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Developmental Biology 278 (2005) 526–541

DEVELOPMENTAL BIOLOGY

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Genomes & Developmental Control

SOX7 is an immediate-early target of VegT and regulates Nodal-related gene expression in *Xenopus*

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Received for publication 3 August 2004, revised 18 October 2004, accepted 9 November 2004
Available online 17 December 2004

Abstract

In zebrafish, the divergent F-type SOX *casanova* acts downstream of Nodal signaling to specify endoderm. While no *casanova* orthologs have been identified in tetrapods, the F-type SOX, SOX7, is supplied maternally in *Xenopus* (Fawcett and Klymkowsky, 2004. GER 4, 29). Subsequent RT-PCR and section-based in situ hybridization analyses indicate that SOX7 mRNA is localized to the vegetal region of the blastula-stage embryo. Overexpression and maternal depletion studies reveal that the T-box transcription factor VegT, which initiates mesoendodermal differentiation, directly regulates SOX7 expression. SOX7, but not SOX17 (another F-type SOX), binds to sites within the *Xnr5* promoter and SOX7, but not SOX17, induces expression of the Nodal-related genes *Xnr1, Xnr2*, *Xnr4*, *Xnr5*, and *Xnr6*, the homeodomain transcription factor *Mixer*, and the endodermal marker *SOX17β*; both SOX7 and SOX17 induce expression of the pan-endodermal marker *endodermin*. SOX7's induction of Xnr expression in animal caps is independent of Mixer and Nodal signaling. In animal caps, VegT's ability to induce *Mixer* and *Edd* appears to depend upon SOX7 activity. Whole embryo experiments suggests that vegetal factors partially compensate for the absence of SOX7. Based on the antagonistic effects of SOX7 and SOX3 (Zhang et al., 2004. Dev. Biol. 273, 23) and their common binding sites in the *Xnr5* promoter, we propose a model in which competitive interactions between these two proteins are involved in refining the domain of endodermal differentiation.
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Keywords: SOX7; VegT; Mesoendodermal gene regulatory network; Nodal-related proteins; F-type SOXs; *Xenopus*

Introduction

Establishing embryonic germ layers involves maternal factors, cellular asymmetries, inductive interactions, and mechanical movements (Keller et al., 2003; Moon and Kimelman, 1998; Slack, 1993). Because of its unique experimental accessibility, much of our understanding of the regulatory mechanics that underlie vertebrate development is based on studies of the clawed frog *Xenopus laevis*. In *Xenopus*, a number of maternal mRNAs are localized asymmetrically within the oocyte and egg (Heasman et al., 2001; Kloc et al., 2001; Rebagliati et al., 1985). Of these, the T-box type transcription factor VegT—also known as BraT, Xombi, Antipodean, and Xtbx6—plays a central role in defining the developmental trajectory of the early embryo (Kavka and Green, 2000; Loose and Patient, 2004; Showell et al., 2004). Localized to the vegetal cortex of late stage oocytes (Zhang and King, 1996), VegT RNA is essential for mesoendodermal induction (Xanthos et al., 2001; Zhang et al., 1998).

In *Xenopus*, sperm entry leads to the cytoplasmic reorganization of the egg and the preferential stabilization and nuclear localization of β-catenin on the side of the blastula opposite the sperm entry point (Moon and Kimelman, 1998). β-Catenin binds to a number of different proteins, most notably to members of the LEF/TCF family of high mobility group (HMG)-box transcription factors. In *Xenopus*, TCF1, TCF3, and TCF4 are supplied maternally (Roel et al., 2003) and act as transcriptional repressors in the absence of bound β-catenin; stabilization of β-catenin acts to derepress TCF-repressed target genes (Brantjes et al., 2001; Hilton et al., 2003; Houston

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doi:10.1016/j.ydbio.2004.11.008

et al., 2002; Klymkowsky, 1997). A similar situation applies in the sea urchin (Kenny et al., 2003), the zebrafish (Kim et al., 2000; Sumoy et al., 1999), and the mouse (Huelsken et al., 2000; Merrill et al., 2004). In the vegetal region of the early embryo, VegT and β-catenin/TCF combine to activate an array of target genes involved in mesoendodermal and dorsal–ventral specification (see Loose and Patient, 2004). In particular, VegT and activated β-catenin are required for the expression of *Xnr5* and *Xnr6*, which encode Nodal-related proteins (Hilton et al., 2003; Onuma et al., 2002; Rex et al., 2002; Takahashi et al., 2000; Yang et al., 2002) (see below). In turn, Xnr5 and Xnr6 can induce the expression of *Xnr1*, *Xnr2*, and *Xnr4* (Takahashi et al., 2000).

The Nodal-related proteins are members of the transforming growth factor-β (TGFβ) family of secreted proteins (Schier, 2003; Whitman, 2001). Nodals are chordate specific, there is a single Nodal in the mouse, three Nodal-related genes in the zebrafish (*squint*, *cyclops*, and *southpaw*), and five (*Xnr1*, *2*, *4*, *5*, and *6*—this excludes the structurally and functionally divergent *Xnr3*) in *X. laevis* (Schier, 2003). TGFβs regulate gene expression through ligand-regulated serine/threonine kinases and SMADs (Derynck et al., 2003), and interactions between SMADs, β-catenin, and LEF/TCF proteins have been described (Hu et al., 2003; Labbe et al., 2000; Nishita et al., 2000).

In both the early *Xenopus* and zebrafish embryos, signaling via Nodals acts through Mix-related paired-type homeobox-containing transcription factors to specify mesoendodermal differentiation (Henry and Melton, 1998; Kikuchi et al., 2000; Trinh et al., 2003). Many of the effects of depleting maternal VegT RNA can be rescued by injection of mRNAs encoding Nodals, but not by RNA encoding Mixer (Xanthos et al., 2001). *Mixer* appears to be specific to the endodermal specification pathway (Henry and Melton, 1998), whereas Nodals are also involved mesodermal induction (see Schier, 2003). In both *Xenopus* and zebrafish, *SOX17* acts downstream of *Mixer* in the endodermal specification pathway (Henry and Melton, 1997; Schier, 2003; Shivdasani, 2002). *SOX17* plays a conserved role in endodermal differentiation in *Xenopus* (Hudson et al., 1997), zebrafish (Alexander and Stainier, 1999), and mouse (Kanai-Azuma et al., 2002).

SOX-type transcription factors share a conserved HMG-box, minor groove DNA-binding domain. The SOX family is large and SOXs have been placed into 10 types based primarily on HMG box sequence (Bowles et al., 2000). SOX proteins bind to specific DNA sequences, characterized by a core consensus sequence ATTGTT, although flanking sequences and accessory factors are often involved in SOX–DNA interactions (van Beest et al., 2000; Weiss, 2001; Wilson and Koopman, 2002). In addition, binding to "off-consensus" sequences has been reported (Luster and Rizzino, 2003). In *X. laevis*, there are two distinct SOX17 paralogs, SOX17α and SOX17β; two alloalleles of SOX17α, SOX17α1, and SOX17α2 have also been identified (Clements et al., 2003; Hasegawa et al., 2002).

Our interest in SOXs was sparked by the observation that SOX17 (an F-type SOX) and SOX3 (a B-type SOX) antagonize the β-catenin-dependent process of dorsal axis specification in *Xenopus* (Zorn et al., 1999). β-Catenin regulates gene expression through its interactions with LEF/TCF-type transcription factors. While the accepted consensus SOX binding site ATTGTT is a subset of LEF/TCF-binding consensus sequence $^{A}/_{T}$ $^{A}/_{T}$ $^{C}/_{G}$ AA $^{A}/_{T}$ $^{C}/_{G}$ (Klymkowsky, 2004; van Beest et al., 2000), it appears that the ability of SOXs to antagonize β-catenin signaling is not due to a direct competition between SOXs and TCFs for DNA binding sites. Rather, two distinct mechanisms have been identified. First, a number of SOX proteins, including SOX1, SOX2, SOX3, SOX9, and SOX17, bind to β-catenin (Akiyama et al., 2004; Kan et al., 2004; Sinner et al., 2004; Zhang et al., 2003; Zorn et al., 1999; unpublished observation). Their binding to β-catenin presumably enables them to compete with LEF/TCFs for this critical transcriptional cofactor. Second, the regulatory region of the β-catenin/TCF-regulated *Xnr5* gene contains distinct binding sites through which SOXs act to modulate gene expression (Zhang et al., 2003, 2004).

A divergent F-type SOX, encoded by the gene *casanova*, is a key mediator of endodermal differentiation in zebrafish (Alexander et al., 1999; Aoki et al., 2002; Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). *Casanova* acts downstream of Nodal/Mixer and upstream of SOX17 (Schier, 2003; Shivdasani, 2002) in the endodermal pathway. While no *casanova* orthologs have been identified in tetrapods, the F-type SOX, SOX7, is expressed maternally in *Xenopus* (Fawcett and Klymkowsky, 2004) in late stage mouse oocytes (Taniguchi et al., 1999) and parietal endoderm (Kanai-Azuma et al., 2002; Murakami et al., 2004). Studies on human SOX7 indicate that it, like SOX3 and SOX17, can inhibit the β-catenin-induced activation of the TCF-responsive TOPFLASH reporter in culture cell assays (Takash et al., 2001).

In the course of studies on the later larval functions of SOXs, we examined the regulation and early embryonic functions of the F-type SOX, SOX7. RT-PCR studies indicate that SOX7 mRNA is localized to the vegetal hemisphere of the early embryo and its expression is induced by, and dependent upon, VegT. SOX7 can induce endodermal markers in *Xenopus* animal caps and can circumvent the inhibition of endodermal differentiation produced by a dominant-negative version of VegT in intact embryos. We present evidence that SOX7, like SOX3, is a direct regulator of *Xnr5* and is required for VegT-induced expression of endodermal markers in animal caps.

Materials and methods

Oocytes, embryos, and animal caps

Fertilized *Xenopus* eggs were generated following established laboratory protocols (Dent et al., 1989; Sive

et al., 2000). Embryos were staged according to Nieuwkoop and Faber (1967). Animal caps were generated from stage 8 embryos using a Gastromaster™ (Xenotech) as described previously (Zhang et al., 2003). In experiments involving hormone activation of chimeric polypeptides in animal caps, caps were first treated for 30 min with the protein synthesis inhibit emetine (10 μg/ml) (Sigma), and then with dexamethasone (2 μM) (Sigma), as described by Clements and Woodland (2003) (see also Sive et al., 2000). For RNA and injection experiments, fertilized eggs and two-cell stage embryos were injected with 10–20 nl of solution following established laboratory protocols (Zhang et al., 2003). Embryos were injected with 1 ng/embryo of SOX7-GFP, SOX7ΔC-EnR, or CerS RNAs, 0.5 ng/embryo Mixer or Mixer-EnR RNAs, or 50–100 pg/embryo VegT or VegT-EnR RNAs. Oocyte VegT RNA depletion experiments were carried out by Jing Yang and Peter Klein (HHMI/U. Penn Med. School) as described by Zhang et al. (1998) using an antisense oligonucleotide (5–7.5 ng injected per oocytes) directed against the VegT mRNA; they supplied us with RNAs or cDNA from stage 10.5 control and experimentally manipulated embryos.

Plasmids and RNAs

The full-length coding region of SOX7 (Fawcett and Klymkowsky, 2004) was subcloned into the pCS2mt-GFP plasmid to form pCS2.SOX7-GFP; the myc-tag region of the pCS2mt-GFP plasmid was removed. For use in morpholino-rescue experiments, an alternative version of SOX7 was constructed using PCR and subcloned to form pCS2.altSOX7-GFP. In this alternative version of SOX7, the wild-type sequence ATG.ACT.ACC.CTG.ATG.GGA.T (against which a morpholino was directed-see below) was changed to ATG.AC**G**.AC**A**.**T**T**A**.ATG.GG**G**.T; these changes are silent with respect to coding. The region of SOX7 encoding amino acids 1–116 was amplified and subcloned into pCS2-SOX3ΔC-EnR-myc (Zhang et al., 2003) to form pCS2-SOX7ΔC-EnR-myc. The plasmid encoding VegT-EnR (originally BraT-EnR) (Horb and Thomsen, 1997) was supplied by Gerry Thomsen (SUNY, Stony Brook); pCS2-VegT plasmids were supplied by Mary Lou Ling (U. Florida) (Zhang and King, 1996) and Janet Heasman (U. Cincinnati); the mt-VegT-GR (glucocorticoid regulated domain) plasmid was constructed using a VegT-GR plasmid made by Dan Kessler (U. Penn), it is similar to the VegT-GR plasmid described by Clements and Woodland (2003); the VegT-GR sequence was subcloned by Jing Yang and Peter Klein (U. Penn) into pCS2mt to form pCS2mt-VegT-GR; pCS2-Mixer and pCS2-Mixer-EnR plasmids (Henry and Melton, 1998) were supplied by Doug Melton (Harvard). A plasmid encoding SOX17β was supplied by Aaron Zorn (U. Cincinnati); the coding sequence was amplified and subcloned into the pCS2mycG and pCS2mt-GFP plasmids (Klymkowsky et al., 1999) to form pCS2mG-SOX17β and pCS2mt-SOX17β-GFP. RNA was synthesized from linearized plasmids using Ambion mMessage mMachine transcription kits. Based on the sequence of SOX7 (Fawcett and Klymkowsky, 2004; Shiozawa et al., 1996), we designed a fluorescein-conjugated morpholino directed against the 5′ UTR and coding sequence [5′-ATCCCATCAGGGTAGT**CAT**TATTCC-3′] (the start coding binding site is underlined). A standard unlabeled control morpholino was purchased from GeneTools, Inc. and used in control injections. Fertilized eggs were injected with 20 ng/embryo morpholino. In whole embryo experiments, embryos were injected at multiple animal and vegetal sites.

In situ hybridization, imaging, and quantitative RT-PCR

Digoxygenin-labeled antisense RNA probes against *SOX3*, *SOX7*, *SOX17*, *Xbra*, and *endodermin* mRNAs were prepared and in situ hybridization was carried out following standard laboratory protocols (Sive et al., 2000). In some cases, fixed embryos were cut longitudinally along the animal–vegetal axis prior to hybridization. All photographs were taken using a Nikon CoolPix 995 Camera on an Inverted Leica M400 Photomicroskop. Images were manipulated using Macromedia Fireworks Software using the “auto levels” and “curves” functions only. Quantitative RT-PCR was carried out as described in Zhang et al. (2003) following the method of Kofron et al. (2001). Primers used for RT-PCR are listed in Table 1.

DNA fishing and immunochemistry

Biotinylated-DNA oligonucleotide affinity analysis (DNA fishing) was carried out using stage 8/9 embryo lysates, the SOX-SOX/TCF (SS/T) binding region of the *Xnr5* promoter, and the MUT2 and MUT3 DNA molecules, as described previously (Gabrielsen et al., 1989; Zhang et al., 2003). Briefly, biotinylated DNAs were bound to streptavidin-agarose beads (Sigma). For each experimental condition, 100–300 μl of embryonic lysate (5–15 embryo equivalents) was made 1× in binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 12% glycerol, 0.5 mM EDTA, 0.1% Triton X-100, 1 mM DTT, and 0.5 μg/ml Herring Sperm DNA). After a 10-min incubation, 50 μl of DNA-streptavidin agarose beads was added, incubated with lysate for 15 min at room temperature with constant, gentle mixing; the beads were then recovered by centrifugation, washed twice with binding buffer, and bound proteins were eluted with 2× SDS-sample buffer, denatured, and analyzed by SDS-PAGE and immunoblot. The antiSOX3c and antiTCF3n antibodies (Zhang et al., 2003) were used to visualize endogenous SOX3 and TCF3, respectively; SOX7-GFP and SOX17-GFP poly-

Table 1
PCR primer sets for RT-PCR

Gene	Primer sequences	Source
VegT		
Endodermin (Edd)	F 5′-AGC AGA AAA TGG CAA ACA CAC-3′ R 5′-GGT CTT TTA ATG GCA ACA GGT-3′	(Sasai et al., 1996)
Mixer	F 5′-CAC CAG CCC AGC ACT TAA CC-3′ R 5′-CAA TGT CAC ATC AAC TGA AG-3′	(Engleka et al., 2001)
Brachury (Bra)	F 5′-GCT GGA AGT ATG TGA ATG GAG-3′ R 5′-TTA AGT GCT GTA ATC TCT TCA-3′	(Agius et al., 2000)
Xnr1	F 5′-TGG CCA GAT AGA GTA GAG-3′ R 5′-TCC AAC GGT TCT CAC TTT-3′	(Kofron et al., 1999)
Xnr2	F 5′-ATC TGA TGC CGT TCT AAG CC-3′ R 5′-GAC CTT CTT CAA CCT CAG CC-3′	(Takahashi et al., 2000)
Xnr4	F 5′-TTA CAA GAT GCT GCA CAC TCC-3′ R 5′-AAC TCT GCA TGT ATG CGT GG-3′	(Takahashi et al., 2000)
Xnr5	F 5′-TCA CAA TCC TTT CAC TAG GGC-3′ R 5′-GGA ACC TCT GAA AGG AAG GC-3′	(Yang et al., 2002)
Xnr6	F 5′-TCC AGT ATG ATC CAT CTG TTG C-3′ R 5′-TTC TCG TTC CTC TTG TGC CTT-3′	(Takahashi et al., 2000)
SOX7[a]	F 5′-CCT CAT GCT CTG AAG ATT GCC-3′ R 5′-GGT CAA TGG TGT CAT AAT GG-3′	This work
SOX7 coding	F 5′-CCC ATC GTA GAC TAC CCT CAT GGG ATC C R 5′-CCC GTC GAC TAG[b] GAA ACA CTA TAA CTG	This work
SOX11	F 5′-GAA GGC GCC AGT ATG TAC GAG G R 5′-GCT ACA AGA GCC TAA GTC CTT G	This work
SOX17β	F 5′-CAG GTG AAG AGG ATG AAG AG-3′ R 5′-CAT TGA GTT GTG GCC CTC AA-3′	(Engleka et al., 2001)
Ornithine decarboxylase (ODC)	F 5′-CAG CTA GCT GTG GTG TGG-3′ R 5′-CAA CAT GGA AAC TCA CAC-3′	(Agius et al., 2000)

[a] These primers were used in all experiments, except for those described in Fig. 1F. Both primer pairs amplify SOX7 plasmid DNA and fail to amplify SOX7ΔC-EnR plasmid DNA, which does not contain the sequence recognized by the reverse primer.
[b] Silent polymorphism between sequence reported by Shiozawa et al. (1996) and the cDNA we isolated (Fawcett and Klymkowsky, 2004); underlined sequences were added for cloning purposes.

peptides were visualized using an anti-GFP antibody (Invitrogen).

Results

SOX7 was originally isolated from a *Xenopus* ovarian cDNA library by Shiozawa et al. (1996). SOX7 mRNA is present maternally and throughout early embryogenesis (Fawcett and Klymkowsky, 2004). A simple BLASTp search (Fig. 1A) revealed the presence of an unambiguous ortholog of SOX7 in zebrafish (GenBank Accession #AY423014). The zebrafish gene *casanova* encodes a SOX protein whose HMG box is most similar to that of the F-type SOXs (Figs. 1A and B) (Sinner et al., 2004). In zebrafish, *casanova* acts downstream of the Nodals *Cyclops* and *Squint*, and *Mixer* (bon), and upstream of *SOX17* in the endoderm specification pathway (Fig. 1C) (Shivdasani, 2002).

To determine whether SOX7 might play an analogous role in *X. laevis*, we extended our characterization of its expression in early embryonic stages. Originally, we had concluded, based on whole-mount in situ hybridization, that SOX7 RNA was uniformly distributed in the early blastula stage embryo (Fawcett and Klymkowsky, 2004). This conclusion appears to be incorrect due to weak nonspecific staining by the anti-sense SOX7 probe. When stage 8/9 embryos were bisected along the animal–vegetal axis prior to in situ hybridization, it was clear that SOX7 RNA staining, while weak, was localized to the vegetal hemisphere (Fig. 2C). It is worth noting that in their original characterization of SOX7, Shiozawa et al. reported that they were unable to visualize in situ staining for SOX7 at any embryonic stage. Strong vegetal staining was found for SOX17 RNA (Fig. 2B), while SOX3 RNA appears to be restricted to the animal region (Fig. 2A). This result was confirmed by cutting blastula stage embryos into animal caps and vegetal masses; these were analyzed by RT-PCR either immediately or after culturing until control embryos reached stage 16 (Fig. 2D); in both cases, SOX7 RNA was found to be restricted to the vegetal region. Similar results were obtained using a second primer pair directed against the full coding region of SOX7 (Fig. 2E). In contrast to SOX7, and like SOX3, SOX11 mRNA (Hiraoka et al., 1997) was localized to the animal hemisphere of blastula stage embryos (Fig. 2D).

SOX7 induces endodermal markers

To determine whether SOX7 could induce endodermal differentiation, fertilized eggs were injected with in vitro

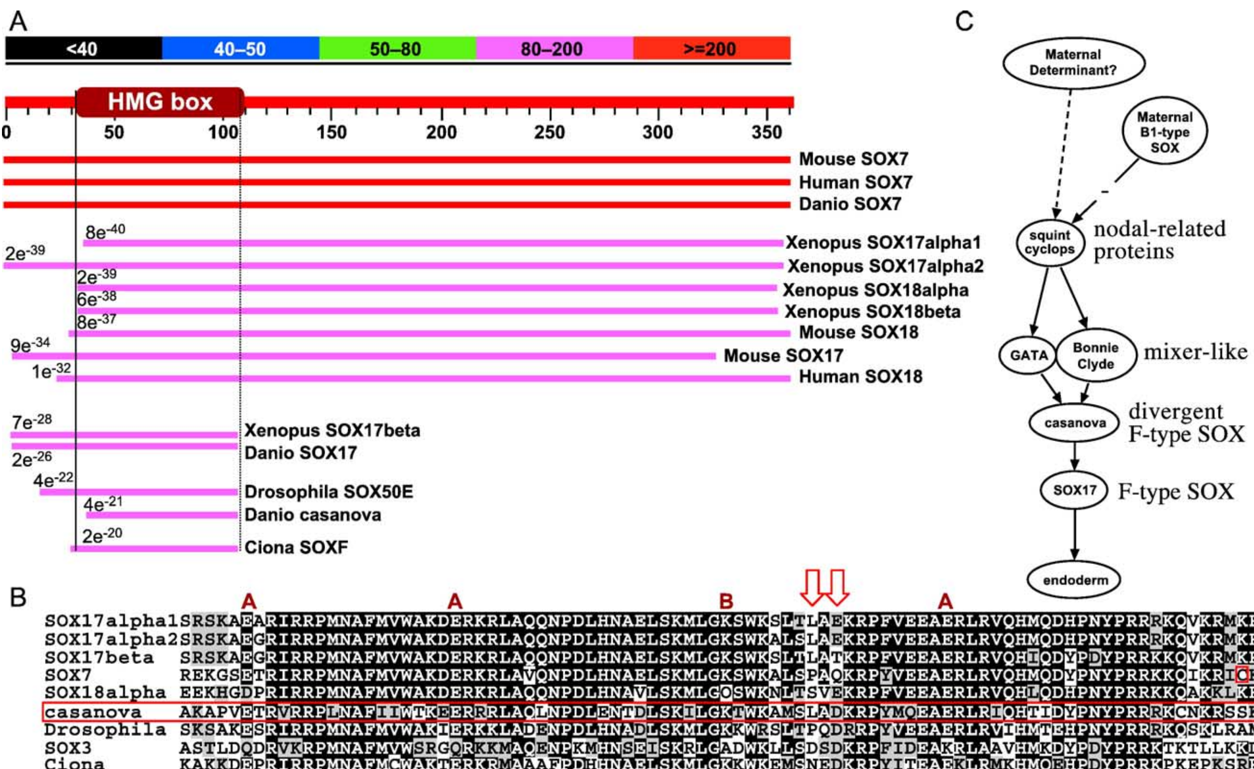

Fig. 1. SOX7 in *Xenopus* and casanova. (A) GenBank was searched with BLASTp using the *X. laevis* SOX7 coding region; SOX7 orthologs (red) were identified in mouse, human, zebrafish (Danio); other F-type SOXs were also recognized (pink), including *Xenopus* SOX17 (α and β) and SOX18 (α and β), *Drosophila melanogaster* SOX50E, and the sea squirt *Ciona intestinalis* SOXF. In part B, the HMG box of these proteins are displayed, together with that of the *Xenopus* B1 type SOX, SOX3; acidic ("A") and basic ("B") residues common to the F-type SOXs, and altered in SOX3, are indicated. Although divergent, the casanova protein falls within the F-type group. It is noteworthy that *Xenopus* SOX7 differs from other *Xenopus* F-type SOXs in the presence of a proline and glutamine residues within the HMG box (open red arrows). (C) In zebrafish (diagram modified from Shivdasani, 2002), *casanova* acts downstream of Nodal and Mixer-like proteins and upstream of SOX17 to mediate endodermal differentiation.

synthesized and capped RNAs encoding SOX7-GFP. At approximately stage 11, embryos were sorted by fluorescence, fixed, and stained for the pan-endodermal marker endodermin (*Edd*) (Sasai et al., 1996). In essentially every SOX7-GFP RNA-injected embryo, there was a clear increase in the strength and extent of *Edd* expression (Figs. 3B and C) compared to uninjected embryos (Fig. 3A). Two well-characterized, cell-autonomous inhibitors of the endodermal/endodermin pathway have been described. The first is an EnR-repression domain chimera of VegT (Horb and Thomsen, 1997), which has been shown to specifically block the expression of both endodermal (Fig. 3D) and mesodermal (*Xbra*) (Fig. 3G) markers in a manner similar to that observed in maternal VegT-RNA-depleted embryos (see Engleka et al., 2001). The second is the Mixer-engrailed repression chimera described by Henry and Melton (1998). Expression of this polypeptide in whole embryos specifically blocks the expression of *Edd* (Fig. 3E), but does not block, and appears to increase the levels of *Xbra* (Fig. 3H) (Henry and Melton, 1998). To test the role of SOX7 in the embryo, we constructed a similar SOX7 repression chimera, SOX7ΔC-EnR-myc, in which the C-terminal domain of SOX7, downstream of the HMG box, was replaced by a myc-epitope-tagged, engrailed transcriptional repression domain. When RNA encoding SOX7ΔC-EnR-myc was injected into fertilized eggs, it suppressed *Edd* (Fig. 3F) and increased *Xbra* (Fig. 3I) expression in a manner similar to the effects produced by Mixer-EnR. QPCR analysis of SOX7ΔC-EnR-myc and Mixer-EnR RNA-injected embryos indicated that *Xbra* expression was increased ~2-fold by both reagents (data not shown). To begin to place SOX7 in the endoderm specification pathway, we examined embryos injected at the one-cell stage with both VegT-EnR and SOX7-GFP RNAs. Compared to VegT-EnR RNA-injected embryos (Figs. 3D and J), coinjections of VegT-EnR and SOX7-GFP RNAs lead to the robust expression of *Edd* (Figs. 3K and L), suggesting that *SOX7* acts downstream of *VegT* in the regulation of *Edd* expression.

SOX7 induces endodermal differentiation in animal cap explants

To extend our initial whole embryo observation, we examined the effects of SOX7-GFP RNA injection on the behavior of ectodermal explants (animal caps). Each of these experiments has been repeated at least twice and

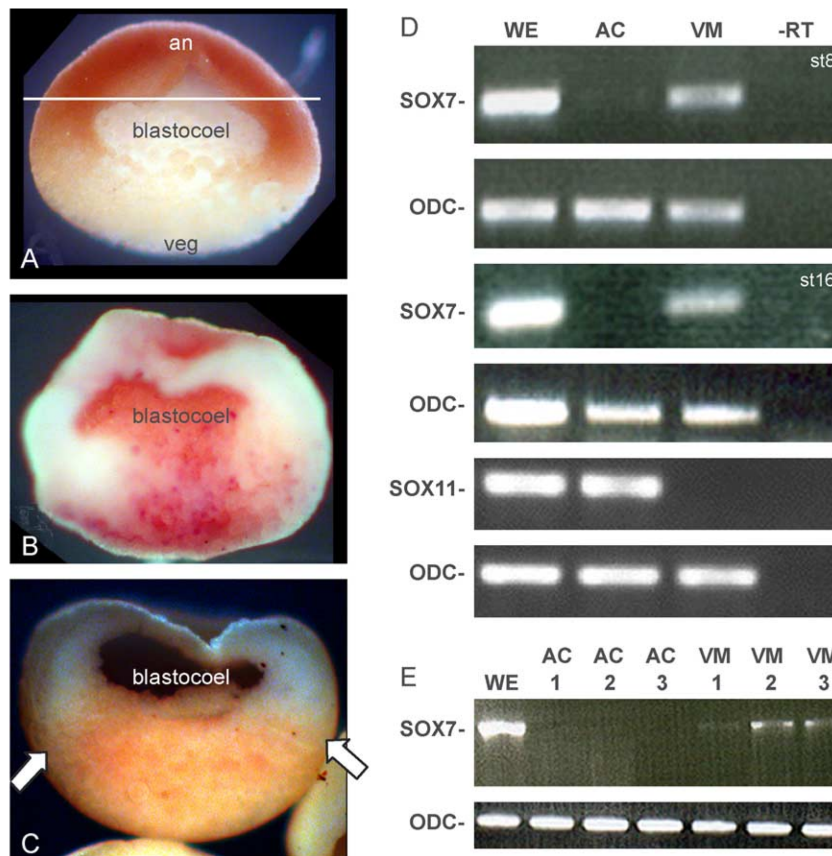

Fig. 2. Localization of SOX7 RNA. In situ hybridization of longitudinally bisected blastula stage embryos reveals the animal hemisphere concentration of SOX3 (A) RNA, while SOX17 RNA is localized to the vegetal region (B). While staining for SOX7 RNA was consistently quite weak, it was clearly restricted to the vegetal hemisphere (C). Embryos at stage 8 were dissected into animal caps (AC) and vegetal masses (VM) (indicated by white line in part A) and then analyzed for SOX7 or SOX11 mRNA by RT-PCR (D) either immediately (st8) or after they had been cultured until control embryos reached stage 16 (st16). Primers for ornithine decarboxylase (ODC) were used as a control for cDNA quality; the absence of reverse transcriptase (-RT) was used to control for the presence of contaminating DNA. At both time points, SOX7 mRNA was restricted to the vegetal mass; under similar conditions, SOX11 mRNA was restricted to the animal hemisphere. (E) A second set of SOX7-specific primers (Table 1 "SOX7 coding") was used to confirm the vegetal localization of SOX7 RNA. Animal caps and vegetal masses were prepared at stage 8 and analyzed either immediately ("1"), after 2 h ("2"), or when control embryos had reached stage 27 ("3"); these primers amplified a fragment of the expected size (~1kb) that was again restricted to the vegetal mass.

often four to more times. Normally, animal caps differentiate into atypical ectoderm/epidermis. As described previously (Clements and Woodland, 2003; Clements et al., 1999; Engleka et al., 2001; Henry and Melton, 1998; Horb and Thomsen, 1997; Hudson et al., 1997; Zhang and King, 1996), injection of VegT RNA induces the expression of *SOX17* (Fig. 4A), *Mixer* (Fig. 4B), *Xbra*, and *Edd* (Fig. 4C). The effects of SOX7 mRNA injection differ from those of VegT in that SOX7 does not induce *Xbra* expression (Fig. 4C). SOX7 RNA induced the expression of *SOX17β* when injected at levels as low as 100 pg per embryo (Fig. 4D).

Mixer appears to act between Nodals and SOX17 in the endoderm specification pathway (Henry and Melton, 1998; Kofron et al., 2004; Shivdasani, 2002). The pattern of gene expression induced by SOX7 in animal caps indicates that it acts upstream of *Mixer*. In animal caps, Sinner et al. (2004) found that SOX17 induces expression of *Xnr4*, but not *Xnr1*, *Xnr2*, or *Mixer* (Fig. 4B). In contrast, SOX7 induced the expression of *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, and *Mixer* (Figs. 4B and E). SOX7's ability to induce all five *Xnr* genes was not altered by coinjection of Mixer-EnR; in the presence of Mixer-EnR, SOX7 induced the expression of *Mixer*, but not the downstream genes *Edd* or *SOX17* (Fig. 4E). At this point we exploited the Nodal-specific inhibitor CerS. CerS, a truncated form of the Cerberus protein, binds to and inhibits the Xnrs but does not inhibit other TGFβ family members (e.g., Activin, BMP, Derriere, and Vg1) (Agius et al., 2000; Piccolo et al., 1999; Takahashi et al., 2000). SOX7 was able to induce *Xnr1*, *2*, *4*, *5*, and *6* expression in the presence of CerS (Fig. 4E). That the CerS protein was active was demonstrated by its ability to inhibit SOX7 induction of *Mixer*, *SOX17β*, and *Edd* expression (Fig. 4E), which are known to depend upon Nodal signaling (see Engleka et al., 2001). Based on these results, SOX7 appears to act upstream of the Nodals in the endodermal specification pathway. SOX7 ability to activate *Mixer* expression appears to be indirect and requires active Nodal signaling.

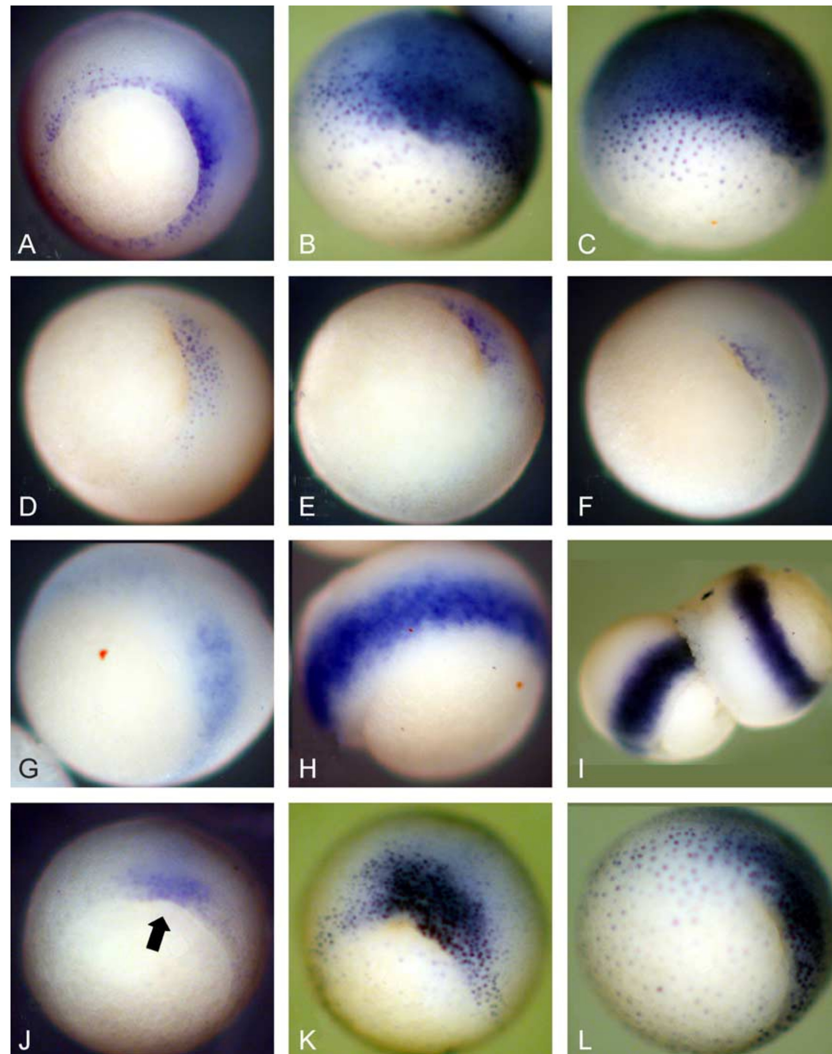

Fig. 3. SOX7 induces the endodermal marker endodermin. Fertilized eggs were injected with RNA encoding SOX7-GFP (B and C), allowed to develop to stage ~11, and were then stained for Edd RNA. Compared to uninjected embryos (A), the extent and intensity of *Edd* staining was greatly increased in SOX7-GFP RNA-injected embryos. We compared the effects of injecting embryos with RNAs encoding VegT-EnR (D and G), Mixer-EnR (E and H), and SOX7ΔC-EnR-myc (F and I). All three EnR-repressor chimeras produce a similar decrease in the level and extent of *Edd* expression (D–F). In contrast, while VegT-EnR suppressed *Xbra* expression (G), both Mixer-EnR (H) and SOX7ΔC-EnR-myc (I) appeared to increase the level of Xbra expression; this increase was confirmed by QPCR (data not shown). When SOX7-GFP and VegT-EnR RNAs were injected together, the level and extent of *Edd* expression (K and L) was increased compared to the level observed in control (A) or VegT-EnR RNA injected embryos (D and J).

SOX7 is an "immediate-early" target of VegT

In *Xenopus*, the mesoendoderm specification pathway is initiated by maternal VegT (see Loose and Patient, 2004; Shivdasani, 2002). We therefore examined the relationship between VegT and SOX7 expression. In animal caps, VegT, but not SOX17, induces the expression of *SOX7* (Fig. 5A). VegT was able to induce the expression of *SOX7*, but not of *SOX17β*, in the presence of either Mixer-EnR (Fig. 5B) or CerS (Fig. 5C). To determine whether VegT's induction of *SOX7* expression is direct or indirect, we injected fertilized eggs with RNA encoding a hormone-regulated version of VegT, mt-VegT-GR. At stage 8/9, animal caps were prepared. Thirty minutes prior to treatment with the activating hormone dexamethasone, animal caps were treated with the protein synthesis inhibitor emetine. Upon exposure to dexamethasone, mt-VegT-GR-expressing animal caps express *SOX7*, *SOX17β*, *Edd*, and *Xbra*; none of these genes were expressed in the absence of dexamethasone (Fig. 5D). Target genes that are activated in the absence of new protein synthesis are referred to as immediate-early targets. *SOX7*, like *SOX17β*, appears to be an immediate-early target of VegT, whereas *Xbra* and *Edd* are not (Figs. 5D and E).

To determine whether SOX7 RNA levels are normally regulated by maternal VegT, we obtained cDNA from VegT-depleted embryos generated by Jing Yang and Peter Klein (U. Penn). In their experiments, they used an antisense phosphothioate oligonucleotide identical to that used pre-

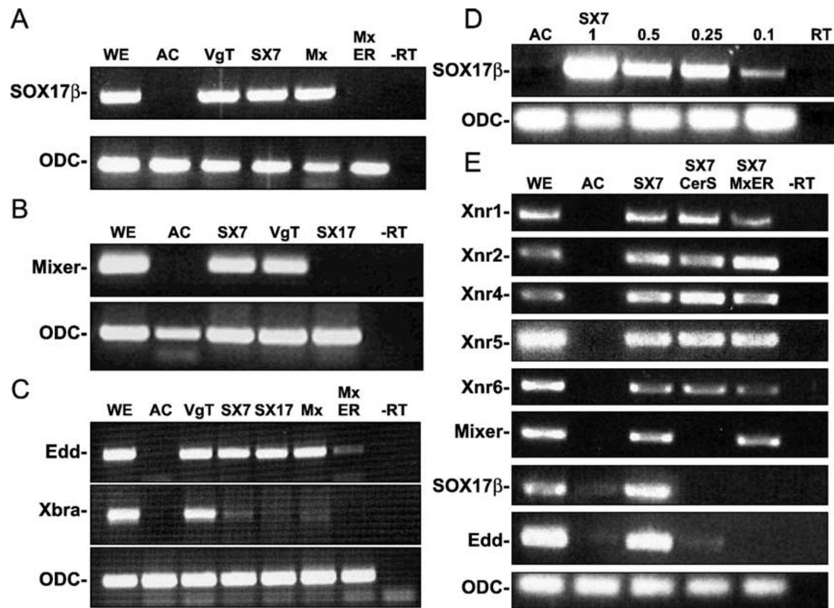

Fig. 4. SOX7 induction of endodermal markers in animal caps. The expressions of *SOX17β Mixer*, *Xbra*, and *Edd* were analyzed by RT-PCR in whole embryos (WE), animal caps from uninjected embryos (AC), or animal caps derived embryo embryos injected with RNAs encoding either VegT (VgT), SOX7-GFP (SX7), Mixer (Mx), Mixer-EnR (MxER), or SOX17β (SOX17β). ODC was used as a control for RNA quality. (A) VegT, SOX7, and Mixer RNAs induced expression of *SOX17β* in animal caps. (B) Both SOX7 and VegT induce *Mixer* expression, whereas SOX17 did not. (C) While VegT, SOX7, SOX17, and Mixer induce *Edd*, only VegT was effective at inducing *Xbra*. (D) The expression of *SOX17β* by could be detected in animal caps derived from fertilized eggs injected with as little as 0.1 ng of SOX7-GFP RNA. (E) SOX7 was able to induce expression of *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5*, and *Xnr6* in animal caps. The ability of SOX7 to induce the Xnrs was not inhibited by the coinjection of RNAs encoding either Mixer-EnR (0.5 ng) or CerS (0.6 ng); Mixer-EnR inhibited SOX7 induction of *SOX17β* and *Edd*; CerS blocked SOX7 induction of *Mixer*, *SOX17β*, and *Edd*.

viously by Heasman et al. (2001), Houston et al. (2002), Xanthos et al. (2001), and Zhang et al. (1998). Using QPCR, we found a correlation between the reduction in VegT RNA levels and reduction in SOX7 RNA levels (Fig. 5F). In the most severe of the four cases analyzed, a ~90–95% reduction in VegT RNA leads to a ~80% reduction in SOX7 RNA level; as expected, SOX17β RNA levels were also decreased in VegT RNA-depleted embryos.

SOX7 mediates VegT activation of the endodermal differentiation pathway in animal caps

To determine whether SOX7 is required for the VegT-induced induction of *Mixer* and *Edd*, fertilized eggs were injected with VegT mRNA (0.1 ng/embryo) alone or together with either SOX7ΔC-EnR RNA (1 ng/embryo). The presence of SOX7ΔC-EnR-myc inhibited VegT's ability to induce both *Mixer* and *Edd*, but had only a mild effect on *SOX17β* expression (Fig. 6A). As an alternative to the use of a dominant-negative polypeptide, we designed and tested a modified antisense oligonucleotide (morpholino) directed against 6 bases of the 5′ UTR and the first 19 bases of the SOX7 mRNA. When injected together SOX7-GFP RNA, this morpholino completely inhibited the accumulation of the SOX7-GFP polypeptide (Fig. 6B). Using QPCR, it was clear that both the SOX7 morpholino and SOX7ΔC-EnR RNA inhibited VegT's ability to induce *Mixer* and *Edd* expression in animal caps, but had no dramatic effect on VegT-induced *Xbra* expression and only modest effects on *SOX17β* expression (Fig. 6C); injection of a control morpholino (20 ng/embryo) had no effect on VegT-induced expression of *Mixer*, *SOX17*, or *Edd* (Fig. 6D). The effects of both the morpholino and SOX7ΔC-EnR could be suppressed by coinjection of RNA encoding an alternative version of SOX7-GFP, altSOX7-GFP (Fig. 6D); altSOX7-GFP contains five silent changes in the nucleotide sequence designed to disrupt interactions with the SOX7 morpholino (see Materials and methods). The failure of either "antiSOX7" reagent to block VegT induction of *SOX17β* expression is likely to be due to the fact SOX17β is a direct target of VegT regulation (Fig. 5D and E) (Clements and Woodland, 2003; Engleka et al., 2001). Occasionally, we did experiments in which both the SOX7MO and SOX7ΔC-EnR-myc produced a decrease in VegT-induced SOX17β expression (data not shown), presumably due to the fact that the maintenance of SOX17 expression is dependent upon Nodal signaling (Engleka et al., 2001).

In whole embryos, injection of the SOX7 morpholino produced a mild but discernable effect on later aspects of development, the most obvious being a reduction in visible blood (Fig. 6E). These phenotypic effects are much less severe that those seen in embryos injected with RNAs encoding the dominant-negative forms of SOX7 (Fig. 6E) (Table 2). This type of behavior is reminiscent of the behavior of *SOX18* in the mouse, where dominant mutations produce a severe effect, while mice homozygous for a null mutation in *SOX18* display only a mild phenotype (Pennisi

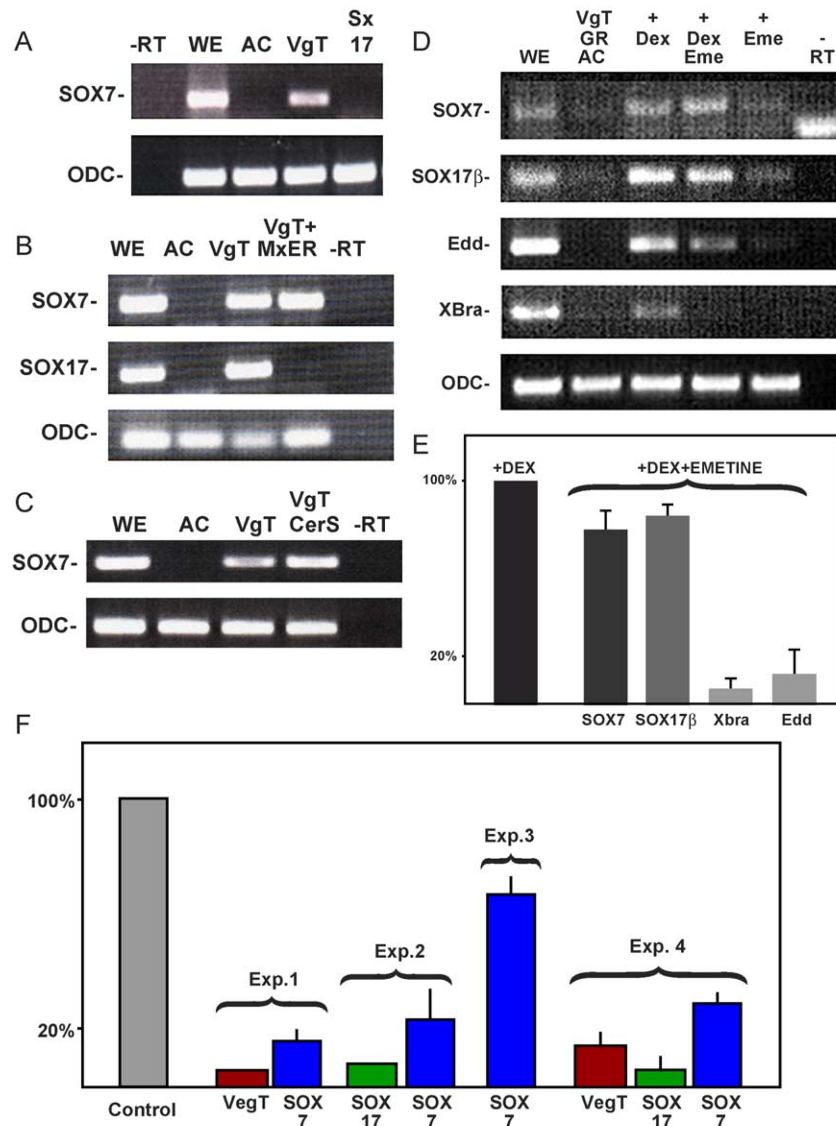

Fig. 5. VegT regulation of SOX7 expression. Fertilized eggs were injected with RNAs encoding either VegT (VgT), SOX17β (Sx17), VegT and Mixer-EnR (MxER), or VegT and CerS. Animal caps were prepared at stage 8 and analyzed when control embryos reached stage 11 (~2 h later) for SOX7 or SOX17β RNA by RT-PCR, using ODC as a control for RNA quality. (A) VegT, but not SOX17, induced SOX7 expression under these conditions. (B) The VegT-induced expression of SOX7 was not inhibited by Mixer-EnR; VegT's induction of SOX17β expression was inhibited by Mixer-EnR. (C) VegT-induced expression of SOX7 was not inhibited by the Nodal inhibitor CerS. (D) To determine if SOX7 was a direct target of VegT regulation, we injected fertilized eggs with RNA encoding mt-VegT-GR; animal caps were prepared at stage 8. In the absence of dexamethasone, there was no expression of endodermal markers of SOX7 ("VgT GR AC"); in the presence of dexamethasone (2 μM) (+Dex), *SOX7*, *SOX17β Edd*, and *Xbra* were induced. Animal caps were treated with the protein synthesis inhibitor emetine either alone (+Eme) (10 μg/ml) or with emetine followed by emetine and dexamethasone (+Dex Eme). The level of SOX7 and SOX17 RNA was similar to that seen in animal caps treated with dexamethasone alone; in contrast, the levels of both *Edd* and *Xbra* were greatly reduced. Treatment with emetine alone had little reproducible effect on any of the markers. (E) These conclusions were confirmed using QPCR. The levels of SOX7 and SOX17β RNAs in dexamethasone-treated, VegT-GR-expressing animal caps were similar to those found in VegT-GR-expressing animal caps treated with emetine and dexamethasone; levels of Xbra and Edd RNAs were greatly reduced by the presence of emetine. (E) To determine if VegT normally regulates SOX7 expression, oocytes were depleted of VegT mRNA by anti-sense oligonucleotide injection; embryos were prepared and RNA was isolated at stage ~10.5. Four separate experiments were examined and RNA levels determined by QPCR. In experiment 1, the level of VegT and SOX7 RNAs was characterized; in experiment 2, SOX17 and SOX7 RNA levels were characterized; in experiment 3, only SOX7 RNA was characterized; and in experiment 4, the levels of VegT, SOX17β, and SOX7 RNAs were characterized. In each case, there was a decrease in SOX7 RNA associated with the depletion of maternal VegT RNA.

et al., 2000a,b). One candidate for such a rescuing factor is zygotic SOX18, whose expression can be detected in stage 9/10 embryos by RT-PCR (Hasegawa et al., 2002; unpublished observation). It is also possible that there are SOXs or other proteins in the vegetal hemisphere of the early *Xenopus* embryo that mitigate the phenotypic effects of reducing SOX7 protein levels. The effects produced by SOX7ΔC-EnR RNA injection were similar to those originally reported by Hudson et al. (1997) using a SOX17-engrailed repressor chimera at levels that inhibited

endodermal, but not mesodermal marker expression. At higher levels, at which mesodermal marker expression was inhibited, SOX17-EnR induces a gastrulation defect; gastrulation defects were not observed in SOX7ΔC-EnR-myc RNA-injected embryos, although gastrulation was often delayed (data not shown). The SOX7ΔC-EnR phenotype is also similar to that reported by Clements et al. (2003) using morpholinos directed against either SOX17α or SOX17β alone, but less severe than the phenotype produced when combinations of morpholinos directed against both SOX17α and β were used together—it is worth noting, however, that these authors were not able to rescue these most severe "combined morpholino" phenotypes with SOX17 RNA injection and so they may, at least in part, reflect nonspecific effects.

Table 2
Late stage SOX7ΔC-EnR and SOX7MO phenotypes

	Normal (%)	Defective (%)	Other (%)[a]	*N*
Uninjected	59 (98)	0 (0)	1 (2)	60
SOX7MO (20 ng total)	30 (34)	55 (63)	3 (3)	87
SOX7ΔC-EnR (1 ng total)	12 (18)	51 (77)	3 (5)	66

Embryos were injected with either SOX7ΔC-EnR mRNA or SOX7 morpholino into both animal and vegetal sites at the one- to two-cell stage. Embryos were scored at the tailbud stage. Defective embryos successfully completed gastrulation and displayed phenotypes quite similar to the images presented in Fig. 6E.

[a] Denotes embryos that died prior to tailbud stage.

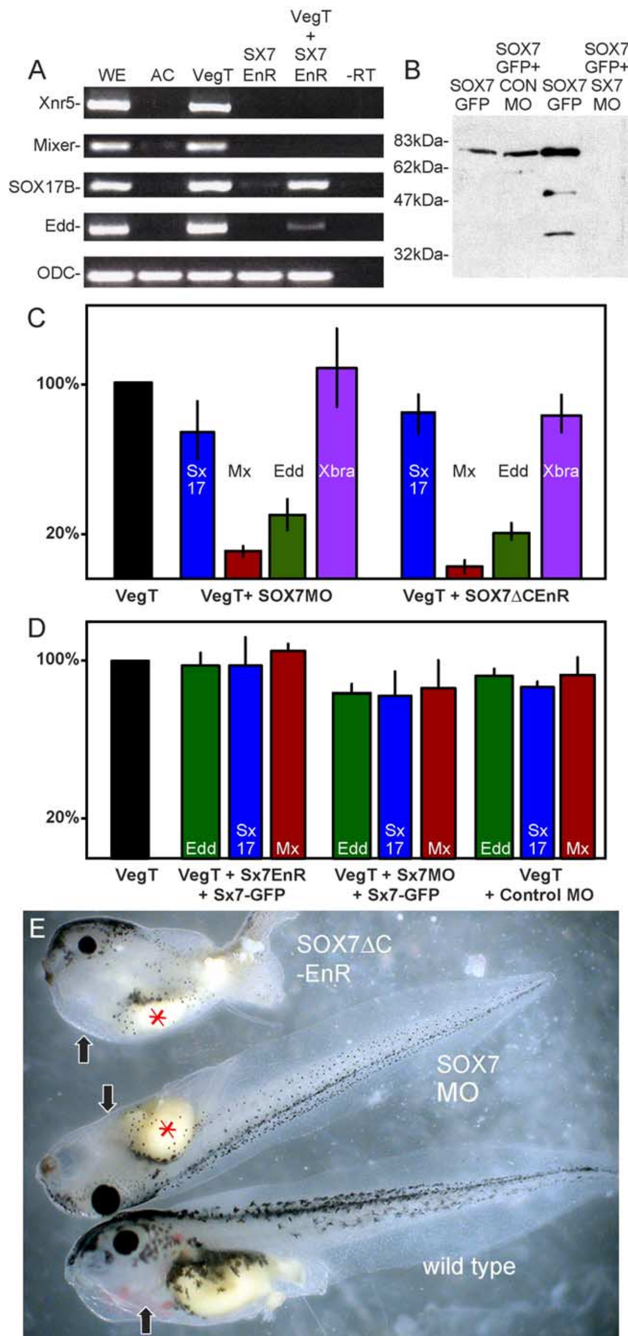

Fig. 6. SOX7 is required for VegT induction of Mixer and Edd in animal caps. (A) Animal caps were prepared from fertilized eggs injected with either VegT RNA, SOX7ΔC-EnR-myc RNA, or both VegT and SOX7ΔC-EnR-myc RNAs. At stage 10, the animal caps were analyzed for the expression of *Xnr5*, *Mixer*, *SOX17β*, and *Edd* RNAs. The presence SOX7ΔC-EnR-myc ('Sx7EnR') blocked the expression of *Xnr5* and *Mixer*, reduced *Edd* expression, and had a minor effect on *SOX17β* expression. (B) The efficacy of the SOX7 morpholino was tested by injecting fertilized eggs with SOX7-GFP RNA either alone (SOX7GFP), or sequentially with SOX7-GFP RNA and a control morpholino (SOX7GFP+CON MO) or SOX7-GFP RNA and the SOX7 morpholino (SOX7GFP+SOX7 MO). At stage 8/9, embryos were analyzed by immunoblot using an anti-GFP antibody; the SOX7 morpholino completely suppressed the accumulation of SOX7-GFP. (C) QPCR of animal caps derived from VegT RNA-injected embryos (100 pg/embryo) expresses Mixer ('Mx'), SOX17β ('Sx17'), endodermin ('Edd'), and Xbra ('Xbra') (see part A). These levels were arbitrarily set to 100%. The expressions of *Mixer* and *Edd*, but not *SOX17β* or *Xbra*, were suppressed by coinjection of either a SOX7 morpholino (SOX7MO) (20 ng/embryo) or SOX7ΔC-EnR RNA (1 ng). (D) The effects of both the SOX7 morpholino and SOX7ΔC-EnR on VegT induction were rescued by injection of 1 ng altSOX7-GFP RNA; injection of a control morpholino (20 ng) had no effect on VegT's induction of *Edd*, *SOX17β*, or *Mixer* expression. In later stage embryos (E), injection of SOX7MO (20 ng total injected at four sites, two animal and two vegetal at the two-cell stage) produced a decrease in blood formation (arrows) and mild effects on gut size (*) and craniofacial development; injection of 1 ng SOX7ΔC-EnR RNA produced a more severe, phenotype; notably blood (arrow) also appeared to be absent in these embryos.

SOX7, but not SOX17, binds to SOX3-binding sites in the Xnr5 promoter

Adjacent to one of two functional, but off-consensus TCF sites in the *Xnr5* promoter (Hilton et al., 2003) are two SOX binding sites, SOXa and SOXb (Fig. 7A) (Zhang et al., 2003). We refer to this region as the SS/T. Previously, we found that SOX3 binds specifically to the SOX sites in the SS/T; binding was abolished when the SOXa and SOXb sites were mutated or when a nonbiotinylated competitor oligonucleotide was added to the embryonic lysates. SOX3 acts as a repressor of *Xnr5* and *Xnr6* expression (Zhang et al., 2003, 2004); SOX7 acts as an activator of *Xnr5* and *Xnr6* expression, while SOX17 does not (Fig. 4). To determine if SOX7 binds to the SS/T, fertilized eggs were

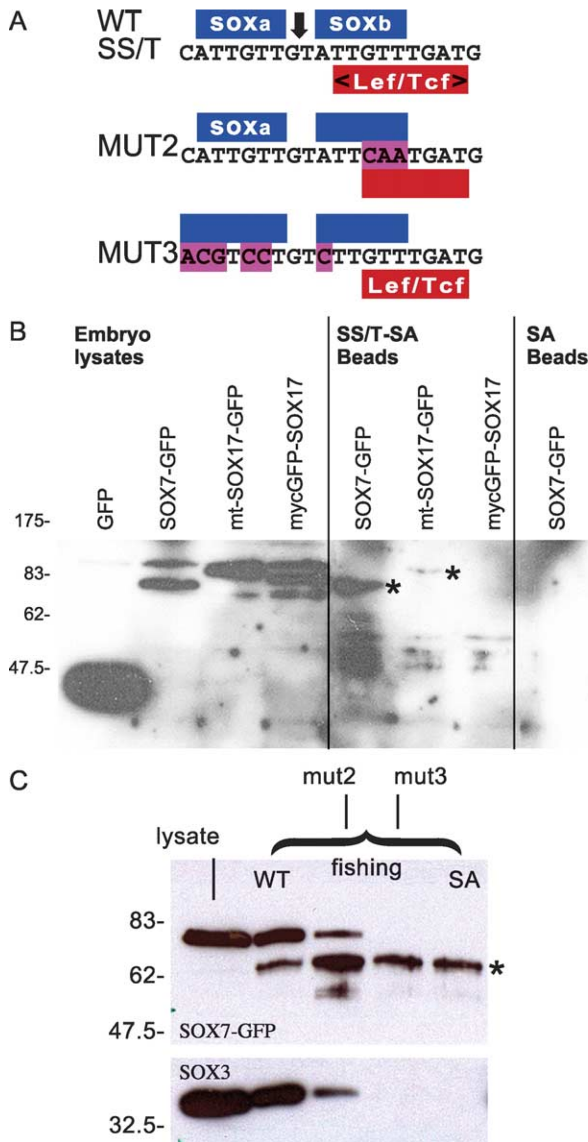

Fig. 7. SOX7, SOX17, and SOX3 binding to the SS/T region of the Xnr5 promoter. (A) Within the Xnr5 promoter is a region, the SS/T, that contains two SOX and a TCF (LEF/TCF) binding site. In the mut2 sequence, the SOXb and TCF sites have been removed, in mut3 both SOX sites have been removed, but the TCF site is intact (Zhang et al., 2003). (B) Fertilized eggs were injected with RNAs encoding either GFP, SOX7-GFP, mycGFP-SOX17β, or mt-SOX17β-GFP, and allowed to develop until stage 8/9, at which point embryo lysates were prepared and analyzed by DNA fishing using a wild type SS/T double-stranded DNA (Zhang et al., 2003). The level of expression of the exogenous polypeptides in the embryo lysates (left panel) was assayed by immunoblot with an anti-GFP antibody. SOX7-GFP bound to the wild-type SS/T domain; binding of mtSOX17β-GFP and mycGFP-SOX17β was weak ("*") or undetectable. No binding of either SOX7-GFP or the SOX17-GFP polypeptides was observed in the absence of DNA (SA Beads). (C) Lysates derived from SOX7-GFP RNA-injected eggs were incubated with streptavidin-agarose-bound wild type (WT), mut2, or mut3 DNAs. As in the case of endogenous SOX3, visualized using the antiSOX3c antibody, SOX7-GFP did not bind to mut3 (which contains a TCF binding site, but no intact SOX sites); binding to mut2 (which contains a single SOX site) was reduced. A nonspecific band, recognized by the anti-GFP antibody (*), binds to streptavidin beads in the absence of DNA (SA); in different experiments, this band can be seen in lysates derived from uninjected embryos (data not shown).

injected with SOX7-GFP, mycGFP-SOX17β, or myc-SOX17β-GFP RNAs; lysates were prepared from stage 8/9 embryos and analyzed using wild type and mutated forms of SS/T. SOX7-GFP bound to the target SS/T DNA while SOX17 binding was weak to undetectable (Fig. 7B). To determine if SOX7 displays the same binding site specificity as SOX3, we compared the binding of exogenous SOX7-GFP and endogenous SOX3 to wild type and mutated versions of the SS/T. Both SOX7-GFP and SOX3 bind to wild type and MUT2 DNAs; the binding to MUT2, which contains only a single SOX site (Fig. 7A), was reduced compared to binding to the wild-type sequence, with its two SOX sites (Fig. 7C). Neither SOX3 nor SOX7 binds to the MUT3 sequence (Fig. 7C), from which both SOX binding sites have been removed but which retains a functional TCF binding site (Fig. 7A) (Zhang et al., 2003), indicating that neither SOX3 nor SOX7 binds with high affinity to at least this type of TCF site.

Discussion

The idea of the "developmental hourglass" (Richardson, 1999) accounts for the diversity in early embryonic mechanisms that converge on a common 'phylotypic' stage. Diverse starting points can lead to morphologically similar intermediates during embryogenesis (see Grbic et al., 1996; Kauffman and Raff, 2003). At the same time, there is a remarkable degree of conservation between the early embryonic gene regulatory networks of different organisms (Hinman et al., 2003). A particularly well-conserved regulatory system is the one that regulates endodermal specification in vertebrates (Schier, 2003; Shivdasani, 2002). In zebrafish, *Xenopus*, and mouse, the F-type SOX, SOX17, is necessary for endodermal differentiation. In *Xenopus* and zebrafish, maternal components lead to the expression of Nodal-related proteins; Nodal signaling appears to act through Mixer-like proteins that, together with GATA factors, activate SOX17, which in turn is required to turn on *endodermin* and other endodermal genes (Fig. 8) (Loose and Patient, 2004; Shivdasani, 2002). In zebrafish, the maternal components responsible for the initial induction of Nodal signaling are not yet known; given that the Nodal Squint is supplied maternally in zebrafish (see Engleka et al., 2001), it is possible that no such early nodal inducer, analogous to VegT in *Xenopus*, is necessary.

SOX7 is no Casanova

Mutational analysis identified *casanova* as a component of the zebrafish endodermal pathway, acting downstream of Nodals and Mixer, and required for SOX17 expression and endodermal differentiation (Alexander et al., 1999; Aoki et al., 2002; Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). Sequence analysis suggests that the casanova protein is unique to zebrafish. Nevertheless,

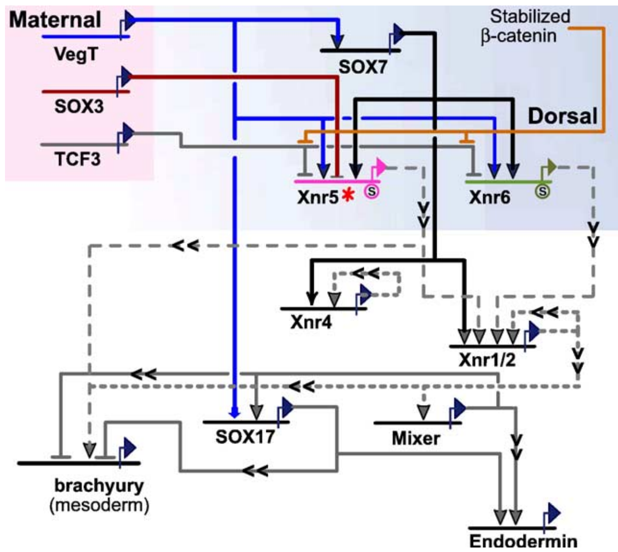

Fig. 8. A place for SOX7 in the mesoendodermal gene regulatory network. Based on the experiments presented here, we can place SOX7 as a direct target of VegT regulation. In animal caps, SOX7 acts upstream to activate transcription of the *Xnr1*, *2*, *4*, *5*, and *6* genes. Within the intact embryo, maternal TCFs repress the expression of *Xnr5* and *Xnr6*; TCF repression is relieved by activation of β-catenin following fertilization (Hilton et al., 2003; Yang et al., 2002). In animal caps, VegT's induction of *Mixer* and *Edd* depends upon SOX7; the weaker phenotype observed in SOX7 morpholino compared to SOX7ΔC-EnR RNA-injected embryos suggests that in the vegetal hemisphere there are either SOX7-like factors or that in the vegetal hemisphere VegT can at least partially circumvent the need for SOX7. Given that SOX3 and SOX7, but not SOX17, bind to the same sites in the Xnr5 promoter ("*"), it is possible that the two proteins antagonize each other's effects on Xnrs and other as yet unidentified genes. This figure is modified from Loose and Patient (2004); their interactive web site can be found at http://www.nottingham.ac.uk/genetics/staff/rogerpatient/networks/mesendoderm/.

casanova has an F-type SOX HMG box (Fig. 1B) and contains the conserved C-terminal β-catenin binding domain characteristic of F-type SOXs (Sinner et al., 2004), both of which supported our initial working hypothesis that *Xenopus* SOX7 might act as a functional analog of *casanova*.

Unlike *casanova*, however, *Xenopus* SOX7 is supplied maternally (Fawcett and Klymkowsky, 2004). In later stage *Xenopus* embryo, SOX7 is expressed in regions of the developing brain, neural crest, and circulatory system. We originally concluded, based on whole-mount in situ hybridization staining, that the SOX7 mRNA was uniformly distributed throughout the early blastula stage. This conclusion was not supported by more sensitive RT-PCR analysis of blastula stage animal caps and vegetal masses or by in situ staining of bisected embryos (Fig. 2); both indicate that SOX7 RNA is localized to the vegetal region of the blastula stage embryo, while SOX3 and SOX11 RNAs are localized to the animal hemisphere. It should be pointed out that there may well be other asymmetrically distributed SOX RNAs and proteins within the early *Xenopus* embryo. Recently, the B2-type SOX, SOX21, has been found to be present maternally in the zebrafish and to act as a transcriptional repressor (Argenton et al., 2004). In contrast to the ventralizing effects of injecting SOX3 or SOX17 RNAs in *Xenopus* (Zorn et al., 1999), injection of SOX21 RNA dorsalizes zebrafish embryos (Argenton et al., 2004). Whether other SOXs are present in the early *Xenopus* embryo and, if so, whether they are asymmetrically distributed remains to be determined.

Given the role of zebrafish *casanova* in endodermal differentiation, we were encouraged to find that injection of RNA encoding a SOX7-GFP polypeptide led to an increase in the expression of the pan-endodermal marker *Edd*, while injection of RNA encoding a SOX7-engrailed transcriptional repressor domain chimera suppressed *Edd* expression (Fig. 3). Taken together, these data indicate that wild-type SOX7 is a transcriptional activator (in contrast to SOX3 that behaves as a transcriptional repressor) and that SOX7 activates the endodermal pathway (Figs. 3 and 4) while SOX3 represses it (Zhang et al., 2003, 2004).

It was in this light that comparing the effects of the SOX7ΔC-EnR-myc polypeptide to those of dominant-negative forms of VegT and Mixer was informative. Previous studies demonstrated that VegT-EnR is a specific inhibitor of VegT function (Horb and Thomsen, 1997) and that the phenotype that it produces is quite similar to that produced by the oligonucleotide-mediated depletion of maternal VegT RNA (see Engleka et al., 2001 and references therein). Similarly, the dominant-negative, engrailed chimera version of Mixer, Mixer-EnR, also appears to be a specific inhibitor of Mixer function (Henry and Melton, 1998). Whereas VegT-EnR suppressed expression of both endodermal and mesodermal differentiation, Mixer-EnR suppresses *Edd* expression but appears to enhance *Xbra* expression (Henry and Melton, 1998). Likewise, a EnR-version of SOX17, which acts downstream of Mixer, suppresses *Edd* expression and activates *Xbra* expression (Hudson et al., 1997). The phenotype produced by SOX7ΔC-EnR is similar to that produced by Mixer-EnR and SOX17-EnR (Hudson et al., 1997), that is, SOX7ΔC-EnR suppressed *Edd* expression but increased *Xbra* expression (Fig. 3; data not shown). These data suggested that *SOX7* normally acts in the same endodermal specification pathway as do *Mixer* and *SOX17*.

To better place SOX7's activity within the endoderm specification pathway, we compared the ability of SOX7, SOX17, and Mixer to induce *Edd* in animal caps (Fig. 4). These studies unambiguously place SOX7 upstream of both *Mixer* and *SOX17* in the endodermal pathway. Perhaps the key experiment is illustrated by Fig. 4E; in contrast to SOX17, SOX7 induced all five of the nodal-related genes in *Xenopus*, *Xnr1*, *2*, *4*, *5*, and *6*, when Nodal signaling was suppressed by the presence of the Nodal-specific inhibitor CerS. Previously, Takahashi et al. (2000) reported that *Xnr5* and *Xnr6* expressions are induced cell autonomously and that they in turn regulate the expression of the other Xnrs, while Sinner et al. (2004) reported that *Xnr4*, but not *Xnr1* or *Xnr2*, was induced by SOX17. To our knowledge, SOX7

is the only protein that has been shown to regulate all five "true" nodal-related proteins in the absence of Nodal signaling.

It is worth noting, however, that these results were obtained in animal caps and that animal caps represent a special, uninduced ectodermal environment that is molecularly distinct from that of the normal "endoderm-producing" region of the embryo. It is possible that factors present in the vegetal hemisphere modify or modulate the activity of SOX7. An example of such a region-specific behavior is supplied in Henry and Melton's (1998) study of *Mixer*; they found that Mixer-EnR suppressed expression of *Edd* when the RNA was injected into intact embryos, but did not inhibit *Edd* expression in vegetal explants, indicating that different regions of the embryo have different regulatory environments. What is clear, however, is that SOX7 does not behave analogously to zebrafish *Casanova*—SOX7 regulates Nodals rather than being regulated by them.

SOX7 as a mediator of VegT's endodermal specification activity in animal caps

Maternal VegT sits at the top of the mesoendodermal gene regulatory network in *Xenopus* (see Loose and Patient, 2004). Localized to the vegetal hemisphere, VegT directly activates the expression of both *SOX17α* and *β* (Clements and Woodland, 2003; Engleka et al., 2001; Yasuo and Lemaire, 1999) and SOX7 (this work). Clements and Woodland (2003) have argued, based on VegT-GR studies, that *Xnr5*, *Mix.1*, *Mixer*, and *Derriere* are also direct targets of VegT activation and that they are regulated both by VegT and a labile, VegT-dependent transcriptional repressor. In contrast, Engleka et al. (2001) found that VegT's induction of *Mixer* is indirect and mediated by Nodal signaling. As far as we can discern, a demonstration of functional VegT-responsive sites within a target promoter has been established only for the *Xnr5* gene (Hilton et al., 2003) and even in this study no direct evidence for VegT-DNA binding was presented. What is necessary are data that directly demonstrate VegT binding to gene-specific regulatory sequences. A full analysis is likely to involve DNA microarray studies using hormone-regulated versions of VegT, together with detailed promoter and DNA-binding studies. In any case, endodermal fate is not irreversibly established until early gastrula stages (Heasman et al., 1984; Wylie et al., 1987) and final endodermal fate determination depends upon Nodal-mediated signaling (Chang and Hemmati-Brivanlou, 2000; Clements et al., 1999; Engleka et al., 2001; Kofron et al., 1999; Yasuo and Lemaire, 1999).

SOX7, SOX3, VegT, and nodal regulation in animal caps

Given the clear requirement for SOX7 in the mediation of VegT-induced activation of endodermal differentiation in animal caps (Figs. 6A, C, and D), we were surprised by the relative mild embryonic phenotype produced by the SOX7 morpholino (Fig. 6E). The fact that injection of a dominant-negative form of SOX7, SOX7ΔC-EnR-myc, produces significantly more severe phenotype suggests one obvious explanation—the presence of compensatory proteins. This type of behavior has been seen for SOX18 in the mouse, where dominant mutations in SOX18 lead to cardiovascular defects (Pennisi et al., 2000b), while mice homozygous for a null mutation in SOX18 have little discernable phenotype (Pennisi et al., 2000a). Such a phenotypic pattern suggests the presence of gene products that can partially or completely perform the functions of the absent gene product.

In *Xenopus*, the SOX7ΔC-EnR-myc RNA-induced phenotype is similar to the phenotype produced by injection of morpholinos directed against either SOX17α or SOX17β, or the injection of a SOX17-EnR chimera under conditions where *Edd*, but not *Xbra*, expression is suppressed (Clements et al., 2003; Hudson et al., 1997) (a fuller characterization of the late stage SOX7ΔC-EnR phenotype is currently in preparation). The SOX7ΔC-EnR-myc-induced phenotype is less severe than that observed when the SOX17-EnR RNA is injected at levels that inhibit mesoderm differentiation or when multiple SOX17 morpholinos are used together; under these conditions, embryos fail to complete gastrulation (Clements et al., 2003; Hudson et al., 1997). SOX7ΔC-EnR-myc RNA-injected embryos complete gastrulation (although it is delayed) (Table 2). There are, however, reasons to suspect that the SOX17-associated gastrulation phenotypes are not specific; they are associated with high levels of SOX17-EnR that inhibit *Xbra* expression and, in the morpholino experiments, they cannot be rescued by injection of SOX17 RNA (Clements et al., 2003).

Placing SOX7 in the mesoendodermal GRN

While SOX7 is sufficient to induce *Edd* expression in both intact embryos (Fig. 3) and animal caps (Fig. 4), the question remains, is SOX7 necessary? If SOX7 alone were essential for endoderm formation, the phenotypes of SOX7ΔC-EnR-myc RNA and SOX7 morpholino-injected embryos should be similar; clearly they are not. We take this as evidence that there are compensatory factors that mitigate the loss of SOX7, but whose functions are blocked by SOX7ΔC-EnR. While other vegetally localized SOXs are attractive candidates, there are also other possibilities. One is that at high levels, VegT together with other vegetally localized factors can induce Nodal expression in the absence of SOX7 or SOX7-like activity. This may be mechanistically similar to failure of Mixer-EnR to suppress *Edd* expression in vegetal blastomeres (see Henry and Melton, 1998).

Given the similar inhibitory effects of the SOX7 morpholino and SOX7ΔC-EnR-myc RNA on VegT's ability to activate *Mixer* and *Edd* expression in animal caps (Figs. 6C and D), the hypothetical, vegetal "detour around SOX7" factors appear to be missing in the animal hemisphere. One obvious difference between animal and vegetal hemisphere (although there are certainly many more) is the presence of

high levels of SOX3 RNA and protein in the animal hemisphere (Fig. 2C) (Penzel et al., 1997; Zhang et al., 2003). That SOX7 and SOX3 are direct regulators of *Xnr5* expression is suggested, but not proven, by the fact that both polypeptides bind to the SS/T region of the *Xnr5* promoter (Fig. 7). The apparent failure of SOX17 to bind to these sites, while surprising given the similarities between F-type SOX HMG box sequences (Fig. 1B), does provide an explanation for SOX17's failure to induce *Xnr5*.

It is tempting to hypothesize that part of VegT's ability to activate the endodermal gene cascade in animal caps is due to its ability to induce SOX7 expression, which in turn antagonizes the repressive effects of SOX3 (Zhang et al., 2003, in press). In this light, it is worth noting that in animal caps VegT has also been found to activate β-catenin signaling, which then cooperates with VegT to activate *Xnr* expression (Rex et al., 2002). The mechanism of VegT's activation of β-catenin signaling could involve its ability to induce *Xwnt8* expression (Zhang and King, 1996), which in turn should activate β-catenin. *Xnr1*, *2*, *4*, *5*, and *6* alone can, in turn, activate the mesoderm marker *Xbra* (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000).

Placing all of these observations into a self-consistent and coherent gene regulatory network (Loose and Patient, 2004) (Fig. 8) is not straightforward, and perhaps at this point, not possible. As currently formulated, the *Xenopus* mesoendodermal gene regulatory network map does not readily take into account regional, and functionally significant, differences within the *Xenopus* embryo, nor have all the players, and regulatory interactions between them, been identified. Nevertheless, given the fact that SOX7 is an immediate-early, that is, direct, target of VegT regulation and can induce Nodal-related gene expression in the absence of Nodal signaling, SOX7, perhaps together with functionally similar proteins, appears to play an important role in early embryonic patterning.

Acknowledgments

We are particularly grateful to Jing Yang, Peter Klein, and Janet Heasman for supplying us with cDNA from maternal VegT-depleted embryos; we thank Aaron Zorn, Gerry Thomsen, Doug Melton, Eddy DeRobertis, Mary Lou King, Jing Yang, and Janet Heasman for plasmids. SRF received support from the University of Colorado, Boulder Undergraduate Research Opportunity Program, funded in part by the HHMI. This work was funded by NIH grant GM54001 to MWK.

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