ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

DEVELOPMENTAL BIOLOGY

Developmental Biology 261 (2003) 412–425

www.elsevier.com/locate/ydbio

Tight regulation of SpSoxB factors is required for patterning and morphogenesis in sea urchin embryos

Alan P. Kenny,[1] David W. Oleksyn, Laurel A. Newman, Robert C. Angerer, and Lynne M. Angerer*

Department of Biology, University of Rochester, Rochester, NY 14627, USA

Received for publication 3 April 2003, revised 29 May 2003, accepted 29 May 2003

Abstract

Previous studies in sea urchin embryos have demonstrated that nuclearization of β-catenin is essential for initial steps in the specification of endoderm and mesenchyme, which are derived from vegetal blastomeres. This process begins at the 4th and extends through the 9th cleavage stage, an interval in which the SpSoxB1 transcription regulator is downregulated by β-catenin-dependent gene products that include the transcription repressor SpKrl. These observations raise the possibility that SpSoxB1 removal is required to allow vegetal development to proceed. Here we show that elevated and ectopic expression of this factor suppresses differentiation of all vegetal cell types, a phenotype that is very similar to that caused by the suppression of β-catenin nuclear function by cadherin overexpression. Suppression of vegetal fates involves interference at the protein-protein level because a mutation of SpSoxB1 that prevents its binding to DNA does not significantly reduce this activity. Reduction in SpSoxB1 level results in elevated TCF/Lef-β-catenin-dependent expression of a luciferase reporter gene in vivo, indicating that in the normal embryo this protein suppresses the primary vegetal signaling mechanism that is required for specification of mesenchyme and endoderm. Surprisingly, normal expression of SpSoxB1 is required for gastrulation and endoderm differentiation, as shown by both morpholino-mediated translational interference and expression of a dominant negative protein. Similar gain-of-function and loss-of-function assays of a closely related factor, SpSoxB2, demonstrate that it, too, is required for gastrulation and that its overexpression can suppress vegetal development. However, significant phenotypic differences are apparent in the two perturbations, indicating that SpSoxB1 and SpSoxB2 have at least some distinct developmental functions. The results of all these studies support a model in which the concentration of SpSoxB factors must be tightly regulated along the animal-vegetal axis of the early sea urchin embryo to allow β-catenin-dependent specification of endoderm and mesenchyme cell fates as well as to activate target genes required for gastrulation.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Morpholino; Misexpression; Animal-vegetal axis

Introduction

SpSoxB1 is the prototype member of a set of positive transcription factor activities that function very early and asymmetrically along the animal-vegetal axis of the sea urchin embryo (Kenny et al., 1999). We have termed these the animalizing transcription factors (ATFs) (Angerer and Angerer, 2000) because they initially activate transcription of reporter constructs in all blastomeres except those derived from the vegetal-most cells, the micromeres (Kenny et al., 1999; Kozlowski et al., 1996; Wei et al., 1999). Specifically, SpSoxB1 was identified as an essential regulator of the *SpAN* promoter, which is activated by the eight-cell stage in all blastomeres and then is abruptly inactivated in the micromeres of the 16-cell embryo (Reynolds et al., 1992). During the next four cleavage cycles, transcription of *SpAN* remains strong in animal blastomeres, which are derived from the mesomeres and animal progeny of the macromeres (veg_1 tier), but is progressively reduced in the progeny of the vegetal derivatives of the macromeres (veg_2 tier), which overlie the micromeres. SpSoxB1 is likely to be

* Corresponding author. Fax: +1-585-275-2070.
E-mail address: lang@mail.rochester.edu (L.M. Angerer).
[1] Current address: Department of Pediatrics, Golisano Children's Hospital at Strong, Strong Memorial Hospital, University of Rochester, 601 Elmwood Avenue, Rochester, NY 14627, USA.

0012-1606/03/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0012-1606(03)00331-2

the primary regulator of this spatial pattern of transcription because it is essential for expression of *SpAN* transgenes (Kozlowski et al., 1996) and its distribution in nuclei between 4th and 8th cleavage strikingly parallels the accumulation of *SpAN* mRNA (Kenny et al., 1999). We have pursued studies on the function of SpSoxB1 because it is one of the transcription factors that accumulates earliest and with an animal bias in blastomere nuclei and it therefore may regulate early events in patterning cell fates along the animal-vegetal axis.

As the concentration of SpSoxB1 is reduced progressively in nuclei around the vegetal pole between cleavage and mesenchyme blastula stages, β-catenin simultaneously enters these same nuclei (Logan et al., 1999). Nuclearization of β-catenin is required for specification of both endoderm and mesenchyme fates (Logan et al., 1999; McClay et al., 2000; Wikramanayake et al., 1998). The apparent coordination of the advancing wave of β-catenin with the retraction of the SpSoxB1 domain raised the possibility that these two phenomena are causally related. Strong support for this idea came recently when we discovered *SpKrl (Strongylocentrotus purpuratus Krüppel-like)*, a direct target of β-catenin/TCF-Lef regulation. SpKrl is a repressor that causes the downregulation of SpSoxB1: Loss of SpKrl by morpholino oligonucleotide antisense interference expands both the domain and level of nuclear SpSoxB1 protein accumulation while gain of SpKrl function by mRNA injection strongly suppresses the level of this ATF (Howard et al., 2001). These results led us to propose that removal of SpSoxB1, and presumably other ATFs, is a prerequisite for endoderm and mesenchyme differentiation and reinforced the idea that SpSoxB1, like nuclear β-catenin, might be an important regulator of cell fates along the A-V axis.

Several facts suggest that SpSoxB1 functions in ectoderm differentiation. It accumulates predominantly in ectoderm nuclei of late gastrulae and plutei (Kenny et al., 1999). Embryos in which all vegetal differentiation is blocked by cadherin overexpression consist of poorly differentiated ectodermal balls that contain high levels of SpSoxB1 in all nuclei (Howard et al., 2001). On the other hand, transient function of SpSoxB1 might also be required for some vegetal fates because it persists at relatively high concentrations in the nuclei of macromere progeny during cleavage, which is a critical period for mesendoderm specification. For example, activation of the canonical wnt/β-catenin signaling pathway before, but not after, the 60-cell stage promotes mesendoderm development (Hörstadius, 1973; Vonica et al., 2000).

Here we have used several different loss-of-function assays to analyze SpSoxB1's role in patterning along the animal-vegetal axis of the sea urchin embryo. We also describe parallel studies on a closely related factor, SpSoxB2, which is expressed in a pattern very similar to that of SpSoxB1. We show that both SpSoxB proteins are required for gastrulation. Interestingly, ectopic and elevated expression (mis- and overexpression, MOE) of either of these factors by mRNA injection also strongly antagonizes differentiation of endoderm and mesenchyme and, in the case of SpSoxB1, even differentiation of the maternally determined primary mesenchyme cells (PMCs). This effect results from interference with the function of β-catenin-TCF/Lef since, in SpSoxB1 knockdown embryos, the transcriptional activity of a reporter gene driven by these factors is elevated. These results support our previous proposal (Angerer and Angerer, 2000; Howard et al., 2001) that nuclear β-catenin-dependent removal of SpSoxB factors from vegetal blastomeres is required for their specification.

Materials and methods

Embryo cultures

Adult sea urchins (*S. purpuratus*) were obtained from Charles M. Hollahan (Santa Barbara, CA). Embryos were cultured as described previously (Angerer and Angerer, 1981).

In situ hybridization

These assays were carried out as described previously (Angerer et al., 1987) using 5-μm sections of selected developmental stages and ^{33}P-labeled antisense RNA probes for *SpSoxB1* and *SpSoxB2* mRNAs (1.0×10^5 dpm/ng). The probes represent the complete open reading frame for each mRNA (~2 kb) and the slides were washed at high stringency ($T_m - 5$–10°C) to avoid signals resulting from cross-hybridization of the conserved HMG domain sequence (83% nucleic acid identity in 240 bp). Identical mRNA distributions were also obtained using probes lacking the HMG domain, confirming that the patterns illustrated in this study, which were obtained with full-length probes, are gene specific.

Constructs

The sequences containing the entire SpSoxB1 and SpSoxB2 open reading frames were transferred as *Eco*RI fragments from pBluescript (pBS) to pSP64T-clone [T-clone (Angerer et al., 2000)]. An mRNA template encoding a 43-amino acid, 5′ deletion of SpSoxB1, Δ5′SoxB1, was produced by polymerase chain reaction (PCR), using a forward primer containing an *Eco*RI site (underlined below), the SpSoxB1 translation initiation site, and sequence starting with amino acid residue 44 at the 5′ end of the DNA binding (HMG) domain (5′-CCG GAA TTC ATC ATG GCG GCC GCC CCG GAC AGA GTA AAA AGG-3′) and a reverse, plasmid-specific primer. In construction of the clone encoding the SpSoxB1-DBD (DNA binding domain), which includes the 142 N-terminal amino acid residues, forward and reverse primers contained *Eco*RI sites (underlined) and sequences at the N-terminus of SpSoxB1 or just

downstream from the DBD, respectively (forward: 5′-CCG GAA TTC ATC ATG GCG GCC GCC ATG TCT GTT CCT GGT GTA-3′, reverse: 5′-CCG ATC GAA TTC GGC GGC CGC TTA CTT CTT CAA CAG GGT CTT-3′). For both Δ5′SoxB1 and SpSoxB1-DBD, the *Eco*RI-digested amplimers were inserted into T-Clone. Capped, polyadenylated mRNAs were synthesized using the Sp6 mMessage-mMachine kit (Ambion) from templates linearized at an *Xba*I site just downstream of the poly(T) region of the T-clone vector. SpSoxB1** was produced by mutating two amino acid residues in the DNA binding domain to leucine, as indicated in Fig. 1. These nucleotide substitutions were made sequentially, using different PCR methods because the initial substitution did not reduce binding to the anticipated extent: The V-to-L mutation was achieved by using the technique of gene splicing by overlap extension (Horton et al., 1989). Two partially overlapping *SpSoxB1* fragments were generated with vector primers and primers containing the desired mutation (underlined nucleotides) (primer extending downstream: 5′-GGG CCT TTT GAG TCT GTC CGG GTT G-3′; primer extending upstream: 5′-CCC GGA CAG ACT CAA AAG GCC CAT G-3′). These fragments were mixed and PCR was used to generate full-length mutated SpSoxB1 sequence, which was subsequently amplified with the outside vector primers. The F-to-L change was effected by using the QuikChange Site-Directed Mutagenesis procedure (Stratagene, Inc.). The primers extending downstream and upstream were 5′-CGG AAA AGA GGC CGC TTA TCG ACG AGG C-3′ and 5′-GCC TCG TCG ATA AGC GGC CTC TTT TCC G-3′, respectively. All changes were verified by sequencing. RNA was synthesized from these templates and the corresponding proteins were translated in vitro using the TnT reticulocyte lysate system (Promega). Electrophoretic mobility shift assays were performed as described previously (Kenny et al., 1999).

Luciferase assays

Topflash (Korinek et al., 1997) was a kind gift from Dr. Frank Costantini, Columbia University. Luciferase assays were carried out as follows: Two to three hundred embryos were collected by centrifugation and resuspended in 35 μl of Cell Culture Lysis Reagent (Promega) freshly diluted five-fold from the stock solution. After incubation for 30 min at room temperature with occasional mixing, 10 μl was removed for quantitation of Topflash DNA by slot-blot hybridization. The remainder of the lysis reaction was clarified by centrifugation at 13,000 rpm for 5 min at 4°C in an Eppendorf centrifuge and the supernatant was transferred to 5-ml polystyrene assay tubes (Sarstedt). Fifty microliters of Luciferase Assay Reagent (Promega) was injected automatically by a luminometer (Auto-Lumat; EGG-Berthold) and, after a 2-s delay, emitted light was measured over a 10-s interval. DNA slot-blot signals were quantitated by phosphorimagery and used to normalize luciferase values for transgene levels.

Egg injection

Sea urchin eggs were dejellied in pH 4.6–4.9 artificial seawater and injected with 2 pl of synthetic mRNAs (~2.5×10^6 molecules of *SpSoxB1* or *SpSoxB1-DBD*, 0.62×10^6 molecules of *Δ5′SoxB1*) in 30% glycerol. For morpholino antisense knockdown experiments, 0.8 pmol of control, SpSoxB1, or SpSoxB2 morpholino was injected to achieve a final embryo cytosolic concentration of approximately 4 μM. The sequences of these morpholinos were: Control, 5′-CCTCTTACCTCAGTTACAATTTAT A-3′; SpSoxB1, 5′-CCAGGAACAGACATTTTGGTCAGTC-3′ (covering −11 to +14 with respect to AUG); SpSoxB2, 5′-CCCCATCGCAGAGTCCATCATCATC-3′ (covering −1 to +24 with respect to AUG). For rescue of the phenotype, synthetic *Δ5′SoxB1* message at 0.5 μg/μl was coinjected with the SpSoxB1 morpholino (SpSoxB1M). Only batches of eggs that yielded control embryos (injected only with 30% glycerol) with more than 80% survival and normal development were analyzed. Only phenotypes produced in >90% of surviving embryos are reported here. The misexpression and morpholino knockdown experiments were performed in at least five different embryo batches; the experiments in which the SpSoxB1M phenotype was rescued by mRNA coinjection were performed twice.

Immunostaining

Embryos were fixed with a solution containing 4% paraformaldehyde in PBST (1× PBS, 0.2% Tween 20) at room temperature for 10 min, washed three times in PBST and incubated for 1 h in blocking solution [PBST containing 3 mg bovine serum albumin (BSA)/ml (Boehringer Mannheim)]. Embryos were incubated for 2 h in blocking solution containing one or two primary antibodies diluted as follows: anti-SpSoxB1 (1:1000), CyIII-conjugated mouse anti-6e10 (1:250, provided by Dr. Charles Ettensohn, Carnegie Mellon University), mouse anti-Endo1 (1:10) and mouse anti-EctoV (1:10) (both provided by Dr. David McClay, Duke University), and rabbit anti-Spec1 antibody (1:1000) (provided by Dr. William Klein, U.T. M.D. Anderson Cancer Center). After washing three times in PBST, embryos were incubated in blocking solution for 15 min, and in a secondary antibody solution for 1 h. Secondary antibody dilutions used were as follows: 1:500 goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (Zymed) for SpSoxB1 and Spec1 antibodies; 1:250 goat anti-mouse fluorescein (Zymed) for Endo1; and 1:500 CyIII-conjugated goat anti-mouse antibody (Jackson Labs) for anti-EctoV antibody. Embryos were then washed one time in PBST containing 2 μg DAPI/ml (Sigma), followed by three washes in PBST. Embryos were mounted on poly-L-lysine (Sigma)-coated coverslips for confocal microscopy. Fluorescent signals were captured by sequential scanning by using a LeicaTS confocal microscope.

```
B1   HsSox2   DRVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGAEWKLLSETE
B1   SpSoxB1  DRVKRPMNAFMVWSRGQRRKLSQENPKMHNSEISKRLGAEWKLLSEDE
                L
B2   SpSoxB2  DHVKRPMNAFMVWSRGQRRKLAQENPKMHNSEISKRLGAEWKLLSEDD
B2   ChSox21  DHVKRPMNAFMVWSRAQRRKMAQENPKMHNSEISKRLGAEWKLLSEAE
              *.**************.****..**********************  .

B1   HsSox2   KRPFIDEAKRLRALHMKEHPDYKYRPRRKTKTLMKKDK
B1   SpSoxB1  KRPFIDEAKRLRAVHMKEHPDYKYRPRRKTKTLLKKDK
                 L
B2   SpSoxB2  KRPFIDEAKRLRALHMKEHPDYKYRPRRKPKSLMKRDK
B2   ChSox21  KRPFIDEAKRLRAMHMKEHPDYKYRPRRKPKTLLKKDK
              *************.*************** *.*.*.**
```

Fig. 1. SpSoxB1 and SpSoxB2 are closely related in the HMG box, which mediates DNA binding. They are assigned to the B class of Sox transcription factors because of the high level of sequence conservation within and immediately C-terminal to this domain (boxed sequence). Comparisons are shown to vertebrate SpSoxB class factors [human, (Hs)Sox2, and chicken, (Ch)Sox21]. To produce the SpSoxB1** sequence (see Fig. 5), two leucine substitutions were introduced to replace a valine and phenylalanine, as indicated by L below the SpSoxB1 sequence.

Results

The expression pattern of SpSoxB2 is similar to that of SpSoxB1, another B class Sox factor

SpSoxB1 and SpSoxB2 are closely related DNA binding proteins with very similar expression patterns in the early sea urchin embryo. SpSoxB1 is one of several transcription factors, the ATFs, whose concentrations are modulated in a similar manner along the animal-vegetal axis during cleavage stages (reviewed by Angerer and Angerer, 2002). This change is dynamic during the period of fate specification of vegetal blastomeres, suggesting that the ATFs may have important developmental functions. Included in this group is a closely related factor, SpSoxB2 (Kenny et al., 1999; Kozlowski, 1997). SpSoxB1 and SpSoxB2 are members of the B class of Sox factors and are 94% identical in their HMG-Box DNA binding domains (accession numbers AF157389 and AF157388, respectively). They also share a short sequence just downstream from this domain, which is conserved with other SoxB factors (Fig. 1, boxed sequence). Otherwise, the sequences of SpSoxB1 and B2 are completely divergent. SpSoxB1 and B2 proteins are assigned to the B1 and B2 subclasses, respectively, because of specific conserved amino acids in the HMG box, which are indicated in Fig. 1 by ovals and diamonds. SpSoxB2 and chicken (Ch) Sox21, another B2 subclass factor, have several other short regions of amino acid sequence identity in the C-terminal half, raising the possibility that they are orthologs.

The spatial expression pattern of *SpSoxB2* is very similar

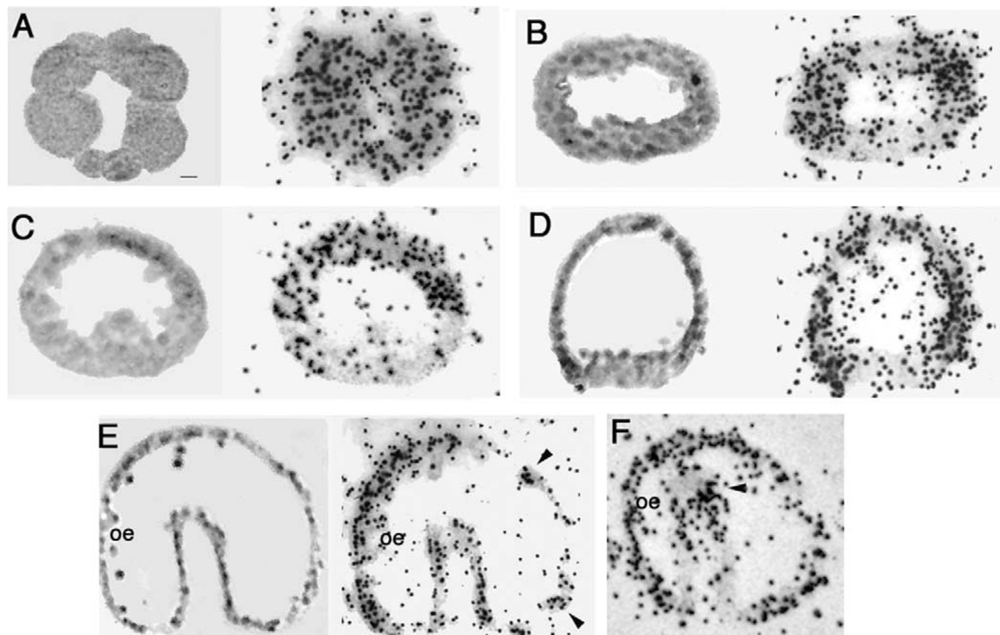


Fig. 2. *SpSoxB2* mRNA accumulation patterns. Bright-field images of embryos and autoradiographic grain distributions are shown side-by-side (except for panel F). (A) 16-cell embryo; (B) early blastula; (C) early mesenchyme blastula; (D) late mesenchyme blastula; (E) gastrula; (F) gastrula probed for *SpSoxB1* mRNA. Arrowheads in E indicate putative pigment cells within the aboral ectoderm that are enriched in *SpSoxB2* transcripts. Arrowheads in F indicate cells in the foregut region that express *SpSoxB1* transcripts at gastrula stage. In all panels embryos are oriented with the animal pole at the top. Scale bar = 10 μm.

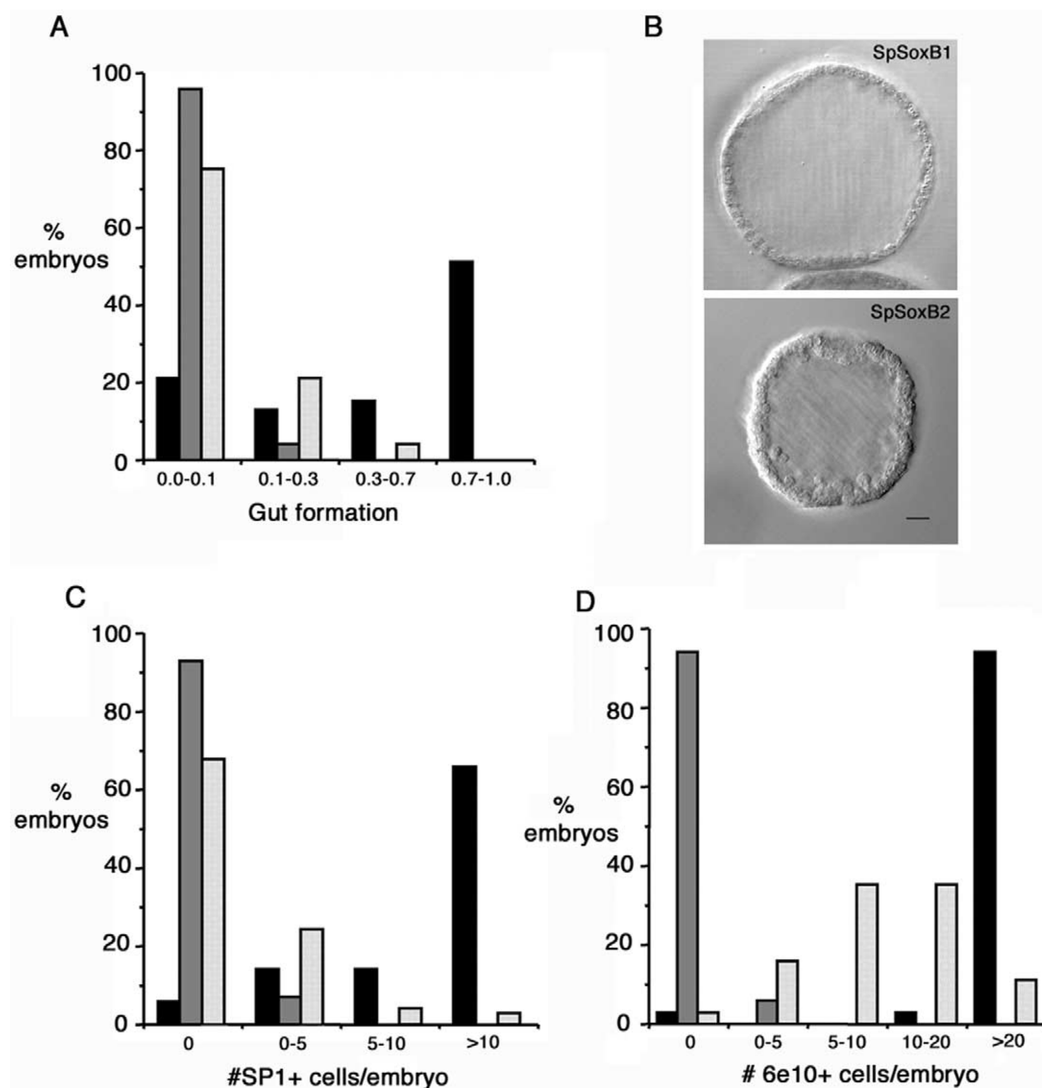


Fig. 3. Misexpression of either SpSoxB1 or SpSoxB2 suppresses vegetal differentiation. (A, C, and D) Fertilized eggs were injected with glycerol (control, black bars), SpSoxB1 mRNA (dark gray bars), or SpSoxB2 mRNA (light gray bars) and allowed to develop for 48 h. Thirty to fifty embryos of each type were then scored for vegetal differentiation: (A) Extent of gut formation, where 1.0 represents an archenteron extending completely across the blastocoel: 0–0.1 (no or small vegetal indentation), 0.1–0.3 (small vesicles or tubes), 0.3–0.7 (incomplete archentera), or 0.7–1.0 (complete or nearly complete archentera). Severe suppression of gut formation by both SpSoxB1 and SpSoxB2 was verified in four different egg batches. (B) DIC microscopic images of severe phenotypes caused by misexpression of SpSoxB1 (upper) and SpSoxB2 (lower). Scale bar = 10 μm. (C) Percentage of embryos containing the indicated number of pigment cells (SP1-positive)/embryo. (D) Percentage of embryos containing the indicated number of primary mesenchyme cells (6e10-positive)/embryo.

to that of *SpSoxB1* (Fig. 2). *SpSoxB2* maternal transcripts are uniformly distributed in the egg (not shown) and early cleaving embryo (Fig. 2B, 16-cell stage) but become depleted in vegetal blastomeres during very early blastula stages (Fig. 2C). A few cells around the animal pole of early (~180-cell) blastulae also lack these RNAs, which provides the earliest known molecular indication that animal pole cells have begun to be specified by this stage (Fig. 2B). By the hatching blastula stage, animal pole cells begin to express *SpSoxB2*, while signals in the vegetal plate and ingressed primary mesenchyme cells remain low, a pattern that continues through the late mesenchyme blastula stage (Fig. 2D). At the gastrula stage, *SpSoxB2* transcripts have accumulated to highest concentration in the oral ectoderm (oe) and also are present throughout the archenteron and in a few cells scattered throughout the aboral ectoderm (Fig. 2E, arrowheads), which probably are pigment cells. *SpSoxB1* RNA distributions up to the blastula stage have been reported previously (Kenny et al., 1999). At the gastrula stage, *SpSoxB1* transcripts also are detectable in the ectoderm and in the archenteron (Fig. 2F) but, unlike *SpSoxB2* mRNAs, they are concentrated in the foregut region (arrowhead) and are uniformly distributed throughout the ectoderm.

SpSoxB1 and SpSoxB2 can antagonize vegetal fate specification

The gradual downregulation of SpSoxB1 mRNA and protein in the presumptive vegetal plate is reciprocal to the advancing wave of nuclearization of β-catenin (Angerer and Angerer, 2000, 2002; Kenny et al., 1999). *SpKrl*, a β-catenin/TCF-Lef target gene, is essential for *SpSoxB1* downregulation because SpSoxB1 disappears from the nuclei of embryos misexpressing SpKrl and increases in nuclei of embryos in which SpKrl translation is blocked by a morpholino antisense oligonucleotide (Howard et al., 2001). Since nuclear β-catenin and SpKrl are both required for mesendoderm development, we proposed that reduction in the levels of SpSoxB1, and probably of other animalizing transcription factors, is an important component of this process. A prediction of this model is that mis- or overexpression (MOE) of SpSoxB1 or B2 in vegetal blastomeres would block endoderm and mesenchyme differentiation. To test this idea, mRNAs encoding these factors (1–3 pg/embryo; 0.5 to 1.5 $\times$ 10^6 molecules) were injected at the one-cell stage, producing an elevation of *SpSoxB1* transcripts in the embryo of approximately 6- to 20-fold. [*SpSoxB1* mRNA is present at about 80,000 transcripts in the egg and early embryo, as estimated by comparing radioactive in situ hybridization signals to those for *SpHE* mRNA, whose abundance is known (Reynolds et al., 1992).] Since *SpSoxB2* mRNA is less abundant than SpSoxB1 message (Kenny et al., 1999), the extent of its overexpression is correspondingly higher. The effect of SpSox MOE on gastrulation was monitored by estimating the length of the archenteron just as gastrulation was completing for the majority of control, glycerol-injected embryos. This stage was chosen to maximize the sensitivity of the measurement while minimizing the effects of possible regulation and/or recovery as the exogeneously supplied mRNA decayed. The frequency distributions of embryos having no/very reduced (0–10%), partial (10–30%; 30–70%), or nearly completed/normal archentera (70–100%) are shown in Fig. 3A. Either SpSoxB1 (dark gray bars) or B2 (light gray bars) MOE strongly suppressed gut formation compared to that in control, glycerol-injected, embryos (black bars). In the most severe phenotypes, embryos consisted almost entirely of cuboidal epithelial cells (Fig. 3B), and were very similar morphologically to those in which β-catenin function is blocked by injection of cadherin mRNA (Li et al., 1999; Logan et al., 1999; Howard et al., 2001; Ransick et al., 2002). Embryos injected with SpSoxB1 mRNA were usually more expanded and the epithelia of these embryos were thinner than those of embryos injected with SpSoxB2 mRNA. They also often lacked ingressed mesenchyme cells. To analyze this latter phenotype more completely, embryos were stained for molecular markers specific for pigment cells, which are secondary mesenchyme derivatives (Fig. 3C, SP1; Gibson and Burke, 1985), and for PMCs (Fig. 3D, 6e10 antibody kindly supplied by Dr. C.A.

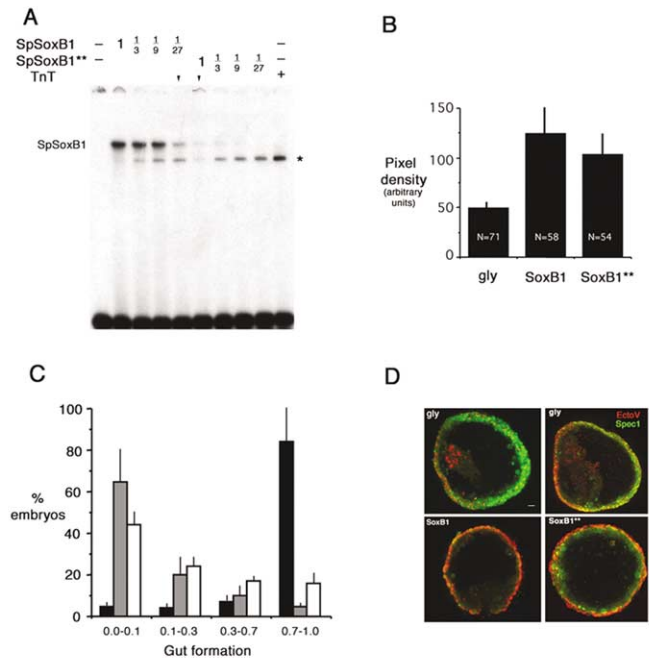


Fig. 4. Mutations in SpSoxB1 that abrogate its ability to bind DNA do not inhibit its ability to suppress vegetal differentiation. (A) The effect of two amino acid substitutions (see Fig. 1) on the DNA binding affinity of SpSoxB1 were tested in EMSAs. Dilution series of equal amounts of in vitro translated SpSoxB1 and SpSoxB1** proteins were tested for binding to the SpSoxB1 site from the *SpAN* promoter (Kenny et al., 1999). Comparison of the amount of complex formed in lanes indicated by arrowheads shows that SpSoxB1** binding is reduced about 100-fold. (*) indicates a complex formed between the probe and a protein present in the rabbit reticulocyte lysate (TnT). (B) Embryos misexpressing SpSoxB1 or SpSoxB1** were immunostained for SpSoxB1 and concentrations in nuclei of mesenchyme blastulae were estimated by measuring confocal pixel densities. (C) The extent of gut formation was scored as described in the legend to Fig. 3. Glycerol (black bars); SpSoxB1 (dark gray bars); SpSoxB1** (white bars). Values are the averages obtained from embryos derived from three different egg batches. (D) Embryos were immunostained for both Spec1 (green) and EctoV (red). Spec1 staining was variable among embryos injected with either SpSoxB1 or SpSoxB1** mRNA, generally reduced and distributed radially about the animal-vegetal axis. This, in combination with radial expression of EctoV, indicates that the ectoderm is poorly differentiated. Scale bar = 10 μm.

Ettensohn). Compared to glycerol-injected control embryos, the number of pigment cells/embryo was sharply reduced by either *SpSoxB* mRNA. However, embryos injected with *SpSoxB1* message had many fewer PMCs than did those injected with *SpSoxB2* mRNA at doses that suppressed gut formation and pigment cell differentiation to similar extents (compare Fig. 3D with 3C and 3A). When development was allowed to continue for another day, clusters of cells appeared in the blastocoels of some embryos, which presumably had started to recover after decay of the exogenously supplied *SpSoxB* mRNA. Immunostaining showed that these clusters included PMCs, pigment cells, and a few cells expressing the mid- and hindgut marker, Endo1 (not shown). Most cells of embryos overexpressing either SpSoxB factor formed epithelial spheres of poorly differentiated ectoderm, which lacked polarity as shown by the ab-

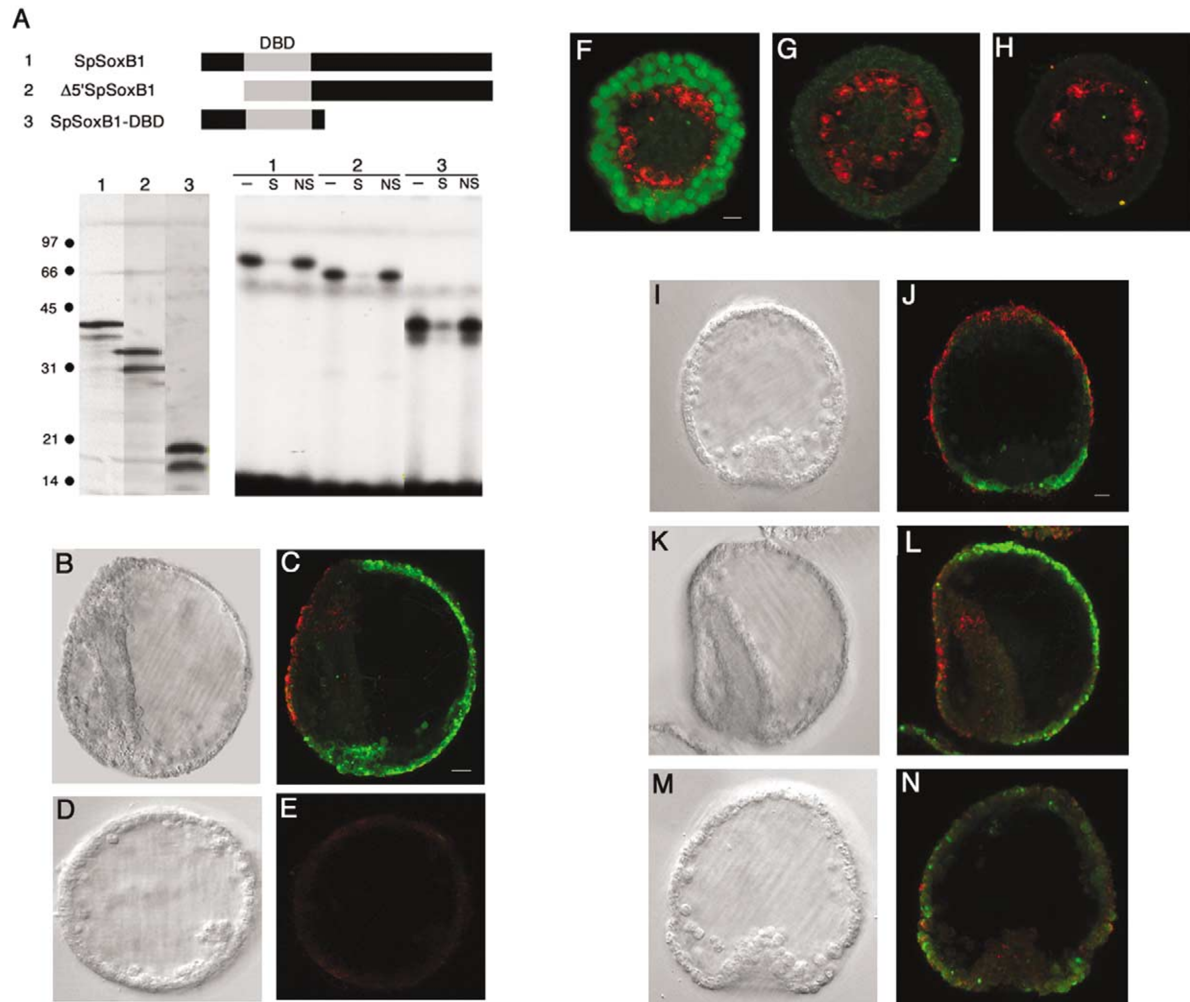


Fig. 5. Loss-of-function assays show that SpSoxB1 and SpSoxB2 are both required for gastrulation and normal ectoderm differentiation. (A) Diagram of SpSoxB1 protein variants: (1) full-length SpSoxB1; (2) N-terminal truncation of SpSoxB1; and (3) SpSoxB1 C-terminal half truncation resulting in a protein consisting mostly of the DNA binding domain. Below, left, a sodium dodecyl sulfate–polyacrylamide gel showing that the in vitro translation products are the sizes expected for these three proteins. Below, right, EMSAs show that the proteins translated in vitro bind similarly to a well-characterized Sox site (Kenny et al., 1999). −, probe alone; S and NS, probe in combination with a 500-fold molar excess of specific or nonspecific unlabeled competitor, respectively. (B–E) Embryos injected with control morpholino (B and C) or SpSoxB1-DBD (D and E) and stained for EctoV (red) and Spec1 (green), which label normal differentiated oral and aboral ectoderm, respectively; (F–H) Verification that antisense interference effectively knocks down SpSoxB1 protein levels. Mesenchyme blastulae that were injected at the one-cell stage with glycerol (F and H) or SpSoxB1 morpholino (G) are shown viewed from the vegetal pole. Embryos were doubly stained with an antibody specific for SpSoxB1 (F and G) or preimmune serum (H) and for the PMC-specific marker, 6e10. (I and J) SpSoxB1 morpholino injection results in radialized 3-day embryos that lack invaginated guts and express EctoV (red) and Spec1 (green) in regions that are radially symmetric around the animal-vegetal axis, rather than polarized along an oral-aboral axis. (K and L) Coinjection of SpSoxB1 morpholino and SpSoxB1 mRNA lacking the morpholino target sequence (in A, protein 2) completely rescues gut formation and oral-aboral polarity. (M and N) SpSoxB2 morpholino injection results in 3-day-old embryos with severely reduced guts and that fail to express EctoV or Spec1. Scale bar = 10 μm.

sence of localized immunostaining for the oral and aboral ectoderm-specific proteins SpGsc (Angerer et al., 2001) (not shown) and Spec1 (Carpenter et al., 1984) (see below). EctoV did accumulate uniformly in the ectoderm of these embryos, a phenomenon that is characteristic of arrested early ectoderm (Angerer et al., 2001). This arrest of ectoderm differentiation could result either from a direct effect of elevated SpSoxB levels on ectodermal cells or from interference with vegetal signals that are known to be required for ectoderm patterning (reviewed by Davidson, 1998; Brandhorst and Klein, 2002; Angerer and Angerer, 2002). The demonstrated ability of SpSoxB factors to antagonize development of vegetal cell types in the sea urchin embryo suggests that the observed dynamic down regulation of SpSoxB1, and probably that of other ATFs, is necessary for vegetal development.

The phenotype produced by SpSoxB1 misexpression is largely independent of DNA binding

Although MOE of either SpSoxB1 or SpSoxB2 severely suppresses vegetal differentiation, the only sequence con-

served between these two proteins is the DNA binding domain. Because SpSoxB factors and TCF-Lef (which mediates β-catenin activity) can bind to some of the same *cis* elements, it was possible that elevated expression of SpSoxB factors suppresses vegetal fates by competitively inhibiting TCF-Lef binding to DNA. To test this idea, substitutions were introduced at the two amino acid residues shown underlined in Fig. 1. The mutant protein, referred to as SpSoxB1**, and SpSoxB1 were translated in vitro. EMSAs show that the binding of SpSoxB1** to a known functional SpSoxB1 *cis* element (Kenny et al., 1999) was reduced by approximately two orders of magnitude (Fig. 4A; compare lanes indicated by arrows). After injection of equivalent levels of mRNA, confocal microscopy of immunostained embryos showed that very similar levels of wild-type and mutant proteins were present in mesenchyme blastulae. This level was two- to three-fold higher than that of endogenous SpSoxB1 in control embryos (Fig. 4B). Formation of gut was efficiently suppressed by SpSoxB1** (Fig. 4C, open bars), whose effect was only slightly weaker than that of wild-type SpSoxB1 (Fig. 4C, gray bars). The SpSoxB1 and B1** phenotypes were very similar when the dose of SpSoxB1** mRNA injected was increased just two-fold (data not shown). Significant reductions in numbers of pigment cells and PMCs were also observed in embryos injected with SpSoxB1** mRNA (data not shown) and ectoderm was again poorly differentiated (Fig. 4D). The levels of expression of the aboral ectoderm markers were highly variable and signals were not restricted to any specific region of the embryos. Most embryos expressed EctoV, which often reflects lack of aboral ectoderm differentiation or arrest at an early stage of ectoderm differentiation (Angerer et al., 2001; Angerer and Angerer, 2002). Because the DNA binding affinity of SpSoxB1** was reduced by several orders of magnitude, yet the mRNA dose differences required to elicit very similar phenotypes were small, we conclude that, in MOE assays, SpSoxB1 suppresses vegetal differentiation primarily through protein-protein interactions.

SpSoxB1 suppresses TCF/Lef-mediated transcriptional activation in the normal embryo

The finding that SpSoxB1 MOE produced a phenotype that was indistinguishable from that caused by cadherin injection raised the possibility that SpSoxB1 can interfere with the function of components of the canonical Wnt signaling pathway. However, it was also possible that elevated levels in MOE assays could produce nonspecific effects unrelated to what occurs in the normal embryo. To test whether endogenous levels of SpSoxB1 were sufficient to antagonize this pathway, we asked whether TCF-Lef/β-catenin activity increased in embryos when SpSoxB1 translation was inhibited with a morpholino antisense oligonucleotide (SpSoxB1M). Experiments demonstrating the efficacy of this approach and the developmental consequences of loss of SpSoxB factors are presented below (Figs. 5–7). We monitored TCF-Lef/β-catenin activity in embryos with Topflash, a transgene that contains 4 TCF-Lef sites and the *c-fos* basal promoter upstream of the firefly luciferase gene (Korinek et al., 1997). Four different batches of eggs were injected either with glycerol or mRNA encoding stable (nonphosphorylatable) β-catenin as positive controls, or with SpSoxB1 morpholino. The embryos were assayed at several different stages, ranging from early blastula (16 h; ~250 cells) to mesenchyme blastula (24 h; 400 cells). During this interval maternal SpSoxB1 protein has turned over completely (our unpublished observations), but there is still significant overlap of zygotic SpSoxB1 and β-catenin in the nuclei of macromere progeny. As shown in Table 1, in each of these batches exogenously supplied β-catenin resulted in an increase in luciferase activity/Topflash transgene (1.5- to 5.3-fold), as expected. Most importantly, knockdown of SpSoxB1 resulted in a significant enhancement of Topflash promoter activity (3- to 10-fold). Thus, normal levels of SpSoxB1 are sufficient to limit β-catenin/Tcf-Lef function in early sea urchin embryos.

SpSoxB1 and SpSoxB2 are necessary for gastrulation and ectoderm differentiation

The finding that the SpSoxB factors can strongly antagonize vegetal development raised the possibility that blocking their function might lead to the expansion of the mesendoderm territory. As an initial test of this idea, we introduced mRNA encoding a truncated protein consisting of only the SpSoxB1 DNA binding domain and a few N-terminal amino acid residues (SoxB-DBD; Fig. 5A, construct 3). This peptide is expected to behave as a dominant negative by competing for DNA binding by both SpSoxB1 and B2 proteins, which have similar specificity (Kenny et al., 1999); our unpublished observations). As shown in Fig. 5A, SpSoxB-DBD and SpSoxB1 that are translated in vitro bind similarly to the SpSoxB1 site in the *SpAN* promoter (samples 3 and 1, respectively). Injection of SpSoxB1-DBD mRNA produced a strong phenotype: When control embryos reached the gastrula stage, mRNA-injected embryos consisted of epithelial balls containing some cells in the blastocoel (Fig. 5D), most of which expressed the PMC-specific marker 6e10 (data not shown). The epithelium of these embryos was morphologically undifferentiated and expression of markers for differentiated aboral (Spec1, green) and oral (EctoV, red) ectoderm (Fig. 5C) was always sharply reduced and often undetectable (Fig. 5E). Surprisingly, gastrulation did not occur and only a few cells expressing the Endo1 endoderm marker were scattered in the vegetal pole region at 3 days post fertilization (data not shown). This may reflect the beginning of recovery after turnover of exogenously supplied SpSoxB1-DBD protein. Assuming that SpSoxB1-DBD interferes with DNA binding of only SpSoxB1 and other closely related factors, these results led to the unexpected conclusion that, while MOE of

SpSoxB factors can suppress vegetal development and SpSoxB1 limits activity of the β-catenin/TCF-Lef pathway during blastula stages, SpSoxB factors also are required for gastrulation in normal embryos.

To test specifically the separate requirements for SpSoxB1 and B2 factors, we injected morpholinos into one-cell zygotes to block translation of the corresponding mRNAs present in the egg and embryo (Heasman et al., 2000; Summerton and Weller, 1997). We have recently used this approach successfully to inhibit the production of several other transcription factors (Angerer et al., 2001; Howard et al., 2001). Injection of the SpSoxB1M (Fig. 5G) effectively eliminated SpSoxB1 (green) from nuclei of mesenchyme blastulae, as shown by comparison to control embryos injected with glycerol (compare Fig. 5G and F); signals were similar to those obtained with preimmune serum (Fig. 5H). These stacks of confocal images of embryos viewed from the vegetal pole illustrate that the vegetal-most cells, the PMCs (red), which have just ingressed into the blastocoel, differentiate in the absence of SpSoxB1. This is not unexpected since SpSoxB1 protein rapidly disappears from the PMC lineages starting at the 16-cell stage (Kenny et al., 1999).

SpSoxB1M produced a striking and highly reproducible morphological alteration that was very similar to the phenotype caused by the dominant negative SpSoxB-DBD protein. Greater than 95% of the embryos failed to gastrulate and appeared to consist primarily of ectoderm and some ingressed cells (Fig. 5I and J; compare to control morpholino-injected embryo of the same age, Fig. 5B and C). This phenotype was attributable specifically to the loss of SpSoxB1 because simultaneous injection of a synthetic mRNA encoding SpSoxB1 but lacking the morpholino target sequence (Δ5′SpSoxB1; Fig. 5A, construct 2) completely rescued gastrulation (Fig. 5K and L). The rescuing protein contained amino acid residues 45 to 345 and its binding to the *SpAN* SpSoxB1 site was comparable to that of the full-length protein and of the SpSoxB1-DBD protein (Fig. 5A, compare lane 2 with lanes 1 and 3). Low concentrations of injected *Δ5′SpSoxB1* mRNA had no detectable effect on the SpSoxB1M phenotype. Higher concentrations rescued essentially normal embryos (Fig. 5K and L), which contained complete guts and also formed coelomic pouches (not shown), which do not normally develop until late in embryogenesis. The morphology of both the SpSoxB1M-injected and rescued embryos was very reproducible. Approximately 100 injected embryos from each of two different egg batches were examined in detail and in each case greater than 95% of the embryos showed the same phenotype. As expected, RNA concentrations greater than those required for rescue elicited the SpSoxB1 misexpression phenotype described above (Fig. 3).

The phenotypes generated by SpSoxB1M and SpSoxB-DBD differed in the ectoderm. Knockdown of SpSoxB1 did not prevent robust expression of either EctoV or Spec1 (Fig. 5J). However, these ectodermal territories were abnormally positioned, in that a large EctoV-positive region (red), radially symmetric about the animal-vegetal axis, encompassed the animal 2/3 of the embryo and a smaller, adjacent torus of Spec1-positive cells (green) surrounded the vegetal pole. These two regions did not overlap significantly and they aligned along the A-V axis, rather than the O-A axis, as in normal embryos (Fig. 5C). Coinjection of Δ5′SpSoxB1 synthetic mRNA also rescued this aspect of the morpholino phenotype and completely restored normal O-A polarity (Fig. 5L). It is likely that ectoderm differentiation does not proceed very far in embryos that lack SpSoxB1 and that restoration of uniformly distributed SpSoxB1 allows it to continue past this early arrest point.

The phenotype produced by SpSoxB1M is not as severe as that generated by misexpression of SpSoxB1-DBD. One likely source of this difference is that the function of SpSoxB1 maternal protein might be antagonized by SpSoxB1-DBD but is not affected by SpSoxB1M-mediated translational interference after fertilization. When embryos are injected with SpSoxB1M, SpSoxB1 protein is reduced but still detectable in nuclei during cleavage stages. It is likely that most of this is persisting maternal protein (Kenny et al., 1999). By 7–8th cleavage, its level in animal nuclei is reduced to approximately 10% of that in mock-injected embryos, as measured by confocal pixel densities; by the hatching/early mesenchyme blastula stage no nuclear SpSoxB1 is detectable (Fig. 5G). A second possible contributing factor is that dominant negative SpSoxB1 might also interfere with SpSoxB2 function, which could be distinct from that of SpSoxB1 because these proteins are completely divergent outside of their DNA binding domains. To test the separate role of SpSoxB2, its translation was blocked with a morpholino. The resulting phenotype (Fig. 5M and N) was very similar to that produced by the dominant negative SpSoxB1 protein (Fig. 5D and E). Again, embryos did not gastrulate and only a few 6e10-positive cells ingressed into the blastocoel. However, unlike embryos injected with SpSoxB1M, expression of the ectoderm markers was strongly suppressed by SpSoxB2M (Fig. 5N). Collectively, these results show that SpSoxB1 and SpSoxB2 are each required for gastrulation and that they probably have at least partially distinct functions in ectoderm differentiation.

To investigate further the state of differentiation of ectoderm in embryos lacking SpSoxB1 or SpSoxB2, we assayed expression of SpGsc, which is the earliest known marker restricted to oral ectoderm. *SpGsc* encodes a key transcriptional repressor that is required for early steps in oral ectoderm specification: Loss of SpGsc allows all ectoderm cells to continue differentiating toward aboral fate, whereas ectopic SpGsc expression suppresses differentiation of this cell type (Angerer et al., 2001). As shown in Fig. 6, SpGsc accumulates in oral ectoderm nuclei of control embryos (top panel), but is undetectable in nuclei of embryos lacking either SpSoxB1 (middle panel) or SpSoxB2

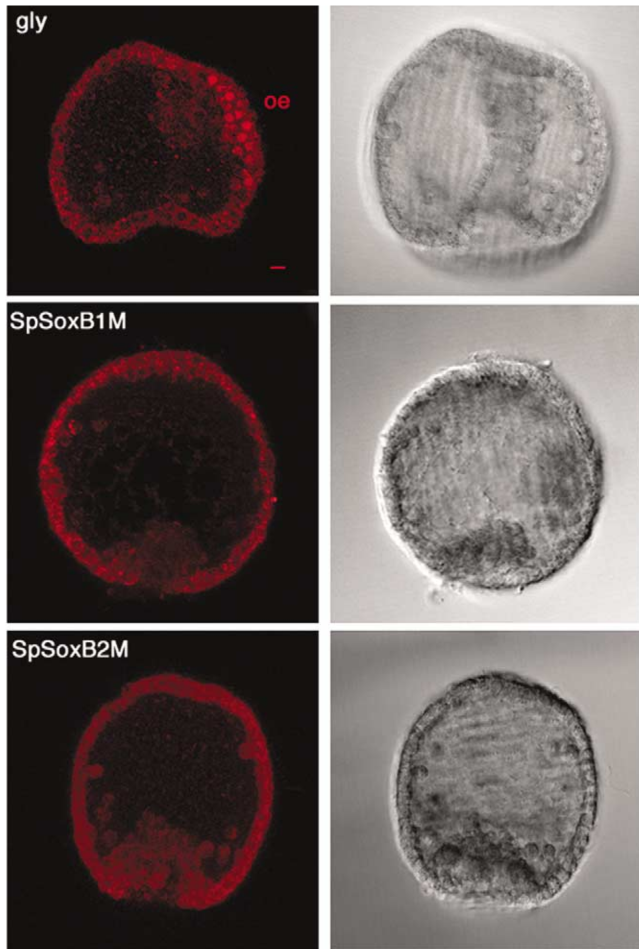


Fig. 6. SpSoxB1 and B2 are required for expression of the SpGsc transcription factor, which regulates oral ectoderm fate. Embryos were injected with glycerol (control) or with SpSoxB1 or SpSoxB2 morpholino as indicated in the panels. When control embryos reached the early gastrula stage, they were stained with polyclonal antiserum against SpGsc. This protein accumulates specifically in oral ectoderm (oe) nuclei of control embryos, but is undetectable in those of morpholino-injected embryos from the same batch. Scale bar = 10 μm.

(bottom panel). These results suggest that specification of oral ectoderm requires both SpSoxB factors.

To determine the identity of cells that had ingressed into the blastocoel in SpSoxB1M- and SpSoxB2M-injected embryos, we stained for epitopes found on primary mesenchyme cells (PMC), on two secondary mesenchyme derivatives, pigment and muscle cells, and on differentiated endoderm. In Fig. 7 are compared embryos injected with glycerol (A–C), with SpSoxB1M (D–F), or with SpSoxB2M (G–I). PMCs (red) and pigment cells (green) were observed in gastrula stage embryos deficient in either SpSoxB1 or SpSoxB2 (Fig. 7D–F). Among different batches of morpholino-injected embryos, the number of pigment cells/embryo ranged from several to more than 20. Although the reason for this variation is not clear, one possibility is that SpSoxB1 is required at a low level during cleavage stages for pigment cell specification and that in some batches of morpholino-injected embryos maternal SpSoxB1 persists in macromere progeny at levels sufficient for this to occur. At the pluteus stage, SpSoxB1M-injected embryos also expressed the late secondary mesenchyme marker, muscle myosin (red), in 5–10 cells that had entered the blastocoel (Fig. 7E). These embryos were also stained with DAPI, which appears green in these images. Control embryos had between 6 and 14 circumesophageal cells that expressed muscle myosin at this stage (Fig. 7B). In contrast, embryos lacking SpSoxB2 rarely contained myosin-positive cells and those that did had at most only one or two such cells (Fig. 7H). In some batches of embryos lacking SpSoxB1, the late marker of mid- and hindgut, Endo1 (Fig. 7C), was expressed in a variable number of cells near the vegetal pole, including some that had entered the blastocoel and others that remained in the epithelial wall (Fig. 7F). EndoI-positive cells were also observed in SpSoxB2M embryos (Fig. 7I), but these were usually fewer in number. As is the case for all SpSoxB1M embryos reported in this study, no SpSoxB1 protein could be detected in nuclei after the early blastula stage (Fig. 7F). Thus, the expression of these relatively late mesenchyme and endoderm markers is not attributable to recovered synthesis of SpSoxB1 at late stages. We presume that the knockdown is equally efficient for SpSoxB2, but no antibody for this protein is yet available.

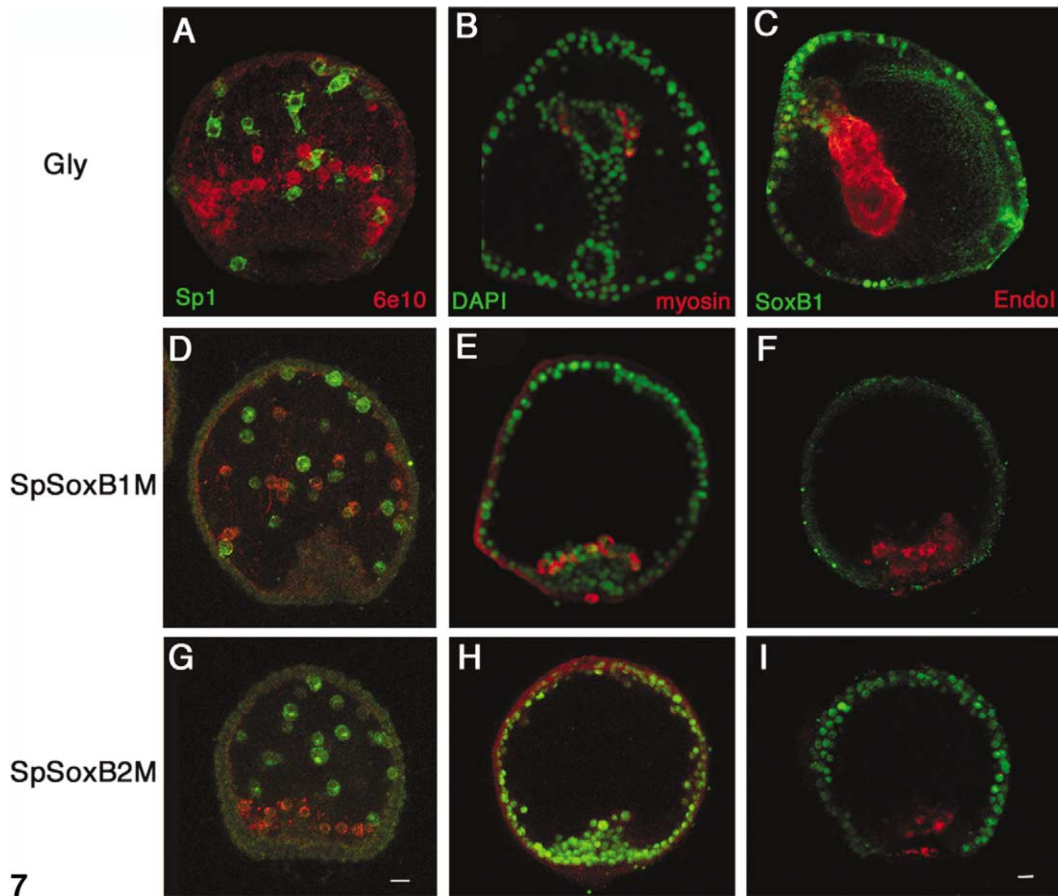
In summary, these results show that the differentiation of PMCs, pigment cells, and some endoderm cells occurs when the levels of SpSoxB1 and SpSoxB2 are strongly reduced. Appearance of muscle cells, a late differentiating SMC cell type, appears to be more dependent on SpSoxB2 than on SpSoxB1, but whether this indicates a specific requirement of this cell type for SpSoxB2 or results from developmental arrest in SpSoxB2-deficient embryos is not yet clear.

The findings that SpSoxB1 (and SpSoxB2) are not required for mesenchyme differentiation but are required for gastrulation led us to examine more closely how long SpSoxB1 protein persists in nuclei of presumptive endoderm. As shown in Fig. 8, the concentration of SpSoxB1 is high in the nuclei of presumptive endoderm cells (white and corresponding black arrowheads) until these cells begin to involute during gastrulation, at which time its concentration

Table 1
Fold increases in luciferase activity[a]

Expt	Embryo age	Stable β-catenin	SoxB1 morpholino
1	20 h	1.5	3.2
2	24 h	3.3	7.4
3	16 h	3.4	10.2
4	16 h	5.3	5.3
Mean		3.4	6.5

[a] Luciferase activities resulting from transcription of the Topflash transgene were normalized to Topflash DNA levels determined by slot-blot hybridization in embryos injected with glycerol (control), stable β-catenin mRNA (test), or SoxB1 morpholino (test). Fold increases are the ratios of normalized test and control values.



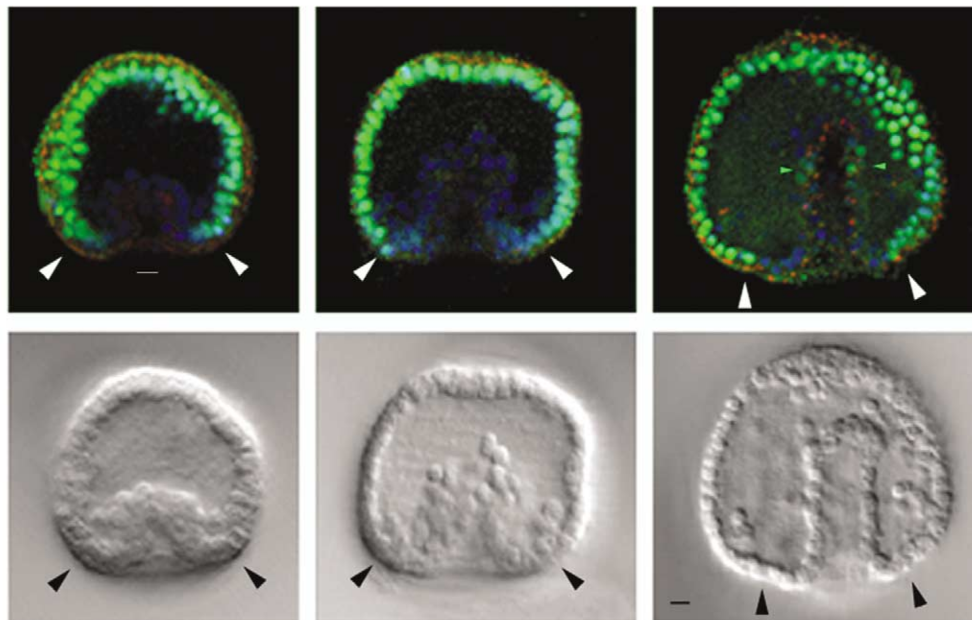


Fig. 7. Vegetal cell type differentiation is not prevented by SpSoxB1 or SpSoxB2 morpholinos. Embryos were injected at the one-cell stage with glycerol (A–C), SpSoxB1M (D–F), or SpSoxB2M (G–I). (A, D, and G) Gastrula stage control embryos (2-day) were stained with antibodies specific for pigment cells (green; SP1) and PMCs (primary mesenchyme cells) (red; 6e10). (B, E, and H) At 3 days (pluteus stage for controls), embryos were stained with an antibody for muscle myosin (red) and for DNA (DAPI; green). (C, F, and I) Three-day-old embryos were stained for SpSoxB1 (green) and EndoI (red; mid- and hindgut marker). Scale bar = 10 μm.

Fig. 8. SpSoxB1 persists in nuclei of presumptive endoderm until cells begin to involute during gastrulation. Embryos at early (left), mid (middle), and late (right) gastrula stages were stained with an antibody specific for SpSoxB1 and with DAPI and the merged confocal images are shown in the top row. As the SpSoxB1/DNA ratio decreases, the color of nuclei shifts from green to blue. Arrowheads for early and mid-gastrula-stage embryos mark corresponding positions of cells that will become part of the archenteron. Arrowheads for the late gastrula mark the probable position of the final ectoderm-endoderm boundary. Scale bar = 10 μm.

drops dramatically. In some embryos very low levels of staining are detectable in the nuclei of cells that have just passed through the blastopore. This observation is consistent with the possibility that SpSoxB1 has an autonomous function(s) in endoderm precursors in promoting gastrulation movements. It does not exclude the possibility that it has non-cell-autonomous functions in promoting gastrulation through its function in ectoderm cells, as discussed below.

Discussion

Recent work from a number of laboratories supports a model in which the initial steps in patterning of cell fates along the animal-vegetal axis of the sea urchin embryo depend on the activities of β-catenin in vegetal blastomeres and a set of transcription factors in the animal hemisphere, termed the animalizing transcription factors (ATFs) (reviewed by Angerer and Angerer, 2000, 2002; Brandhorst and Klein, 2002; Davidson et al., 2002). Starting at the 16-cell stage and continuing until the beginning of morphogenesis after hatching, β-catenin accumulates in nuclei of progressively more animal blastomeres of the vegetal hemisphere, while expression of the ATFs, including SpSoxB1, SpSoxB2, and SpEts4 (Wei et al., 1999), as well as that of their immediate target genes [the VEB genes *SpAN* and *SpHE* (Reynolds et al., 1992)] is coordinately diminished in the same cells (Kenny et al., 1999). The region in which this dynamic interplay of animalizing and vegetalizing factors takes place includes the veg_2 tier and vegetal derivatives of the veg_1 tier of blastomeres, which cells are fated to form the endoderm and secondary mesenchyme. We have proposed that the balance of ATF factors and nuclear β-catenin is important in specifying these tissues (Angerer and Angerer, 2002). Previously, we have shown that elimination of nuclear β-catenin leads to dramatic upregulation of SpSoxB1 throughout the embryo. Conversely, progressive clearance of SpSoxB1 from vegetal cells is dependent on nuclear β-catenin and is mediated, at least in part, by several direct targets of β-catenin that encode transcriptional repressors [SpKrl (Howard et al., 2001) and pmar1 (Oliveri et al., 2003)].

In this study we have tested these ideas further by perturbing the concentrations of two of the ATFs SpSoxB1 and SpSoxB2. Our studies support a major prediction of the above model, i.e., that ATFs antagonize the function of the vegetal organizer. First, overexpression of SpSoxB1 converts the embryo to an epithelial ball that lacks all vegetal cell types; SpSoxB2 overexpression has a similar effect, but PMCs are present. Second, knockdown of SpSoxB1 leads to an increase in β-catenin/TCF-Lef activity, suggesting that SpSoxB1 protein normally limits the magnitude and range of activity of the vegetal signaling mechanism. However, the SpSoxB factors also play a positive role in development of mesendoderm, since each is required for gastrulation, as shown by morpholino-mediated knockdown experiments. Below we suggest that this role may be played out both autonomously within the mesendoderm and nonautonomously through regulation of gene activity in the early ectoderm. These results show that tight regulation of SpSoxB levels in time and space is critical for normal development of the sea urchin embryo.

The role of SpSoxB factors in gastrulation and ectoderm development

Loss of either SpSoxB1 or SpSoxB2 alters ectoderm differentiation and prevents gastrulation. In contrast, neither SpSoxB1 nor SpSoxB2 is essential for differentiation of PMCs and pigment cells, two of the most vegetal derivatives, consistent with the observation that in normal embryos SpSoxB1, and presumably SpSoxB2, are cleared first from these nuclei. Both SpSoxB factors are essential for gut formation, but whether they function in endoderm specification, in later differentiation and morphogenesis, or in all of these processes is not firmly established. The experiments we have presented do not include functional tests of endoderm specification. However, much has been learned recently about the gene regulatory network that supports endomesoderm specification and differentiation (http://www.its.caltech.edu/~mirsky/endomes.html) and these data do not currently support an essential role for SpSoxB1 in general endoderm specification. Analysis by real-time PCR shows that expression of core genes in the endomesoderm gene regulatory network is not affected by loss of SpSoxB1 function (although these same genes are markedly affected by misexpression). Furthermore, a few cells differentiate sufficiently to express Endo1, which is a relatively late product of mid- and hindgut. If SpSoxB1 is involved in endoderm specification, then perhaps different presumptive endoderm cells have quantitatively different requirements for SpSoxB1, which in some cases may be met by maternal SpSoxB protein. Differential requirement could occur if different cells with the same eventual fate arise from progenitors with different initial concentrations of the relevant regulatory molecules and, perhaps, signaling inputs. This idea is consistent with the highly regulatory nature of endomesoderm patterning. Alternatively, SpSoxB1 may function in a subroutine of the regulatory network and be required for specification of some endoderm cell types, but not others. In addition, SpSoxB2 may have a partially overlapping function that can support some vegetal development in the absence of SpSoxB1. Further work is required to determine the exact position of the SpSoxB factors in the regulatory network and their requirement for production of different cell types in the gut.

Alternatively or additionally, SpSoxB1 target genes expressed in early ectoderm may provide signals or substrates required for gastrulation. For example, transcription of *SpAN* (Kozlowski et al., 1996; Kenny et al., 1999) is

strongly dependent on direct regulation by SpSoxB1. *SpAN* is expressed in the ATF domain of the very early blastula and encodes a tolloid-like metalloprotease that is secreted to the apical extracellular matrix. The integrity of this matrix is known to be critical for the cell movements of gastrulation because these can be blocked by antibodies that bind to either fibropellin (Burke et al., 1991) or hyalin (Adelson and Humphreys, 1988). Interestingly, SpAN can modify hyalin via proteolytic cleavage (Howard, E., unpublished observations), which provides a potential link between continued SpSoxB1 action in the ectoderm and gastrulation movements. More detailed understanding of the role of SpSoxB factors in gastrulation will require identifying other target genes.

Since the major site of expression of SpSoxB factors is in presumptive ectoderm, it is not surprising that they are essential for normal differentiation of these tissues. However, some of the effects of loss of SpSoxB function may also be indirect and reflect a role for these factors in endomesoderm because ectoderm differentiation and oral-aboral polarity require signaling from vegetal blastomeres (reviewed in Brandhorst and Klein, 2002; Angerer and Angerer, 2002). In embryos lacking SpSoxB2, presumptive ectoderm arrests at a very early stage before it is molecularly patterned. Embryos depleted of (zygotic) SpSoxB1 are radialized, with a large animal region that expresses EctoV above a narrow torus of cells expressing Spec1. This arrangement is orthogonal to the alignment of tissues expressing these markers in normal, more differentiated embryos and might therefore be interpreted as a reorientation of the oral-aboral axis to align with the animal-vegetal axis. However, SpSoxB1 knockdown embryos fail to activate expression of SpGsc, which is an essential early step in the differentiation of oral ectoderm (Angerer et al., 2001). Therefore, we favor an alternate interpretation of this phenotype in which most of the ectoderm is blocked at an early stage and accumulates EctoV, while the more vegetal cells can proceed to the next stage and express aboral markers (see Angerer and Angerer, 2002, for further discussion of stages in ectoderm differentiation). In this interpretation, the SpSoxB1M phenotype reflects a failure to establish O-A polarity rather than misalignment of this axis. It is particularly interesting that uniform expression of SpSoxB1 by mRNA injection can rescue this polarity.

SpSoxB factors can antagonize vegetal development

Injection of mRNA encoding either SpSoxB1 or SpSoxB2 antagonizes specification of vegetal cell types. The finding that SpSoxB1 can also inhibit the development of PMCs was surprising because the fate of these cells is thought to be determined by the 16-cell stage through nuclearization of β-catenin. All nuclei of normal eight-cell embryos have equivalent high levels of SpSoxB1 protein. The next asymmetric cleavage of the vegetal blastomeres is quickly followed by significant depletion of SpSoxB1 in the micromeres (Kenny et al., 1999). The ability of SpSoxB1 to block PMC development supports the view that early downregulation of SpSoxB1 at the 16-cell stage is essential for the early fate specification of these cells. Strong support for this conclusion is now provided by the demonstration that activity of a β-catenin/TCF-Lef-dependent promoter is greatly enhanced when the SpSoxB1 concentration is reduced by morpholino-mediated translational interference. Although the exact mechanism by which SpSoxB1 antagonizes β-catenin function is not yet known, it is not simply by competing with TCF-Lef for binding to shared *cis* regulatory elements because the SpSoxB1** protein, which lacks significant DNA binding activity, produces the same phenotype as does the wild-type protein. Such a role for regions of SpSoxB factors outside their DNA binding domains is interesting in light of a report that several different *Xenopus* Sox proteins can interact directly with β-catenin, thereby preventing its interaction with TCF-Lef (Zorn et al., 1999). If SpSoxB proteins shared this property, it could explain why they antagonize vegetal differentiation, which is completely dependent on β-catenin/TCF-Lef activity (Logan et al., 1999; Wikramanayake et al., 1998; Vonica et al., 2000). As in the case of SpSoxB1 and B2, the *Xenopus* Sox proteins are dissimilar outside their DNA binding domains. In GST pulldown assays, we found that, although both SpSoxB proteins translated in vitro could bind to *Xenopus* β-catenin, they did not bind as well as did *Xenopus* Sox17β nor did they bind detectably to sea urchin (*S. purpuratus* or *L. variegatus*) β-catenin in similar assays (Levine, A., unpublished observations). In mammalian cells, several HMG box-containing transcription factors, HBP1 (Sampson et al., 2001) and Sox7 (Takash et al., 2001), also can suppress Wnt signaling. In the case of HBP1, this effect does not depend on its DNA binding ability but instead is thought to be due to a direct interaction that interferes with TCF-Lef's binding to DNA. Additional studies are required to determine the mechanism by which SpSoxB factors inhibit vegetal cell fate specification in sea urchin embryos.

Acknowledgments

We would like to thank Drs. Eric Davidson and Eric Howard for stimulating discussions and Drs. Charles Ettensohn, William Klein, and David McClay for antibodies. This study was supported by an NIH grant to R.C.A. (GM25553). A.P.K. was a trainee in the medical scientist training program, NIH grant T32 GM07356.

References

Adelson, D.L., Humphreys, T., 1988. Sea urchin morphogenesis and cell-hyalin adhesion are perturbed by a monoclonal antibody specific for hyalin. Development 104, 391–402.

Angerer, L.M., Angerer, R.C., 1981. Detection of poly A+ RNA in sea urchin eggs and embryos by quantitative in situ hybridization. Nucleic Acids Res. 9, 2819–2840.

Angerer, L.M., Angerer, R.C., 2000. Animal-vegetal axis patterning mechanisms in the early sea urchin embryo. Dev. Biol. 218, 1–12.

Angerer, L.M., Angerer, R.C., 2002. Patterning the sea urchin embryo: gene regulatory networks, signaling pathways and cellular interactions. Curr. Top. Dev. Biol. 53, 159–198.

Angerer, L.M., Cox, K.H., Angerer, R.C., 1987. Demonstration of tissue-specific gene expression by in situ hybridization. Methods Enzymol. 152, 649–661.

Angerer, L.M., Oleksyn, D.W., Levine, A.M., Li, X., Klein, W.H., Angerer, R.C., 2001. Sea urchin goosecoid function links fate specification along the animal-vegetal and oral-aboral embryonic axes. Development 128, 4393–4404.

Angerer, L.M., Oleksyn, D.W., Logan, C.Y., McClay, D.R., Dale, L., Angerer, R.C., 2000. A BMP pathway regulates cell fate allocation along the sea urchin animal-vegetal embryonic axis. Development 127, 1105–1114.

Brandhorst, B.P., Klein, W.H., 2002. Molecular patterning along the sea urchin animal-vegetal axis. Int. Rev. Cytol. 213, 183–232.

Burke, R.D., Myers, R.L., Sexton, T.L., Jackson, C., 1991. Cell movements during the initial phase of gastrulation in the sea urchin embryo. Dev. Biol. 146, 542–557.

Carpenter, C.D., Bruskin, A.M., Hardin, P.E., Keast, M.J., Anstrom, J., Tyner, A.L., Brandhorst, B.P., Klein, W.H., 1984. Novel proteins belonging to the troponin C superfamily are encoded by a set of mRNAs in sea urchin embryos. Cell 36, 663–671.

Davidson, E.H., 1998. Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. Development 125, 3269–3290.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P.Y., Revilla, R., Rust, A.G., Pan, Z., Schilstra, M.J., Clarke, P.J., Arnone, M.I., Rowen, L., Cameron, R.A., McClay, D.R., Hood, L., Bolouri, H., 2002. A genomic regulatory network for development. Science 295, 1669–1678.

Gibson, A.W., Burke, R.D., 1985. The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. Dev. Biol. 107, 414–419.

Heasman, J., Kofron, M., Wylie, C., 2000. β-Catenin signaling activity dissected in the early *Xenopus* embryo: a novel antisense approach. Dev. Biol. 222, 124–134.

Hörstadius, S., 1973. Experimental Embryology of Echinoderms. Clarendon Press, Oxford.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R., 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61–68.

Howard, E.W., Newman, L.A., Oleksyn, D.W., Angerer, R.C., Angerer, L.M., 2001. *SpKrl*: a direct target of β-catenin regulation required for endoderm differentiation in sea urchin embryos. Development 128, 365–375.

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.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., Clevers, H., 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/−colon carcinoma. Science 275, 1784–1787.

Kozlowski, D.J., 1997. Ph.D. thesis, University of Rochester, Rochester, NY.

Kozlowski, D.J., Gagnon, M.L., Marchant, J.K., Reynolds, S.D., Angerer, L.M., Angerer, R.C., 1996. Characterization of a *SpAN* promoter sufficient to mediate correct spatial regulation along the animal-vegetal axis of the sea urchin embryo. Dev. Biol. 176, 95–107.

Li, X., Wikramanayake, A.H., Klein, W.H., 1999. Requirement of SpOtx in cell fate decisions in the sea urchin embryo and possible role as a mediator of beta-catenin signaling. Dev. Biol. 212, 425–439.

Logan, C.Y., Miller, J.R., Ferkowicz, M.J., McClay, D.R., 1999. Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. Development 126, 345–357.

McClay, D.R., Peterson, R.E., Range, R.C., Winter-Vann, A.M., Ferkowicz, M.J., 2000. A micromere induction signal is activated by β-catenin and acts through Notch to initiate specification of secondary mesenchyme cells in the sea urchin embryo. Development 127, 5113–5122.

Oliveri, P., Davidson, E.H., McClay, D.R., 2003. Activation of pmarl controls specification of micromeres in the sea urchin embryo. Dev. Biol. 258, 32–43.

Ransick, A., Rast, J.P., Minokawa, T., Calestani, C., Davidson, E.H., 2002. New early zygotic regulators expressed in endomesoderm of sea urchin embryos discovered by differential array hybridization. Dev. Biol. 246, 132–147.

Reynolds, S.D., Angerer, L.M., Palis, J., Nasir, A., Angerer, R.C., 1992. Early mRNAs, spatially restricted along the animal-vegetal axis of sea urchin embryos, include one encoding a protein related to tolloid and BMP-1. Development 114, 769–786.

Sampson, E.M., Haque, Z.K., Ku, M.C., Tevosian, S.G., Albanese, C., Pestell, R.G., Paulson, K.E., Yee, A.S., 2001. Negative regulation of the Wnt-beta-catenin pathway by the transcriptional repressor HBP1. EMBO J. 20, 4500–4511.

Summerton, D., Weller, D., 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. 7, 187–195.

Takash, W., Canizares, J., Bonneaud, N., Poulat, F., Mattei, M.G., Jay, P., Berta, P., 2001. SOX7 transcription factor: sequence, chromosomal localisation, expression, transactivation and interference with Wnt signalling. Nucleic Acids Res. 29, 4274–4283.

Vonica, A., Weng, W., Gumbiner, B.M., Venuti, J.M., 2000. TCF is the nuclear effector of the beta-catenin signal that patterns the sea urchin animal-vegetal axis. Dev. Biol. 217, 230–243.

Wei, Z., Angerer, L.M., Angerer, R.C., 1999. Spatially regulated SpEts4 transcription factor activity along the sea urchin embryo animal-vegetal axis. Development 126, 1729–1737.

Wikramanayake, A.H., Huang, L., Klein, W.H., 1998. Beta-catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. Proc. Natl. Acad. Sci. USA 95, 9343–9348.

Zorn, A.M., Barish, G.D., Williams, B.O., Lavender, P., Klymkowsky, M.W., Varmus, H.E., 1999. Regulation of Wnt signaling by Sox proteins: XSox17 alpha/beta and XSox3 physically interact with beta-catenin. Mol. Cell 4, 487–498.