

## RESEARCH ARTICLE

# Short-range Wnt5 signaling initiates specification of sea urchin posterior ectoderm

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## ABSTRACT

The border between the posterior ectoderm and the endoderm is a location where two germ layers meet and establish an enduring relationship that also later serves, in deuterostomes, as the anatomical site of the anus. In the sea urchin, a prototypic deuterostome, the ectoderm-endoderm boundary is established before gastrulation, and ectodermal cells at the boundary are thought to provide patterning inputs to the underlying mesenchyme. Here we show that a short-range Wnt5 signal from the endoderm actively patterns the adjacent boundary ectoderm. This signal activates a unique subcircuit of the ectoderm gene regulatory network, including the transcription factors *Irxa*, *Nk1*, *Pax2/5/8* and *Lim1*, which are ultimately restricted to subregions of the border ectoderm (BE). Surprisingly, *Nodal* and *BMP2/4*, previously shown to be activators of ectodermal specification and the secondary embryonic axis, instead restrict the expression of these genes to subregions of the BE. A detailed examination showed that endodermal Wnt5 functions as a short-range signal that activates only a narrow band of ectodermal cells, even though all ectoderm is competent to receive the signal. Thus, cells in the BE integrate positive and negative signals from both the primary and secondary embryonic axes to correctly locate and specify the border ectoderm.

**KEY WORDS:** Wnt5, Sea urchin, VEGF, Ectoderm, Specification

## INTRODUCTION

In an embryo, tissue boundaries form when cells of two different gene regulatory states are juxtaposed. These cells can interact with each other, and such interactions often have important consequences for development (Dahmann et al., 2011). For example, these interactions often prevent the mixture of the different cell types, thereby maintaining the boundary even in the face of cell proliferation and tissue morphogenesis (Mellitzer et al., 1999; Xu et al., 1999). In addition, cells at a boundary often act as a signaling center, directing patterning of other nearby tissues (Dahmann et al., 2011). One of the most basic, and curiously, least-studied boundaries in deuterostomes is the demarcation between the endoderm and ectoderm. Both germ layers arise from a single epidermal sheet, yet by gastrulation these two tissues are clearly different at a molecular level. The boundary between these two cell types remains stable throughout gastrulation and will ultimately become the anus.

Here, the sea urchin was used as a model to study the formation of the ectoderm-endoderm boundary. The posterior half of this

embryo is specified as endomesoderm by Wnt and Delta-Notch signaling (Croce et al., 2011; Sethi et al., 2009; Sherwood and McClay, 1999; Sweet et al., 2002), and a decade of experiments have established an advanced gene regulatory network (GRN) modeling the specification of these tissues up to the time of ingression of the skeletogenic cells into the blastocoel (Davidson et al., 2002; Rafiq et al., 2012; Revilla-i-Domingo et al., 2007). The remaining anterior (animal) cells become ectoderm and neural ectoderm. Ectoderm is believed to be the default state of the embryo in the absence of any inductive signals, based on the observation that if nuclear accumulation of  $\beta$ -catenin is blocked, the embryos form a dauer blastula composed of ectoderm and neural ectoderm (Yaguchi et al., 2006).

Whereas the endomesoderm is patterned by a series of short-range signals in the posterior (vegetal) half of the embryo (Davidson, 1989; McClay, 2011), the ectoderm is primarily patterned by long-distance signals acting along the dorsoventral (oral-aboral) axis. With the exception of the neurogenic domain at the animal pole, territories within the ectoderm are established by asymmetric TGF $\beta$  signaling (Angerer et al., 2000; Duboc et al., 2004). Initially, *Nodal* signaling is activated on the ventral (oral) side of the embryo. *Nodal* activates oral fates and activates *BMP2/4*, which is needed for aboral fates. *Nodal* and *BMP* act as positive regulators of oral and aboral ectoderm specification, respectively. Without these signals expression of markers in these territories is lost and most ectodermal cells assume a ciliary band fate (Angerer et al., 2000; Bradham and McClay, 2006; Saudemont et al., 2010).

Although the mechanisms initially specifying both endoderm and ectoderm fate are understood, how the boundary between these two tissues is established is not clear. At a molecular level, endoderm can first be distinguished from endomesoderm by the expression of *Brachyury* by 8 hours post fertilization (hpf) when the embryo is still an indistinct hollow ball of epithelial cells (Croce and McClay, 2010). By mesenchyme blastula stage, *Brachyury*, *FoxA* and nuclear  $\beta$ -catenin are expressed at the boundary of the endoderm and ectoderm (Logan et al., 1999; Croce and McClay, 2010). Once established, the border with the ectoderm remains stable. How the precise position of this boundary is established remains unknown. At 12–14 hpf gastrulation begins, and the ensuing tissue rearrangements provide a massive challenge to boundary integrity. What mechanisms maintain the boundary in the face of these challenges? Although the endoderm-ectoderm boundary has not been studied previously in deuterostomes, maintenance of other boundaries such as the midbrain-hindbrain boundary and the boundaries between somites requires signaling between the two juxtaposed tissues in order to prevent admixture and for initiation of subsequent developmental programs (Mellitzer et al., 1999; Sato et al., 2002; Watanabe et al., 2007; Watanabe et al., 2009; Xu et al., 1999). We sought to determine if similar mechanisms were working at the conserved boundary between ectoderm and endoderm.

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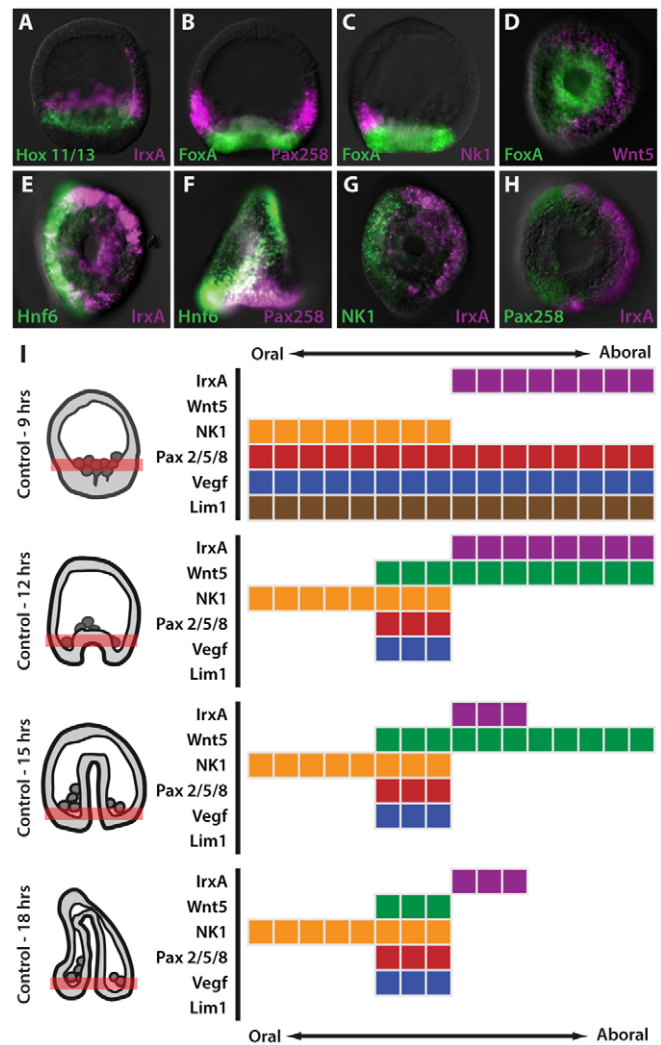
In this report we characterize the origin and specification of the posterior ectoderm-endoderm boundary in the urchin, showing that it requires inputs both from endodermal cells along the animal-vegetal axis and from Nodal and BMP2/4 on the oral-aboral axis. Cells in the ectoderm at the boundary, hereafter called border ectoderm (BE), express a group of basic helix-loop-helix (bHLH) and homeodomain transcription factors including *IrxA*, *Nk1*, *Pax2/5/8* and *Lim1*. These genes have dynamic spatial expression profiles and many are restricted to subregions of the BE. Surprisingly, perturbations showed that Nodal and BMP2/4 restricted the expression of BE genes rather than activating them, indicating that the BE utilizes TGF $\beta$  signaling in a very different way from the rest of the ectoderm. Several lines of evidence then supported the hypothesis that the signal(s) necessary for activating the BE comes from the endoderm. A detailed examination of this signaling showed that endodermal *Wnt5* is necessary for activation of BE specification. Our results demonstrate that *Wnt5* acts as a short-range inter-blastomere signal, activating the BE subcircuit in a narrow band of adjacent ectodermal cells. The results show further that cells in the BE integrate different types of positional information from both the primary and secondary embryonic axes in order to correctly locate the production site of signals needed for skeleton formation to take place.

## RESULTS

### Dynamic expression of markers within the border ectoderm

The position of the border ectoderm was established with two-color *in situ* hybridization. Markers of the BE region were expressed in the ectoderm immediately adjacent to the endodermal markers *FoxA* and *Hox11/13b* (Fig. 1A-D). Depending on the stage, some partially colocalized with the ciliary band, which is orthogonal to and intersects the BE on the right and left side (Fig. 1E,F). *IrxA* was expressed opposite to *Nk1* and *Pax2/5/8*, indicating that it was confined to the aboral BE, whereas *Nk1* and *Pax2/5/8* were expressed in the oral BE (Fig. 1G,H). These results were confirmed at later stages, when the morphology of the embryo could be used to determine oral/aboral position (supplementary material Fig. S1).

Expression of the six BE marker genes selected was dynamic. Fig. 1I graphically displays the expression domain of each gene at stages every 3 hours from mesenchyme blastula through gastrulation (supplementary material Fig. S1 shows the *in situ* data summarized in Fig. 1I). Preliminary expression profiles for several of these genes have been observed in other species of sea urchin, suggesting that the BE is a conserved region of the echinoderm ectoderm (Czerny et al., 1997; Duloquin et al., 2007; Saudemont et al., 2010; Su et al., 2009). The earliest marker expressed in the BE was *Lim1*. It was expressed throughout the BE beginning at 8 hpf and was diminished by gastrulation. Expression of four other genes in the BE was detected at 9 hpf. At that time, *Pax2/5/8* and VEGF, two of the BE genes tracked, were broadly expressed throughout the BE. By contrast, *Nk1* and *IrxA* were expressed on the oral and aboral sides of the BE, respectively. By 12 hpf gastrulation had begun, and genes in the BE exhibited more restricted expression patterns. *Nk1* and *IrxA* remained on opposite sides of the embryo. VEGF was expressed, along with *Pax2/5/8* at the BE-ciliary band intersection. At 12 hpf a sixth gene, *Wnt5*, was also expressed in the aboral BE. Before this time *Wnt5* was expressed in the endoderm. At 15 hpf, *IrxA* expression was restricted to two lateral patches aboral relative to *Pax2/5/8*. At prism stage (18 hpf), VEGF expression was reduced in the BE, and *Wnt5* expression had become restricted to two lateral patches, located oral relative to *IrxA*.



**Fig. 1. The posterior ectoderm-endoderm border is a unique region of the sea urchin ectoderm.** (A-D) Two-color *in situ* hybridizations. (A) *Hox11/13b* (endoderm) is adjacent to *IrxA* (ectoderm) (9 hpf), lateral view. (B-D) *FoxA* (endoderm) is adjacent to *Pax2/5/8*. (C) *Nk1* and (D) *Wnt5* (ectoderm at 12 hpf). (E) *Hnf6* (ciliary band) abuts *IrxA* (ectoderm) (vegetal view), (F) *Hnf6* overlaps *Pax2/5/8* (lateral view, 12 hpf). (G) Oral BE marker *Nk1* is opposite aboral BE marker *IrxA* (vegetal view, 12 hpf). (H) Lateral BE marker *Pax2/5/8* abuts aboral marker *IrxA*. (I) The expression pattern of each gene in the BE is summarized from *in situ* data (supplementary material Fig. S1). As the expression patterns changed as development progressed, the expression domain of each gene at stages from mesenchyme blastula through gastrulation is shown. On the left the graphic shows a lateral view of embryos with the BE depicted as colored bar. To the right the linear extent of expression of a gene along the oral-aboral (ventrodorsal) axis of the BE. The anteroposterior axis is not shown in the graphic. This layout approximates patterns of expression as well as stages. In each case, the data in supplementary material Fig. S1 show the actual *in situ* patterns that are summarized in the graphic and figures.

### TGF $\beta$ signals restrict expression of border ectoderm markers

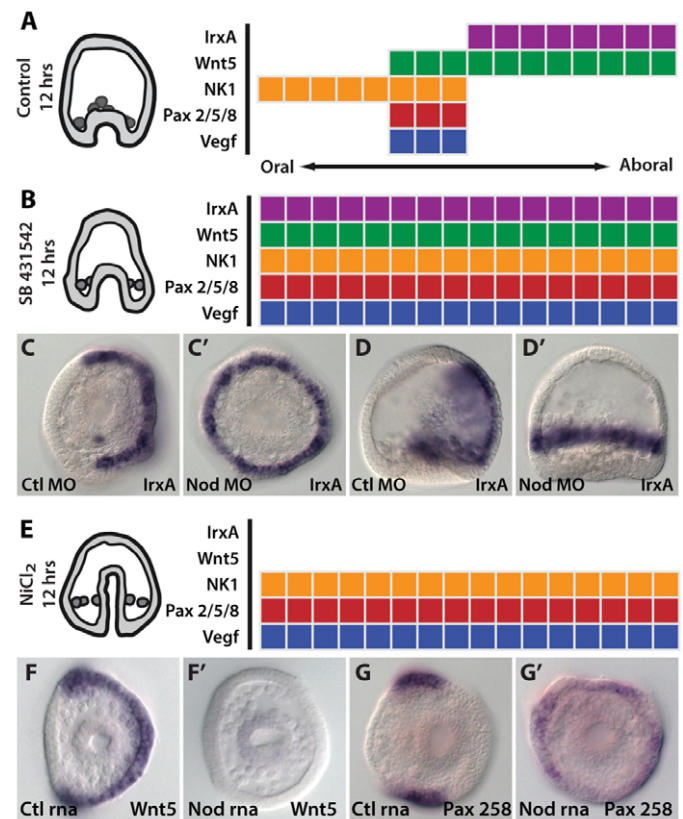
As specification of most sea urchin ectoderm is activated along the oral-aboral (ventrodorsal) axis by TGF $\beta$  signaling (Angerer et al., 2000; Duboc et al., 2004; Duboc et al., 2010), the expression of BE genes was assessed in embryos where Nodal signaling through the

Alk4/5/7 receptor was disrupted by SB 431542 (Inman et al., 2002; Saudemont et al., 2010; Sethi et al., 2009). Surprisingly, when Nodal signaling was inhibited (Fig. 2A,B; supplementary material Fig. S1), BE gene expression expanded along the oral-aboral axis, indicating that, unlike more anterior ectoderm, BE is inhibited by TGF $\beta$  signaling. The territorial expansion persisted throughout gastrulation and was not simply a delay. Additionally, the late restriction of IrxA and Wnt5 to lateral patches also required Nodal signaling (supplementary material Fig. S2). To confirm these results, we examined IrxA expression in embryos injected with a Nodal morpholino antisense oligonucleotide (MO). Knockdown of Nodal led to expression of IrxA around the entire BE, consistent with the SB treatment results (Fig. 2C,C'). A second domain of IrxA expression was also observed in the greater aboral ectoderm at early gastrula. There, IrxA expression was lost in Nodal MO and SB treatments (Fig. 2D,D'), suggesting that at least two cis-regulatory modules control IrxA expression, one that is activated as a consequence of Nodal signaling (aboral anterior ectoderm region, indirectly through Nodal activation of BMP) and the other that represses IrxA in response to Nodal signaling (in the oral BE region).

To further challenge these results Nodal signaling was expanded to see if that would result in the loss of aboral BE genes. Sea urchin embryos treated with NiCl<sub>2</sub> exhibit expanded Nodal signaling and develop with exclusively oral ectoderm fates (Hardin et al., 1992). As predicted, when Nodal expression was expanded as a result of NiCl<sub>2</sub> treatment, expression of the aboral BE genes IrxA and Wnt5 was lost (compare Fig. 2A and 2E; supplementary material Fig. S1). Early expression of Wnt5 in the endoderm was not affected by expanded Nodal expression (supplementary material Fig. S1). The oral BE gene Nk1 expanded to a complete ring in these embryos. This is consistent with a lack of BMP signaling in oralized embryos (Duboc et al., 2004; Saudemont et al., 2010). Pax2/5/8 and VEGF expression was unrestricted with respect to the oral-aboral axis as well, further suggesting that Nodal, directly or indirectly, normally restricts their expression in the BE. As an independent test, Nodal mRNA was injected into the embryo, and expression of BE genes was assayed by *in situ* analysis. As in NiCl<sub>2</sub> treatments, Wnt5 BE expression was lost in these embryos and expression of Pax2/5/8 became unrestricted (Fig. 2F,G). Reciprocally, in embryos where BMP2/4 signaling was perturbed with an MO (supplementary material Fig. S1), expression of the aboral marker Wnt5 did not change (Nodal was still expressed to restrict Wnt5 expression to the aboral side) but the oral marker Nk1 expanded to the aboral BE, demonstrating that BMP represses oral BE genes on the aboral side. Thus, TGF $\beta$  signaling acts to restrict, rather than to activate, BE gene expression.

### Endodermal inputs activate border ectoderm specification

As TGF $\beta$  signaling territorially restricted BE gene expression rather than activating it, we sought another source for the activating signal(s). We reasoned that those signals could: (1) originate from the endoderm; (2) originate from the animal pole; or (3) could be unrestricted, and BE gene expression might be restricted by later signals. The latter two hypotheses were unlikely, because both required isolated animal halves to express BE genes, and this was not observed (Fig. 3A-A'''; VEGF was expressed in vegetal but not animal halves). We therefore focused on endoderm as a potential source of a signal for BE specification. Hox11/13b is at or near the top of the endoderm GRN (Peter and Davidson, 2010), so we asked if the BE was affected when Hox11/13b was knocked down. In Hox11/13b morphant embryos, we observed a loss of both IrxA and



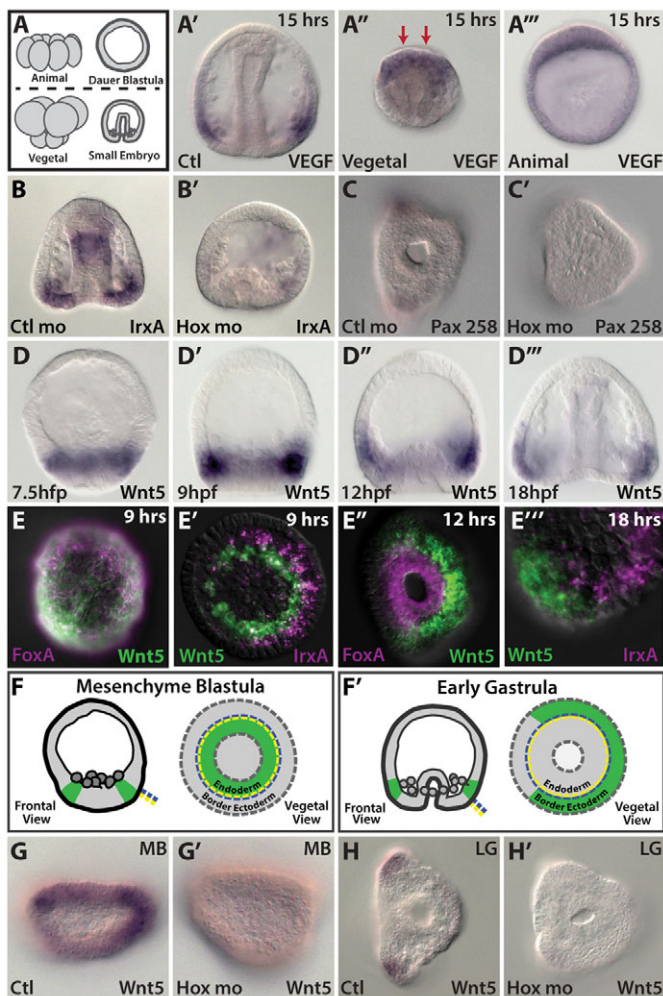
**Fig. 2. Perturbations to TGF $\beta$  signaling disrupt BE specification.**

(A,B) Control versus changes to BE gene expression after treatment with SB 431542 at 12 hpf. BE gene expression became unrestricted along the oral/aboral axis when Nodal signaling was blocked. (C-D') Nodal MO knockdown expanded expression of IrxA throughout the entire BE but eliminated IrxA expression from the more anterior aboral ectoderm region (12 hpf, vegetal view in C,C', lateral view in D,D'). (E) Changes in BE gene expression after increasing Nodal signaling with NiCl<sub>2</sub>. Aboral BE gene expression was lost, whereas oral BE gene expression expanded. (F-G') Nodal mRNA injection eliminated BE Wnt5 expression, and expanded Pax2/5/8 expression (15 hpf, vegetal views).

Pax2/5/8 expression in the BE (Fig. 3B-C'). These results suggested that an endodermal signal, expressed downstream of Hox11/13b, was necessary for border formation.

A preliminary survey showed that the signaling ligand Wnt5 was expressed first in endoderm, before moving to BE after gastrulation began. Fig. 3D-D''' shows the expression of Wnt5 from 7.5 to 18 hpf. Early Wnt5 expression colocalized with the endodermal marker FoxA (Peter and Davidson, 2010), and was adjacent to the BE marker IrxA (Fig. 3E,E'). After gastrulation began at 12 hpf, Wnt5 expression was adjacent to FoxA (Fig. 3E''). Wnt5 expression also changed from a full ring before gastrulation to an aboral crescent and then to two patches on either side of the border ectoderm (Fig. 3E''', showing an enlargement of one patch). The movement from endodermal to ectodermal expression is diagrammed in Fig. 3F,F'. Wnt5 expression depended on Hox11/13b function. Both endodermal and ectodermal Wnt5 expression was lost in Hox11/13b-MO-treated embryos (Fig. 3G-H').

To test the hypothesis that Wnt5 was the endodermal signal, a morpholino was designed to block the translation of Wnt5 mRNA (Wnt5 MO). At 24 hpf, control injected embryos had completed gastrulation and produced a normally patterned skeleton ( $n=48/50$



**Fig. 3. Endoderm is required for BE activation.** (A-A''') Micro-surgically separated animal and vegetal halves at the 16-cell stage were allowed to develop until late gastrula, when BE markers had been activated in control embryos for several hours. VEGF expression was assayed in each half-embryo. Isolated animal halves formed dauer blastulae and did not express VEGF ( $n=7/7$ ), whereas vegetal halves expressed VEGF ( $n=8/8$ , red arrows). (B-C') At 18 hpf, morpholino targeting of Hox11/13b showed that endoderm specification was required for BE expression of IrxA and Pax2/5/8. (D-E''') Wnt5 expression moved from the endoderm to the ectoderm concurrent with the onset of gastrulation. Wnt5 expression at 7.5, 9, 12 and 18 hpf appeared to be at the ectoderm-endoderm boundary (frontal views). Two-color *in situ* hybridization showed that before gastrulation, at 9 hpf, Wnt5 colocalized with the endoderm marker FoxA and also at 9 hpf, its expression abutted the BE marker IrxA (vegetal views). After gastrulation began, at 12 hpf, Wnt5 and FoxA were expressed in adjacent cell populations (vegetal views). By prism stage (18 hpf), Wnt5 marked a patch of cells in the lateral BE independent of IrxA. (F,F') Wnt5 expression was dynamic, changing from a ring inside the endoderm before gastrulation (9 hpf) to a crescent in the ectoderm once gastrulation had begun (12, 18 hpf). (G-H') *In situ* hybridization showing both early and late domains of Wnt5 expression required input from Hox11/13b in the endoderm.

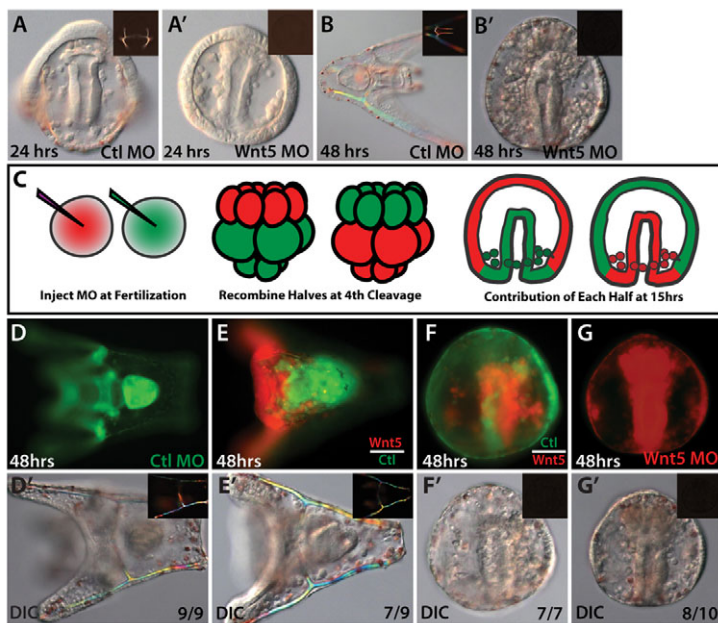
embryos). However, Wnt5-MO-injected embryos had not produced any skeleton, even though gastrulation had been completed and mesenchyme cells were visible in the blastocoel (Fig. 4A,A';  $n=47/51$  embryos). By 48 hpf, control embryos had reached the larval stage normally, whereas differentiation of the skeleton in Wnt5-MO-injected embryos never occurred (Fig. 4B,B'). The specificity of the morpholino was demonstrated by a rescue

experiment. Co-injection of *in vitro* synthesized Wnt5 mRNA (insensitive to Wnt5 MO inhibition) with the Wnt5 MO rescued vegetal skeletal structures (supplementary material Fig. S2). In those rescues animal skeletal structures were present but abnormal, consistent with the patterning consequences of ectopic Wnt5 expression (see below). To examine the function exclusively of endodermal Wnt5 we created chimeric embryos where only the animal or vegetal half of the embryo contained the Wnt5 MO (Fig. 4C). As the endomesoderm derives entirely from the vegetal half (Davidson et al., 1998), this should separate the two Wnt5 domains. When the Wnt5 MO was confined to the animal half, a normal pluteus larva formed (compare Fig. 4D,D' with 4E,E') ( $n=7/9$ ). However, when the Wnt5 MO was confined to the vegetal half of the embryo, patterning at the boundary was disrupted (compare Fig. 4F,F' with 4G,G') ( $n=7/7$ ) and these embryos were indistinguishable from those containing the MO in all cells. Thus Wnt5 from the endoderm affects boundary formation.

Next, we showed that disruption of Wnt5 signaling blocked expression of the other BE ectoderm markers (Fig. 5). Pax2/5/8, VEGF and IrxA expression were lost in Wnt5-MO-treated embryos (Fig. 5A-C'). Loss of VEGF probably explains the lack of skeleton in Wnt5-MO-injected embryos. Similarly, ectodermal Wnt5 expression required input from endodermal Wnt5 (Fig. 5D,D'). Expression of Pax 2/5/8 was also lost in chimeric embryos containing the Wnt5 MO in the vegetal, but not the animal, half (Fig. 5E,E').

We hypothesized that the endodermal Wnt5 acted as a short-range signal. If this were true, we predicted that expansion of Wnt5 signaling would lead to an expanded boundary and broad activation of BE genes throughout the ectoderm. This hypothesis was tested by misexpressing Wnt5 mRNA, or a control mRNA, throughout the entire embryo. At 24 hpf control embryos were early pluteus larvae, with normal patterning of the boundary and associated skeletal structures (Fig. 6A). Embryos injected with Wnt5 mRNA exhibited an unusual phenotype, in which an ectopic chain of skeletogenic mesenchyme cells reached over the animal pole of the embryo, just underneath the ciliary band (Fig. 6A';  $n=43/46$  embryos). We confirmed this novel phenotype by staining with the skeletogenic-mesenchyme-specific antibody 1D5 (Fig. 6B,B'). As predicted, the expression domains of BE genes expanded in Wnt5-misexpressing embryos. In control embryos Pax2/5/8 was expressed in two lateral patches of BE, sitting overtop of the lateral clusters of mesenchyme cells. In Wnt5-RNA-injected embryos Pax2/5/8 expression expanded in an arc over the animal pole of the embryo but remained narrowly restricted along the oral-aboral axis (Fig. 6C-C'''). A similar expression pattern was seen for VEGF (Fig. 6D-D''', arrows indicating ectopic staining). Finally, expression of both IrxA (Fig. 6E-E''') and Nk1 (Fig. 6F-F''') expanded from the endoderm boundary throughout the ectoderm, but remained restricted to the dorsal and ventral territories, respectively.

The pattern observed was consistent with an expansion of Wnt5 signaling assuming the continued presence of the repressive effects of Nodal and BMP2/4 seen earlier. We therefore hypothesized that BE markers should be expressed throughout the ectoderm in embryos with both expanded Wnt5 activity and disrupted TGF $\beta$  signaling. We tested this by examining Pax2/5/8 expression in embryos both injected with Wnt5 mRNA and treated with SB431542. Pax2/5/8 was expressed normally in control embryos, expressed in an arc over the animal pole in Wnt5-mRNA-injected embryos, and expressed throughout the ectoderm in embryos treated with both Wnt5 mRNA and SB (Fig. 7B-B''). This result further demonstrated the independent effects of these two signaling systems on posterior ectoderm specification.



**Fig. 4. Perturbation of Wnt5 expression blocks BE formation.** (A-B') When expression of Wnt5 was blocked with a morpholino (A',B'), embryos appeared arrested at late gastrula and produced neither skeleton (see insets) nor posterior ectoderm. This block persisted after 2 days of development, indicating that it was not simply a delay. (C) Strategy for producing chimeric embryos containing Wnt5 MO in only the animal or vegetal half. (D-G') Phenotypes observed in control and chimeric embryos. Fluorescence and differential interference contrast (DIC) images shown are of the same embryo, indicated as D,D',E,E', etc. When the Wnt5 MO was confined to the animal half, embryos were indistinguishable from controls. However, when the MO was confined to the vegetal half, embryos exhibited the same phenotype as seen when the entire embryos contained the MO.

## DISCUSSION

These results suggest a model in which initially all ectodermal cells are competent to receive the Wnt5 signal. In normal embryos, endodermal Wnt5 acts as a short-range signal, activating BE genes in a narrow band of posterior ectodermal cells. TGF $\beta$  signaling then restricts expression of these genes to subregions of the BE (Fig. 7A, left). When extra Wnt5 is provided, all ectodermal cells receive the activating input for BE. However, because the activation of Nodal and BMP are unaffected by expanded Wnt5 signaling, BE gene expression remains restricted along the secondary axis of the embryo (Fig. 7A, center). When TGF $\beta$  signaling is also disrupted, BE markers are expressed throughout the ectoderm (Fig. 7A, right). The role played by TGF $\beta$  signaling in restricting BE gene expression is similar to the role these signals play in limiting ciliary band specification along the oral-aboral axis (Yaguchi et al., 2010). Our data indicate that a subcircuit of the GRN is activated in BE cells in response to Wnt5 signaling (Fig. 7C). This circuit is activated by endodermal Wnt5, which acts through an as yet unknown Wnt receptor to induce the expression of genes in the BE including Nk1, Pax 2/5/8, Vegf, IrxA and Wnt5. The expression of each BE gene is then restricted to specific subterritories by Nodal and BMP2/4. Because these signals are expressed before BE restriction occurs, repressors must exist downstream of these signals to mediate their effects on transcription factors in the BE [one such repressor is likely to be Not1 (Li et al., 2012)]. Those modeled repressors or other components of the circuit then stabilize the border ectoderm territories following the later inactivation of Nodal and BMP signaling.

The GRN subcircuit(s) active in the BE may function to refine the expression boundaries of genes that are initially more broadly expressed. BE genes, including Pax2/5/8, IrxA and VEGF, are first expressed throughout the BE but then rapidly become restricted to subdomains of the BE. In other systems genes of the Iroquois, Nkx, Pax, Lim, Msx and Fox families function as repressors, often working to establish boundaries between differently fated populations of cells within contiguous tissues. A good example of this is found in the dorsoventral patterning system at work in the vertebrate neural tube. In vertebrates, the neural tube is patterned on the dorsoventral axis by opposing gradients of BMP and SHH (Liem et al., 1995; Roelink et

al., 1994). In response, a series of homeodomain (HD) and bHLH transcription factors are activated in specific subregions of the neural tube. Adjacent pairs of transcription factors act as mutual repressors, creating the sharp expression boundaries observed along the dorsoventral axis of the neural tube (Briscoe and Ericson, 2001). A similar regulatory logic appears to apply to the sea urchin BE as well. Our results indicate a broad activation signal is combined with a series of spatially restricted transcriptional repressors that subdivide the different regions within the BE. Detailed GRN analysis has not yet been done, but one prediction is that at least some of these transcription factors work as mutual repressors in the BE to restrict territorial expression.

Perturbations to Wnt5 suggest that it signals directly to the border ectoderm, and an important question is how that signal is transduced. In other systems, Wnt5 is thought to signal primarily through the noncanonical, or  $\beta$ -catenin-independent, Wnt signaling pathway, where it plays a role in directing cell migration and convergent extension (Nishita et al., 2010). However, Wnt5 has also been reported to activate the canonical pathway in certain contexts (Mikels and Nusse, 2006). In the sea urchin, nuclear  $\beta$ -catenin is necessary for endoderm, but not ectoderm, formation (Cameron and Davidson, 1997; Logan et al., 1999). Thus, Wnt5 probably acts through a  $\beta$ -catenin-independent pathway to activate BE genes.

A second question logically follows – what is the receptor(s) active in the ectoderm through which Wnt5 signals? Wnt ligands are known to signal through Frizzled (Fzd) receptors, and the sea urchin genome contains four Fzds comprising four of the five deuterostome frizzled families (Bhanot et al., 1996; Croce et al., 2006a). Perturbations to two of these genes, Fzd5/8 and Fzd1/2/7, lead to defects in mesoderm and endoderm specification, respectively (Croce et al., 2006b; Lhomond et al., 2012). Frz1/2/7 is also expressed in the ectoderm, and perturbations to Fzd1/2/7 cause defects in skeleton formation. However, the phenotype of these embryos is less severe than that of Wnt5 perturbations, suggesting that if Fzd1/2/7 conducts the Wnt5 signal, other co-receptors may also function.

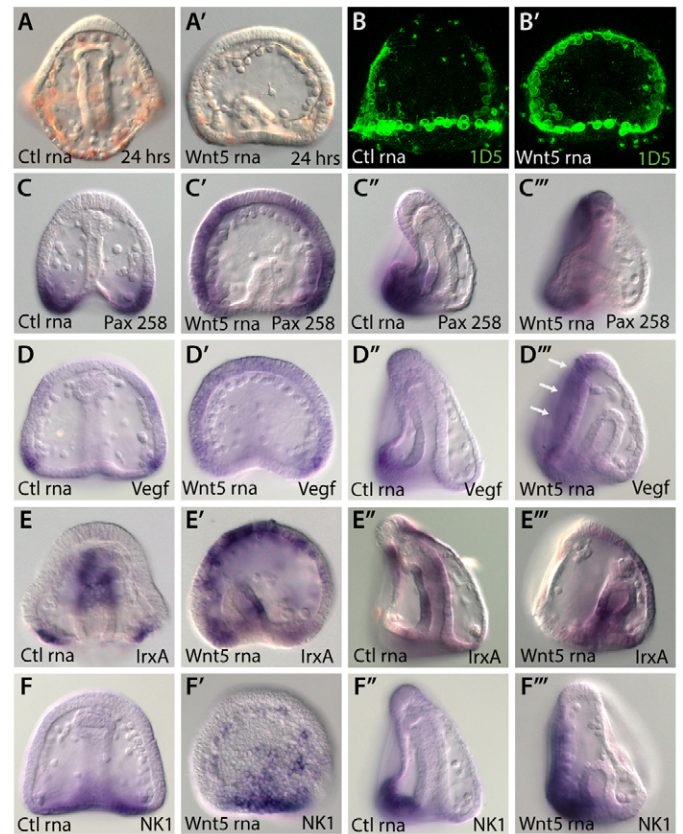
Wnt5 also has been reported to have a highly conserved interaction with the orphan tyrosine kinase receptor ROR, which acts in many developmental contexts, including skeletal and nervous



**Fig. 5. BE markers are lost in Wnt5 MO.** (A-D') At 15 hpf, Wnt5-MO-injected embryos did not express BE markers, including Pax2/5/8, IrxA and VEGF, or ectodermal Wnt5. (E,E') Chimeric embryos showed Wnt5 was required in the vegetal half for Pax2/5/8 expression.

system development (Green et al., 2008). ROR1/2 double-knockout mice exhibit a nearly identical phenotype to Wnt5a knockout mice, indicating that RORs are likely to be the key mediator of Wnt5a signaling in that system (Ho et al., 2012). Wnt5-ROR signaling is believed to be a noncanonical Wnt signaling pathway; however, the phenotypes of ROR double-knockout mice do not substantially overlap with Van Gogh double-knockout mice, indicating that it does not act through the planar cell polarity pathway (Ho et al., 2012; Song et al., 2010). One ROR receptor is encoded in the sea urchin genome, but the gene has otherwise not been studied in sea urchins. It will be important to determine if the interaction with Wnt5 is conserved and if it does indeed mediate Wnt5 activation of BE genes.

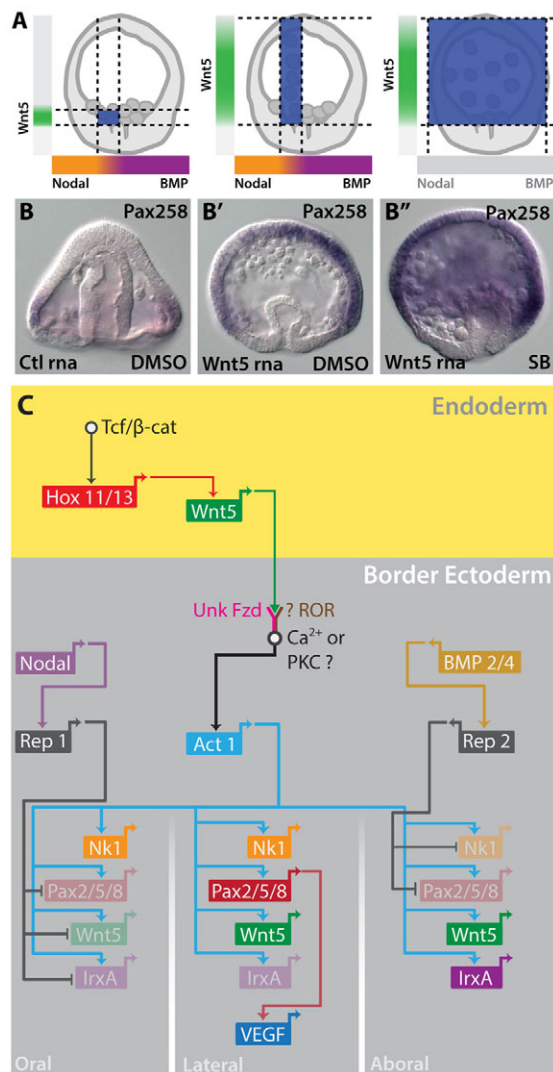
Sea urchin endomesoderm is segregated into nonskeletogenic mesenchyme and endoderm by Delta/Notch signaling (Croce and McClay, 2010; Sherwood and McClay, 1997; Sherwood and McClay, 1999; Sweet et al., 2002). Prolonged exposure of Veg2 progeny that remain in contact with Delta-expressing skeletogenic mesoderm cells activates specification of the nonskeletogenic mesoderm and separates these cells from the endoderm (Croce and McClay, 2010). Because Delta/Notch acts as a short-range signal, it can effectively segregate the two neighboring groups of cells. Similarly, activation of the BE, which is at most four cells wide, requires a signal that acts over a relatively short range. Our data from Wnt5-misexpressing embryos indicate that Wnt5 acts in such



**Fig. 6. Wnt5 acts as a short-range signal.** (A,A') Wnt5 mRNA expression throughout the embryo caused misplacement of an ectopic chain of mesenchyme cells that reached over the animal pole (24 hpf). These cells produced ectopic skeletal spicules. (B,B') The ectopic cells expressed the skeletogenic marker 1D5. Images show a frontal view (24 hpf). (C-F''') By late gastrula Wnt5 misexpression led to expanded BE gene expression. When Wnt5 was misexpressed, BE genes were no longer restricted to the BE. Instead they were observed in territories reaching all the way to the animal pole. However, the ventrodorsal restriction of each gene was not changed. This was true for Pax2/5/8, VEGF, IrxA and Nk1. For each gene, frontal and lateral views of both control and morpholino embryos are shown.

a manner. Indeed, the entire ectoderm appears competent to receive this signal, and in Wnt5-misexpressing embryos BE genes are activated in the ectoderm without restriction along the animal-vegetal axis. Wnt ligands are known to be lipid modified, and this dramatically reduces their diffusion and activity as long-range signals (MacDonald et al., 2009). In systems where Wnts act in this manner, other accessory proteins such as Reggie-1 are required, possibly to package the ligands into a less hydrophobic form (Katanaev et al., 2008). Consistent with these results, endogenous Wnt5a appears to not be released into the media in mammalian cell culture systems and wild-type cells are unable to rescue Wnt5a<sup>-/-</sup> cells even when cultured in close proximity (Ho et al., 2012). These authors conclude that Wnt5a signaling may require close contact for effective signaling. That is consistent with the conclusion of this work, in which endodermal Wnt5 acts as a short-range signal to activate BE fates in a narrow band of ectodermal cells.

Much of the current understanding of ectoderm specification in vertebrates has been gleaned from studying amphibians, where dorsoventral specification distinguishes between neural and epidermal fates. Ectodermal cells receiving BMP become epidermis. The dorsal ectoderm, however, is protected from these signals by



**Fig. 7. Model for BE specification.** (A) A model comparing BE gene activation in normal embryos with those where Wnt5 activity is expanded. Lateral views of each embryo are shown, with the extent of Wnt5 activity along the AP ( $y$ -axis), shown in green, TGF $\beta$  signaling along the oral-aboral axis ( $x$ -axis) in orange/purple, and the activation of BE markers shown in blue. Perturbations to Wnt5 do not change oral-aboral axis specification, and BE markers expand only along the AP axis within the ciliary band. When Wnt5 is expanded and TGF $\beta$  signaling is eliminated, BE markers are expressed without restriction along either axis. (B-B'') Pax2/5/8 *in situ* staining at 18 hpf in normal embryos, those where Wnt5 mRNA has been misexpressed, and those treated with both Wnt5 mRNA and SB431542. (C) Model of the GRN subcircuit active in the BE region. This network integrates positional information from both Wnt5 and TGF $\beta$  in order to segregate the various subregions of the BE. Repressors 1 and 2 must exist in order to mediate the activity of Nodal and BMP, but have not been identified yet.

secreted inhibitors such as Noggin and Chordin (Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1994; Smith et al., 1993). As a result, a region of the dorsal ectoderm assumes a neural fate. Wnt signaling then further refines neural fates along the anteroposterior axis, distinguishing brain from spinal cord (Bouwmeester et al., 1996). This model appears true for other vertebrates and sea urchins (Muñoz-Sanjuán and Brivanlou, 2002; Range et al., 2013). Our results suggest that an additional layer of

complexity exists in this system. Signaling from the endoderm distinguishes the BE from the remaining ectoderm in the sea urchin, and in mammals signaling from the anterior endoderm distinguishes the anterior neurectoderm from more posterior neurectoderm (Beddington and Robertson, 1999). In both cases Wnt signaling is involved.

The BE region appears to have at least two major functions in the embryo. First, because their specification history is unique, cells in the BE have the potential for providing territory-specific patterning information. The BE is a rich source of patterning information for the skeletogenic mesenchyme (Armstrong et al., 1993; Duloquin et al., 2007; Guss and Etensohn, 1997; Röttinger et al., 2008), and perturbations to BE specification disrupt skeleton patterning. Second, specification of the BE probably includes regulatory components necessary to enforce the ectoderm-endoderm boundary, which remains distinct through gastrulation and beyond. Immiscibility at a boundary is frequently achieved via signaling (Dahmann et al., 2011). For example, alternate rhombomeres in the zebrafish hindbrain are segregated by repulsive Eph-Ephrin signaling (Xu et al., 1999). In the sea urchin, a clean boundary between the ectoderm and endoderm is maintained throughout gastrulation and beyond, based on marker expression. How this boundary remains stable has yet to be shown. In the future, the ease of embryological intervention and advanced imaging will allow the BE to be studied in detail and make the sea urchin an attractive system in which to address formation and function of this conserved boundary.

## MATERIALS AND METHODS

### Embryos, surgery and drug treatments

Adult *Lytechinus variegatus* were collected at the Duke University Marine Lab (Beaufort, NC, USA) or were purchased from Sea Life (Tavernier, FL, USA). Adult sea urchins were spawned by intracoelomic injection of 0.5 M KCl. Once fertilized, embryos were cultured in artificial seawater (ASW) at 20–23°C. Microsurgical manipulations including mesomere transplants and animal/vegetal recombinations were performed at the 16-cell or 32-cell stage. Donor embryos were injected with either a rhodamine or fluorescein dextran lineage tracer so that the different clones could be followed later in development. Microsurgery was performed with fine glass needles and Narishige micromanipulators. SB 431542 (Sigma) was used at 10 to 50  $\mu$ M and added to cultures after fertilization. NiCl<sub>2</sub> was used at 50–100  $\mu$ M and added to cultures after fertilization. For both SB and NiCl<sub>2</sub> treatments, minimal effective doses were used and these were determined by dose-response experiments.

### MO and mRNA microinjection

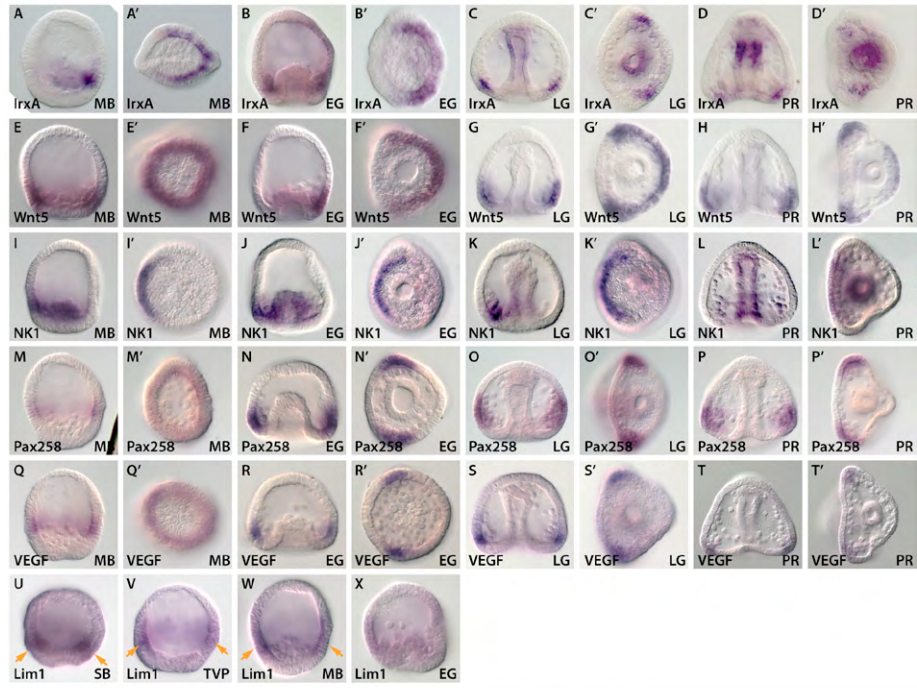
MOs were obtained from GeneTools. MO sequences are as follows: Wnt5-MO (5'-CGCTGGCAGACAAAGGGCGACTCGA-3'), Nodal-MO (5'-TGCATGGTTAAAAGTCCTTAGAGAT-3'), BMP2/4-MO (5'-GACC-CCAATGTGAGGTGGTAACCAT-3'), Hox11/13b-MO (5'-AAGCCTGT-TCCATACCTATCTGCAT-3'), Control-MO (5'-CCTCTTACCTCAGT-TACAATTTATA-3') (Bradham and McClay, 2006; Bradham et al., 2009; Peter and Davidson, 2010). MOs were used at the following concentrations: Wnt5, 1.0 mM; Nodal, 0.3 mM; BMP2/4, 0.5 mM; Hox11/13b, 0.75 mM; Control, at concentration equal to experimental. Wnt5 CDS, Wnt5-DN and mem-RFP were cloned in pCS2 and mRNA was synthesized *in vitro* using the mMessage mMachine kit (Ambion). Nodal mRNA was made as previously described (Bradham and McClay, 2006). For injection, mRNAs were diluted in 20% glycerol in diethylpyrocabonate-treated H<sub>2</sub>O with or without a fluorescent lineage marker as indicated in the text. Concentrations used were as follows: Wnt5 mRNA, and mem-RFP mRNA were injected at 300–500 ng/ $\mu$ l; Nodal mRNA was injected at 100–250 ng/ $\mu$ l. The precise concentration was slightly different depending on the batch of urchins, and was determined by dose-response experiments. Control mRNA (mem-RFP) was used at the same concentration as the corresponding experimental





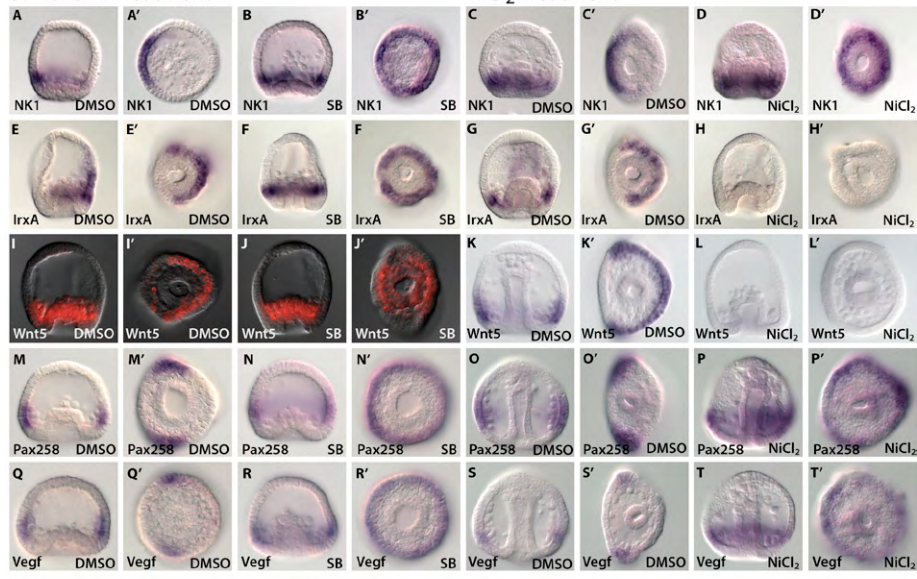
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Control Expression



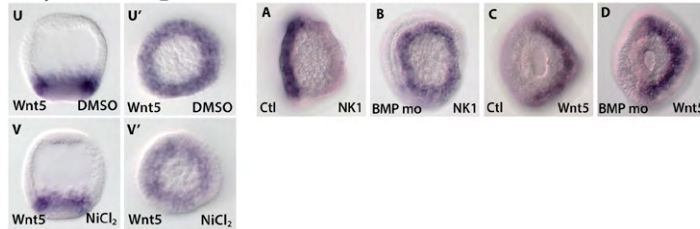
SB 431542 Treatment

NiCl<sub>2</sub> Treatment



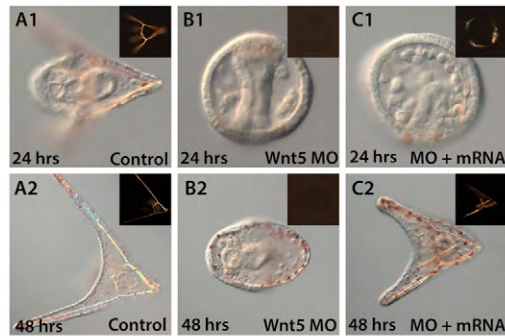
Early Wnt5, NiCl<sub>2</sub>

BMP MO Treatment

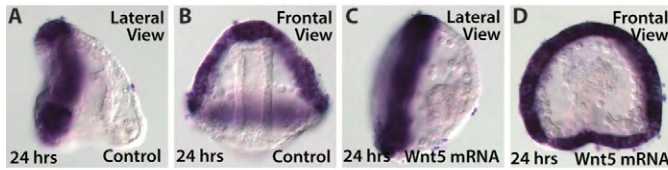


**Fig. S1. Expression patterns of BE genes from 9 to 18 hpf.** *In situ* hybridization was used to detect expression patterns of IrxA (A-D'), Wnt5 (E-H'), NK1 (I-L'), Pax2/5/8 (M-P'), VEGF (Q-T') and Lim1 (U-X). Expression patterns are shown in front/lateral views as well as vegetal views for each gene. Each gene is shown at the following stages: 9 hpf (MB), 12 hpf (EG), 15 hpf (LG) and 18 hpf (Prism); except for Lim1 which is shown at earlier stages, as indicated. These data are summarized in the graphic shown as Fig. 1H. Expression of BE genes following perturbations to TGFbeta signaling. SB 431542 was used to disrupt Nodal signaling. NiCl<sub>2</sub> was used to activate Nodal signaling throughout the ectoderm. Expression of each BE gene was assayed by *in situ* hybridization under each condition during gastrulation, when BE expression was normally restricted to sub-regions of the BE. These results are shown for IrxA (A-D'), Wnt5 (E-H'), NK1 (I-L'), Pax2/5/8 (M-P') and VEGF (Q-T'). For each gene/perturbation combination, frontal or lateral, and vegetal, views are shown. These results are summarized in Fig. 2A,B and E. We also assayed the effect of NiCl<sub>2</sub> on endodermal Wnt5 at mesenchyme blastula, and observed no changes in expression (U-V'). Effect of BMP2/4 MO perturbations on BE gene expression. The oral BE marker NK1 expanded when BMP activity was blocked. The aboral marker WNT5 was unaffected.

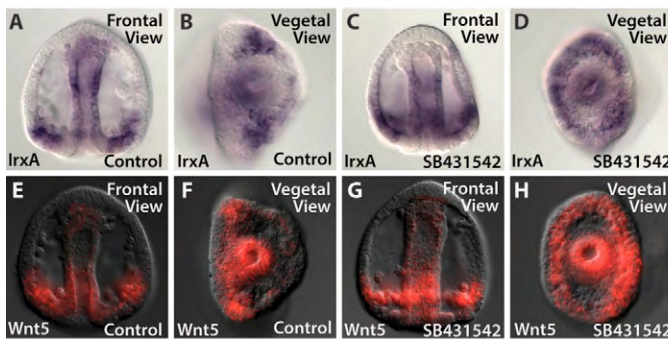
## Rescue of Wnt5 MO



## Hnf6 expression in Wnt5 RNA injected embryos



## Late restriction of IrxA and Wnt5 requires Nodal



**Fig. S2. Rescue of the Wnt5 MO with mRNA not sensitive to the MO.** Control injected embryos developed normally at 24 and 48 hpf (A1 and A2). Wnt5 MO injected embryos arrested at late gastrula and had not produce skeleton at 48 hpf (panels B1 and B2). Embryos receiving both Wnt5 MO and mRNA were delayed, but had produced a robust skeleton by 48 hpf (panels C1 and C2). Inserts show birefringent skeletons in control and rescued embryos. Effect of Wnt5 rna injection on Ventral-Dorsal Specification Hnf6 staining on Wnt5 rna injected embryos at 24 hpf. Frontal and lateral views are shown. No changes in Hnf6 expression were observed indicating specification of the DV axis by TGFB signaling was not disrupted. Late restriction of IrxA and Wnt5 required Nodal. *In situ* hybridization for IrxA (A-D) and Wnt5 (E-H) at 18 hpf in embryos treated with SB431542. Frontal and vegetal views are shown. Late restriction of IrxA and Wnt5 to lateral patches of the BE was not observed in SB treatment. Instead, expression was radial.