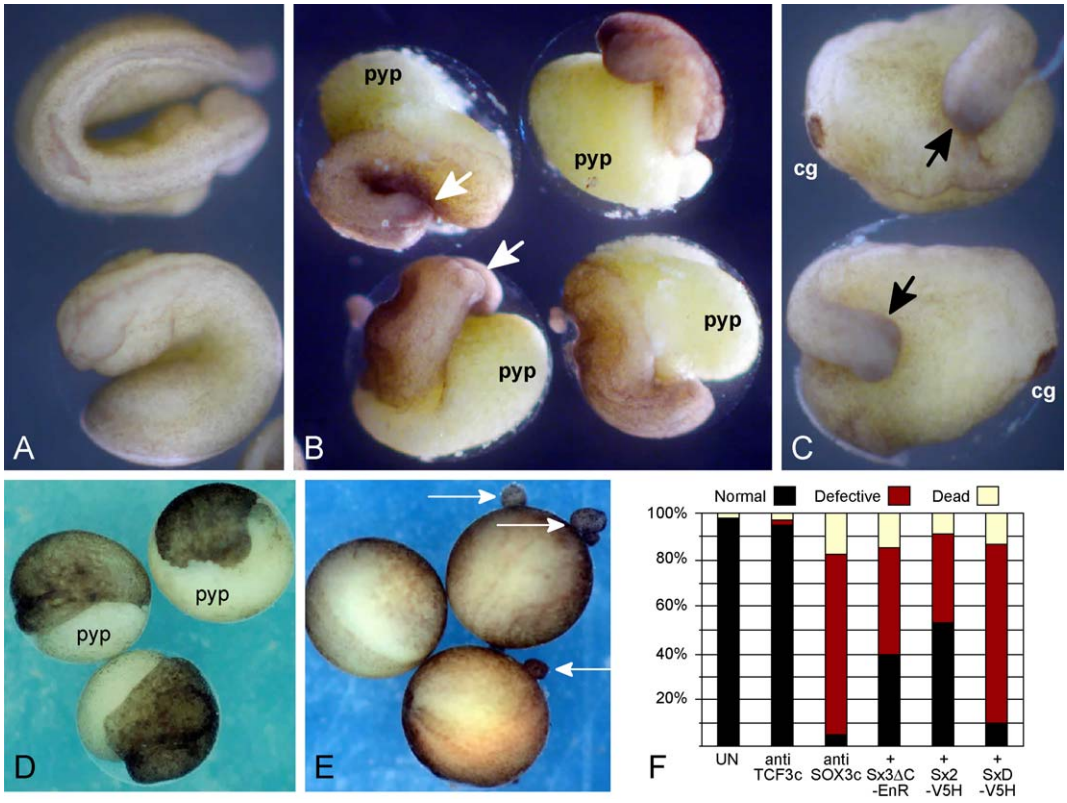

Fig. 2. AntiSOX3c effects and phenotypic rescue. Fertilized *Xenopus* eggs were injected with 50 ng of either antiTCF3c (A) or antiSOX3c (B–E) antibody and then allowed to develop until uninjected embryos (not shown) reached stage 25 (A–C) or 13/14 (D–E). AntiTCF3c-injected embryos developed normally. In the most severely effected antiSOX3c-injected embryos (B, D), gastrulation was completely aberrant. The embryos displayed a protruding yolk plug ("pyp") and often an elongated animal cap (arrows). When antiSOX3c-injected embryos successfully completed gastrulation (C), there was a distinct head, with a cement gland ("cg") and a posterior protrusion (arrows). To examine the specificity of the antiSOX3c phenotype, fertilized eggs were injected before (F) or after (data not shown) antibody injection with RNAs encoding SOX3ΔC-EnR-myc (E), SOX2V5H (not shown), or SOXDV5H (not shown). Compared to embryos injected with antiSOX3c alone, a significant percentage of embryos was phenotypically rescued by SOX3ΔC-EnR-*myc* or SOX2V5H RNA injection; SOXDV5H did not rescue the antiSOX3c phenotype (F). Injection artifacts (small protruding blebs) are indicated by white arrows in part E.

regions and missing in others (Figs. 3B, D). The *Xenopus* ortholog of *wnt11* is expressed in a pattern quite similar to, although weaker than, that of *brachyury* (Figs. 3E, F).

Table 1
AntiSOX3c rescue by SOX3ΔC-EnR and SOX2V5H in *Xenopus*

Injection	Amounts (ng per embryo)	*N*	Phenotype(%)		Dead(%)
			Normal (%)	Defect (%)	
Uninjected	NA	117	115 (98%)	0	2 (2%)
antiTCF3c	50	102	97 (95%)	2 (2%)	3 (3%)
antiSOX3c	50	161	8 (5 %)	126 (78%)	27 (17%)
antiSOX3c+ SOX3ΔC-EnR	50	121	49 (40%)	54 (45%)	18 (15%)
	0.6				
antiSOX3c+ SOX2-V5H	50	125	66 (53%)	38 (38%)	11 (9%)
	0.6				
antiSOX3c+ SOX3ΔC-VP16	50	132	8 (6%)	77 (76.5%)	23 (17.5%)
	0.6				
antiSOX3c+ SOXD-???	50	79	8 (10%)	60 (76%)	11 (14%)
	0.62				

Expression of wnt11 is directly regulated by *brachyury* (Tada and Smith, 2000). antiSOX3c injection disrupted the pattern of *wnt11* expression (Fig. 3G). For both *brachyury* and *wnt11*, there was often a dramatic animal-poleward displacement of the expression domain away from the blastopore (Figs. 3B, D, G—arrows), a pattern never observed in uninjected or antiTCF3c-injected embryos. The blastopore itself, however, was still formed in an at least superficially normal manner.

Goosecoid encodes a homeobox-containing protein expressed in dorsal (head) mesoderm (Blumberg et al., 1991). In uninjected (Fig. 3H) or antiTCF3c (Fig. 3I) injected gastrula stage embryos, *goosecoid* RNA was restricted to a domain corresponding to the Spemann organizer (Cho et al., 1991). In antiSOX3c-injected embryos, the region of *goosecoid* expression is larger and more diffuse (Fig. 3J). While expression of goosecoid inhibits brachyury expression, overexpression of brachyury does not alter goosecoid expression (Artinger et al., 1997). It is possible that the increase in goosecoid expression is responsible in part for the irregularity in brachyury expression observed in antiSOX3c-injected embryos.

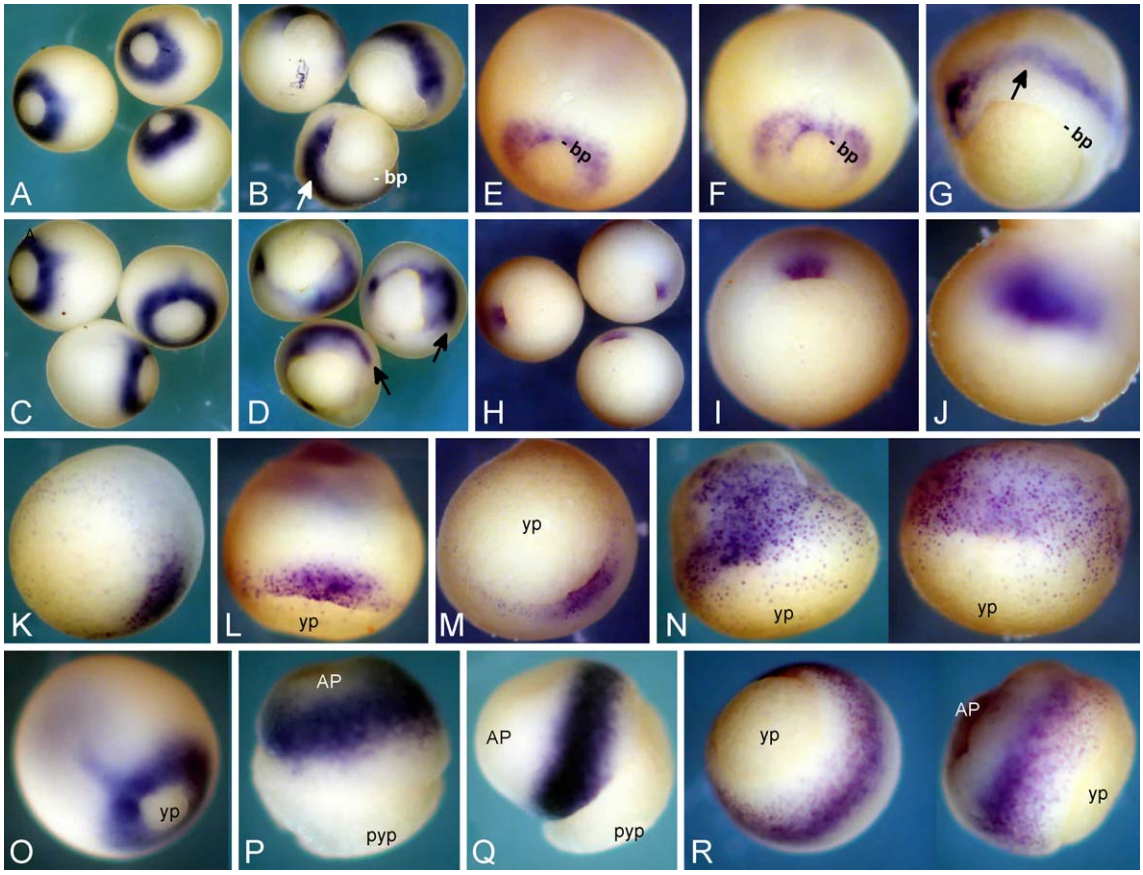

Fig. 3. AntiSOX3c effects on early mesodermal and endodermal markers. Uninjected (A), antiTCF3c-injected (C), and antiSOX3c-injected (B, D) embryos were stained in situ for *brachyury* RNA at stage 12. *Brachyury* RNA is normally found in a ring around the blastopore and more faintly along the nascent notochord (A, C). Injection of antiSOX3c disrupts this pattern of expression; *brachyury* staining was irregular and often displaced toward the animal pole of the embryo (arrows in B and D; the position of the blastopore is indicated by "-bp"). The normal pattern of *wnt11* expression is similar to that of *brachyury*, although typically less intense (E, uninjected; F, antiTCF3c). Injection of antiSOX3c produced an animalward displacement of the *wnt11* domain (G, arrow), away from the blastopore ("-bp"). The effect of antiSOX3c injection on the distribution of *goosecoid* RNA was somewhat subtler. Normally expressed in the region of the Spemann organizer (H, uninjected; I, antiTCF3c-injected), the *goosecoid* RNA domain is noticeably expanded and more diffuse in antiSOX3c-injected (J) embryos stained at stage 11. *Endodermin* RNA is a pan-endodermal marker. It is initially expressed primarily in the Spemann organizer region (K, uninjected; L, M, antiTCF3c-injected); in antiSOX3c-injected embryos (N), the *endodermin* RNA domain is dramatically expanded. In contrast to the other markers, *endodermin* RNA appears be concentrated in nuclei. Given the nonuniform distribution of injected antibody observed in embryos injected at the one-cell stage (data not shown), we injected both blastomeres of 2-cell embryos a total of 50 ng of antiSOX3c (i.e., 25 ng per blastomere). Control (O) and antiSOX3c-injected embryos (P–R) were stained for *brachyury* (O–Q) or *wnt11* (R) RNA when control embryos had reached stage 12/13. In antiSOX3c-injected embryos, the *brachyury* and *wnt11* expression domains were displaced toward the animal poll of the embryo ('yp," yolk plug; "pyp," protruding yolk plug; "AP," animal pole).

Endodermin is a pan-endodermal marker that encodes a secreted α2-macroglobulin-like protein (Sasai et al., 1996). In the intact embryo, *endodermin* expression is initially restricted to the region of the Spemann organizer (Fig. 3K) and later extends around the blastopore (Sasai et al., 1996). Endodermin expression is unaffected by antiTCF3c injection (Figs. 3L, M) but was expanded animally in response to antiSOX3c (Fig. 3N). Antibody injected at the 1-cell stage is not uniformly distributed within later stage embryos (data not shown). To examine the effects of a more uniform distribution of injected antibody, both blastomeres of the 2-cell embryo were injected with antiSOX3c (25 ng per blastomere, 50 ng per embryo total). When controls had reached stage 12/13, embryos were fixed and examined by in situ hybridization for *brachyury* (Figs. 3O–Q), *wnt11* (Fig. 3R), and *endodermin* (data not shown). In each case, the domain of marker expression was greatly expanded, particularly toward the animal pole and away from the blastopore. In the case of *brachyury*, the effect was particularly dramatic, with expression often occurring within a distinct bandlike domain of the animal cap in exogastrulating embryos (Figs. 3P, Q). Taken together, the aberrations in *brachyury*, *endodermin*, *goosecoid*, and *wnt11* expression appear sufficient to account for the gastrulation defects observed in antiSOX3c-injected embryos.

SOX3 binds to sites within the *Xnr5* promoter, and injection of antiSOX3c leads to an approximately two- to threefold increase in *Xnr5* RNA levels (Zhang et al., 2003; see below). Seeing as *Xnr5* is the earliest known zygotic target of the VegT/β-catenin system (Yang et al., 2002), we

examined whether the phenotypic effects of antiSOX3c injection could be rescued by the injection of RNA encoding Cerberus-short (CerS). Cerberus, a secreted protein that antagonizes Wnt, BMP, and nodal signaling (Piccolo et al., 1999), is required for head formation in *Xenopus* (Silva et al., 2003). CerS is an engineered form of the Cerberus protein that specifically inhibits nodal signaling (Piccolo et al., 1999). While high levels of CerS RNA injection produced an aberrant phenotype on their own, it was possible to rescue the antiSOX3c phenotype is a significant percentage of embryo by using lower levels of CerS RNA. When antiSOX3c-injected embryos were injected with 300 pg per embryo CerS RNA, approximately 40% of embryos appeared to develop normally (Figs. 4A–C, I; Table 2).

Analysis of CerS-rescued, antiSOX3c-injected embryos by whole-mount immunocytochemistry (Figs. 4D–G) or immunoblot for injected antibody (Fig. 4H) revealed no obvious difference in the amount or general distribution of antiSOX3c antibody between aberrant and rescued embry-

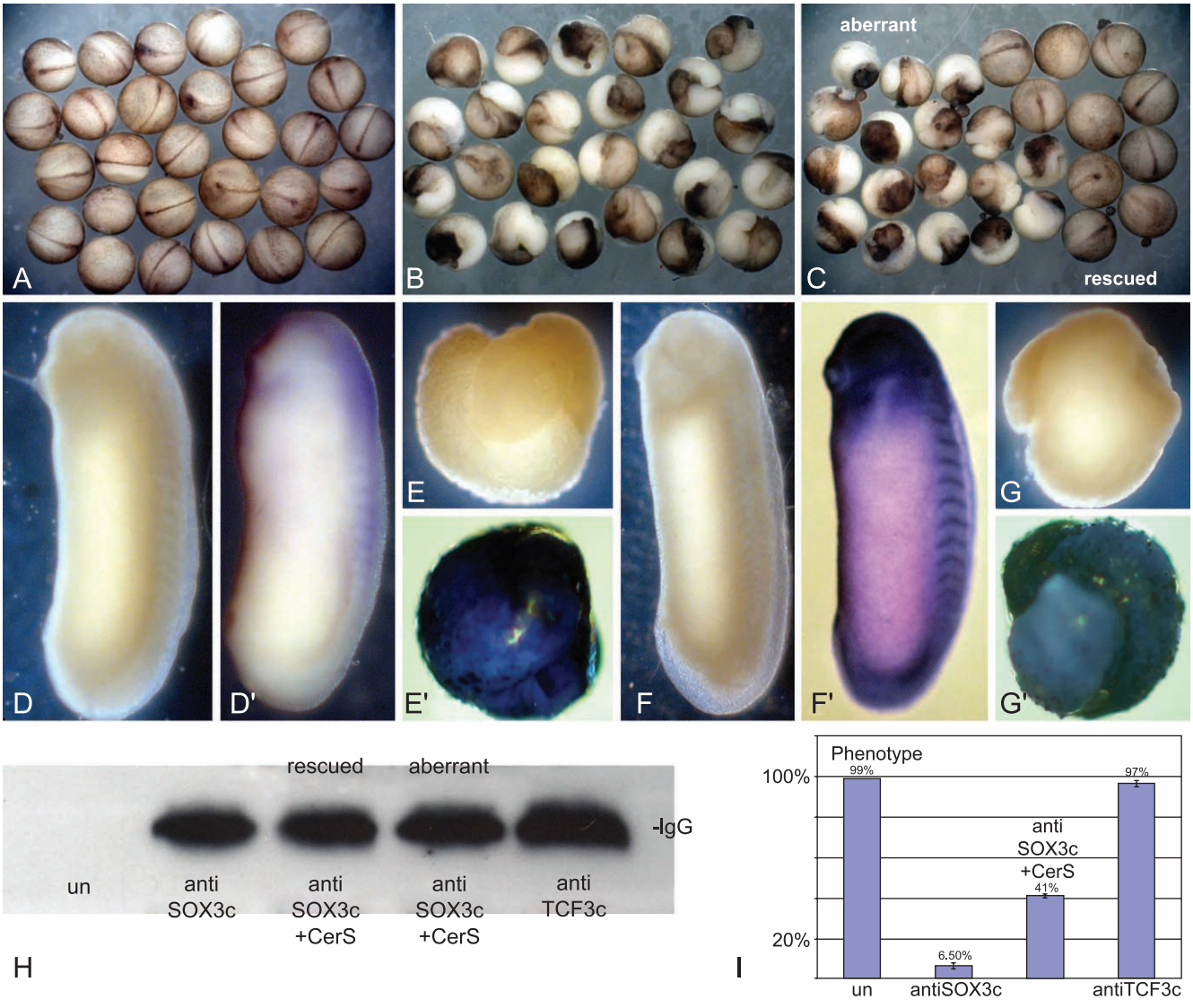

Fig. 4. CerS rescue of the antiSOX3c phenotype. Uninjected (A, D), antiTCF3c-injected (not shown), antiSOX3c alone (B, E), or antiSOX3c/CerS RNA-injected embryos (C, F–G) were analyzed. A group comparison of the phenotypes of antiSOX3c-alone (B) and antiSOX3c/CerS RNA-injected embryos (C) illustrates the uniformity of the antiSOX3c effect and the essentially complete rescue observed in approximately 40% of antiSOX3c/CerS RNA-injected embryos. These data are plotted in graphical form in part I. To confirm that rescued embryos contained antibody, embryos were fixed, bleached (D–G), and stained for rabbit immunoglobulin (D′–G′). Uninjected embryos (D, D′) are unstained. All embryos injected with antiSOX3c (E–G) were darkly stained for rabbit immunoglobulin (E′–G′). Embryos injected with both antiSOX3c and CerS RNA fell into two distinct groups, those that appeared essentially normal (F) and yet contained antiSOX3c antibody (F′) and those that were aberrant (G, G′). Since the morphology of the embryo greatly effects the apparent intensity of antirabbit immunoglobulin staining, we chose groups of five uninjected, antiTCF3c-injected, antiSOX3c-injected, normal-appearing antiSOX3c/CerS RNA-injected, and abnormal antiSOX3c/CerS RNA-injected embryos. They were homogenized, and one embryo equivalent per lane was analyzed by SDS–PAGE/immunoblot for rabbit immunoglobulin (H). No staining ("IgG," marks position of immunoglobulin heavy chain) was observed in uninjected embryos; the level of immunoglobulin in antiSOX3c, antiSOX3c + CerS RNA, and antiTCF3c-injected embryos appears quite similar.

Table 2
AntiSOX3c rescue by CerS in *Xenopus*

Injection	Amount (ng per embryo)	*N*	Phenotype(%)		Dead(%)
			Normal(%)	Defect(%)	
Uninjected	NA	290	287 (99%)	0	3 (1%)
antiTCF3c	50	173	168 (97%)	2 (1%)	3 (2%)
antiSOX3c	50	318	21 (6.6%)	267 (83.9%)	51 (9.5%)
antiSOX3c + CerS	50 0.3	216	89 (41.2%)	108 (50%)	19 (9%)

os. CerS RNA injection reduced the levels of *Xnr5* and *Xnr6* RNAs in antiSOX3c-injected embryos to levels similar to that found control embryos (Fig. 5A), suggesting that nodal activity is required to maintain *Xnr5/6* expression (Onuma et al., 2002). SOX17 is a marker of endoderm differentiation and induces expression of endodermin in animal cap experiments (Hudson et al., 1997). The initiation of SOX17 expression appears to be directly controlled by VegT, but the maintenance of expression is dependent upon nodal signaling (Clements and Woodland, 2003; Xanthos et al., 2001). Injection of antiSOX3c leads to an approximately twofold increase in SOX17β RNA levels, as measured by quantitative RT–PCR, and this increase was blocked by the injection of CerS RNA (Fig. 5A).

The dramatic effect of CerS RNA on the antiSOX3c phenotype leads us to examine whether antiSOX3c acted on genes downstream of nodal-related proteins in the endoderm specification pathway. In *Xenopus*, both the homeobox-containing protein Mixer (Henry and Melton, 1998) and

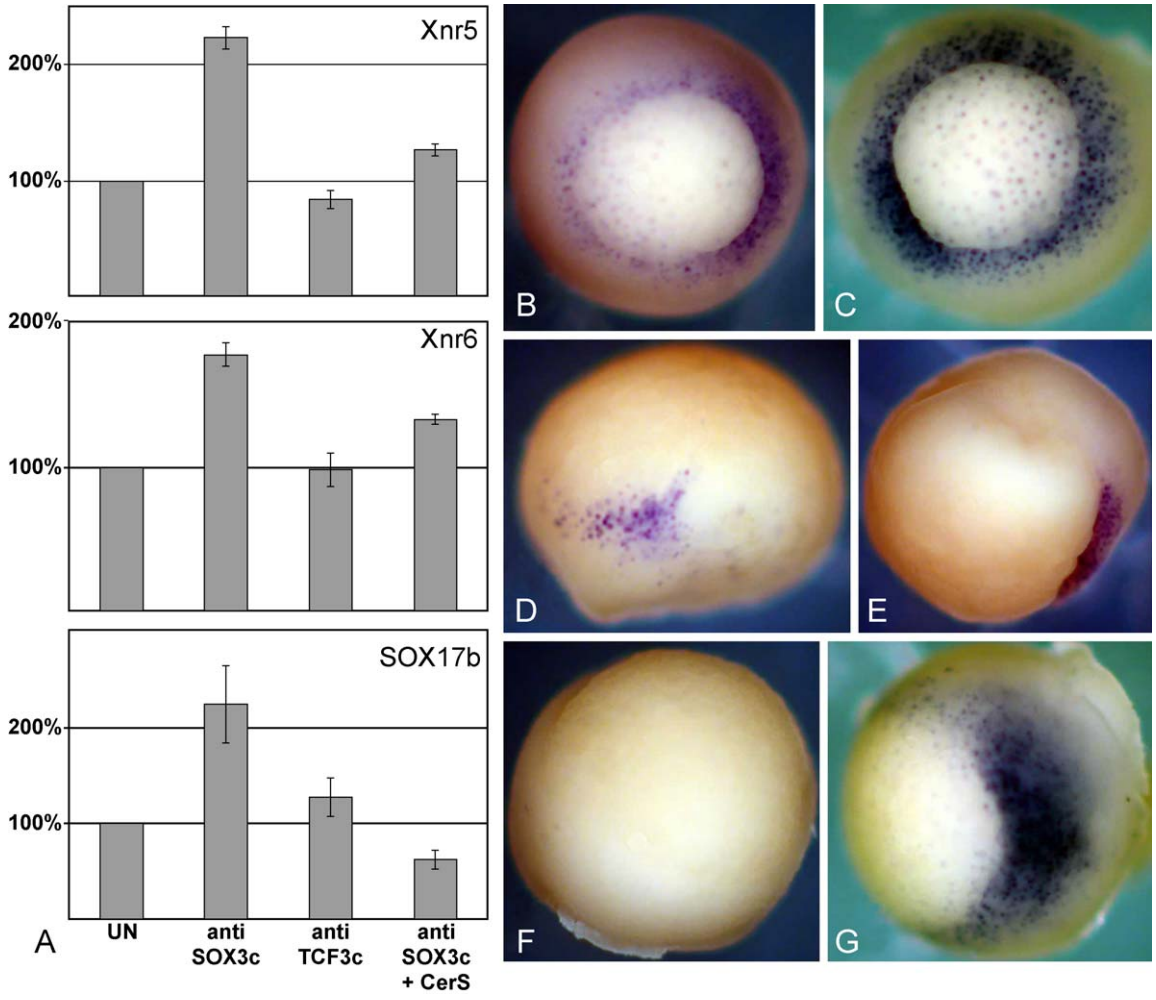

Fig. 5. (A) Quantitative RT–PCR analyses of Xnr5, Xnr6, and SOX17β were carried on uninjected ("un"), antiSOX3c-injected, antiTCF3c-injected, and antiSOX3c + CerS RNA (300 pg per embryo)-injected embryos. Embryos were injected with antibody at the 1-cell stage. In each case, injection of antiSOX3c produced an increase in target RNA levels that was reduced by the injection of CerS RNA. Xnr5 and Xnr6 analyses were done on embryos at stage 8; SOX17β analysis was done with embryos at stage 11. The effects of inhibitors of the endoderm specification pathway acting downstream of Xnr5 and 6 were analyzed by in situ hybridization analysis for endodermin in antiSOX3c-injected embryos. Uninjected embryos, fixed at approximately stage 11, display the standard pattern of endodermin expression, concentrated in the organizer region (B). Injection of antiSOX3c leads to an increase in the strength and extent of the endodermin signal (C). Injection of RNA encoding a dominant negative form of Mixer, Mixer-EnR, led to a dramatic reduction in endodermin staining in antiSOX3c-injected embryos (D and E). Similarly, injection of a morpholino directed against SOX17α2 greatly reduced endodermin staining in antiSOX3c-injected embryos (F), whereas injection of a control morpholino (G) had no effect. Quantitative date for these experiments is shown in Table 3.

Table 3
SOX17α2 MO and Mixer-EnR rescue of antiSOX3c/Edd phenotype

Injection[a]	*N*	Increased Edd[b]	Normal or reduced Edd[c]
antiSOX3c (50 ng)	36	30	6 (17%)
antiSOX3c+SOX17α2MO (20 ng)	45	5	40 (89%)
antiSOX3c+control MO (20 ng)	35	28	7 (20%)
antiSOX3c+mixer-EnR (0.5 ng)	72	30	42 (58%)

[a] RNA or morpholino was injected into the fertilized egg; antiSOX3c was injected into both blastomeres of 2-cell stage embryos. Embryos were analyzed by in situ for Edd at stage 10.
[b] Embryos were classed as "increased Edd" if there was an increase in the domain of Edd expression compared to wild-type (uninjected) embryos.
[c] Embryos were classified as "normal or reduced Edd" if domain of Edd was normal or reduced compared to wild-type (uninjected) embryos.

SOX17 (Hudson et al., 1997) are involved in the expression of endodermin. We compared uninjected embryos stained for endodermin (Fig. 5B) with embryos injected with antiSOX3c alone (Fig. 5C), antiSOX3c, and an RNA encoding a dominant negative form of Mixer, Mixer-EnR (Figs. 5D, E), antiSOX3c, and a morpholino previously against SOX17α2 (Fig. 5F) or antiSOX3c and a control morpholino (Fig. 5G; Table 3). antiSOX3c injection increases the strength and extent of the endodermin expression domain. Both Mixer-EnR and SOX17α2 morpholino decreased endodermin expression in antiSOX3c-injected embryos. Since both Mixer and SOX17α2 act downstream of nodal signaling in *Xenopus* (Shivdasani, 2002), these results indicate that the effects of antiSOX3c on endodermin expression are primarily at the level of nodal expression.

Studies in the zebrafish

A search of the published literature revealed the presence of a maternally expressed B1-type SOX, SOX31 (Girard et al., 2001), and a B2-type SOX, SOX21 (Rimini et al., 1999), in the zebrafish. Another zebrafish B1-like SOX, SOX19, has been identified (Vris and Lovell-Badge, 1995), but its expression pattern has not been described. A search of the Ensembl *Dario rerio* whole genome assembly (Version 15.2.1) revealed the presence of putative SOX1 (SOX1a and SOX1b), SOX2, and SOX3 orthologs (Table 4). Alignment of the C-termini of these sequences reveals

Table 4
Zebrafish B1-type SOX sequences

Alignment Name	Ensembl/NCBI identifier	Molecular weight
DrSOX3	ENSDARP00000004152	33417
DrSOX2	ENSDARP00000018856	34755
DrSOX1a	ENSDARP00000020525	36253
DrSOX1b	ENSDARP00000026510	36437
DrSOX31	AJ404687	32218
DrSOX19	P47792	45270
XlSOX3	Y07542	34034

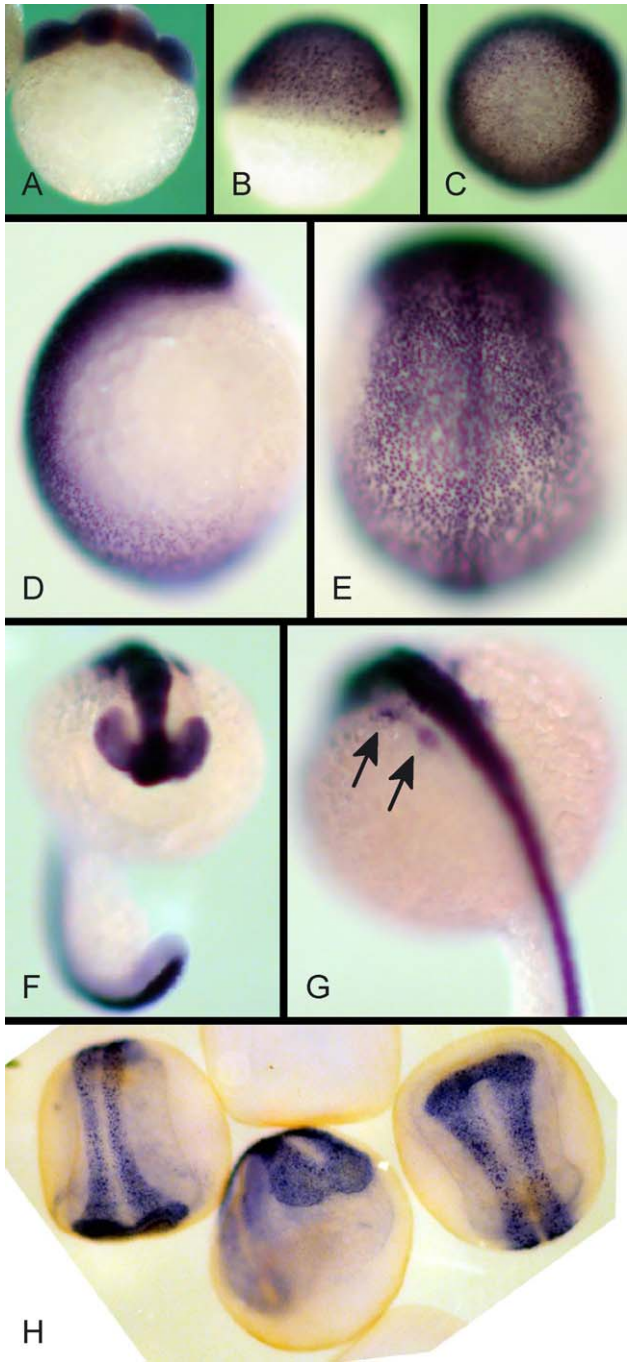

Fig. 6. antiSOX3c stains zebrafish embryos. Zebrafish (A–G) and *Xenopus* embryos (H) were stained in whole mount with antiSOX3c. At the 8-cell stage (A, lateral view) staining is restricted to the animal blastomeres. By 8 hpf/50% epiboly (B, lateral view; C, animal pole view) staining remains uniform throughout the animal cap. By 11 hpf (2-somite stage) (D, E), staining was strong and restricted to the neural plate; the punctate, nuclear localization of the staining is clearly visible. By 24 hpf (F, G), the neural tube and developing nervous system are strongly and uniformly stained. Peripheral staining (arrows in part G) has not been identified unambiguously but may correspond to fin buds. For purposes of comparison, *Xenopus* embryos at stage 18 were stained with antiSOX3c (H). These embryos were photographed after clearing; staining is nuclear and restricted to the neural plate.

similarities between *Xenopus* SOX3 and the zebrafish B1-type SOXs that are comparable to, or greater than, that between *Xenopus* SOX2 and SOX3 (Fig. 1B). Immunoblot analysis of early stage zebrafish embryos with the anti-SOX3c antibody revealed a single band of approximately 34 kDa (Fig. 1C), consistent with the predicted molecule weights of B1-type zebrafish SOX proteins (Table 4).

Zebrafish embryos were strongly stained by antiSOX3c from the earliest stages examined; staining was restricted to the animal blastomeres (Figs. 6A, B). Early on, antiSOX3c staining appeared to be primarily cytoplasmic, but it became increasingly nuclear as development proceeded (Figs. 6C, E). No staining whatsoever was seen when the primary antibody was omitted (data not shown). During early gastrulation, staining becomes restricted to the germ ring (Fig. 6C). Following gastrulation staining was restricted to the neural plate (Figs. 6D, E), much like the pattern of antiSOX3c staining seen at an analogous stage in *Xenopus* (Fig. 6H). At later stages, antiSOX3c staining of zebrafish embryos was concentrated within the neural tube and peripheral structures that may be fin buds (Figs. 6F, G). The pattern of antiSOX3c staining in the early zebrafish embryo is similar to that described for SOX31 but diverges from SOX31 in later neurula stages (Girard et al., 2001).

Fertilized zebrafish eggs injected with antiSOX3c antibody produced phenotypes that were reminiscent of those seen in *Xenopus*. Unlike in *Xenopus*, zebrafish embryos that do not gastrulate die. At high concentrations (15 ng per embryo), antiSOX3c injection led to high levels of embryonic death (Table 4). Reducing the amount of injected antibody to 12 ng per embryo led to a higher percentage of embryos that gastrulated successfully, although the level of embryonic death was still high (approximately 50% in many experiments). In those embryos that gastrulated successfully, the embryonic axis was shortened and irregular (Fig. 7A). Embryos injected with similar concentrations of antiTCF3c showed no overt phenotype and no increase in embryonic death (Table 4). As with *Xenopus*, expression of

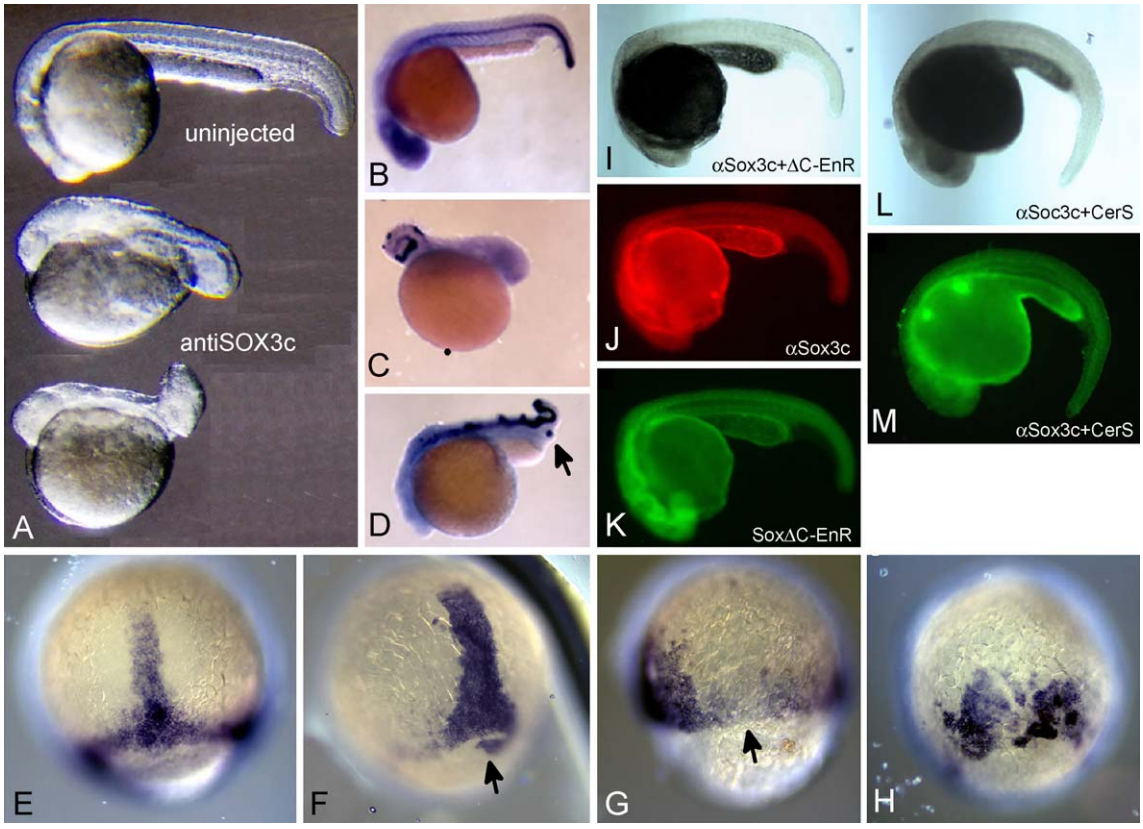

Fig. 7. Effects of antiSOX3c and rescue in zebrafish: Embryos were injected with 12 ng of antiSOX3c and cultured until 24 hpf. About 50% of embryos that were injected with antiSOX3c did not survive gastrulation. Of the remaining embryos (A), most had a distinct head but displayed a shortened body axis as compared to uninjected or antiTCF3c-injected (not shown) embryos, suggesting what may be a notochord defect. In fact, there were clear disruptions in the pattern of *brachyury* in situ staining in antiSOX3c-injected embryos (C, D) compared to control (B) and antiTCF3c-injected (not shown) embryos. The expression of *brachyury* was irregular and noncontiguous in antiSOX3c-injected embryos. Arrow in part D points to isolated region of *brachyury* expression. Embryos undergoing gastrulation (80–90% epiboly) also showed disruptions in *brachyury* expression. Compared to uninjected controls (E) in which expression is uniform around the blastopore and the nascent notochord, the domain of *brachyury* expression was thickened (F), disrupted (G, H), and often did not extend completely around the blastopore (arrow in G) in antiSOX3c-injected embryos. In rescue experiments (I—M), embryos were injected with antiSOX3c and either SOX3ΔC-EnR-*myc* (I–K) or CerS (L–M) RNAs. In the SOX3ΔC-EnR-*myc* rescue experiments, antibody was injected first together with a red fluorescent marker (J), RNA was injected 30 min later with a green fluorescent marker (K). For the CerS rescue experiments, antibody and RNA were injected together with the green fluorescent marker (M). Quantitative data for these experiments are shown in Table 5.

Table 5
AntiSOX3c effects in zebrafish[a]

Injection	Antibody (ng per embryo)	RNA (ng per embryo)	No. of embryos	No. of dead
AntiTCF3c	12	NA	@24 hpf—42/43 normal (one mild tail shortening)	0/12
AntiSOX3c+LFD[#]	15	NA		15/15
antiSOX3c+LFD	12+	NA	@ 6–14 hpf—20/54 thickened, disrupted T expression, little extension	34/54
antiSOX3c+LFD	12+	NA	@24 hpf—27/55 strong phenotype	28/55
antiSOX3c+LFD+ 30'+SOX3ΔC-EnR+LRD	12	500 pg	30/35 full-rescue (high LFD/LRD fluorescence) (lower fluorescence: three others partial rescue)	
antiSOX3c+ CerS+LFD	12	300 pg	10/34[a] full-rescue high-level LFD/antibody	
antiSOX3c+ CerS+LFD	12	120 pg	18/22 full-rescue	0/12

#Embryos injected with higher concentration of antiSOX3c antibody were all dead before gastrulation.
+Even at the lower level of 12 ng/embryo, approximately 50% of the embryos did not survive gastrulation. This death phenotype is not observed with injection of other RNAs or the antiTCF3c antibody, and so appears to be due directly to the inhibition of normal gastrulation antiSOX3. Of those embryos that gastrulate, those that contained large amounts of LFD/antibody showed strong phenotypes; embryos with lower LFD/antibody levels seemed grossly normal.
[a] All sets of injection experiments were done at least three times.

brachyury, the product of the no tail (*ntl*) locus (Schulte-Merker et al., 1994), was aberrant in a high percentage of antiSOX3c-injected embryos (Figs. 7B–H). At 6–14 h post-fertilization, staining for brachyury was thickened and disrupted (Figs. 7F–H) compared to uninjected (Fig. 7E) or antiTCF3c-injected controls (not shown) and remained irregular in embryos that survived gastrulation (Figs. 7B–D). The defects observed in embryos injected with anti-SOX3c could be rescued by the injection of RNAs encoding either *Xenopus* SOX3ΔC-EnR (7I-K) or CerS (Figs. 7L, M; Table 4). As in the case of *Xenopus*, higher levels of CerS RNA (300 pg per embryo) were less effective at rescuing the antiSOX3c effect than were lower levels (120 pg per embryo) (Table 5).

To determine whether antiSOX3c injection altered levels of nodal-related protein expression in the zebrafish, we

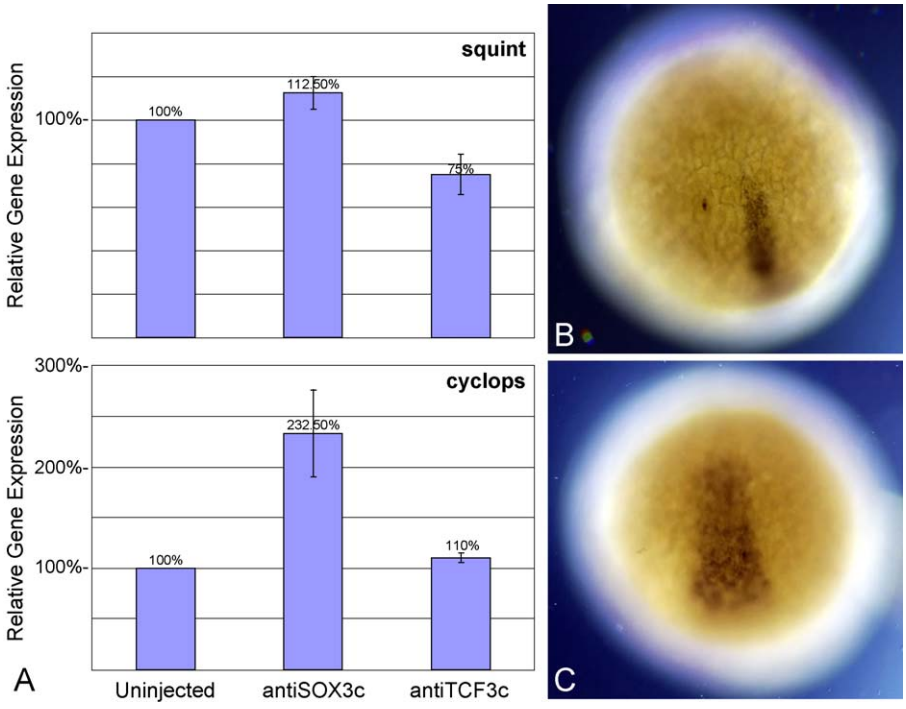

Fig. 8. AntiSOX3c injection increases cyclops RNA accumulation in zebrafish. (A) Zebrafish embryos were injected with antiSOX3c (6 ng) or antiTCF3c (6 ng) and allowed to develop to 50% epiboly (approximately 8 hpf) at which point their RNA was extracted and analyzed by quantitative RT–PCR using EF1α as a standard. While the observed effects on squint RNA level were minimal, cyclops RNA level increased over twofold in response to antiSOX3c injection. No increase in the level of either squint or cyclops RNA was observed in antiTCF3c-injected embryos. AntiTCF3c (B)- and antiSOX3c (C)-injected embryos were fixed at tailbud stage (10 hpf) and stained for cyclops and squint (data not shown). There was a clear increase in the cyclops expression domain in antiSOX3c-injected embryos.

carried out quantitative RT–PCR and in situ hybridization analyses. Two nodal-related genes, *squint* and *cyclops*, are expressed in the early zebrafish embryo (Schier, 2003). Embryos were injected with 6 ng of antiSOX3c or antiTCF3c antibody and analyzed at 50% epiboly. Injection of antiSOX3c led to a clear increase in *cyclops* RNA levels but had little effect on *squint* RNA levels (Fig. 8A). A similar result was found by in situ hybridization analysis; there was a clear and reproducible increase in the domain of *cyclops* expression (Figs. 8B, C) but little or no change in the level or extent of *squint* expression (data not shown).

Discussion

A striking and conserved feature of the B1 type SOX proteins is their maternal nature and asymmetrical distribution in metazoan embryos, including the ascidian *Halocynthia roretzi* (Miya and Nishida, 2003), hemichordate *Saccoglossus kowalevskii* (Lowe et al., 2003), the sea urchin *Strongylocentrotus purpuratus* (Kenny et al., 1999), the zebrafish (Girard et al., 2001; this work), the frog *Xenopus* (Penzel et al., 1997; Zhang et al., 2003), and the mouse (Avilion et al., 2003). The asymmetry in maternal and early zygotic B1-type SOX distribution is particularly interesting given that B1-type SOXs can antagonize β-catenin-mediated axis specification in *Xenopus* (Zhang et al., 2003; Zorn et al., 1999a) and sea urchin (Kenny et al., 2003). The β-catenin/TCF system is itself phylogenetically ancient and is involved in axial patterning from hydra (Hobmayer et al., 2000) to the mouse (Huelsken et al., 2000; Merrill et al., 2003).

The mechanism of SOX–β-catenin antagonism depends upon the specific system under study (see Klymkowsky, 2004). In the early *Xenopus* embryo, the binding of SOX3 to sites within promoter of the β-catenin–TCF target gene *Xnr5* appears to be essential for its antagonistic effects (Zhang et al., 2003). In contrast, another of the β-catenin antagonistic SOXs originally identified by Zorn et al. (1999a), SOX17, does not display detectable binding to the *Xnr5* promoter under these conditions (Basta and Klymkowsky, unpublished). Given that antiSOX3c inhibits SOX3 DNA binding (Zhang et al., 2003), it is a uniquely valuable reagent for studying the functions of the maternal protein.

The most dramatic effect of antiSOX3c injection in *Xenopus* is the disruption of normal gastrulation (Fig. 2). The effect appears to be specific since it can be rescued at reasonable frequency (approximately 40%) by the injection of RNAs encoding a version of SOX3, SOX3ΔC-EnR, and by SOX2 (Fig. 2; Table 1). While SOX2 is recognized weakly by antiSOX3c, the SOX3ΔC-EnR polypeptide lacks the epitope against which the antiSOX3c antibody was generated. SOX3ΔC-EnR acts like the wild-type SOX3 polypeptide with respect to its effects on *Xnr5* RNA accumulation (Zhang et al., 2003), suggesting that in the early *Xenopus* embryo, SOX3 acts as transcriptional repressor, at least of *Xnr5*. The antiSOX3c phenotype was not rescued by an the analogous "transcriptional activator" version of SOX3, SOX3ΔC-VP16, nor by injection of RNA encoding the unrelated SOX, SOXD (Table 1). SOXD, unlike SOX2 and SOX3, does not antagonize β-catenin-mediated dorsal axis specification (Klymkowsky, 2004; Zhang et al., 2003). Finally, another factor that argues that the antiSOX3c effect is specific is that it is complementary to the effects produced by the overexpression of SOX3. Injection of SOX3 RNA ventralizes embryos (Zhang et al., 2003; Zorn et al., 1999a), while those antiSOX3c-injected embryos that gastrulate are dorsalized (Fig. 2C).

SOX3 in the early embryo

In antiSOX3c-injected *Xenopus* embryos that fail to gastrulate, differentiation of the animal cap proceeds with the expression of muscle markers and the appearance of acetylated α-tubulin-expressing ciliated epidermal cells (data not shown). When gastrulation was at least partially successful, neuronal and notochord development occurred (data not shown) and embryos appeared partially hyperdorsalized (Fig. 2). While injected antibody persists into later stage embryos (Fig. 4), there are a number of reasons why a more thorough analysis of the terminal antiSOX3c phenotype is not likely to be particularly revealing or easily interpretable. Most importantly, the major effects of antiSOX3 appear to involve early zygotic gene targets, such as *Xnr5* and *Xnr6* (Zhang et al., 2003), *brachyury*, *wnt11*, *goosecoid*, *SOX17*, and *endodermin* (Figs. 3 and 5).

In *Xenopus*, *brachyury* expression is positively regulated by a secreted Wnt and the downstream β-catenin/TCF system (Vonica and Gumbiner, 2002). The *brachyury* protein is a positively acting regulator of its own expression (Conlon et al., 1996) and that of wnt11 (Tada and Smith, 2000). Both brachyury and wnt11, which acts through β-catenin-independent pathway, are required for normal gastrulation (Kuhl, 2002; Kwan and Kirschner, 2003). In each case, the expression domains of these genes, normally tightly restricted to the region around the blastopore, extend toward the animal pole in antiSOX3c-injected embryos (Figs. 3A–G, O–R). Quantitative RT–PCR analysis of brachyury RNA levels indicates that they increase twofold in response to antiSOX3c injection (data not shown), consistent with SOX3 acting as direct transcriptional repressor or as a β-catenin antagonist. Examination of the rather limited amount of published *brachyury* promoter sequence failed to reveal an overt SOX3 binding site, leaving unresolved whether brachyury expression is directly regulated by SOX3.

Goosecoid encodes a homeobox-containing protein and forms a regulatory circuit with *brachyury*. *Goosecoid* is normally expressed in the dorsal region of the embryo, within the Spemann organizer domain (Blumberg et al., 1991; Cho et al., 1991). antiSOX3c expands the *goosecoid* expression domain (Figs. 3H–J), which in itself would be

expected to suppress *brachyury* expression (Artinger et al., 1997; Latinkic and Smith, 1999). This in fact may provide at least a partial explanation for the irregularities in brachyury expression often observed in antiSOX3c-injected embryos (Figs. 3B, D).

Other targets of SOX3

It is clear that most "simple" changes in gene regulatory systems, such as the inactivation of a specific gene/protein, lead to complex and dynamic changes in the patterns of gene expression. In the case of SOX3, we have begun a DNA microarray-based analysis of this regulatory network. This analysis has revealed at least one gene, *Cyr61*, whose regulation may be relevant to the observed gastrulation defect produced by antiSOX3c injection. In embryos injected with antiSOX3c, *Cyr61* RNA levels are increased over twofold in blastula stage embryos (Zhang, Grow and Klymkowsky, unpublished observation), consist with SOX3 normally acting as a negative regulator of Cyr61 expression. Both up- and down-regulation of *Cyr61* expression have been reported to lead gastrulation defects in *Xenopus* (Latikinic et al., 2003).

At this point, the observed changes in *brachyury*, *wnt11*, *goosecoid*, *Cyr61* appear to be sufficient to produce the gastrulation defect observed in antiSOX3c-injected embryos. Whether the effects of inhibiting SOX3 on the expression of these genes are direct or indirect remains to be determined. It is well established that the VegT-dependent formation of endomesoderm is dependent upon the expression of nodal-related and other TGBβ family members (Agius et al., 2000; Clements et al., 1999; Osada and Wright, 1999; Rex et al., 2002; Schier, 2003; Takahashi et al., 2000; Xanthos et al., 2001). The central role of nodal-related proteins in the antiSOX3c phenotype is indicated by the ability of the nodal-specific inhibitor CerS (Agius et al., 2000; Piccolo et al., 1999) to suppress the phenotypic effects of antiSOX3c injection (Figs. 4 and 5; Table 2). Given that at least six distinct nodals are expressed in the early embryo (Onuma et al., 2002; Rex et al., 2002; Takahashi et al., 2000), and that most, if not all, of which can be expected to bind CerS, the detailed molecular interpretation of such a rescue is not completely straightforward.

We can, however, get a strong clue as to the targets of SOX3 regulation in *Xenopus* by focusing on the specification of endoderm. Endoderm, like the mesoderm and ectoderm, is patterned along the dorsal–anterior/ventral–posterior axis. This axial specification is mediated in part by a β-catenin/TCF system through a pathway that involves direct VegT regulation of SOX17 expression (Clements and Woodland, 2003; Engleka et al., 2003; Zorn et al., 1999b). Targets of early nodal activity, for example, Mixer, are required to maintain SOX17 expression and to induce endoderm, as visualized by endodermin expression (Clements and Woodland, 2003; Henry and Melton, 1998; Xanthos et al., 2001). In antiSOX3c-injected embryos, SOX17 RNA levels increase twofold (Fig. 5A), and there is a dramatic expansion of the endodermin expression domain toward the animal pole (Figs. 4K–N and Figs. 5B, C). Both a dominant negative form of Mixer and a morpholino directed against SOX17α2 block antiSOX3c effects on endodermin expression (Figs. 5D–G; Table 3) arguing that antiSOX3c acts upstream of both these genes. Given CerS's ability to rescue the antiSOX3c phenotype, we conclude that in *Xenopus*, SOX3 normally acts to repress expression of *Xnr5* and *Xnr6*, and that the antiSOX3c phenotype is primarily, if not exclusively, due to effects *Xnr5* and *Xnr6* expression (see below).

A conserved role for B1-type SOXs in endoderm formation?

Based on the observed cross-reaction of antiSOX3c with *Xenopus* SOX2 (Fig. 1A) and high degree of sequence similarity between the antiSOX3c epitope and the C-terminal tails zebrafish B1 type SOXs (Fig. 1B), we examined the ability of antiSOX3c to stain zebrafish embryos. antiSOX3c antibody reacts with a single band in immunoblots of early zebrafish embryos (Fig. 1C), and the pattern of antiSOX3c staining (Fig. 6) is analogous to that observed in *Xenopus* and similar to that described for SOX31 (Girard et al., 2001) in the early embryo. antiSOX3c staining was restricted to animal blastomeres; in later stages, staining was restricted to the neural plate (Figs. 6D–G). Injection of the antiSOX3c antibody into zebrafish embryos produced a phenotype analogous to that observed in *Xenopus* (Fig. 7); as in *Xenopus*, the antiSOX3c phenotype could be rescued by injection of RNAs encoding either SOX3ΔC-EnR or CerS

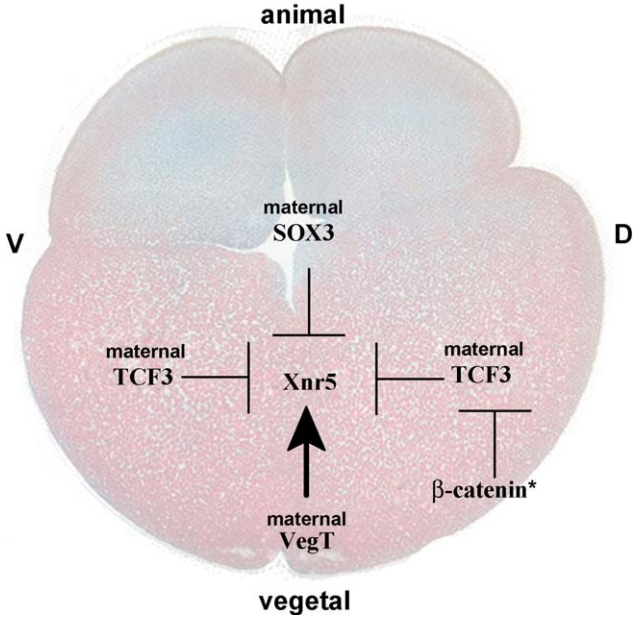

Fig. 9. Cartoon of SOX3 action in *Xenopus*. Based on the ability to rescue antiSOX3c effects by SOX3ΔC-Enr and CerS RNA injection, we propose that SOX3 acts to suppress the expression of Xnr5 in the animal region of the embryo. In the vegetal region, a combination of VegT and stabilized β-catenin ("β-catenin*") acting through TCFs activates Xnr5 expression.

(Fig. 7, Table 5). In particular, the ability of CerS to rescue the phenotype argues for a similar mechanism of action of antiSOX3c in both *Xenopus* and zebrafish, that is, that B1-type SOXs in the early zebrafish embryo act as transcriptional repressors and act specifically to regulate nodal expression. In contrast to *Xenopus*, where six different nodal-related proteins are present in the early embryo (Onuma et al., 2002; Rex et al., 2002), only three nodal-related proteins have been characterized in the zebrafish, squint (NR1), cyclops (NR2), and southpaw (NR3). Of these, only squint and cyclops are expressed in the early embryo (Schier, 2003). Quantitative RT–PCR and in situ analyses (Fig. 8) indicate that *cyclops* is the target of B1-type SOX regulation in zebrafish.

Besides their high degree of sequence conservation across diverse metazoan species, what is striking about the B1 type SOXs is their conserved pattern of maternal and asymmetric expression in deuterostomes. Taken together with the recent work of Avilion et al. (2003) and Kenny et al. (2003), it appears that maternal/early zygotic B1-type SOXs play a key role in the specification of germ layers and embryonic axes (Fig. 9). In the case of *Xenopus* and zebrafish, this process appears to involve regulation of nodal-related genes; whether nodals or another gene family is involved in sea urchin or mouse remains unclear.

Acknowledgments

We thank Matt Grow and Bob Boswell for helpful comments, Rebecca Klymkowsky for editing, and Eddy DeRobertis, Aaron Zorn, Bob Old, David Kimelman, and Wolfgang Driever for plasmids. This work was supported primarily by grant GM54001 from the NIH to MWK, with addition support from the March of Dimes Birth Defects Foundation to MWK, NIH/NIDCR K22DE14200 to KBA. DLS is supported by the Wellcome Trust.

References

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C., De Robertis, E.M., 2000. Endodermal nodal-related signals and mesoderm induction in *Xenopus*. Development 127, 1173–1183.

Artinger, M., Blitz, I., Inoue, K., Tran, U., Cho, K.W., 1997. Interaction of *goosecoid* and *brachyury* in *Xenopus* mesoderm patterning. Mech. Dev. 65, 187–196.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., Lovell-Badge, R., 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 17, 126–140.

Bewley, C.A., Gronenborn, A.M., Clore, G.M., 1998. Minor groove-binding architectural proteins: structure, function, and DNA recognition. Annu. Rev. Biophys. Biomol. Struct. 27, 105–131.

Blumberg, B., Wright, C.V.E., DeRobertis, E.M., Cho, K.W.Y., 1991. Organizer-specific homeobox genes in *Xenopus laevis* embryos. Science 253, 194–196.

Bowles, J., Schepers, G., Koopman, P., 2000. Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev. Biol. 227, 239–255.

Chan, A.P., Etkin, L.D., 2001. Patterning and lineage specification in the amphibian embryo. Curr. Top. Dev. Biol. 51, 1–67.

Chang, H., Brown, C.W., Matzuk, M.M., 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr. Rev. 23, 787–823.

Cho, K.W.Y., Blumberg, B., Steinbeisser, H., DeRobertis, E.M., 1991. Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene *goosecoid*. Cell 67, 1111–1120.

Clements, D., Woodland, H.R., 2003. VegT induces endoderm by a self-limiting mechanism and by changing the competence of cells to respond to TGF-β signals. Dev. Biol. 258, 454–463.

Clements, D., Friday, R.V., Woodland, H.R., 1999. Mode of action of VegT in mesoderm and endoderm formation. Development 126, 4903–4911.

Conlon, F.L., Sedgwick, S.G., Weston, K.M., Smith, J.C., 1996. Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. Development 122, 2427–2435.

Cunliffe, V., Smith, J.C., 1992. Ectopic mesoderm formation in *Xenopus* embryos caused by widespread expression of a *brachyury* homologue. Nature 358, 427–430.

Dent, J.A., Polson, A.G., Klymkowsky, M.W., 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. Development 105, 61–74.

Engleka, M.J., Craig, E.J., Kessler, D.S., 2003. VegT activation of Sox17 at the midblastula transition alters the response to nodal signals in the vegetal endoderm domain. Dev. Biol. 237, 159–172.

Gimlich, R.L., 1986. Acquisition of developmental autonomy in the equatorial region of the *Xenopus* embryo. Dev. Biol. 104, 117–130.

Girard, F., Cremazy, F., Berta, P., Renucci, A., 2001. Expression pattern of the Sox31 gene during zebrafish embryonic development. Mech. Dev. 100, 71–73.

Goldin, S.N., Papaioannou, V.E., 2003. Paracrine action of FGF4 during periimplantation development maintains trophectoderm and primitive endoderm. Genesis 36, 40–47.

Heasman, J., Wessely, O., Langland, R., Craig, E.J., Kessler, D.S., 2001. Vegetal localization of maternal mRNAs is disrupted by VegT depletion. Dev. Biol. 240, 377–386.

Henry, G.L., Melton, D.A., 1998. Mixer, a homeobox gene required for endoderm development. Science 281, 91–96.

Hilton, E., Rex, M., Old, R., 2003. VegT activation of the early zygotic gene *Xnr5* requires lifting of Tcf-mediated repression in the *Xenopus* blastula. Mech. Dev. 120, 1127–1138.

Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C.M., von Laue, C.C., Snyder, P., Rothbacher, U., Holstein, T.W., 2000. WNT signalling molecules act in axis formation in the diploblastic metazoan hydra. Nature 407, 186–189.

Houston, D.W., Kofron, M., Resnik, E., Langland, R., Destree, O., Wylie, C., Heasman, J., 2002. Repression of organizer genes in dorsal and ventral *Xenopus* cells mediated by maternal XTcf3. Development 129, 4015–4025.

Hudson, C., Clements, D., Friday, R.V., Stott, D., Woodland, H.R., 1997. Xsox17α and -β mediate endoderm formation in *Xenopus*. Cell 91, 397–405.

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., Birchmeier, W., 2000. Requirement for β-catenin in anterior–posterior axis formation in mice. J. Cell Biol. 148, 567–578.

Kao, K.R., Elinson, R.P., 1988. The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. Dev. Biol. 127, 64–77.

Kenny, A.P., Kozlowski, D., Oleksyn, D.W., Angerer, L.M., Angerer, R.C., 1999. SpSoxB1, a maternally encoded transcription factor asymmetrically distributed among early sea urchin blastomeres. Development 126, 5473–5483.

Kenny, A.P., Oleksyn, D.W., Newman, L.A., Angerer, R.C., Angerer, L.M., 2003. Tight regulation of SpSoxB factors is required for patterning and morphogenesis in sea urchin embryos. Dev. Biol. 261, 412–425.

Kishi, M., Mizuseki, K., Sasai, N., Yamazaki, H., Shiota, K., Nakanishi, S.,

Gurdon, J.B., 2000. Requirement of Sox2-mediated signaling for differentiation of early *Xenopus* neuroectoderm. Development 127, 791–800.
Klymkowsky, M.W., 2004. Wnt signaling networks and embryonic patterning. In: Savaneger, P. (Ed.), Rise and Fall of Epithelial Phenotype. Landes Biosciences, Georgetown. Vol. in press.
Kuhl, M., 2002. Non-canonical Wnt signaling in *Xenopus*: regulation of axis formation and gastrulation. Semin. Cell Dev. Biol. 13, 243–249.
Kumano, G., Smith, W.C., 2002. Revisions to the *Xenopus* gastrula fate map: implications for mesoderm induction and patterning. Dev. Dyn. 225, 409–421.
Kwan, K.M., Kirschner, M.W., 2003. Xbra functions as a switch between cell migration and convergent extension in the *Xenopus* gastrula. Development 130, 1961–1972.
Lane, M.C., Sheets, M.D., 2002. Rethinking axial patterning in amphibians. Dev. Dyn. 225, 434–447.
Latinkic, B.V., Smith, J.C., 1999. Goosecoid and mix.1 repress *brachyury* expression and are required for head formation in *Xenopus*. Development 126, 1769–1779.
Love, J.J., Li, X., Case, D.A., Giese, K., Grosschedl, R., Wright, P.E., 1995. Structural basis for DNA bending by the architectural transcription factor LEF-1. Nature 376, 791–795.
Lowe, C.J., Wu, M., Salic, A., Evans, L., Lander, E., Stange-Thomann, N., Gruber, C.E., Gerhart, J., Kirschner, M., 2003. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. Cell 113, 853–865.
Merrill, B.J., Pasolli, H.A., Polak, L., Rendl, M., Garcia-Garcia, M.J., Anderson, K.V., Fuchs, E., 2003. Tcf3: a transcriptional regulator of axis induction in the early embryo. Development (Epub).
Mertin, S., McDowall, S.G., Harley, V.R., 1999. The DNA-binding specificity of SOX9 and other SOX proteins. Nucleic Acids Res. 27, 1359–1364.
Miya, T., Nishida, H., 2003. Expression pattern and transcriptional control of SoxB1 in embryos of the ascidian *Halocynthia roretzi*. Zool. Sci. 20, 59–67.
Onuma, Y., Takahashi, S., Yokota, C., Asashima, M., 2002. Multiple nodal-related genes act coordinately in *Xenopus* embryogenesis. Dev. Biol. 241, 94–105.
Osada, S.I., Wright, C.V., 1999. *Xenopus* nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. Development 126, 3229–3240.
Pandur, P.D., Sullivan, S.A., Moody, S.A., 2002. Multiple maternal influences on dorsal–ventral fate of *Xenopus* animal blastomeres. Dev. Dyn. 225, 581–587.
Penzel, R., Oschwald, R., Chen, Y., Tacke, L., Grunz, H., 1997. Characterization and early embryonic expression of a neural specific transcription factor xSOX3 in *Xenopus laevis*. Int. J. Dev. Biol. 41, 667–677.
Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., DeRobertis, E., 1999. The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature 397, 707–710.
Rex, M., Hilton, E., Old, R., 2002. Multiple interactions between maternally-activated signalling pathways control *Xenopus* nodal-related genes. Int. J. Dev. Biol. 46, 217–226.
Rimini, R., Beltrame, M., Argenton, F., Szymczak, D., Cotelli, F., Bianchi, M.E., 1999. Expression patterns of zebrafish sox11A, sox11B and sox21. Mech. Dev. 89, 167–171.
Sasai, Y., 2001. Roles of Sox factors in neural determination: conserved signaling in evolution? Int. J. Dev. Biol. 45, 321–326.
Sasai, Y., Lu, B., Piccolo, S., De Robertis, E.M., 1996. Endoderm induction by the organizer-secreted factors chordin and noggin in *Xenopus* animal caps. EMBO J. 15, 4547–4555.
Scaffidi, P., Bianchi, M.E., 2001. Spatially precise DNA bending is an essential activity of the sox2 transcription factor. J. Biol. Chem. 276, 47296–47302.
Schier, A.F., 2003. Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. 19, 589–621.
Schulte-Merker, S., van Eeden, F.J., Halpern, M. El, Kimmel, C.B., Nusslein-Volhard, C., 1994. No tail (*ntl*) is the zebrafish homologue of the mouse T (*brachyury*) gene. Development 120, 1009–1015.
Shivdasani, R.A., 2002. Molecular regulation of vertebrate early endoderm development. Dev. Biol. 249, 191–203.
Silva, A.C., Filipe, M., Kuerner, K.M., Steinbeisser, H., Bela, J.A., 2003. Endogenous Cerberus activity is required for anterior head specification in *Xenopus*. Development 130, 4943–4953.
Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early Development of *Xenopus laevis*: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Slack, J.M.W., 1991. From Egg to Embryo: Regional Specification in Early Development Cambridge Univ. Press, Cambridge.
Smith, J.C., Price, B.M., Green, J.B., Weigel, D., Herrmann, B.G., 1991. Expression of a *Xenopus* homolog of *brachyury* (T) is an immediate-early response to mesoderm induction. Cell 67, 79–87.
St Amand, A.L., Klymkowsky, M.W., 2001. Cadherins and catenins, Wnts and SOXs: embryonic patterning in *Xenopus*. Int. Rev. Cytol. 203, 291–355.
Tada, M., Smith, J.C., 2000. Xwnt11 is a target of *Xenopus* brachyury: regulation of gastrulation movements via dishevelled, but not through the canonical Wnt pathway. Development 127, 2227–2238.
Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J., Asashima, M., 2000. Two novel nodal-related genes initiate early inductive events in *Xenopus* Nieuwkoop center. Development 127, 5319–5329.
Uchikawa, M., Kamachi, Y., Kondoh, H., 1999. Two distinct subgroups of group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken. Mech. Dev. 84, 103–120.
van Beest, M., Dooijes, D., van De Wetering, M., Kjaerulff, S., Bonvin, A., Nielsen, O., Clevers, H., 2000. Sequence-specific high mobility group box factors recognize 10–12-base pair minor groove motifs. J. Biol. Chem. 275, 27266–27273.
Vonica, A., Gumbiner, B.M., 2002. Zygotic Wnt activity is required for *brachyury* expression in the early *Xenopus laevis* embryo. Dev. Biol. 250, 112–127.
Vris, S., Lovell-Badge, R., 1995. The zebrafish ZF-SOX19 protein: a novel member of the Sox family which reveals highly conserved motifs outside of the DNA-binding domain. Gene 153, 275–276.
Westerfield, M., 1994. The Zebrafish Book University of Oregon Press, Eugene, OR.
Xanthos, J.B., Kofron, M., Wylie, C., Heasman, J., 2001. Maternal VegT is the initiator of a molecular network specifying endoderm in *Xenopus laevis*. Development 128, 167–180.
Yang, J., Tan, C., Darken, R.S., Wilson, P.A., Klein, P.S., 2002. β-catenin/Tcf-regulated transcription prior to the midblastula transition. Development 129, 5743–5752.
Yuan, H., Corbi, N., Basilico, C., Dailey, L., 1995. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev. 9, 2635–2645.
Zernicka-Goetz, M., 2002. Patterning of the embryo: the first spatial decisions in the life of a mouse. Development 129, 815–829.
Zhang, J., Houston, D.W., King, M.L., Payne, C., Wylie, C., Heasman, J., 1998. The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. Cell 94, 515–524.
Zhang, C., Basta, T., Jensen, E.D., Klymkowsky, M.W., 2003. The β-catenin/VegT-regulated early zygotic gene *Xnr5* is a direct target of SOX3 regulation in *Xenopus*. Development 130, 5609–5624.
Zorn, A.M., Barish, G.D., Williams, B.O., Lavender, P., Klymkowsky, M.W., Varmus, H.E., 1999a. Regulation of Wnt signaling by Sox proteins: XSox17α/β and XSox3 physically interact with β-catenin. Mol. Cell 4, 487–498.
Zorn, A.M., Bulter, K., Gurdon, J.B., 1999b. Anterior endomesoderm specification by Wnt/β-catenin and TGF-β signaling pathways. Dev. Biol. 209, 282–297.