Development 128, 365-375 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV4527

SpKrl: a direct target of β-catenin regulation required for endoderm differentiation in sea urchin embryos

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Accepted 17 November 2000; published on WWW 11 January 2001

SUMMARY

Localization of nuclear β-catenin initiates specification of vegetal fates in sea urchin embryos. We have identified *SpKrl*, a gene that is activated upon nuclear entry of β-catenin. *SpKrl* is upregulated when nuclear β-catenin activity is increased with LiCl and downregulated in embryos injected with molecules that inhibit β-catenin nuclear function. LiCl-mediated *SpKrl* activation is independent of protein synthesis, indicating that *SpKrl* is a direct target of β-catenin and TCF. Embryos in which SpKrl translation is inhibited with morpholino antisense oligonucleotides lack endoderm. Conversely, *SpKrl* mRNA injection rescues some vegetal structures in β-catenin-deficient embryos. SpKrl negatively regulates expression of the animalizing transcription factor, SpSoxB1. We propose that SpKrl functions in patterning the vegetal domain by suppressing animal regulatory activities.

Key words: Sea urchin embryo, β-Catenin, Zinc finger, Endoderm, Morpholino, Transcriptional repressor, Sox

INTRODUCTION

The animal-vegetal (A-V) axis of the sea urchin embryo is established before fertilization. Classical experiments showed that animal and vegetal halves of bisected eggs have different developmental potentials, since only vegetal halves inherit the maternally derived program required for mesendoderm tissues while animal halves form poorly differentiated pre-ectoderm (Hörstadius, 1973; Maruyama et al., 1985). Several recent reports demonstrate a central role for the β-catenin/TCF pathway in specification of vegetal fates. β-catenin enters vegetal nuclei during cleavage stages when vegetal fates are specified (Logan et al., 1999). Elevation of nuclear β-catenin levels by injection at one-cell stage of mRNA encoding a stabilized form of β-catenin (Wikramanayake et al., 1998) or inactive GSK3β kinase (Emily-Fenouil et al., 1998) results in vegetalized embryos that resemble the phenotype caused by inhibition of GSK3β by LiCl treatment. Conversely, depletion of β-catenin by elevating GSK3β kinase levels (Emily-Fenouil et al., 1998) or by preventing its nuclear entry with C-cadherin (Wikramanayake et al., 1998; Logan et al., 1999) animalizes embryos. Results of experiments modulating TCF activity also are consistent with its proposed role in this pathway (Huang et al., 2000; Vonica et al., 2000). A dominant negative *Xenopus* TCF that lacks the β-catenin-binding domain blocks vegetal tissue differentiation, while embryos expressing a constitutively active *Xenopus* TCF variant develop with an excess of vegetal tissues. A sea urchin factor, SpTCF/Lef, mediates similar effects (Huang et al., 2000); for simplicity, in this paper we will refer to this transcription factor as TCF-Lef, since its sequence is equally similar to those of TCF and Lef. The fact that expression of *Xenopus* dominant negative TCF blocks the activity of LiCl implies that essential early functions of this classic vegetalizing agent are mediated by β-catenin through TCF-Lef-responsive promoters (Vonica et al., 2000).

The Wnt signaling pathway is involved in early specification of cell fates in diverse embryos (Moon and Kimelman, 1998). In *Xenopus* embryos, the function of β-catenin in dorsalizing mesoderm is mediated, at least in part, by the transcription factors encoded by *siamois* (Brannon et al., 1997) and *twin* (Laurent et al., 1997). In murine embryos β-catenin activates the gene for brachyury, which also is essential for mesoderm specification (Arnold et al., 2000). Similarly, likely targets in sea urchin embryos include genes encoding transcription factors that activate vegetalizing genes and/or repress animalizing genes. A requirement for repressors of animalizing functions is suggested by our recent observations that expression of the transcription factor, SpSoxB1, is gradually extinguished in the vegetal plate (Kenny et al., 1999) as mesendoderm is patterned and that persistent expression of *SpSoxB1* converts vegetal blastomeres to a primitive ectoderm state (A. Kenny, D. W. O., L. M. A. and R. C. A., unpublished observations).

We expected that β-catenin/TCF-Lef target genes would be progressively activated in vegetal blastomeres between 16-cell and early blastula stages, following closely the vegetal-to-animal wave of entry of β-catenin into the nuclei of these cells, which starts in the micromeres (Logan et al., 1999). To identify candidates, we combined a subtractive hybridization screen, sequencing of the resulting partial cDNAs, and in situ

hybridization in order to identify vegetally restricted, early zygotic mRNAs. This approach identified *SpKrl* (*Strongylocentrotus purpuratus Krüppel-like*), a gene similar in its Zn-finger DNA-binding domain to *Drosophila Krüppel*. Here we show that the level of *SpKrl* expression correlates with upregulation or downregulation of β-catenin. Moreover, we provide evidence that *SpKrl* is a direct target of β-catenin regulation because its transcriptional activation does not require synthesis of an intermediary factor. SpKrl is required for endoderm differentiation since inhibition of its translation with morpholine-substituted antisense oligonucleotides prevents gut formation. We show that *SpKrl* behaves as a negative transcriptional regulator whose ectopic expression downregulates the animal transcription factor, *SpSoxB1* (Kenny et al., 1999). These data strongly support a model in which *SpKrl* is one of the early zygotic genes that responds to nuclear β-catenin activity and encodes a factor that acts to promote vegetal differentiation. We propose that it does this by repressing *SpSoxB1*, and perhaps genes encoding other animalizing transcription factors (Angerer and Angerer, 2000; Wei et al., 1999) clearing them from vegetal blastomeres to allow their further specification as endoderm and mesenchyme.

MATERIALS AND METHODS

Embryo and single cell cultures

Adult sea urchins (*S. purpuratus*) were obtained from Charles M. Hollahan (Santa Barbara, CA). Embryos were cultured (Angerer and Angerer, 1981) and cell separation experiments were carried out essentially as described previously (Reynolds et al., 1992). Blastomeres were resuspended in Ca^{2+}-free sea water and cultured in spinner flasks as described (Hurley et al., 1989).

Subtractive hybridization and cloning

Total RNA from 2.5- and 12-hour embryos was isolated using TRIzol reagent (Life Technologies), and poly(A)+ mRNA was isolated from total RNA preparations using oligo dT-cellulose chromatography (Fastrack 2.0, Invitrogen). Suppression subtractive hybridization was performed using a PCR-Select cDNA Subtraction kit (Clontech) according to the manufacturer's protocol, using double-stranded cDNA synthesized from tester (12 hour) and driver (2.5 hour) mRNAs. One sequence (SpKrl) selected in this screen was similar to that of Krüppel-related zinc-finger-containing transcription factors and was extended at both 3′ and 5′ ends with RACE PCR (Gibco BRL, Bethesda MD), using nested primers within the zinc-finger domain. A complete cDNA was obtained by RT-PCR, using a primer in the 3′ untranslated region, Superscript reverse transcriptase (Gibco-BRL) and a high fidelity polymerase (Vent, Gibco-BRL). All experiments described here were carried out with one SpKrl allele (337 amino acids). A second allele has also been sequenced that contains 336 amino acids. The GenBank Accession Number is AF314167.

Effect of LiCl and emetine on accumulation of *SpKrl* mRNA in separated cells

To inhibit protein synthesis, 50 μM emetine (Sigma) was added to dissociated cells (~1000 embryo equivalents/ml) in Ca^{2+}-free sea water (CFSW) at 5 hours (16-cell stage in cultures of intact embryos) and, at 5.5 hours, 30 mM LiCl and radiolabeled amino acids ([^{35}S]methionine/cysteine Promix, Amersham (5 μCi/ml)) were added. At 8.5 hours, cells were collected by centrifugation and dissolved in TRIzol for purification of RNA and total protein as described by the manufacturer (Gibco-BRL).

Synthetic mRNA and antisense morpholino microinjection

The complete *SpKrl* cDNA coding sequence (plus 10 nucleotides 3′UTR and ~165 nucleotides 5′UTR) was cloned in pGEM-T-easy, released with *Eco*R1 and inserted in the expression vector, T-clone (derived from pSP64T (Angerer et al., 2000)) for RNA synthesis. The Engrailed-SpKrl chimera (SpKrl-DBD-Eng) was generated by fusing the SpKrl DNA-binding domain, amplified by PCR with a 5′ primer starting at base 575 that contains a *Bgl*II adapter sequence, downstream and in frame with the Engrailed transcriptional repressor domain (296 amino acids in pXT7 (Fan and Sokol, 1997)) containing a *Bam*HI site at its 3′ end**.** Constructs were verified by sequencing. Synthetic mRNAs were transcribed with Sp6 RNA polymerase (Sp6 mESSAGE mACHINE, Ambion) from templates truncated with *Xba*I, suspended in 30% glycerol, quantitated by spectrophotomety and gel electrophoresis, and microinjected as described previously (Angerer et al., 2000). Either 2 or 6 $\times 10^5$ RNA molecules were injected into each egg. Morpholine-substituted antisense oligonucleotides were obtained from Gene Tools (Corvallis, OR). MKrl-1 and MKrl-2 sequences complementary to sequences in the 5′ UTR of SpKrl mRNA with respect to translational initiation were: MKrl-1, 5′ CGTGATGCTGAATGGCAGTGGAGAC 3′ (–46 to –22); MKrl-2, 5′ CGCCGCGTGTAGACGGTTCATGTCG 3′ (–4 to +21).

The control morpholino was supplied by Gene Tools. Test target mRNAs included GFP mRNA and a message in which the MKrl complementary sequence was inserted in the GFP 5′ UTR. Morpholinos were dissolved in distilled water and 2 pl of a solution containing 200 μM morpholino and 30% glycerol was microinjected into fertilized eggs to give a final concentration in the egg of 2 μM (~2.4×10^8 molecules).

Immunostaining and microscopy

Embryos were fixed in 4% paraformaldehyde and stained with a polyclonal antibody against SpSoxB1 (Kenny et al., 1999) and a monoclonal antibody against a PMC-specific epitope, 6e10. Fluorescent signals were captured by sequential scanning using a LeicaTS confocal microscope.

Hybridization assays

For RNA blots, 1 μg each of 2.5 hour, 12 hour and 24 hour poly(A)+ RNA and 10 μg total 12 hour RNA were electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde, transferred to Genescreen plus (DuPont NEN, Boston, MA), and hybridized with probe labeled with ^{32}P by random priming. Successive washes in 4×SSC, 2×SSC, 1×SSC and 0.1×SSC, each containing 1% SDS, were also carried out at 60°C.

For RNase protection assays, total RNA from normal embryos (10 μg) or from dissociated embryos (2.5 μg) purified with TRIzol reagent (Gibco BRL, Bethesda, MD) was hybridized to a *SpKrl* probe (275 base pairs cDNA, representing sequence 880 to 1155 that includes 129 bases of ORF and 146 bases of 3′UTR) and a *Spec2a* probe as described previously (Hardin et al., 1988).

For in situ hybridization assays, adjacent 5 μm sections of selected developmental stages were hybridized with ^{33}P-labeled RNA probes for *SpKrl* (2.5×10^5 dpm/ng) and *SpHE* (5×10^4 dpm/ng) mRNAs, as described previously (Angerer et al., 1987).

RT-PCR

Embryos were injected separately with synthetic mRNAs encoding cadherin (1.4 pg); dominant negative Lef (0.5 pg; Huang et al., 2000); β-catenin (0.1 pg; Wikramanayake et al., 1998; Logan et al., 1999); or Eng-Lef (0.5 pg, kindly provided by Dr Judith Venuti, Tulane University) and allowed to develop to 16 hours postfertilization. For each sample, RNA was purified from 200 embryos with TRIzol, digested with DNase I (0.5 units/μl, Pharmacia, 37°C, 30 minutes), followed by extraction with organic solvents and ethanol precipitation. Reverse transcription was carried out as described previously (Wikramanayake et al., 1997) with Superscript II (Gibco-BRL) at

Fig. 1. SpKrl is a member of the Krüppel transcription factor subclass. The four zinc-finger repeats of SpKrl (double-headed arrows) are shown aligned with those of *Drosophila* Krüppel and the N-terminal four of the five repeats in *Drosophila* Glass. Cysteine and histidine residues are shaded light gray, and the 'H-C link motifs' diagnostic for Krüppel-like factors are shaded dark gray. The co-linear alignment of the three peptides shown here does not align the most similar repeats of the different proteins.

42°C for 1.5 hours. PCR was carried out with Taq polymerase in the presence of ~20μCi of ^{32}P-dCTP at annealing and elongation temperatures of 55°C and 72°C, respectively. Products were analyzed between cycles 24 and 28 to assure the linearity of amplification. Single products of expected size were obtained for both the SpKrl and *S. purpuratus* mitochondrial (mito) primers: SpKrl forward primer, GCT CTA GAC ATG AAC CGT CTA CAC GCG; SpKrl reverse primer, GCT CCA TGG CAA TCG TTG ACG CTT CGT AGG; Mito forward primer, ACT CTC TCC TCG GAG CTA TA; Mito reverse primer, GTA TAA TTT TTG CGT ATT CGG C.

Quantities of radiolabeled products were measured by phosphorimagery (Molecular Dynamics; ImageQuant).

RESULTS

SpKrl encodes a putative transcription factor containing multiple zinc fingers

A partial *SpKrl* cDNA was isolated by subtractive hybridization to select for early zygotic RNA species not represented in maternal RNA. The limits of the open reading frame were reached as described in Materials and Methods and the predicted peptide sequence consists of 337 amino acid residues, with four zinc-finger motifs at its C terminus (Fig. 1). Database searches reveal a conserved motif between adjacent zinc fingers, called the H-C link (Schuh et al., 1986), which places SpKrl in the Krüppel-like family of transcription factors. The most closely related proteins found using a Blast search were the products of the *Drosophila* genes, *Glass* (P13360) and *Krüppel* (AE003466.1). In common with many Krüppel-related factors, sequence similarity is limited to the zinc finger regions of the DNA-binding domain, and SpKrl has no significant match to known proteins outside this region.

SpKrl is a developmentally regulated early zygotic gene

SpKrl expression was analyzed by RNA blot and RNase protection assays. A single, 1.7 kilobase RNA species was detected in total RNA (Fig. 2A) and in poly A+ RNA (data not shown). Consistent with the design of the subtractive hybridization screen, *SpKrl* message is not detectable for the first 3 to 4 hours after fertilization. Thereafter, the message accumulates transiently to maximal levels at early blastula stage (~16 hours; ~200-cell stage) and then rapidly disappears by early mesenchyme blastula stage (22 hours). RNase protection assays (Fig. 2B) confirm that *SpKrl* transcription is activated at 16-cell stage, when β-catenin can first be observed in vegetal nuclei (Logan et al., 1999). The

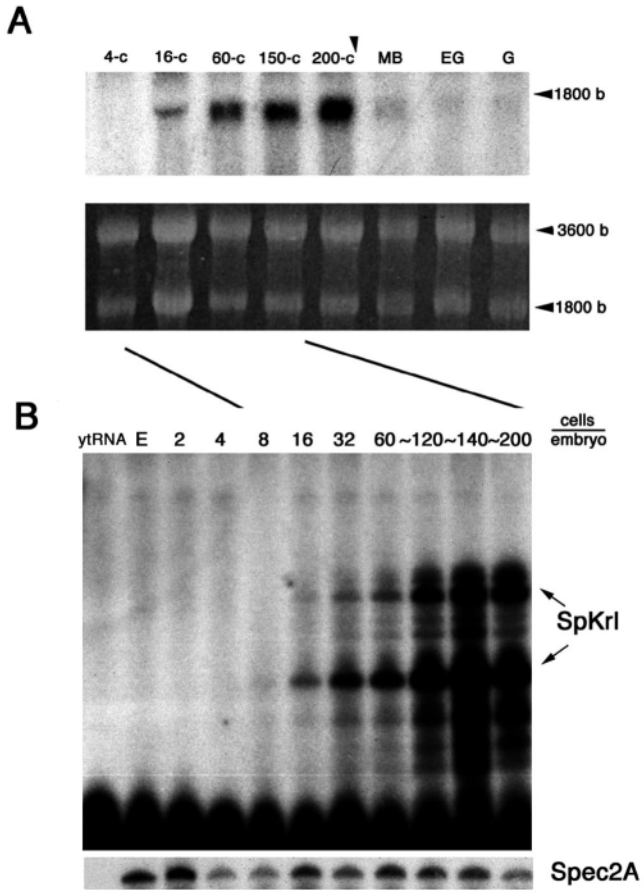

Fig. 2. *SpKrl* mRNA accumulates transiently during cleavage. mRNA abundance was measured by RNA blotting (A) and RNase protection (B) assays. (A) *SpKrl* mRNA levels in total RNA from embryos at four- through 200-cell stages (cell numbers indicated above the lanes), mesenchyme blastula (MB, ~400 cells), early gastrula (EG) and late gastrula (G) stages. The lower panel shows ethidium bromide-stained rRNA bands, which provides a recovery control. (B) The upper and lower panels show *SpKrl* and control *Spec2a* mRNA levels from each cell division during cleavage. P, input probe; ytRNA, yeast tRNA was substituted for embryo RNA.

control *Spec2a* maternal transcripts are present at similar levels in RNA from all stages for which data are shown (Fig. 2B, bottom panel). We conclude that *SpKrl* expression is strictly zygotic and restricted to stages before morphogenesis and overt cell differentiation begin, supporting the possibility that it plays a role in early specification of cell fates.

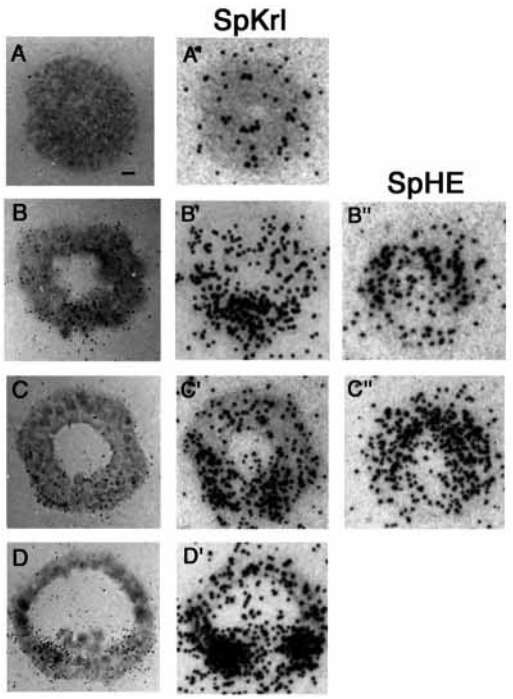

Fig. 3. *SpKrl* mRNA is concentrated in vegetal precursors of endoderm and mesenchyme. A,A′-D,D′) ^{33}P-labeled RNA probe for *SpKrl* mRNA was hybridized to sections of eggs (A) or embryos at 60-cell (B), very early blastula (9 hours, ~180 cell) (C) and early mesenchyme blastula (18 hours, ~300 cells) (D). A-D, brightfield illumination; A′-D′, darkfield illumination and image inversion. (B″,C″) Sections adjacent to those shown in B′ and C′ were hybridized with a probe for *SpHE* mRNA, which accumulates preferentially in nonvegetal blastomeres. Scale bar: 10 μm.

Vegetal cells of developing blastulae are enriched with *SpKrl* mRNA

To determine the *SpKrl* mRNA distribution, in situ hybridization was performed on sections of sea urchin embryos using an antisense, ^{33}P-labeled probe. Because *SpKrl* mRNA is undetectable before 16-cell stage in RNase protection and RNA blot assays, the grains over the egg section represent background (Fig. 3A). Signal was first detectable at the 60-cell stage when it is concentrated on one side of the embryo (Fig. 3B). This region was identified as the vegetal pole because adjacent sections hybridized with *SpHE* probe gave the complementary, nonvegetal pattern (Reynolds et al., 1992). Signals in the animal hemisphere are much lower, but consistently above background.

The *SpKrl* expression pattern is dynamic at later developmental stages. By ~180-cell stage, the region of highest *SpKrl* mRNA concentration includes presumptive endoderm (Fig. 3C). By the hatching blastula stage (18 hours), the *SpKrl* mRNA distribution has modulated to a torus about the vegetal pole (Fig. 3D). This changing pattern is quite similar to that reported for nuclear entry of β-catenin (Logan et al., 1999), raising the possibility that these factors act in a common pathway.

Accumulation of normal *SpKrl* mRNA levels requires cell-cell interactions and is sensitive to perturbations of the Wnt signaling pathway

To test whether *SpKrl* is regulated by β-catenin/TCF-Lef, we determined the effects of treating embryos with LiCl at concentrations known to increase nuclear β-catenin levels and to cause its ectopic entry into nuclei of more animal (veg_1) blastomeres (Logan et al., 1999). RNase protection assays showed that LiCl treatment increases *SpKrl* mRNA levels severalfold (Fig. 4A), whereas maternal *Spec2a* message levels are unaffected. As a second, more direct test (Fig. 4B), we injected mRNA encoding a stabilized form of β-catenin (β-C) that vegetalizes embryos (Wikramanayake et al., 1998; Logan et al., 1999) and compared the resulting *SpKrl* mRNA levels with those of controls (U) using RT-PCR. *SpKrl* mRNA levels were normalized to equal embryo numbers among samples, using a mitochondrial RNA standard. As expected, activation of β-catenin/Lef function in this manner increased *SpKrl* transcript levels. Conversely, SpKrl levels were decreased by treatments that reduce nuclear β-catenin function. These included injecting mRNAs encoding *Xenopus* C-cadherin (Cd; Wikramanayake et al., 1998) or injecting a chimera containing the engrailed active repression domain linked to the sea urchin TCF-Lef DNA-binding domain (Eng-TCF-Lef) or a dominant negative variant of TCF-Lef that cannot bind β-catenin (dnTCF-Lef). The effect of dnTCF-Lef misexpression is relatively small compared with that obtained with Eng-TCF-Lef. While the reason for this is not clear, it may reflect the relative differences in the ability of TCF-Lef and Eng-TCF-Lef to repress target genes.

In normal embryos, zygotic intercellular signaling via SpWnt8 is required for specification of mesendoderm (A. Wikramanayake, personal communication). This suggested that *SpKrl* mRNA accumulation might be reduced if signaling were blocked by dissociating embryos into single cells. This is the case (Fig. 4A); the low level of *SpKrl* transcripts still present may result from the initial entry of β-catenin into vegetal nuclei, which is cell-autonomous (Logan et al., 1999). Importantly, *SpKrl* transcript levels can be restored to those in normal embryos by treating cells of dissociated embryos with LiCl. We conclude that full expression of *SpKrl* requires cell-cell interactions and that LiCl supplies the signal lost by embryo dissociation.

Activation of *SpKrl* transcription by LiCl is independent of protein synthesis

Because *SpKrl* expression is sensitive to LiCl treatment and coincident with nuclear entry of β-catenin, it could be directly activated by β-catenin/TCF-Lef. To test this, we used emetine to inhibit protein synthesis to determine if LiCl could upregulate *SpKrl* expression without the production of an intermediary factor. RNase protection assays were performed on RNA isolated from blastomere cultures treated with combinations of LiCl and emetine. In embryos of this species, emetine arrests protein synthesis quickly (~20 minutes) and efficiently (>98%; Gong and Brandhorst, 1988), and we obtained similar results with cultures of dissociated blastomeres (see Fig. 4C legend).

LiCl treatment rescued *SpKrl* expression in blastomeres treated with protein synthesis inhibitor, as described in Materials and Methods (Fig. 4C). Total *SpKrl* transcript levels as a function of time were measured by RNase protection, and protein synthesis was assessed by determining incorporation of labeled amino acids into total cellular protein. After correction for RNA load (Fig. 4C, bottom), the degree of LiCl-dependent

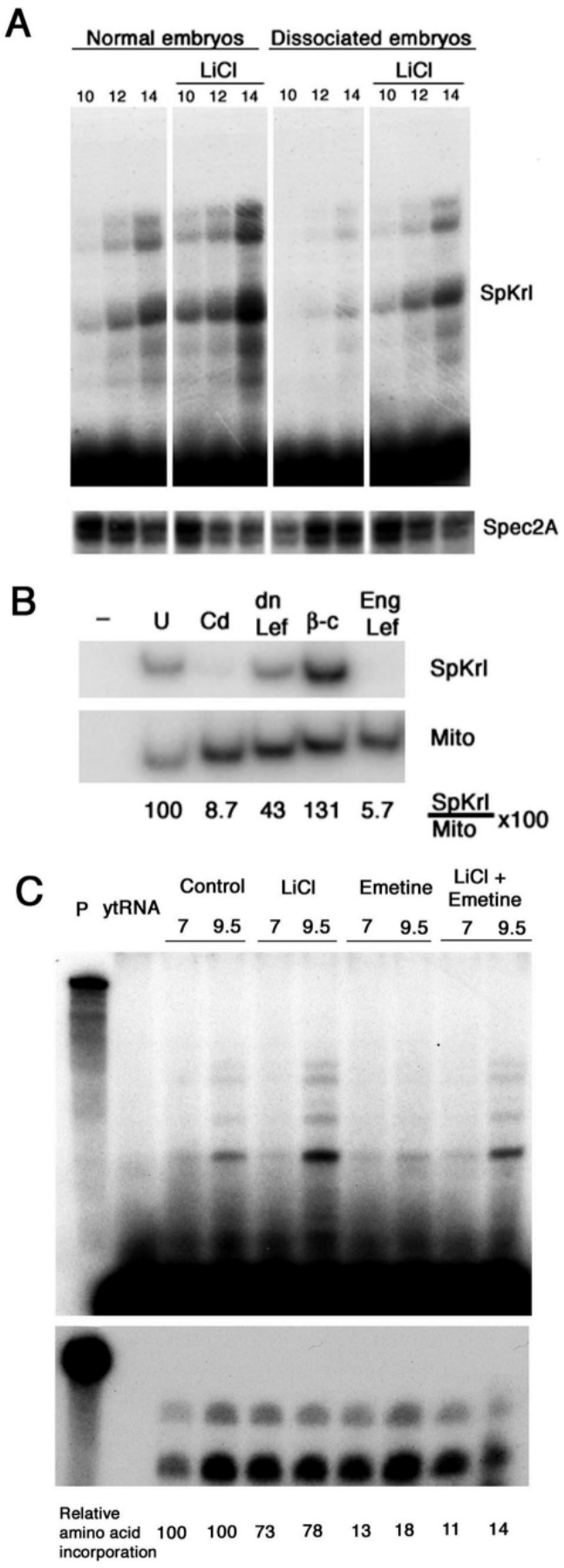

Fig. 4. *SpKrl* is a direct target of the β-catenin signaling pathway. (A) *SpKrl* activation depends on cell-cell interactions and is stimulated by LiCl. Normal embryos or cell suspensions of embryos dissociated at the two-cell stage were cultured with or without 30 mM LiCl added at the two-cell stage. Samples were removed at 10, 12 and 14 hours and assayed by RNase protection for the levels of *SpKrl* mRNA or *Spec2a* mRNA as a load control. Multiple bands observed for *SpKrl* and *Spec2a* are due to sequence polymorphisms between probe and target RNA that lead to fragments partially resistant to RNase cleavage. The exact band pattern varies between experiments in Figs 4A,C, 2B because the target RNAs are from different embryo cultures. (B) Embryos were injected at the one-cell stage with synthetic mRNAs encoding C-cadherin (Cd), dominant-negative TCF-Lef (dnTCF-Lef), constitutively active β-catenin (β-c) or a chimeric repressor targeted to TCF-Lef-binding sites (EngTCF-Lef). U, RNA from uninjected embryos; –, no template. RT-PCR was carried out for 24 cycles and primers specific for *SpKrl* mRNA (SpKrl) or for a mitochondrial RNA (mito) in separate reactions. Relative signals (bottom) were quantitated by phosphorimaging and normalized to equal embryo equivalents using the mitochondrial standard. (C) Upregulation of *SpKrl* transcription by LiCl is independent of protein synthesis. Embryos were continuously dissociated into individual cells beginning at the two-cell stage and cultured normally (control) or with the addition of 30 mM LiCl at 5.5 hours or 50 μM emetine at 5 hours, or both. RNA samples taken at 7 and 9.5 hours were assayed for levels of *SpKrl* (upper panel) or control maternal *Spec2a* (lower panel) mRNAs by RNase protection. P, probe; ytRNA, yeast tRNA substituted for embryo RNA. To estimate inhibition of protein synthesis, embryos were labeled with ^{35}S-methionine/cysteine beginning at 5.5 hours and cpm in protein were determined. The values given below the lanes are cpm incorporated into protein as a percentage of cpm incorporated into controls at the same time.

upregulation of *SpKrl* mRNA accumulation in dissociated cells was not detectably reduced by treatment with emetine. Together these results show that surrogate activation of the β-catenin/Lef pathway by LiCl can substitute for cell-cell interactions and that the activation of *SpKrl* downstream is independent of synthesis of intermediary factors. Because all of the vegetalizing effects of LiCl can be blocked by dominant negative TCF (Vonica et al., 2000), these results strongly support the conclusion that *SpKrl* is a direct target of Wnt/β-catenin signaling.

SpKrl can function as a transcriptional repressor that suppresses animal cell (ectoderm) fates

Expression of stabilized β-catenin or treatment with LiCl produces embryos with a vegetalized phenotype in which animal cells form mesoderm and endoderm instead of ectoderm (Wikramanayake et al., 1998). To determine whether SpKrl and stabilized β-catenin produced similar phenotypes, we analyzed mRNA-injected blastula-stage embryos for level and distribution of SpSoxB1 protein (Fig. 5, green stain), which is a sensitive upstream indicator of animal and vegetal cell fate specification (Angerer and Angerer, 2000). In normal embryos, SpSoxB1 protein initially accumulates to higher levels in nuclei of macromeres and mesomeres than in those of micromeres. Then it is gradually eliminated from vegetal mesendoderm precursors and eventually is restricted to ectoderm (Kenny et al., 1999). We also assayed embryos for the 6e10 epitope (Fig. 5, red staining), which is expressed specifically by PMCs (antibody kindly provided by Dr C. Ettensohn). Immunostaining shows that increasing the level of

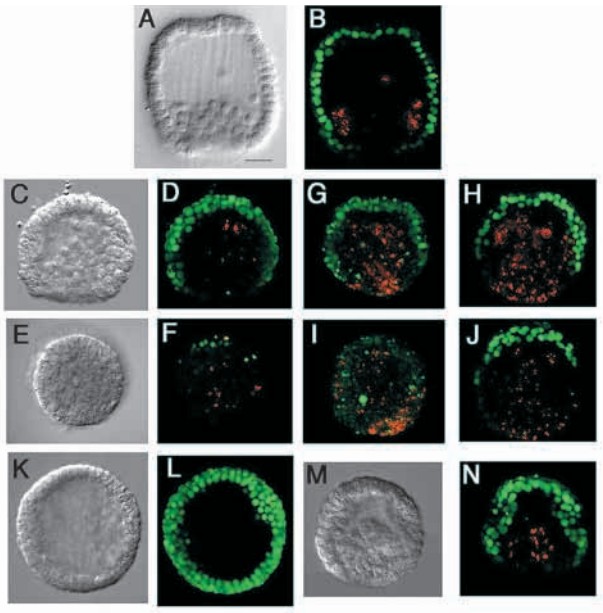

Fig. 5. SpKrl functions downstream of β-catenin to specify vegetal fates. (A-J) Fertilized eggs were injected either with glycerol (A,B) or with the indicated synthetic mRNAs: SpKrl (C,D, lower dose; E,F, higher dose), stable (non-phosphorylatable) β-catenin (G, lower dose; I, higher dose) or a fusion of the DNA-binding domain of SpKrl and the active engrailed repression domain (H, lower dose; J, higher dose). Green: SpSoxB1 transcription factor, which in normal embryos is restricted to presumptive ectoderm nuclei at this stage. Red: PMC-specific 6e10 immunostaining. Injection with each of these mRNAs vegetalizes embryos, reducing SpSoxB1 staining and increasing 6e10 staining. (K,L) Injection with ~6×10^5 mRNA molecules encoding *Xenopus* C-cadherin (template supplied by Dr A. Wikramanayake), which reduces nuclear β-catenin levels, leads to ubiquitous and elevated SpSoxB1 levels and produces embryos consisting solely of oral ectoderm-like (animal) tissue. (M,N) Co-injection of 6×10^5 molecules of mRNA for C-cadherin and 8×10^5 molecules of *SpKrl* mRNA rescues staining of 6e10 and absence of SpSoxB1 staining at the vegetal pole. The epithelial wall consists of a single layer of cells and all images are partial stacks through approximately 30% of the embryo. Scale bar: 20 μm.

SpKrl restricts the SpSoxB1 expression domain towards the animal pole and decreases SpSoxB1 concentration in most nuclei (Fig. 5D,F). Very similar effects on SpSoxB1 levels and distribution in these blastula-stage embryos are caused by expression of activated β-catenin (Fig. 5G,I). Conversely, in both cases, the number of 6e10-positive cells ingressing into the blastocoel increases (Fig. 5C,E), although the concentration of 6e10 is usually lower in *SpKrl* mRNA-injected embryos. This difference in 6e10 expression level may reflect the fact that nuclear β-catenin is required for PMC differentiation, which probably depends on β-catenin target genes other than *SpKrl*.

The SpKrl-mediated reduction in the SpSoxB1 domain size suggests that SpKrl may negatively regulate genes required for animal fates

To test this hypothesis, we compared the activity of native SpKrl to that of a chimeric repressor, SpKrl-DBD-Eng, consisting of a fusion between the *Drosophila* Engrailed repression and SpKrl DNA-binding domains (Fan and Sokol, 1997). The phenotypes produced by injection of mRNAs encoding these two factors are similar (compare Fig. 5D,F with Fig. 5H,J). In both cases, 6e10-positive cells increase in number while the number of SpSoxB1-positive cells decreases. This suggests that the role of SpKrl in patterning fates along the AV axis is largely to antagonize the function of genes required for animal fates.

It is interesting to note that SpSoxB1 expression is most refractory to misexpression of SpKrl, stabilized β-catenin or SpKrl-DBD-Eng in cells that are closest to the animal pole. While the reasons for this are not understood, it is not surprising because this region of presumptive ectoderm has a distinct pattern of gene expression (e.g. Hardin et al., 1988; Yang et al., 1989; Grimwade et al., 1991; Stenzel et al., 1994).

SpKrl mRNA injection restores the polarized SpSoxB1 distribution and rescues micromere fates in embryos depleted of β-catenin

The above results indicate that *SpKrl* is a factor that functions downstream of β-catenin/TCF-Lef and that it suppresses animal cell fates. Conversely, blocking β-catenin function by injection of *Xenopus* C-cadherin mRNA completely eliminates PMC differentiation, as previously reported (Logan et al., 1999), and SpSoxB1 is expressed ubiquitously (Fig. 5K,L). These results predict that supplying exogenous SpKrl might rescue some of the defects caused by blocking nuclear β-catenin accumulation. In fact, this is the case since co-injection of *SpKrl* mRNA (at the same level as in Fig. 5E,F) along with the C-cadherin message (at the same level as in Fig. 5K,L) clears SpSoxB1 from vegetal cells and rescues 6e10-positive cells in the blastocoels of >90% of embryos (three experiments, ~600 total embryos; Fig. 5M,N). Many embryos also contained triradiate spicule rudiments, indicating that these cells execute skeletogenesis, the terminal developmental program of PMCs (data not shown). Thus, SpKrl can both antagonize animalizing activity in the vegetal domain and rescue PMC differentiation in embryos depleted of β-catenin, consistent with its functioning downstream in the β-catenin/TCF-Lef pathway.

Loss of SpKrl function blocks endoderm differentiation

The experiments described above show that *SpKrl* mRNA injections can alter allocation of fates along the AV axis. To determine whether *SpKrl* function is required for correct patterning along this axis in normal embryos, we used antisense inhibition to deprive embryos of SpKrl protein. This method employs oligonucleotides, complementary to the 5′ untranslated leader of the target mRNA, in which a six-membered morpholine ring is substituted for ribose in each residue of a 25-mer (hence, 'morpholinos'). The stable duplex formed blocks mRNA translation initiation (Summerton and Weller, 1997). If inhibition of SpKrl translation is complete, the result is a null phenotype, since there is no detectable maternal *SpKrl* mRNA, and hence no maternal protein.

As a first test of this method in sea urchin embryos, we constructed an artificial mRNA in which the 5′ SpKrl leader sequence was inserted in the corresponding position of green fluorescent protein mRNA (Krl-GFP). This target message (~500,000 copies) was injected into fertilized eggs alone or in combination with a 500-fold sequence excess of morpholino complementary to the SpKrl leader (MKrl-1) or an unrelated

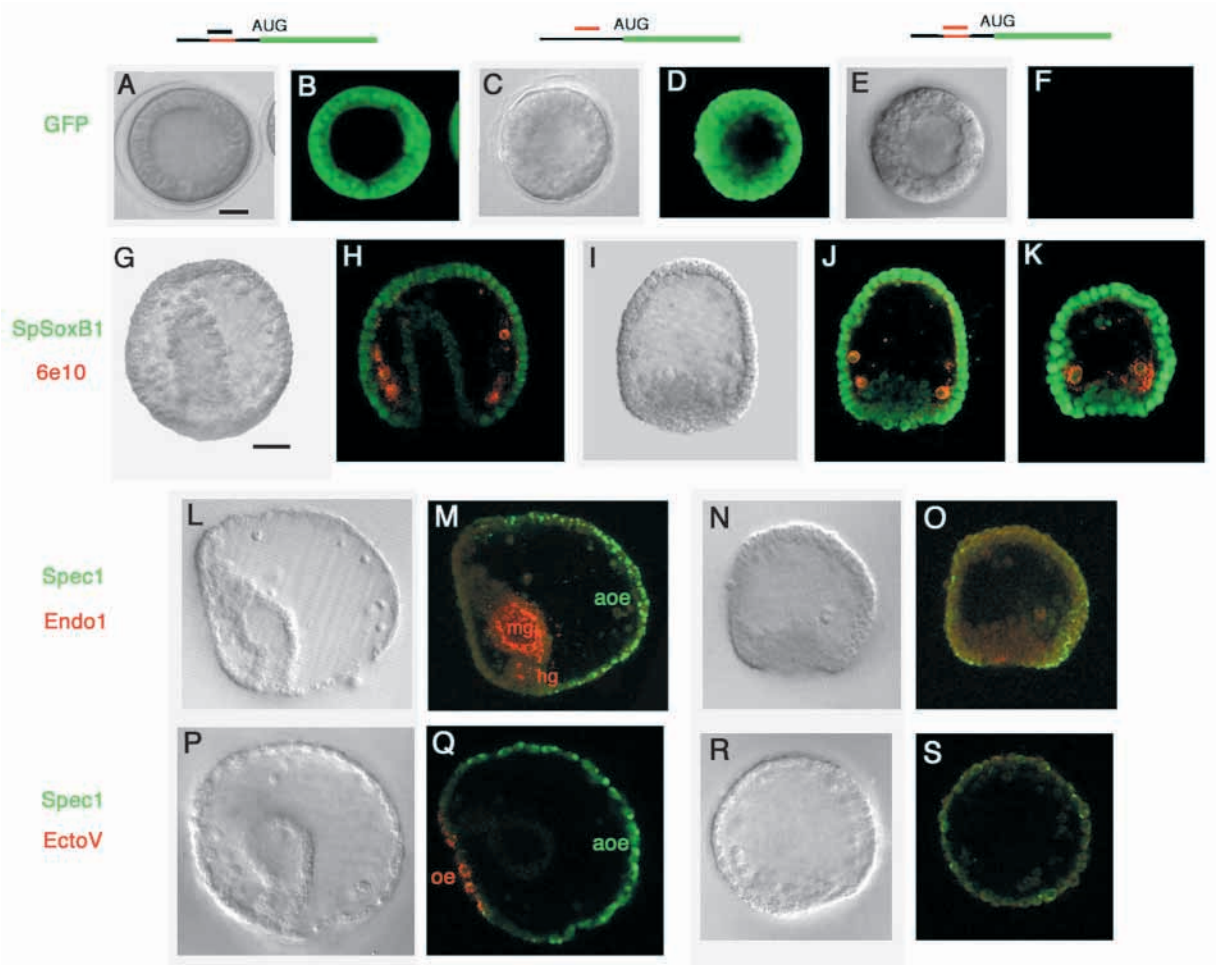

Fig. 6. Loss of SpKrl function blocks archenteron formation. Control and *SpKrl* antisense morpholinos were injected into fertilized eggs to achieve a final concentration of 2 μM. (A-F) SpKrl morpholino-mediated translational inhibition of GFP. Eggs were co-injected with 500,000 copies of mRNA encoding GFP (represented by green lines in diagrams above A-F) that either contained (red line, A,B,E,F) or lacked (black line, C,D) morpholino complementary target sequence in the 5′ UTR. The GFP signal is undetectable in 18-hour hatching blastulae when target and morpholino sequences are complementary (E,F), but strong and equivalent in the two heterologous controls (A-D). (G-K) Embryos injected with two different SpKrl morpholino sequences (MKrl-2, I,J and MKrl-1, K) are indistinguishable, lacking archentera, containing PMCs (red) and a larger fraction of SpSoxB1-positive cells (green). Embryos injected with control morpholino (G,H) develop normally. SpSoxB1 signals in the control embryos are equivalent to those in uninjected embryos, but are reproducibly lower than those in SpKrl morpholino-injected embryos. In H and J or K signal intensities are directly comparable since photomultiplier sensitivities were identical. (L-O) SpKrl morpholino-injected 72-hour embryos still lack a gut and do not express an endoderm (midgut, mg; hindgut, hg) marker (Endo 1, red; N,O), whereas control morpholino-injected embryos do (M). In addition, Spec1 (green), a marker of differentiated aboral ectoderm (aoe), (M, control morpholino) is reduced in SpKrl morpholino-injected embryos (compare M with O). (P-S) Same as L-O, but embryos are stained with an antibody against EctoV, a marker of oral regions of ectoderm (oe), as well as Spec1. All embryos in G-Q are oriented with vegetal poles down; the plane of the optical section of the embryo shown in R,S is orthogonal to the animal-vegetal axis. The images shown in P-S are single optical sections, whereas all the others are stacks of optical sections that include about 25% of the embryo. Scale bars: in A, 20 μm for A-F; in G, 20 μm for G-S.

morpholino (M-c) as a negative control. As shown in Fig. 6B,D, in the absence of a complementary morpholino, the Krl-GFP mRNA produced a strong fluorescent signal in each of the injected embryos (n>100), providing a sensitive test of the ability of MKrl-1 to inhibit its translation. This signal was completely eliminated in a similar number of embryos by MKrl-1 (Fig. 6F). Since signal can be detected with 100-fold less GFP mRNA (not shown), more than 99% of GFP translation was blocked. In contrast, the signal was not detectably reduced by M-c (Fig. 6B) and embryonic development proceeded normally.

Inhibition of endogenous SpKrl translation was carried out using levels of MKrl-1 (2-4 μM in the egg) sufficient to block detectable translation of the much more abundant, exogenously supplied SpKrl-GFP mRNA. At 4 μM, a very consistent phenotype (>90% of embryos) resulted in which endoderm failed to differentiate (Fig. 6J,O). Again no deleterious effects were detectable with the control morpholino. At a concentration of 2 μM morpholino in the egg, the phenotype was slightly less consistent, with a small gut forming in a few embryos. These observations suggest that the morpholino concentrations used here are not far over the threshold required to block SpKrl function. In MKrl-1-treated embryos, PMCs ingressed as in control embryos and expressed the 6e10 epitope (6J,O). In

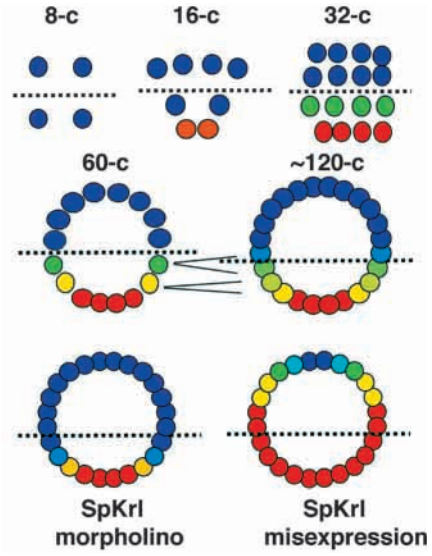

Fig. 7. A model for the role of the βC(SpKrl)/SpSoxB1 ratio in cell fate specification along the sea urchin embryo AV axis. The spectrum of colors of nuclei in different tiers of blastomeres along the animal-vegetal (up-down) axis depicts the ratio of nuclear β-catenin (βC)/SpKrl (red) to SpSoxB1 (blue). For simplicity, only half of the blastomeres of the embryo at each stage are illustrated. At the eight-cell stage, all nuclei have equal concentrations of SpSoxB1 (blue) and no detectable βC; at 16-cell stage, micromeres are born, and acquire βC and lose SpSoxB1 (orange-red); at the 32-cell stage, macromere daughters acquire βC and SpSoxB1 is reduced slightly in these cells (green) but has nearly disappeared from the nuclei of micromere daughters (red); at the 60-cell stage, macromere daughters divide to produce veg_1 and veg_2 tiers of blastomeres that contain, respectively, lower (green) and higher (yellow) βC/SpSoxB1 ratios, as SpSoxB1 retracts and βC advances; this pattern change continues in the 120-cell embryo. When SpKrl levels are depleted by morpholino antisense translational inhibition (bottom left), the spectrum shifts toward the blue (more animal/less vegetal); conversely, when SpKrl levels are elevated by mRNA injection (bottom right), the shift is towards the red (less animal/more vegetal). The horizontal broken line represents the presumptive ectoderm/endoderm border.

addition, they had differentiated pigment cells (data not shown), indicating that at least some of the most vegetal secondary mesenchyme derivatives (Ruffins and Ettensohn, 1996) do not require SpKrl function. When control embryos reached the mid-gastrula stage (Fig. 6G,H), MKrl-1-treated embryos showed no evidence of archenteron invagination (Fig. 6I,J). Nuclei throughout the epithelial portion of these embryos consistently accumulated SpSoxB1 protein to levels higher, on average, than those in presumptive ectoderm of control embryos at temporally equivalent stages (for example, compare Fig. 6H with Fig. 6J,K). At the late gastrula stage, the archentera of normal embryos stained for the late endoderm marker, Endo1 (Fig. 6M), whereas all MKrl-1-treated embryos showed a severe reduction in invaginated tissues and lacked Endo1-positive cells (Fig. 6N,O). Furthermore, these embryos did not express Endo16, a marker initially expressed in the vegetal plate of normal embryos (D. W. O., R. C. A. and L. M. A., unpublished observations). Essentially all embryos injected with a second SpKrl morpholino (MKrl-2; Fig. 6K) also lack archentera, indicating that morpholino-mediated antisense translational inhibition is specific for the intended target mRNA. We conclude that SpKrl is not required for differentiation of the most vegetally derived mesenchymal tissues, but is essential for endoderm differentiation in normal embryos.

The ectoderm of embryos injected with SpKrl morpholino is poorly differentiated. Spec1 (aboral ectoderm) signals are considerably reduced and always lower than those in control embryos (compare the green signals in Fig. 6M and Fig. 6O) and EctoV (oral ectoderm) expression is not detectable (compare the red signals in Fig. 6Q and Fig. 6S). Whether this ectoderm phenotype is a consequence of the lack of vegetal signaling in these endoderm-deficient embryos or results from some SpKrl-dependent process within the presumptive ectoderm is not yet clear.

DISCUSSION

Maternal information specifies the vegetal pole of the sea urchin embryo, and the 16-cell stage micromeres that inherit this information then form an organizing center that regulates patterning of the vegetal plate to form secondary mesenchyme and endoderm. β-Catenin, in its role as a co-activator working with TCF-Lef is a central component of this organizer (reviewed by Angerer and Angerer, 2000). The experiments presented here identify *SpKrl* as a direct target of β-catenin regulation that antagonizes animalizing genes, including *SpSoxB1*, and is required for gut formation.

SpKrl is a direct target of β-catenin/TCF-Lef

We have presented four different lines of evidence that collectively demonstrate that *SpKrl* is a direct target of the β-catenin/TCF-Lef pathway. First, circumstantial evidence indicates that *SpKrl* expression is strictly zygotic and activated just as β-catenin enters vegetal nuclei, and that the wave of *SpKrl* mRNA produced by accumulation and decay strikingly mimics the vegetal-to-animal wave of nuclear entry of β-catenin during cleavage. Second, accumulation of *SpKrl* mRNA meets several criteria expected for regulation by the β-catenin/TCF-Lef pathway: It is strongly inhibited by cell separation that blocks cell-cell interactions, it is augmented by treatment of embryos with LiCl, which has been shown to stimulate the β-catenin/TCF-Lef pathway and it is inhibited by antagonists of this pathway (Logan et al., 1999; Wikramanayake et al., 1998; Huang et al., 2000; Vonica et al., 2000). Third, the embryo phenotypes produced by altering SpKrl activity share features with those caused by the corresponding alterations in β-catenin levels. The ability of SpKrl to rescue aspects of vegetal differentiation in embryos deprived of β-catenin function is consistent with its working downstream of β-catenin in normal embryos. Furthermore, elimination of SpKrl translation by antisense morpholino oligonucleotides creates phenotypes that are similar to those produced when endogenous nuclear β-catenin is downregulated by injection of cadherin mRNA, with an interesting difference discussed below. Fourth, upregulation of *SpKrl* mRNA accumulation by LiCl treatment of dissociated embryos is independent of new protein synthesis, demonstrating that *SpKrl* transcription does not require synthesis of an intermediate transcriptional regulator.

SpKrl is required for gut formation

Using the new morpholino antisense oligonucleotide method for inhibiting translation of specific target mRNAs, we found that embryos lacking SpKrl fail to differentiate endoderm, as measured by lack of both archenteron invagination and

expression of the Endo1 marker. The fact that two different *SpKrl* morpholinos produce the same phenotype strongly supports the specificity of their action. This represents a null phenotype because the same level of SpKrl morpholinos blocked detectable GFP translation from the SpKrl-GFP reporter mRNA, which we estimate was introduced at a level, ~500,000 transcripts per embryo, that is at least tenfold higher than that of endogenous *SpKrl* message at its highest concentration per cell.

Loss of SpKrl function might be expected to affect differentiation of all vegetal derivatives because *SpKrl* mRNA is expressed in all cells of the vegetal plate at some time during the period before mesenchyme blastula stage. However, PMCs and (at least) the pigment cell secondary mesenchyme cell type appear to differentiate normally in *SpKrl* morpholino-injected embryos. This differential requirement for SpKrl in specification of different vegetal cell fates must be considered in the context of the overall mechanism that regulates fates along the A-V axis and the role of SpKrl in that process.

A model for the role of SpKrl in specifying vegetal cell fates

A model for the role of SpKrl in patterning of vegetal fates incorporates two hypotheses. First, we have proposed that a cohort of animalizing transcription factors (ATFs) antagonizes the vegetal organizing center and that the ratio of nuclear β-catenin to the ATFs (βC/ATF) in different blastomeres is an important component of the mechanism that regulates fates along the AV axis (Angerer and Angerer, 2000; Kenny et al., 1999; Wei et al., 1999). SpSoxB1 is the first ATF to be analyzed in detail and has been used in these studies as an indicator of the domain of ATF activity because injection of *SpSoxB1* mRNA drives all cells to animal fates (A. Kenny, D. W. O., R. C. A. and L. M. A., unpublished observations). Conversely, misexpression of stable β-catenin restricts the animal domain (Fig. 5). These observations lead to the second hypothesis, which is that an essential role of SpKrl is to progressively downregulate ATFs. Consistent with a repressor function for SpKrl, injection of *SpKrl* and *SpKrl-DBD-Eng* synthetic mRNAs produces similar phenotypes. *SpKrl* mRNA restricts the domain of SpSoxB1 toward the animal pole and reduces the level of SpSoxB1 protein in most animal nuclei.

Although the available evidence thus supports the idea that the βC/ATF ratio is critical for patterning along the A-V axis of normal embryos, ectopically placed micromeres can induce endoderm formation by animal blastomeres that do not exhibit detectable nuclear β-catenin (Logan et al., 1999). Similarly, the relatively modest increase in *SpKrl* mRNA levels produced by overexpression of β-catenin (Fig. 4B) suggests that components of this pathway acting downstream of β-catenin also may not be mobilized in animal nuclei. These observations suggest that alternative pathways for endoderm specification can be activated in some experimental circumstances (for further discussion, see Angerer and Angerer, 2000).

The central idea of the working model for patterning normal embryos is that the nuclear βC/SpSoxB1 ratio is progressively increased in a vegetal-to-animal wave, thereby allowing macromere progeny to progress from a general mesendoderm fate to more restricted vegetal fates. This further specification clearly involves positive effectors thought to act downstream of β-catenin/TCF-Lef, including zygotic Wnt8 (A. Wikramanake, personal communication) and signals that activate Notch (Sherwood and McClay, 1999; Sweet et al., 1999); it may also require repressors in addition to SpKrl. The gradient in βC/SpSoxB1 ratio is created and progressively modified by a coordinated combination of cellular and molecular mechanisms. These include asymmetric cell division, cleavage plane orientations, level of SpSoxB1 produced by translation of maternal RNA and new zygotic transcription, and the vectorial wave of β-catenin entry into nuclei in the vegetal hemisphere.

The elaboration of the graded ratio of nuclear β-catenin to SpSoxB1, is depicted in Fig. 7 by the spectrum of colors in different nuclei. Through eight-cell stage, no β-catenin can be detected in nuclei and SpSoxB1 is uniformly distributed among them. The asymmetric 4th cleavage establishes macromeres and micromeres as distinct transcriptional territories. Micromeres acquire a smaller (~15-fold) pool of SpSoxB1 as a result of volume differences among these blastomere types and other as yet undefined factors (Kenny et al., 1999). They also are the first blastomeres to acquire detectable nuclear β-catenin (Logan et al., 1999). Consequently, they quickly establish the highest βC/SpSoxB1 ratio and are irreversibly determined to form PMCs. Our results suggest that maternal mechanisms are sufficient to create the required high βC/SpSoxB1 and that transient expression of *SpKrl* is not necessary for this commitment, as revealed by the loss-of-SpKrl function phenotype.

Patterning of vegetal fates then occurs primarily within progeny of the macromeres. Macromere daughters begin to acquire nuclear β-catenin only at 32-cell stage and also retain significant amounts of SpSoxB1, forming a single tier with uniform βC/SpSoxB1. At the next division to 60-cell stage, the more animal (veg_1) and vegetal (veg_2) grand-daughters separate. Veg_2 blastomeres accumulate higher levels of nuclear β-catenin (Logan et al., 1999) and adopt more vegetal fates, while veg_1 progeny have a lower βC/SpSoxB1 and will give rise to endoderm and a small amount of ectoderm. Modulation of βC/SpSoxB1 continues until mesenchyme blastula stage (~400 cells). The loss-of-function phenotype suggests that SpKrl becomes essential for endoderm specification after veg_1 and veg_2 lineages separate and that endoderm (and possibly some SMC) progenitors are most sensitive to perturbation of the βC/SpSoxB1 ratio. The fact that *SpKrl* mRNA accumulates relatively late in animal macromere derivatives is consistent with data from lineage analyses, which show that endoderm is not stably determined until mesenchyme blastula stage (Chen and Wessel, 1996).

The effects of modifying SpKrl level on endoderm differentiation can be interpreted in view of its demonstrated role in altering the βC/SpSoxB1 by downregulating SpSoxB1. Whether SpKrl directly represses *SpSoxB1* transcription remains to be determined. However, the magnitude of the increase in SpSoxB1 levels in animal blastomeres when SpKrl translation is blocked with an antisense morpholino was surprising, since these cells normally contain much lower levels of *SpKrl* mRNA than do vegetal cells. Possible explanations are either that an indirect pathway exists that works in the absence of SpKrl to upregulate SpSoxB1 in animal cells or that SpSoxB1 expression is more sensitive to negative regulation by SpKrl in these cells. Expression of SpKrl throughout the embryo by mRNA injection downregulates both

SpSoxB1 and the size of the presumptive ectoderm domain, increasing the βC/SpSoxB1 ratio at all points along the A-V gradient (Fig. 7, bottom). Conversely, loss of SpKrl function allows over-accumulation of SpSoxB1 and blocks differentiation of endoderm, as is the case in embryos injected with SpSoxB1 mRNA.

The fact that SpKrl function is not required for differentiation of PMCs superficially appears to contradict the finding that *SpKrl* mRNA injection rescues PMCs in embryos depleted of nuclear β-catenin (Fig. 6). The resolution of these effects lies in the different regulatory contexts in which SpKrl works in normal and β-catenin-depleted embryos. In normal embryos maternal mechanisms are sufficient to achieve the βC/SpSoxB1 ratio required for establishing the fates of micromere progeny. In embryos overexpressing cadherin, the concentration of nuclear β-catenin, and consequently that of SpKrl, is reduced, causing *SpSoxB1* to accumulate in micromeres and their progeny at abnormally high levels. In this case, the βC/SpSoxB1 ratio falls below the threshold for micromere development. Injection of *SpKrl* mRNA in these cadherin-expressing embryos downregulates *SpSoxB1* transcription, which, coupled with SpSoxB1 turnover, is sufficient to restore the ratio above micromere fate threshold. In contrast to the situation in the micromere lineage, endoderm precursors in normal embryos have relatively high early SpSoxB1 levels and β-catenin enters their nuclei only relatively late. Thus, the embryo requires a mechanism to reduce SpSoxB1 so that the appropriate βC/SpSoxB1 ratio can be achieved. In cadherin-overexpressing embryos, elevated and persistent SpSoxB1 expression blocks endoderm differentiation. Although *SpKrl* mRNA injection might also have been sufficient to rescue endoderm, this was not observed. Thus, additional regulators, including other β-catenin targets must be required; i.e. SpKrl is necessary but not sufficient for endoderm differentiation. The reason why SpKrl mRNA injection does not result in strong downregulation of SpSoxB1 in the animal-most cells is not clear and use of higher SpKrl concentrations results in embryo lethality. The persistence of SpSoxB1 suggests that other pathways exist for regulating the levels of this factor in animal pole cells.

We have shown that an essential early event in presumptive sea urchin embryo mesendoderm patterning is the activation of zygotic *SpKrl* expression by maternally regulated β-catenin. SpKrl then represses at least one ATF, SpSoxB1, which otherwise would maintain blastomeres in an early pre-ectoderm fate. We imagine that SpKrl-mediated repression is necessary for these blastomeres to acquire competence to respond to other vegetal signals, including those that activate a Notch pathway (Sherwood and McClay, 1999; Sweet et al., 1999) as well as other β-catenin/TCF-Lef effectors (e.g., Wnt8, A. Wikramanayake and W. H. Klein, personal communication). At the same time, SpSoxB1 exhibits a strong antagonism against β-catenin, possibly mediated through direct interactions that interfere with β-catenin/TCF-Lef complex formation, as suggested recently for several *Xenopus* Sox factors (Zorn et al., 1999).

Classical theories proposed that opposing gradients of animalizing and vegetalizing diffusible factors control specification of cell fates along the primary axis of sea urchin embryos (Hörstadius, 1973; Runnström, 1975). While diffusible ligands have been implicated recently in patterning along this axis (Angerer et al., 2000), other recent work, including that reported here, indicates that an earlier asymmetry involves the balance between mutually antagonistic transcription factors, including SpSoxB1 and nuclear β-catenin/SpKrl, whose activities are initially confined to animal and vegetal domains by cell autonomous mechanisms.

Work in the authors' laboratory is supported by grant GM25553 from the National Institutes of Health. E. W. H. was supported by the NIH/NIDCR Dentist Scientist Training Program (NIH grant K16-DE00159). We thank our colleagues for providing the following reagents: C. Ettensohn (6e10 antibody); A. Wikramanayake and W. Klein (β-catenin and C-cadherin clones); and J. Venuti (dnTCF-Lef; EngLef). We appreciate helpful comments on the manuscript by Drs R. Fleming and R. Jiang.

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