SoxB1 downregulation in vegetal lineages of sea urchin embryos is achieved by both transcriptional repression and selective protein turnover

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Summary

Patterning of cell fates along the sea urchin animal-vegetal embryonic axis requires the opposing functions of nuclear β-catenin/TCF-Lef, which activates the endomesoderm gene regulatory network, and SoxB1, which antagonizes β-catenin and limits its range of function. A crucial aspect of this interaction is the temporally controlled downregulation of SoxB1, first in micromeres and then in macromere progeny. We show that SoxB1 is regulated at the level of protein turnover in these lineages. This mechanism is dependent on nuclear β-catenin function. It can be activated by Pmar1, but not by Krl, both of which function downstream of β-catenin/TCF-Lef. At least partially distinct, lineage-specific mechanisms operate, as turnover in the macromeres depends on entry of SoxB1 into nuclei, and on redundant destruction signals, neither of which is required in micromeres. Neither of these turnover mechanisms operates in mesomere progeny, which give rise to ectoderm. However, in mesomeres, *SoxB1* appears to be subject to negative autoregulation that helps to maintain tight regulation of *SoxB1* mRNA levels in presumptive ectoderm. Between the seventh and tenth cleavage stages, β-catenin not only promotes degradation of SoxB1, but also suppresses accumulation of its message in macromere-derived blastomeres. Collectively, these different mechanisms work to regulate precisely the levels of SoxB1 in the progeny of different tiers of blastomeres arrayed along the animal-vegetal axis.

Key words: Wnt, Cell fate specification, Animal-vegetal axis, Sea urchin, Embryo

Introduction

The correct specification and patterning of cell fates along the animal-vegetal axis of sea urchin embryos depend on the balance between the vegetalizing activity of nuclear β-catenin and the opposing activities of a set of transcriptional regulators, termed the animalizing transcription factors or ATFs (reviewed by Angerer and Angerer, 2000). Activation of the endomesoderm gene regulatory cascade begins when β-catenin enters the nuclei of micromeres, shortly after they form at the vegetal pole by the asymmetric fourth cleavage (Davidson et al., 2002a; Logan et al., 1999). Nuclear β-catenin, functioning as a transcriptional co-activator with TCF-Lef, is required both for the differentiation of micromeres and for their acquisition of signaling properties (Logan et al., 1999). During several subsequent cleavages, β-catenin also enters nuclei of the overlying macromere progeny, where it is required for these blastomeres to respond to micromere signals, to activate downstream genes in the endomesoderm network and to relay the inductive cascade (Davidson et al., 2002b). Among the signals that mediate this series of inductions are Delta, Wnt8 and Nodal. Delta/Notch signaling is required for specifying various secondary mesenchyme cell types (Sherwood and McClay, 1999; Sweet et al., 2002; Sweet et al., 1999); Wnt8 is required for some aspects of both primary mesenchyme cell (PMC) and endoderm differentiation (Wikramanayake et al., 2004); and Nodal is required for oral ectoderm specification (Duboc et al., 2004). In addition to these defined signaling pathways, an early signal (ES), whose molecular identity is not known, is transmitted from micromeres, primarily between the 16- and 60-cell stages, and is required for normal endomesoderm development (Ransick and Davidson, 1995). The property of the endomesoderm gene regulatory network (GRN) that is most significant for the present study is the fact that nuclear β-catenin transcriptional function currently stands alone at the top of the hierarchy, because zygotic expression of all members of this GRN depends on its function (Davidson et al., 2002b)

Opposing this β-catenin-dependent vegetal inductive cascade are the ATFs, whose prototypical and most extensively studied member is SoxB1 (Kenny et al., 1999; Kenny et al., 2003). Maternal SoxB1 protein is uniformly distributed among all nuclei through the eight-cell stage, but then rapidly begins to disappear from micromeres soon after they form at the fourth cleavage division. Beginning around the seventh cleavage, SoxB1 also progressively clears from nuclei of macromere progeny. This clearance is necessary because forced continued accumulation of SoxB1 protein in vegetal blastomeres by injection of *SoxB1* mRNA at the one-cell stage blocks

activation of genes in the endomesoderm GRN (L.M.A., R.C.A. and E. Davidson, unpublished) and prevents all vegetal development (Kenny et al., 2003). This phenotype is indistinguishable from that produced by loss of β-catenin nuclear function achieved through cadherin overexpression (Howard et al., 2001; Logan et al., 1999; Wikramanayake et al., 1998). Conversely, morpholino antisense knockdown of SoxB1 protein leads to a large increase in β-catenin-dependent transcriptional activity, as measured by a β-catenin/TCF-Lef-dependent reporter transgene (Kenny et al., 2003). Thus, an important function of SoxB1 in the early embryo is to limit the range of β-catenin activity along the AV axis.

An essential aspect of endomesoderm differentiation, in turn, is the activation of mechanisms for the temporally and spatially controlled downregulation of SoxB1 in vegetal blastomeres. Elimination of SoxB1 from these vegetal lineages is completely dependent on nuclear β-catenin function (Wikramanayake et al., 1998; Logan et al., 1999; Howard et al., 2001). In micromeres, the rapid disappearance of SoxB1 is most likely to be regulated cell-autonomously, whereas in the overlying macromere lineages, clearance at around seventh cleavage depends, at least in part, on a signal from micromeres, because it is inhibited when they are removed (Oliveri et al., 2003). Generation of this signal can be stimulated by Pmar1, a micromere-specific transcription factor, because cells that lack nuclear β-catenin, but are supplied with *Pmar1* mRNA, can induce clearance of SoxB1 protein in neighboring cells (Oliveri et al., 2003). We have reported that SoxB1 protein levels also are sensitive to the presence or absence of the transcriptional repressor Krl. Like *Pmar1*, *Krl* is a β-catenin target gene but, unlike *Pmar1*, *Krl* is expressed in both micromere and macromere progeny (Howard et al., 2001; Minokawa et al., 2004).

Here, we show, using loss-of-function assays, that two different nuclear, β-catenin-dependent, mechanisms operate to downregulate SoxB1 by reducing the level of its mRNA and by promoting turnover of the SoxB1 protein. Neither of these mechanisms depends on Krl, as shown by morpholino knockdown of its translation, suggesting that the previously demonstrated effects of Krl on SoxB1 levels (Howard et al., 2001) are quite indirect. The finding that SoxB1 is selectively degraded in vegetal blastomeres was unexpected and demonstrates a new kind of regulatory mechanism that mediates the antagonism between SoxB1 and β-catenin/Tcf-Lef activity in the endomesoderm GRN. This output may work through Pmar1, as misexpression of this factor can promote SoxB1 turnover in all cells of the embryo. Lastly, we provide evidence for at least two different lineage-specific degradation mechanisms, as the turnover of SoxB1 in macromere progeny, but not in micromere progeny, depends both on its nuclear localization and on sequences in its C-terminal domain. The existence of these two degradation pathways may reflect cell-autonomous regulation of SoxB1 in the micromere lineage and non-autonomous regulation in macromere descendants.

Materials and methods

Embryo cultures

Adult sea urchins (*S. purpuratus*) were obtained from Charles M. Hollahan (Santa Barbara, CA). Microinjected fertilized eggs were cultured in artificial seawater (ASW) in injection Petri plates at 15°C.

Construct preparation

SoxB1-GFP was constructed using SOE (splicing by overlap extension) PCR (Horton et al., 1989) to link the SoxB1 protein-coding region (amino acid residues 1 to 344) in frame with GFP. The SoxB1 fragment was generated by PCR with a forward primer containing an *Xho*I site and N-terminal SoxB1 codons, and a reverse primer containing C-terminal SoxB1 and N-terminal GFP codons. The GFP fragment was generated from a pSP64T.clone construct containing GFP sequence originally derived from Green Lantern (Promega), using an N-terminal GFP forward primer and a reverse primer starting just downstream of the polyadenylation sequence. After linking the SoxB1 and GFP fragments by three rounds of PCR, the fusion was amplified with the outside primers and the amplimer was ligated into pET-15b between the *Xba*I and *Esp*I (blunted) sites. This construct served as the parent plasmid for producing the series of SoxB1-GFP variants described below, all of which lack 5′ and 3′ UTR sequences. All constructs encoding SoxB1-GFP variants were verified by sequencing across junctions and by translation of synthetic mRNAs in vitro; translation of full-length proteins in vivo was verified by green fluorescence.

SoxB1DBD (DNA-binding domain)-GFP was produced by synthesizing SoxB1DBD by PCR as described previously (Kenny et al., 2003). This fragment was then ligated in frame at the *Eco*R1 site of GFP/pSP64T.clone. The template for production of *SoxB1GFP* RNA was made by PCR using a 5′ primer equipped with an Sp6 promoter followed by N-terminal SoxB1 sequence (–12 to +9 with respect to AUG; GCT CAG ACT GAC CAA AAT GTC TGT T), and a 3′ primer downstream of the polydT tract (CCG GAA TTC TGT CTT CTT CAA CAG GGT CTT; the *Eco*R1 site is underlined). For the templates containing a partial SoxB1 sequence C-terminal from the DNA-binding domain (NDBD SoxB1GFP and 3′NLS SoxB1GFP), the forward primers encoded an Sp6 promoter, followed by a translation initiation site (Kozak, 1989), and five SoxB1 codons, starting at either amino acid residue 146 or residue 127, respectively. These constructs lacked any *SoxB1* 5′ or 3′ UTR sequence. The construct encoding SoxB1 lacking the DNA-binding domain was made by deleting codons for 44 amino acid residues from the DBD of the SoxB1GFP/pET-15b plasmid template (residues 83-126) using SOE PCR (Horton et al., 1989). The resulting fragment, retaining the two nuclear localization signals, was amplified using the primers described above for SoxB1GFP. SOE PCR was used to create 3′ SoxB1-GFP deletions in the C-terminal half of the protein: deletions 1, 2 and 1+3 lack amino acid residues 138 to 252, 254 to 344 and 138 to 252 + 326 to 344, respectively. The templates for producing *Krl* (Howard et al., 2001), *cadherin* (Lee and Gumbiner, 1995) and *SoxB1* MO-immune (Kenny et al., 2003) mRNAs have been described previously. SoxB1MO-immune mRNA contains no 5′ *SoxB1* UTR sequence and approximately 1 kb of 3′UTR sequence. The plasmid containing full-length *Pmar1* cDNA sequence in pBK-CMV was a gift from P. Oliveri and E. Davidson (California Institute of Technology, USA).

Microinjection and confocal image analysis

Fertilized eggs were microinjected with synthetic mRNAs or SoxB1MO as described previously (Kenny et al., 2003). Capped, polyadenylated mRNAs were transcribed using the mMessage mMachine kit (Ambion) and purified according to the manufacturer's instructions with DNase digestion and LiCl precipitation. RNA concentrations were chosen that generated sufficient GFP signals, but that were well below the levels that inhibit β-catenin function (Kenny et al., 2003), so that the embryos developed normally. For all SoxB1 variants, approximately 250,000 mRNA molecules were injected. Injection solutions containing RNA, 10 mM Tris-HCl (pH 8.0) and 30% glycerol were filtered by centrifugation through 0.22 μm filters. Shortly after hatching (20-23 hours post-fertilization), at least 30 embryos were examined by fluorescence microscopy for SoxB1-GFP distributions. They were then collected in a thin capillary pipette and

deciliated by passage sequentially through two 100-μl aliquots of cold, hypertonic seawater (Stephens, 1997) in a depression slide. The embryos were then equilibrated in ASW, deposited into a drop of ASW underneath a coverslip and immediately photographed using a Leica TS confocal microscope. Fluorescent GFP signals were overlaid on DIC images using Adobe Photoshop.

Immunostaining and whole-mount in situ hybridization

Embryos were fixed with 4% paraformaldehyde, 1×PBS (phosphate-buffered saline), 0.2% Tween 20, for 15 minutes at room temperature, and stained with a rabbit polyclonal SoxB1 antibody and a CyIII-conjugated mouse anti-6e10 antibody, as described previously (Kenny et al., 2003). Embryos that were analyzed for both *SoxB1* mRNA and protein were first hybridized with digoxigenin-labeled *SoxB1* antisense RNA. The template consisted of a 415-bp *Bal*I/*Nde*I fragment of the *SoxB1* coding sequence inserted into the *Eco*RV site of pBluescript, which was digested with *Xho*I. RNA was synthesized with T3 RNA polymerase in vitro. Hybridization and hybrid detection were carried out according to Minokawa et al. (Minokawa et al., 2004) with the exception that the hybridization time was shortened to one day. After staining with anti-digoxigenin linked to alkaline phosphatase, embryos were immunostained by incubation for 2 hours with anti-SoxB1antibody (1:1000) and 1 hour with goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (1:500; Zymed).

Results

Vegetal cells regulate SoxB1 protein at a post-transcriptional level

Post-transcriptionally regulated clearance of SoxB1 from vegetal blastomeres was detected when all the SoxB1 protein in the embryo was translated from uniformly distributed microinjected mRNA. To create this situation, we eliminated translation of both maternal and zygotic endogenous transcripts by injecting a SoxB1 morpholine-substituted oligonucleotide (SoxB1MO) and co-injecting *SoxB1* synthetic mRNA that lacked sequence complementary to the MO (MO-immune). Immunostaining of embryos injected with SoxB1MO alone showed that, by 12 hours of development, maternal SoxB1 protein was undetectable (data not shown). As previously reported, a reproducible loss-of-function phenotype was produced, in which the archenteron failed to form and the ectoderm was radialized (Kenny et al., 2003). Although *SoxB1* transcripts normally are abundant during the 3 days of development to the prism stage (Kenny et al., 1999), the SoxB1MO very effectively inhibits translation throughout this period (Fig. 1A). Surprisingly, when embryos were co-injected at the one-cell stage with MO and MO-immune *SoxB1* mRNA, SoxB1 protein accumulated in the wild-type pattern, in which it was mostly confined to all of the presumptive ectoderm (Fig. 1B). This observation indicates that the protein must be relatively stable in these cells, as the injected messages turn over by the gastrula stage (2 days; data not shown). By contrast, and as in normal embryos, SoxB1 protein was absent from most macromere derivatives, including nearly all of the archenteron and secondary mesenchyme (Fig. 1B; coelomic rudiments, pharyngeal muscle fibers derived from secondary mesenchyme, and non-primary mesenchyme cells in the blastocoel are indicated by white arrowheads). Interestingly, a low level of SoxB1 protein reappears in a portion of the foregut, which is a normal site of expression after the gastrula stage. The simplest interpretation of this observation is that some translation of endogenous *SoxB1* message escapes suppression at later stages by diminishing concentrations of the MO, and that the post-transcriptional mechanism is no longer active in these cells at the late gastrula stage. As we have documented previously (Kenny et al., 2003), rescue of the SoxB1 loss-of-function phenotype by uniformly distributed *SoxB1* mRNA is surprisingly effective in these doubly injected embryos. As we now show, this is attributable to the fact that embryos have a robust post-transcriptional mechanism that effectively downregulates accumulation of the SoxB1 protein in vegetal lineages, thereby permitting activation of the endomesoderm GRN.

Differential post-transcriptional regulation of SoxB1 in animal and vegetal tissues of these late embryos might simply

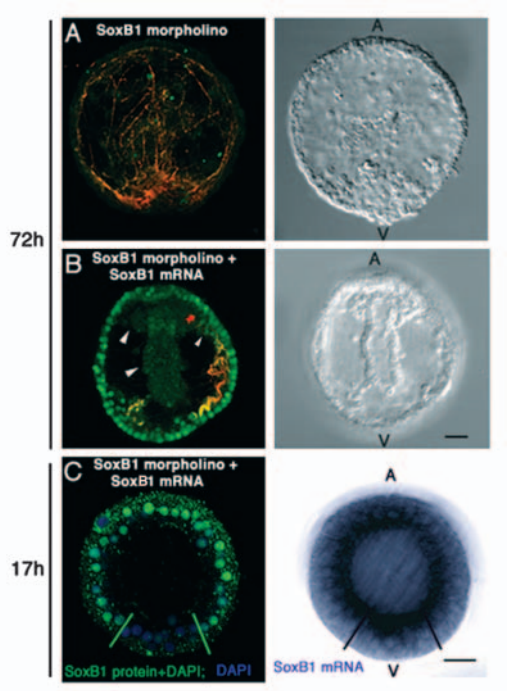

Fig. 1. SoxB1 protein encoded from mRNA microinjected at the one-cell stage does not persist in vegetal cells. (A) Translation of SoxB1 mRNA is efficiently blocked in embryos that develop from zygotes injected with SoxB1MO, as shown by the absence of staining with an antibody specific for SoxB1 (green, compare with B and C). Red staining detects the 6e10 extracellular epitope produced by primary mesenchyme cells. This embryo is at the temporal equivalent of prism stage but has failed to differentiate an archenteron (Kenny et al., 2003). (B) Co-injection of the SoxB1 morpholino and MO-immune *SoxB1* synthetic mRNA rescues differentiation of gut and coelomic rudiments (white arrowheads). SoxB1 protein translated from the microinjected mRNA (green) accumulates and persists in the ectoderm, but does not persist in secondary mesenchyme (white arrowheads) or endoderm (except for low levels in the foregut). (C) An embryo at the hatched blastula stage treated as in B also clears SoxB1 protein (green signal, left) from vegetal blastomeres, despite the fact that the microinjected mRNA is present in them, as shown by whole-mount in situ hybridization to *SoxB1* mRNA in the same embryo (blue signal, right). The embryo is stained for SoxB1 (FITC) and DNA (DAPI); the merged fluorescent signals over SoxB1-positive nuclei are blue-green, whereas those of SoxB1-negative nuclei are blue. Immunofluorescence images are shown on the left; DIC images, right. A and V indicate animal and vegetal poles, respectively. Scale bars: in B, 20 μm for A,B; in C, 20 μm.

be a late aspect of their differentiation that does not operate earlier when cell fates are being specified. In normal embryos, SoxB1 is cleared from vegetal blastomeres by the mesenchyme blastula stage. To determine whether the post-transcriptional mechanism is active in earlier embryos, we examined similar doubly injected embryos at 17 hours post-fertilization. We compared the distribution of *SoxB1* mRNA, which comes from both endogenous and exogenous sources, analyzed by whole-mount in situ hybridization, to that of the SoxB1 protein translated from the MO-immune transcripts, determined by immunostaining. As shown in Fig. 1C, SoxB1 protein is undetectable in the nuclei of vegetal cells, which, however, maintain the same concentration of mRNA as in the rest of the embryo. This indicates that the post-transcriptional mechanism is very robust during the period when SoxB1 protein normally disappears from macromere-derived blastomeres (seventh-eighth cleavage). Furthermore, it demonstrates that the post-transcriptional mechanism operating at this stage does not involve differential turnover of the injected mRNA.

A SoxB1GFP fusion protein is selectively degraded in vegetal cells

The mechanism that prevents accumulation of SoxB1 protein in vegetal cells could be translational or post-translational. To begin to discriminate between these alternatives, we injected mRNA encoding GFP-tagged SoxB1 lacking the 5′ and 3′ UTR sequences, in which translational control sequences nearly always reside. In all experiments in which *SoxB1* mRNAs were injected, the dose was approximately 10-fold lower than the minimum dose that interferes detectably with endomesoderm development, yet high enough to detect GFP fluorescence. Live embryos accumulated this SoxB1-GFP fusion protein in a very reproducible pattern: it was detectable in all nuclei of the embryo beginning at about the eight-cell stage and was partitioned to nuclei of mesomeres, macromeres and micromeres of the 16-cell embryo, in a manner similar to endogenous SoxB1 protein (Fig. 2A). It was cleared from the descendants of micromeres and from most of the macromere progeny by the early mesenchyme blastula stage. A confocal image of a representative embryo is shown in Fig. 2B. This result strongly suggests that the post-transcriptional mechanism that clears SoxB1 from vegetal cells is selective turnover, not translational control.

Preferential turnover of SoxB1 protein in endomesoderm requires nuclear β-catenin function

Embryos in which nuclear entry of β-catenin and, consequently, development of endoderm and mesenchyme are blocked by injection of C-cadherin mRNA accumulate abnormally high levels of SoxB1 protein in all their nuclei and arrest as epithelial spheres of cells that resemble poorly differentiated ectoderm (Howard et al., 2001). To determine whether the molecular signatures of this animalized phenotype include failure of SoxB1 to be degraded in vegetal cells, we co-injected mRNAs encoding SoxB1-GFP and C-cadherin, and analyzed the distribution of the GFP reporter in embryos at the temporal equivalent of the mesenchyme blastula stage. Again the phenotype was consistent in the several dozen embryos analyzed, one of which is shown in Fig. 2C. The fusion protein accumulated uniformly in the nuclei of all cells, indicating that spatially regulated turnover of SoxB1 is an output of the β-catenin-driven endomesoderm GRN. The converse experiment, in which nuclear β-catenin activity is upregulated by microinjection of mRNA encoding a stabilized form, results in vegetalized embryos (Wikramanayake et al., 1998). Increasing doses of stabilized β-catenin progressively restrict endogenous SoxB1 protein to a region around the animal pole (Howard et al., 2001). To determine whether turnover of SoxB1 protein contributes to this spatial regulation, we co-injected *SoxB1-GFP* mRNA (again at a very low dose that does not produce a detectable phenotype) and message encoding stable β-catenin at a level sufficient to vegetalize the embryos. Clearance of

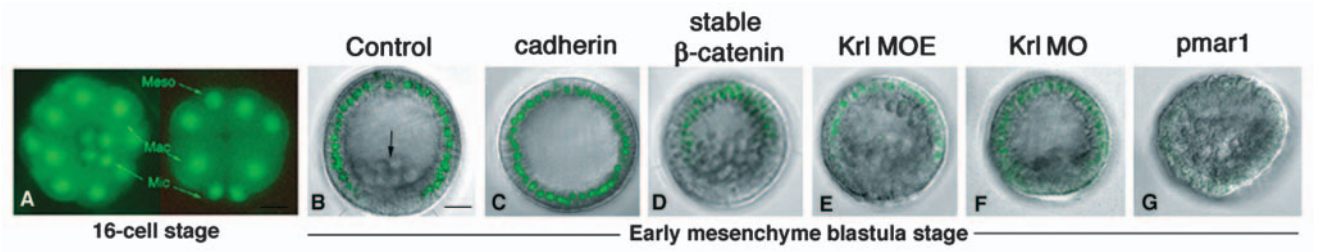

Fig. 2. Vegetal turnover of SoxB1GFP requires nuclear β-catenin and can be driven by Pmar1, but is not affected by Krl. (A) Embryos at the 16-cell stage derived from fertilized eggs that had been injected with mRNA encoding SoxB1-GFP. Images were obtained with a Nikon inverted microscope equipped with epifluoresence optics and Kodak Elite 35 mm slide film. The embryo on the left is shown in a slightly tilted, vegetal pole view that shows the four micromeres, eight macromeres and one mesomere. The embryo is oriented so that mesomeres at the animal pole are up and micromeres at the vegetal pole are down. (B-G) Zygotes were injected with mRNA encoding SoxB1-GFP and the indicated proteins, allowed to develop to the temporal equivalent of mesenchyme blastula stage, deciliated, and fluorescence images were captured from live embryos by laser confocal microscopy. (B) Control embryo injected with *SoxB1-GFP* mRNA and glycerol, demonstrating that the SoxB1-GFP fusion protein mimics the vegetal turnover exhibited by the endogenous SoxB1. The arrow indicates PMCs that have ingressed from the vegetal plate. (C) Embryos in which nuclearization of β-catenin is blocked by co-injection of C-cadherin mRNA do not clear SoxB1GFP from vegetal blastomeres. These embryos lack PMCs (Logan et al., 1999). (D) By contrast, upregulation of nuclear β-catenin activity with mRNA encoding stabilized β-catenin vegetalizes the embryo and also expands the vegetal domain of SoxB1-GFP degradation. (E,F) Mis/overexpression of Krl (E) also appears to expand the SoxB1-GFP degradation domain, consistent with its strong vegetalizing effect (Howard et al., 2001), but knockdown of Krl by means of Krl MO injection (F) does not detectably alter SoxB1 turnover. (G) Mis/overexpression of Pmar1 converts most of the cells to a PMC-like fate and upregulates SoxB1-GFP turnover throughout the embryo. Scale bar: 20 μm.

SoxB1 in these embryos was compared with that in embryos from the same batch injected with *SoxB1-GFP* mRNA alone (Fig. 2, control), when the latter had reached the mesenchyme blastula stage. As shown in Fig. 2D, upregulation of nuclear β-catenin activity expands the vegetal region in which SoxB1-GFP is preferentially degraded. We conclude that nuclear β-catenin is both necessary for SoxB1 protein turnover in normal embryos and can cause ectopic turnover when expressed in more animal blastomeres, although cells closer to the animal pole are more resistant to this effect. It is important to note that because perturbations of nuclear β-catenin concentrations cause dramatic changes in cell fate, the regulation of SoxB1 turnover by β-catenin may be indirect.

SoxB1-GFP degradation in vegetal cells does not depend on Krl, but can be promoted by Pmar1

We next examined the potential roles of two candidate genes, *Krl* and *Pmar1*, in mediating SoxB1 turnover. *Krl* mRNA is expressed in the cells in which SoxB1 is selectively degraded in response to nuclear β-catenin, and accumulates in a vegetal-to-animal wave like that of nuclear β-catenin. MO-mediated loss of Krl function causes endogenous SoxB1 protein to accumulate to higher levels throughout most of the embryo, including some cells within the endomesodermal territory that normally would downregulate this protein. Embryos arrest at a stage that resembles a mesenchyme blastula (Howard et al., 2001). Conversely, mis/overexpression (MOE) of Krl by mRNA injection eliminates SoxB1 protein from almost all cells of the embryo, with the exception of a few near the animal pole (Howard et al., 2001). We therefore tested the effect of altering Krl levels on SoxB1-GFP distributions by injecting *Krl* mRNA or by blocking its translation with a KrlMO. As shown in Fig. 2E,F, neither perturbation detectably affected the clearance of SoxB1-GFP.

Pmar1 is a second candidate for mediating β-catenin-dependent SoxB1 turnover. Embryos mis/overexpressing Pmar1 are extremely vegetalized, with most cells of the embryo being transformed to a PMC-like phenotype by the mesenchyme blastula stage (Oliveri et al., 2002). Micromeres lacking β-catenin nuclear function but supplied with Pmar1 do not accumulate SoxB1 and can induce its downregulation in nearby cells (Oliveri et al., 2003), although it is not known whether this regulation operates at the level of *SoxB1* mRNA or protein. To test whether Pmar1 can affect the stability of SoxB1 protein, mRNAs encoding Pmar1 and SoxB1-GFP were co-injected. As shown in Fig. 2G, SoxB1-GFP is not stable in these embryos, indicating that ectopic expression of Pmar1 can promote SoxB1 turnover. The effect of eliminating Pmar1 was not tested because of Pmar gene redundancy (P. Oliveri and E. Davidson, personal communication).

SoxB1 turnover in macromere derivatives requires functionally redundant sequences within its C-terminal region

SoxB1 contains a highly conserved HMG-box DNA-binding domain (DBD) that is flanked by a short N-terminal (58 amino acid residues) and a much longer C-terminal (207 residues) sequence. At the borders of the DBD are motifs matching the two separate nuclear localization signals that are found in this class of transcription factor (Fig. 3A). To begin mapping the sequences that are required for SoxB1 degradation, we injected mRNAs encoding mutated SoxB1 proteins, each tagged at its C terminus with GFP. At least 30 embryos expressing each construct were analyzed and the results were very reproducible. SoxB1-GFP chimeras lacking most of the sequence C-terminal to the DBD were stable in all cells except the PMCs (Fig. 3B). This suggests that the essential destruction sequences in SoxB1 that are recognized in macromere-derived endomesoderm reside C-terminal from the DBD, and that a different, or additional, degradation mechanism functions in micromere derivatives. Interestingly, each of several different peptides that were partially deleted in the C-terminal region of

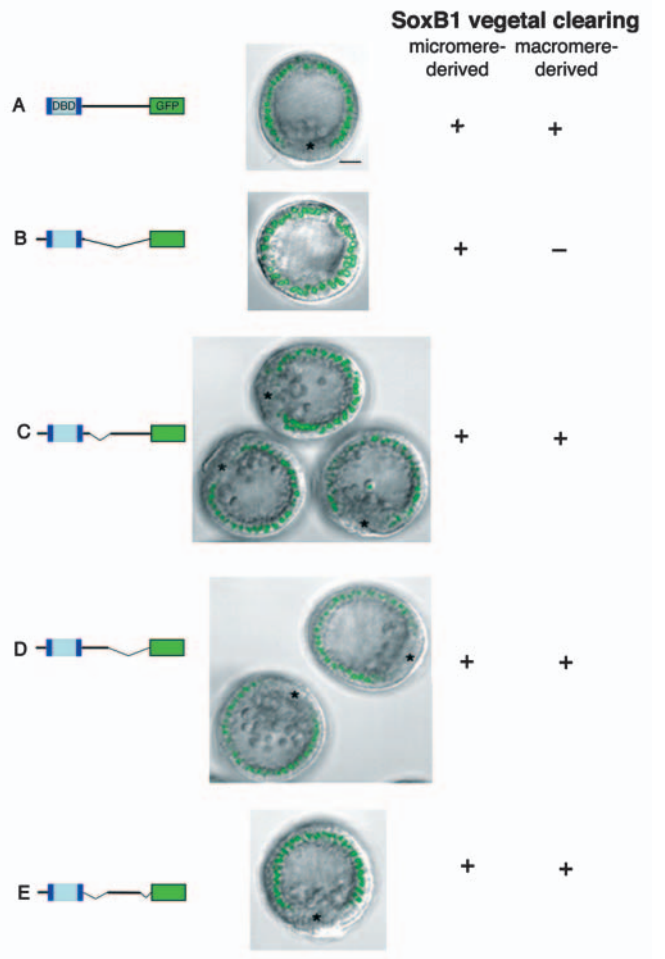

Fig. 3. The SoxB1 C-terminal region contains multiple sequence signals for turnover in macromere lineages that are not required for degadation in micromere progeny. Zygotes were injected with GFP-tagged (green box) SoxB1, from which residues were deleted as indicated by the thinner black lines in the diagrams of the constructs shown on the left. The DNA-binding domain (light blue box) is bordered on each side by a nuclear localization signal motif (dark blue). Embryos (at least 30) were examined for depletion of the protein in micromere derivatives (primary mesenchyme cells) and macromere progeny (the vegetal plate). All embryos treated with the same mRNA showed a consistent phenotype, which is illustrated in the middle column. An asterisk marks the center of the vegetal plate of each embryo. (A) Control, intact SoxB1-GFP. (B) SoxB1-GFP from which almost the entire sequence 3′ of the DNA-binding domain was deleted was not destabilized preferentially in vegetal plate cells. (C-E) Different portions of the C-terminal region are each sufficient to mediate vegetal clearance. Scale bar: 20 μm.

SoxB1 was degraded preferentially in both macromere and micromere progeny (Fig. 3C-E). Although a number of putative phosphorylation motifs are distributed throughout the C-terminal region, the three regions that can mediate turnover do not share a common candidate motif. These results suggest the existence of functionally redundant sequences mediating SoxB1-GFP decay. Although these data do not rule out the formal possibility that turnover is in some way compromised when the GFP and SoxB1 DBD domains are closely spaced, such an effect would be restricted to turnover in endomesoderm derived from macromeres and not from micromeres.

Turnover of SoxB1GFP in macromere progeny requires its entry into nuclei, but not binding to DNA

SoxB1 accumulates primarily in nuclei except during mitosis, when it is released to the cytoplasm (Fig. 4). To test whether SoxB1 turnover requires its nuclear localization, we injected mRNA encoding a GFP fusion lacking the SoxB1 N-terminal region, which bears the DBD, flanking nuclear localization signal (NLS) motifs and the short N-terminal peptide. As shown in Fig. 5A, this peptide remains cytoplasmic and it is degraded only in the PMCs. By contrast, a peptide lacking only the DBD but retaining the NLS motifs is cleared from both macromere and micromere progeny (Fig. 5B). It is not the case that the NLSs themselves contain motifs sufficient to target SoxB1 for degradation, because, as shown above (Fig. 3B), a fusion protein that contains the NLSs but lacks the C-terminal half of SoxB1 is stable in macromere-derived endomesoderm. In addition, introduction of two point mutations that reduce binding of SoxB1 to DNA by two orders of magnitude (Kenny et al., 2003) does not detectably alter the timing or extent of SoxB1 degradation in vegetal cells (data not shown). A construct retaining only the 3′NLS gave intermediate results: both nuclear localization and turnover in macromere derivatives were less efficient (Fig. 5C). These results suggest that, within the macromere progeny, SoxB1 is either degraded in the nucleus, possibly through ubiquitin-dependent nuclear proteasomes, or must be modified there for recognition by the degradation machinery in the cytoplasm. In either case, vegetal clearance of SoxB1 does not require its DNA-binding function.

In summary, SoxB1 turnover in macromere progeny requires nuclear entry and at least one of several functionally redundant sequences in the C-terminal region of the protein, whereas turnover of SoxB1 in the PMCs, derived from micromeres, requires neither.

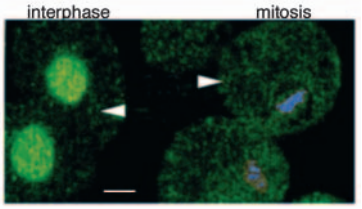

Fig. 4. SoxB1 protein is released into the cytoplasm during mitosis. Two blastomeres of embryos at the 16-cell stage are shown in interphase (left side) or in mitosis (right). Measurements of pixel densities show that the cytoplasmic signal increases 1.7-fold in blastomeres in mitosis (arrowheads). Chromosomes in these cells lack detectable SoxB1 staining, whereas most of the SoxB1 signal in interphase cells is in nuclei. Scale bar: 5 μm.

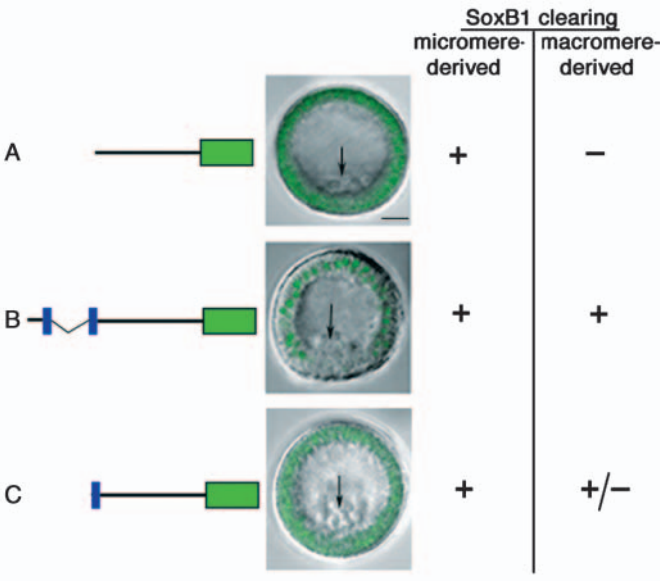

Fig. 5. Entry into nuclei is required for SoxB1 degradation in macromere progeny, but not in primary mesenchyme cells. The assay was performed as described in the legend to Fig. 3. (A) SoxB1-GFP from which the DNA-binding domain and flanking NLS sequences were removed was cleared from micromere derivatives, but not from macromere progeny. (B) SoxB1 deleted of the DNA-binding domain but retaining the NLS sequences is preferentially degraded in both micromere and macromere progeny. (C) SoxB1-GFP deleted of the DNA-binding domain and 5′NLS sequence, but retaining the 3′NLS sequence. The arrows indicate clusters of ingressed primary mesenchyme cells. Scale bar: 20 μm.

SoxB1 may provide a negative feedback on its own mRNA accumulation

Previous studies showed that the distribution of *SoxB1* mRNA modulates from its uniform maternal pattern to a non-vegetal pattern during blastula stages, around the seventh to eighth cleavage (Kenny et al., 1999). If SoxB1 were a positive regulator of its own transcription, then its loss from vegetal cells could account for reduced *SoxB1* transcription in these cells. To test this possibility, embryos were injected with SoxB1MO and *SoxB1* mRNA accumulation was analyzed semi-quantitatively by whole-mount in situ hybridization. As shown in Fig. 6, loss of SoxB1 did not lead to a decrease in signal. Instead, the signal was strongly increased in non-vegetal cells. This result corroborates real-time PCR measurements, which indicate that *SoxB1* mRNA levels in whole embryos increase about tenfold in SoxB1MO embryos (C. Livi, L.M.A. and E. Davidson, unpublished). Thus, these results show that SoxB1 does not positively regulate accumulation of its mRNA and raise the possibility that it functions either directly or indirectly in negative-feedback regulation.

Downregulation of *SoxB1* mRNA in vegetal cells requires nuclear β-catenin, but not Krl

To test whether the reduction in *SoxB1* mRNA in vegetal cells of blastulae requires nuclear β-catenin function, we compared the distribution of this message in embryos injected with C-cadherin mRNA to that in controls injected with glycerol only

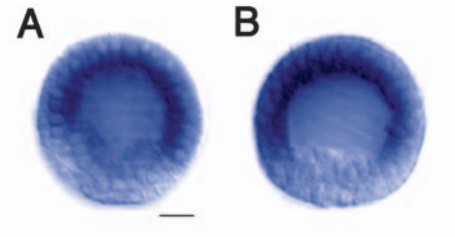

Fig. 6. SoxB1 functions in a negative-feedback loop. Zygotes were injected with glycerol as a control (A) or with SoxB1 MO (B) and allowed to develop to the mesenchyme blastula stage or its temporal equivalent. They were then subjected to whole-mount in situ hybridization with a SoxB1 probe. In order to observe the spatial pattern of expression, the enzymatic signal development was 4-fold longer for controls than for experimental embryos. Signals were consistently and significantly elevated in SoxB1 MO embryos, but only in presumptive ectoderm. Scale bar: 20 μm.

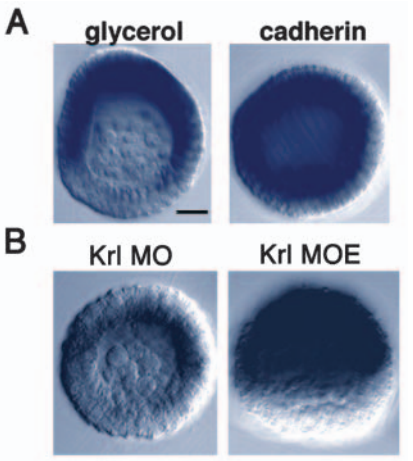

Fig. 7. Downregulation of *SoxB1* mRNA in vegetal blastomeres requires β-catenin, but not Krl. Zygotes were injected with glycerol (control), with mRNA encoding the indicated mRNAs, or with Krl-MO, allowed to develop to the hatched blastula stage, and then assayed by whole-mount in situ hybridization with a *SoxB1* probe. (A) Blocking nuclearization of β-catenin by injection of C-cadherin mRNA blocks downregulation of *SoxB1* mRNA in vegetal blastomeres. (B) Neither knockdown of Krl translation with a KrlMO nor its mis/overexpression by mRNA injection (Krl MOE) detectably affects downregulation of *SoxB1* message in vegetal blastomeres. However, in the animal hemisphere, loss of Krl function leads to a decrease in *SoxB1* mRNA, whereas MOE up regulates it. This effect is likely to reflect the operation of the SoxB1 negative-autoregulatory loop, as discussed in the text and illustrated in Fig. 5. Scale bar: 20 μm.

(Fig. 7A). All cells accumulated approximately equivalent concentrations of *SoxB1* mRNA in the experimental embryos, demonstrating that nuclear β-catenin function is required to downregulate *SoxB1* mRNA in vegetal blastomeres. This effect probably reflects regulation at the level of transcription rather than differential mRNA stability, because, as shown in Fig. 1C, injected *SoxB1* mRNA persists at similar concentrations in animal and vegetal cells of 17-hour blastulae. (However, a caveat to this argument is that the injected mRNA did not contain its complete 3′UTR.)

Repression of *SoxB1* transcription in vegetal cells of normal embryos could be mediated by β-catenin-dependent production of a repressor, by blocking production of an essential activator, or by both of these mechanisms. A good candidate for mediating repression is Krl, because it is expressed in precisely the right cells at the right time, its production is β-catenin-dependent and it has repressor activity (Howard et al., 2001). If Krl had this function, then its MO-mediated knockdown should increase the abundance of *SoxB1* transcripts, at least in the vegetal cells that express Krl. However, neither knockdown of Krl activity nor its MOE by mRNA injection altered the downregulation of *SoxB1* mRNA in vegetal blastomeres (Fig. 7B). Surprisingly, although signals were somewhat variable, knockdown of Krl usually caused a decrease in *SoxB1* mRNA abundance in animal blastomeres, whereas Krl MOE resulted in a dramatic increase in expression in the same domain**.** Thus, Krl does not repress *SoxB1* transcription; instead it can elevate *SoxB1* mRNA levels. It is not clear why the regulatory effect of Krl on SoxB1 protein is negative, while on *SoxB1* mRNA it is positive, given that Krl does not affect SoxB1 vegetal turnover. One possibility suggested by the phenotype of Krl MO embryos (Howard et al., 2001) is that loss of Krl leads to developmental arrest and a general delay in turnover of maternal SoxB1 protein throughout the embryo. Elevated levels of SoxB1 would then lead to reduced mRNA accumulation through negative autoregulation. Misexpression of Krl could have nonspecific effects on multiple genes leading to loss of SoxB1 and consequent increase in *SoxB1* mRNA concentration.

Discussion

In previous work, our laboratory has demonstrated that tight temporal and spatial regulation of SoxB1 levels is essential for correct specification and/or differentiation of the major tissue types arrayed along the animal-vegetal axis of the sea urchin embryo (Kenny et al., 2003). Misexpression of SoxB1 blocks all vegetal differentiation, while loss of SoxB1 function strongly suppresses endoderm differentiation and gastrulation. In addition, both perturbations disrupt ectoderm patterning along the orthogonal oral-aboral axis. Consistent with this stringent regulatory requirement, we provide evidence that at least five different mechanisms regulate SoxB1 levels in micromere, macromere and mesomere lineages. Collectively, these form a regulatory system whose major effects are: (1) to rapidly reduce SoxB1 levels initially in micromeres; (2) to produce a subsequent, more gradual reduction in macromere progeny; and (3) to maintain an essentially constant concentration in nuclei of the remainder of the embryo throughout most of development. Our current understanding of the known mechanisms operating within this regulatory system is summarized in Fig. 8. Although these mechanisms are illustrated in sequence according to the stages at which they are most clearly observed, more than one mechanism may operate at the same stage.

Downregulation of SoxB1 protein concentration is first observable in micromeres, shortly after these blastomeres form at the fourth cleavage (about 5.5 hours). Kenny et al. (Kenny et al., 1999) reported lower SoxB1 concentrations in nuclei of micromeres (see Fig. 8A) and noted that this difference most likely reflected, in part, the ~4-fold lower cytoplasmic volume inherited by micromeres versus macromeres through asymmetric cleavage. In Fig. 4, we document the fact that most

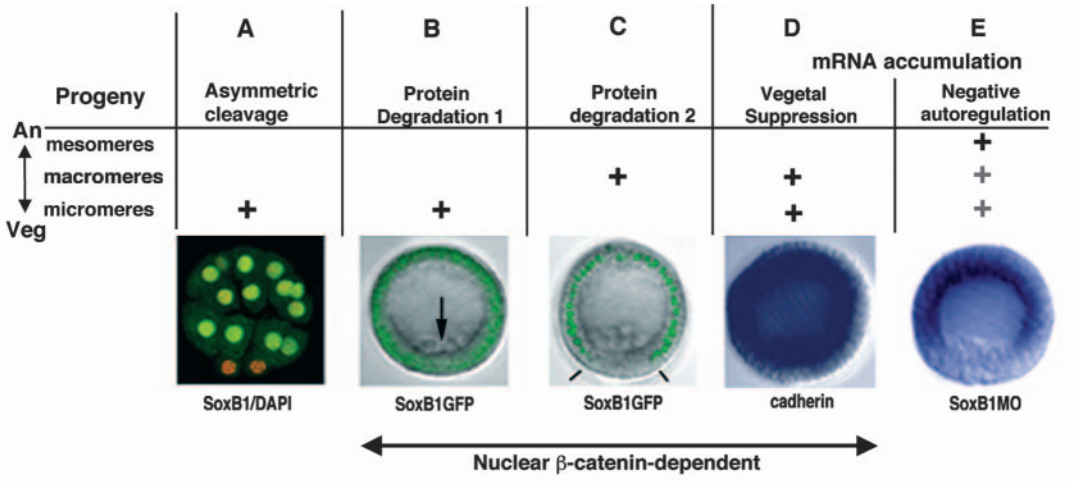

Fig. 8. Summary of evidence for the multiple mechanisms that regulate SoxB1 accumulation along the animal-vegetal axis. (A) Uniformly distributed SoxB1 is asymmetrically partitioned at fourth cleavage among different sized blastomeres in proportion to their cytoplasmic volume. [Image of an embryo doubly stained with SoxB1 antibody and DAPI between 16 and 32-cell stages reproduced, with permission, from Kenny et al. (Kenny et al., 1999)]. (B,C) SoxB1 is selectively degraded in micromeres and macromeres via different mechanisms, both of which depend on nuclear β-catenin function. SoxB1 peptides lacking the NLS sequences or the 3′-terminal region are eliminated from micromeres but not macromres (B), whereas SoxB1 variants retaining the NLSs and at least one out of three regions in the C-terminal domain clear from both micromere and macromere derivatives (C). (D) *SoxB1* mRNA levels remain high in vegetal blastomeres in the absence of nuclear β-catenin. (E) *SoxB1* mRNA concentrations are elevated in animal blastomeres in embryos lacking SoxB1 protein.

SoxB1 protein is released to the cytoplasm during mitosis, and re-enters nuclei during interphase. Thus, SoxB1 is distributed unequally in proportion to cytoplasmic volume. Similarly, we have not detected any inhomogeneity in the concentration of *SoxB1* mRNA in 16-cell embryos, when most transcripts are of maternal origin, and therefore micromeres initially also have a decreased capacity for zygotic translation of new SoxB1 protein.

Our results show that SoxB1 expression also is spatially regulated at the level of protein turnover (Fig. 8B,C). When SoxB1 protein was synthesized from uniformly distributed microinjected mRNA transcripts and detected by immunostaining, it was found to accumulate in animal, but not vegetal blastomeres at the hatching blastula stage (about 17 hours) (Fig. 1C), thus demonstrating post-transcriptional control. When mRNA encoding a SoxB1-GFP protein chimera was injected, fluorescence initially was detectable in all cells of eight- to 16-cell embryos (Fig. 2A), but the protein turned over in both micromere and macromere progeny at the early mesenchyme blastula stage. Analysis of the behavior of partial SoxB1 peptides similarly tagged with GFP provided evidence for a separate protein turnover mechanism that functions only in micromeres: turnover in macromere derivatives requires signals in the C-terminal region and the NLS sequences, whereas neither of these is required for selective degradation in micromere derivatives (Fig. 3B, Fig. 4). Either of these mechanisms could account for the observation by Kenny et al. (Kenny et al., 1999) that the amount of SoxB1 DNA-binding activity per microgram of total protein is about 5-fold lower in whole-cell extracts of micromeres, when compared with extracts of macromeres plus mesomeres. Together, these observations suggest that the micromere-specific mechanism may be activated as early as the 16-cell stage. However, we have not been able to demonstrate this directly by the available assays because turnover of GFP-tagged peptides was not observable until the early mesenchyme blastula stage (Figs 3, 5). Potential explanations for this delay include the possibility that the level of SoxB1-GFP that is required for detection overloads the turnover mechanisms, or that the conformation of the SoxB1-GFP fusion proteins somehow slows the rate of turnover. As misexpression of SoxB1 blocks all vegetal development (Kenny et al., 2003), an early micromere-specific mechanism for removal of SoxB1 could be a crucial feature of activating the endomesoderm GRN.

In normal embryos, *SoxB1* mRNA begins to be downregulated in macromere progeny between seventh and eighth cleavages (10-15 hours) (Fig. 8D). This presumably reflects regulation at the level of transcription, as there is no evidence at the present time for a lower stability of *SoxB1* mRNA in vegetal blastomeres. At the same time, SoxB1 protein begins to disappear from the presumptive secondary mesenchyme and endoderm, starting within the more vegetal blastomeres (Kenny et al., 1999). The exact timing of transcriptional repression versus selective protein turnover, and the relative contributions of these mechanisms to the establishment of polarized SoxB1 distributions are not yet clear. However, it is important to note that the protein turnover mechanism is active and robust in the early blastula. We showed that SoxB1 protein is cleared from embryos that have uniformly distributed microinjected *SoxB1* MO-immune mRNA that is ~3-fold higher than the level of endogenous mRNA in the egg and throughout cleavage (Fig. 1C). The functional significance of SoxB1 protein turnover is suggested by the fact that normal vegetal differentiation proceeds on schedule in these embryos, despite the persistence of elevated levels of uniformly distributed *SoxB1* mRNA. Thus, the embryo appears to have an excess capacity to downregulate SoxB1 in vegetal blastomeres at the level of protein turnover. These observations also can explain why even higher levels of microinjected *SoxB1* message, i.e. about 10-fold above normal

endogenous levels, are required to evoke the animalized misexpression phenotype (Kenny et al., 2003). Together, the available data strongly suggest that the post-translational mechanism serves to clear SoxB1 protein from endomesoderm more rapidly than could be achieved by downregulating mRNA abundance. However, definitive evaluation of the relative importance of the transcriptional and post-translational mechanisms in regulating SoxB1 distributions and normal patterning of fates along the AV axis will require specific inhibition of SoxB1 turnover, which is not yet possible.

Our previous work has shown that reduction of *SoxB1* mRNA levels in vegetal blastomeres is dependent on the function of nuclear β-catenin. In the present work, we have demonstrated that differential turnover of SoxB1-GFP is blocked in embryos in which β-catenin function is blocked by overexpressing C-cadherin. As SoxB1 turnover follows entry of β-catenin into nuclei of macromere progeny, this process could be cell-autonomous. However, available evidence suggests that the link between nuclear β-catenin function and SoxB1 turnover in macromere derivatives is indirect and, at least partially, non cell-autonomous. Oliveri et al. (Oliveri et al., 2003) have reported that efficient SoxB1 clearance from endomesoderm requires a signal from micromeres, as it is diminished when micromeres are removed after the fourth cleavage. This signal was reported to be mediated by Pmar1 function, as micromeres carrying C-cadherin mRNA could downregulate endogenous SoxB1, both in themselves and in nearby blastomeres, if supplied with exogenous *Pmar1* mRNA. Although these experiments demonstrate a possible function for Pmar1 upstream of a signal that promotes loss of SoxB1 in macromere progeny, tests of loss of Pmar1 function will be required to confirm that it is necessary in the normal embryo; to date such tests have been confounded by the multiplicity of Pmar1-related genes. Interestingly, injection of *Pmar1* mRNA also conferred on mesomeres the same capacity, although mesomere derivatives never express Pmar1 in normal embryos. This observation suggests a cell-autonomous role for Pmar1 in the clearance of SoxB1 from micromeres. Our experiments do not reveal whether the early micromere-specific protein turnover mechanism depends on nuclear β-catenin and Pmar1, because over accumulation of *SoxB1* mRNA in cadherin-expressing embryos could overwhelm this mechanism by the early mesenchyme blastula stage, when we carried out our analyses.

Finally, SoxB1 appears to exert a potent negative feedback on its own transcription because *SoxB1* mRNA levels are elevated about 10-fold in SoxB1MO-knockdown embryos (Fig. 6, Fig. 8E) (C. Livi, E. H. Davidson and L.M.A., unpublished). As SoxB1 is a DNA-bending 'architectural factor' without detectable ability to activate transcription independently (Kenny et al., 2001), it might interact with other regulatory factors to directly repress its own expression, or to activate production of an intermediary repressor. The fact that *SoxB1* mRNA increases dramatically in abundance in presumptive ectoderm of SoxB1MO-knockdown mesenchyme blastulae, but is undetectable in all endomesoderm (Fig. 6B, Fig. 8E), suggests that this feedback loop is restricted to ectoderm, whereas other, β-catenin-dependent, mechanisms downregulate *SoxB1* transcription in vegetal blastomeres. We think it likely that this feedback loop serves to limit SoxB1 accumulation in presumptive ectoderm after cleavage, as the rate of cell division decreases. Transcription of several ectoderm-specific genes has been found to decrease significantly during this same period (Gagnon et al., 1992; Lee et al., 1992).

Modulation of transcription factor concentrations via regulated turnover is a relatively unusual developmental mechanism. An example closely related to the selective degradation in early blastomeres described here is provided by the early *C. elegans* embryo, in which germline regulatory protein degradation is activated in somatic blastomeres during the first few asymmetric cleavages (Pellettieri et al., 2003). An example analogous to the antagonism between SoxB1 and nuclear β-catenin has been described in *Xenopus* embryos: in that system selective proteolysis of the homeodomain repressor Xom is activated on the future dorsal side of the embryo, thus preventing it from inhibiting activation of dorsal-specific genes, including *goosecoid* (Zhu and Kirschner, 2002). Targeted protein turnover has also been shown to be a response to various signals, as is probably the case for SoxB1 degradation in macromere progeny. For example, the dorsal/Nfκb cytoplasmic tether cactus/Iκb is degraded in response to signaling through Toll/cytokine receptors (reviewed by Belvin and Anderson, 1996) and the stability of β-catenin itself is regulated through Wnt signaling (for a review, see Nelson and Nusse, 2004). Our understanding of β-catenin-dependent pathways has expanded dramatically in recent years with most emphasis placed on regulatory target genes, encoding either transcription factors or signaling molecules. To our knowledge, this is the first description of a β-catenin-dependent pathway that promotes the turnover of a key developmental regulator of transcription, the SoxB1 protein of the sea urchin embryo.

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Note added in proof

Using GFP-tagged β-catenin, Weitzel et al. (Weitzel et al., 2004) recently demonstrated a pattern of protein stability along the animal-vegetal axis of sea urchin embryos that is reciprocal to that of SoxB1.

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