Long- and short-range signals control the dynamic expression of an animal hemisphere-specific gene in *Xenopus*

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Abstract

Little is known of the control of gene expression in the animal hemisphere of the *Xenopus* embryo. Here we show that expression of *FoxI1e*, a gene essential for normal ectoderm formation, is expressed regionally within the animal hemisphere, in a highly dynamic fashion. In situ hybridization shows that *FoxI1e* is expressed in a wave-like fashion that is initiated on the dorsal side of the animal hemisphere, extends across to the ventral side by the mid-gastrula stage, and is then turned off in the dorsal ectoderm, the neural plate, at the neurula stage. It is confined to the inner layers of cells in the animal cap, and is expressed in a mosaic fashion throughout. We show that this dynamic pattern of expression is controlled by both short- and long-range signals. Notch signaling controls both the mosaic, and dorsal/ventral changes in expression, and is controlled, in turn, by Vg1 signaling from the vegetal mass. *FoxI1e* expression is also regulated by nodal signaling downstream of VegT. Canonical Wnt signaling contributes only to late changes in the *FoxI1e* expression pattern.

These results provide new insights into the roles of vegetally localized mRNAs in controlling zygotic genes expressed in the animal hemisphere by long-range signaling. They also provide novel insights into the role of Notch signaling at the earliest stages of vertebrate development.
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Keywords: FoxI1e; Xenopus; Ectoderm; Animal gene expression

Introduction

The *Xenopus* blastula is conventionally divided into three regions, with respect to both cell fate and gene expression. Cells in the vegetal region give rise to the embryonic endoderm. They inherit the maternally encoded transcription factor VegT, which activates the synthesis of nodals. These, in turn, induce mesoderm in the adjacent marginal region. Cells in the animal region form the ectoderm. It is not known how this fate is initiated. In addition to forming the germ layers, the three blastula regions each become patterned to form the axes of the body, by the expression of dorsal and ventral genes.

This picture of gene expression in the blastula tends to be regarded as dynamic with respect to time, but static with respect to each region of the blastula; a linear progression within each

region of transcriptional initiation, leading to specification to enter a particular lineage, or to exhibit a particular type of cell behavior. We show here that in fact, gene expression is highly dynamic within the animal region. The forkhead transcription factor *FoxI1e* is expressed in the animal hemisphere, starting at the blastula stage. It is required for the expression of genes in both the early neural and ectodermal lineages, and for the later differentiation of the epidermis (Mir et al., 2007). It is also required to repress mesoderm specification in the animal hemisphere (Suri et al., 2005). Here we show that its expression pattern changes rapidly in both time and space within the animal hemisphere. Expression is initiated dorsally at the blastula stage, then spreads to encompass the ventral region of the animal hemisphere by the mid-gastrula stage. At the early neurula stage, expression is lost from the dorsal ectoderm cells that form the neural plate. During this sequence, its expression domain also changes with respect to the layers of the animal cap. First, it is restricted to the inner cells of the animal cap at the blastula

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stage, and then to a layer of cells between the inner and outer layers of the animal cap at the gastrula stage. Furthermore, throughout this temporal and spatial progression, *FoxI1e* expression is always in a mosaic pattern, with positive cells interspersed with non-expressing cells.

These observations revealed a previously unsuspected regionalization of the animal hemisphere. The total expression domain of *FoxI1e* extends across the animal cap, excluding the outer cells at the blastula stage, and both inner and outer cells at the gastrula stage, and all the time is restricted to only some cells, but not others, in this expression domain. During the late blastula to mid-gastrula stages, the expression of *FoxI1e* moves across this expression domain in a dorsal to ventral direction. This pattern is completely different from the expression patterns of other animally localized transcripts previously reported, such as ectodermin (Dupont et al., 2005) and Xlim5 (Toyama et al., 1995; Houston and Wylie, 2003), which are not mosaic, nor dynamic with respect to the dorsal–ventral axis. We therefore set out to identify some of the factors that might control these spatial and temporal changes.

We found that both long- and short-range signals control this pattern of expression. First, mosaic expression is controlled by Notch signaling. Both gain- and loss-of-function experiments showed that Notch signaling represses *FoxI1e* expression, and that loss of Notch signaling causes expression of *FoxI1e* in all cells of its total expression domain, and eliminates both the temporal and spatial progression of expression within this domain. This raised the issue of how Notch signaling is controlled in the animal hemisphere. It could be intrinsic, or controlled at long-range from the other developing germ layer regions. We show that control of *FoxI1e* expression by Notch is, in turn, controlled by the TGF-β signal, Vg1, whose mRNA is maternally encoded, and inherited only by the vegetal cells of the embryo (Melton, 1987). We also find that the level of *FoxI1e*, but not its mosaic distribution, is controlled by nodal signaling downstream of VegT, a vegetally localized, maternally encoded transcription factor (Zhang and King, 1996).

These data reveal multiple levels of control of early zygotic genes in *Xenopus*. First, long-range signals from the vegetal cells of the embryo, as well as short-range signals through the Notch pathway, combine to control both amount and position of expression of animal-specific genes during the blastula and gastrula stages. Second, none of these signals controls the "total expression domain" of *FoxI1e*, which remains confined to the group of cells that normally express it during its changing temporal expression at the blastula and gastrula stages. Instead, they control the temporal and spatial sequence of expression within this domain.

Materials and methods

Oocytes and embryos

Oocytes were generated for host transfer experiments by manual defolliculation of surgically removed ovary as previously described (Heasman et al., 1991). Culture and injections were carried out in L15-based oocyte culture medium (OCM). The length of the culture period between injection and host

transfer varied by experiment. All mRNA injections were cultured overnight. VegT-depleted embryos were generated using a morpholino oligo [5′-CCCGACAGCAGTTTCTCATTCCACG-3′], and cultured for 3 days after injection. Vg1 depletions were carried out using the oligo Vg1c as previously described (Birsoy et al., 2006), with 4 days of culture after injection. *Xotch* was depleted using AS14MP [5′-GGAAGGGCTCAGCGCTAC-3′], with 3 days of culture. For rescue experiments, mRNA was injected on the last day of culture, before progesterone treatment, or at the 2-cell stage after fertilization. Eggs were collected in high-salt solution, fertilized in vitro with isolated testis, and cultured in 0.1× MMR. Staging was according to Nieuwkoop and Faber (1967). Dissections were performed at stage 9 or stage 10 on agarose-coated dishes in 1× MMR and then cultured in OCM. β-Catenin-depleted embryos were generated using a morpholino as previously described (Heasman et al., 2000). Synthetic mRNAs encoding β-catenin, BMP4, cmBMP7, β-galactosidase, NICD, Su(H)-DBM, and Vg1 were generated using Ambion mMessage mMachine kits.

Real-time RT–PCR

Total RNA was extracted as previously described (Zhang et al., 1998). Unless otherwise indicated, input was 2 whole embryos, 3 marginal zones, or 5 vegetal masses per sample. cDNA was synthesized using oligo dT primers, and semi-quantitative real-time RT–PCR was carried out using the LightCycler system as described (Kofron et al., 2001). *Ornithine decarboxylase* (*ODC*) was used as a loading control, and all values were normalized to *ODC* levels. In all cases, water-only and reverse transcriptase-negative controls failed to produce specific products. Each experiment was repeated a minimum of three times in independent experiments to verify reproducibility of results. Primer sequences were: *Siamois*: U: 5′-CTGTCCTACAAGAGACTCTG-3′, D: 5′-TGTTGACTGCAGACTGTTGA-3′. *Xnr3*: U: 5′-CTTCTGCACTAGATTCTG-3′, D: 5′-CAGCTTCTGGCCAAGACT-3′. *FoxI1e*: U-5′-GCACCTGCTGTGGTTCATAA-3′, D-5′-CACCACTG-TAGTGCGTCAGAA-3′. *Xotch*: U: 5′-AGTAACCCGTGCAAAAATGG-3′, D: 5′-AGCTTCCGGTAAATCCAGGT-3′. *ESR-1*: U: 5′-TGGCAAAACTGGAA-CAGGAT-3′, D: 5′-TGGGATACAACAGGGAGCTT-3′.

Whole-mount in situ hybridization, membrane staining, and Red-Gal staining

In situ hybridizations were carried out as described by Harland (1991), with a probe concentration for *FoxI1e* of 5 μg/ml, and using BMB Purple (Roche) as the alkaline phosphatase substrate. For membrane staining, embryos were stained for *FoxI1e*, sectioned, and stained with Alexa-488-conjugated Wheat Germ Agglutinin (Molecular Probes) at 0.01 μg/μl in PBS+0.1% Tween-20 for 30 min. For lineage labeling, embryos injected with 50 pg of nuclear β-galactosidase mRNA in the A1 blastomere were fixed for 1 h at room temperature in MEMFA, stained with Red-Gal (Research Organics) for 20 min at 37 °C, and fixed for another hour at room temperature before in situ hybridization for *FoxI1e*.

Results

FoxI1e is expressed in a dorsal to ventral wave, in a subset of cells in the animal cap

In previous work, we found that *FoxI1e* expression was mosaic in the early embryo (Mir et al., 2007). This could be due either to an asynchronous onset of expression, leading to a more stable homogeneous expression pattern, or it could be that gene expression in the animal hemisphere is not uniform, but controlled in more complex ways than previously thought. To distinguish between these possibilities, we carried out in situ hybridization on embryos from the late blastula stage (stage 9) to the mid-gastrula stage (stage 11). We found that expression in all embryos began in a localized manner above the equator on

one side of the embryo, and then spread across the animal cap (Fig. 1A). The site of onset of expression was identified as the dorsal side by injection of β-galactosidase mRNA into the 2 dorsal animal cells at the 8-cell stage. The β-Gal signal consistently co-localized with *FoxI1e* expression (Fig. 1B, 92%, *n*=71), showing that *FoxI1e* expression begins on the dorsal side of the embryo, and spreads ventrally. To confirm this finding, we dissected early gastrulae into dorsal and ventral halves, and used real-time RT–PCR to compare the amount of *FoxI1e* expression. We found that *FoxI1e* mRNA was indeed expressed at higher levels in dorsal halves at the late blastula stage (Fig. 1C).

The animal cap consists of two cell layers—an epithelial, or outer layer, and an inner, sensorial layer (Chalmers et al., 2003). From late blastula to early gastrula stages, *FoxI1e* was expressed only in the sensorial layer. By the mid-gastrula stage, however, when expression had spread to the ventral side of the embryo, there was no signal in either the inner or the outer cell layers of the cap. Instead, there was expression in between the two layers.

These observations raise the question of how such an unexpected pattern of gene expression in the animal hemisphere is controlled.

Late, but not early expression of FoxI1e is controlled by Wnt-dependent dorsal axis specification

Since *FoxI1e* expression begins dorsally, we wanted to know if its initial expression is controlled by the Wnt signal transduction pathway, which is known to activate dorsal axis formation in *Xenopus*. To test this, we either depleted β-catenin (β-Cat) on the dorsal side of the embryo [by injecting the two dorsal cells at the 4-cell stage with 20 ng morpholino oligo (Heasman et al., 2000)], or overexpressed β-Cat on the ventral side (by injection of 100 pg β-Cat mRNA into the two ventral cells at the 4-cell stage). Embryos were dissected at the early gastrula stage into dorsal and ventral halves, and *FoxI1e* mRNA levels measured by real-time RT–PCR. In embryos injected with β-Cat MO, there was a reduction in the expression of the direct targets *Xnr3* and *Siamois* in dorsal halves, and in mRNA-injected embryos, *Xnr3* and *Siamois* were upregulated in ventral halves, indicating that the manipulations affected the embryos as expected (Figs. 2A, B). However, we found that regardless of the experimental treatment, *FoxI1e* was always expressed at higher levels in the dorsal halves than in the ventral halves. Also, the overall level of *FoxI1e* expression in whole embryos was not consistently affected by β-Cat MO or mRNA
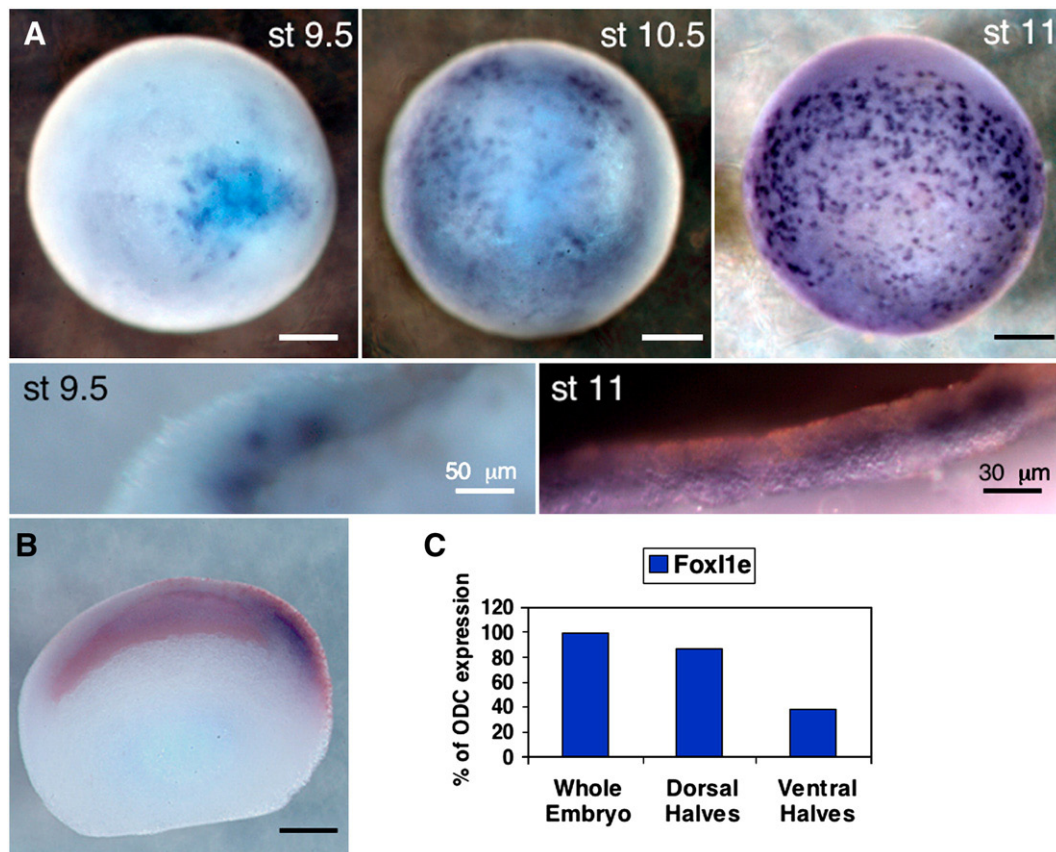

Fig. 1. FoxI1e is expressed in a dorsal to ventral wave in the blastula and gastrula stages. (A) In situ hybridization for FoxI1e shows initial staining at stage 9.5 on one side of the embryo, and then spreading across the embryo. Its expression is always mosaic. (B) Embryos injected in the two dorsal, animal blastomeres at the 4-cell stage with 50 pg of β-Gal mRNA were stained with Red-Gal before in situ hybridization, showing the initial expression is on the dorsal side of the embryo. (C) Embryos were dissected into dorsal and ventral halves at stage 10 and frozen for real-time PCR. FoxI1e expression is enriched on the dorsal side at stage 10. Results are normalized to ODC expression levels. (D) Stage 11 embryos were stained for FoxI1e and sectioned. Staining with Wheat Germ Agglutinin defines a small population of FoxI1e-positive cells between the sensorial and epithelial layers of the ectoderm. Scale bars represent 200 μm, unless otherwise noted.

(Figs. 2C–F). These results show that the spatial regulation of *FoxI1e* expression at the blastula and gastrula stages is independent of canonical Wnt signaling.

One of the downstream effects of Wnt signaling on the dorsal side of the embryo is inhibition of BMP signaling (Baker et al., 1999). We therefore assayed the effects of altered BMP sig-

naling on *FoxI1e* expression. To test the effect of increased BMP signaling on *FoxI1e*, 500 pg of mRNA encoding BMP4 was injected into manually defolliculated oocytes, which were fertilized using the host transfer method after overnight culture. The effect of blocking BMP signaling was tested by injection of mRNA encoding the endogenous BMP inhibitor Noggin, at
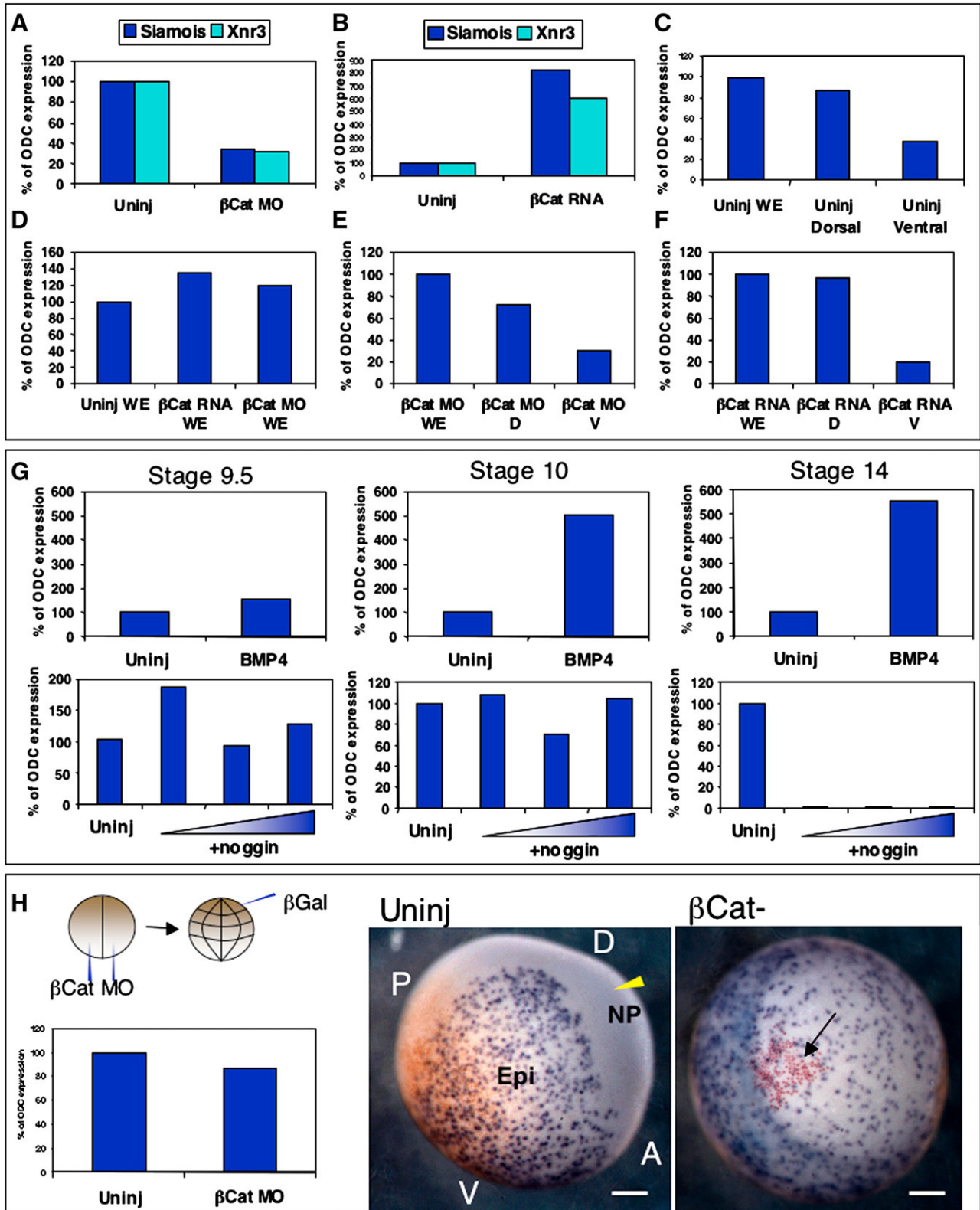
doses between 10 and 500 pg (Zimmerman et al., 1996) into embryos at the 2-cell stage. In both groups, the levels of *FoxI1e* mRNA were measured by real-time RT–PCR.

Fig. 2G shows that, at the late blastula stage (stage 9.5), neither increased nor decreased BMP signaling consistently affected the level of *FoxI1e* expression, suggesting that the initiation, and the initial level of *FoxI1e* expression on the dorsal side is not controlled by BMP signaling. At the early gastrula stage, loss of BMP signaling did not change *FoxI1e* expression levels, but increased BMP signaling dramatically upregulated *FoxI1e* expression. At the neurula stage (stage 14), loss of BMP signaling caused complete loss of *FoxI1e* expression, and increased BMP signaling enhanced its expression (Fig. 2G). These data show that initiation of expression on the dorsal side, and the control of its initial level of expression, is not controlled by BMP inhibition. However, the subsequent increase in expression on the ventral side, and loss on the dorsal side, in the neural plate, is controlled by BMP signaling.

Next, we analyzed the spatial expression of *FoxI1e* in β-catenin-depleted embryos. Embryos were injected at the 2-cell stage with 40 ng β-catenin MO, and dorsal cells marked by the injection of 50 pg of mRNA encoding nuclear β-galactosidase (β-gal) in a dorsal animal cell (cell A1) at the 32-cell stage. At stage 14, real-time RT–PCR showed similar levels of expression between β-catenin-depleted and control whole embryos. Localization of *FoxI1e* expression was assayed by in situ hybridization, combined with Red-Gal staining for the β-gal marker of dorsal cells. In uninjected embryos, *FoxI1e* expression was visible in the epidermis, but absent from the presumptive neural plate. In β-Cat MO-injected embryos, however, cells marked by Red-Gal staining that would normally have become part of the CNS and switched off *FoxI1e* still had positive *FoxI1e* staining (Fig. 2H).

Taken together, these data show that neurula stage *FoxI1e* expression is dependent on dorsal–ventral embryonic patterning initiated by canonical Wnt signaling, and propagated through the ectoderm by modulation of BMP signaling, but that the initial dorsal expression of *FoxI1e* is independent of this pathway.

VegT and downstream Nodals, as well as the maternal TGF-β ligand Vg1, control the level of FoxI1e expression in the animal hemisphere

FoxI1e was initially identified in this project by its upregulation in embryos depleted of VegT, in which a 12-fold upregulation of expression was seen in the vegetal mass (Mir et al., 2007). Although consistent, this upregulation comprises only a small fraction of the increase in overall expression in the whole embryo, suggesting that VegT may play a greater role in controlling expression in the animal hemisphere. To test this, we carried out in situ hybridization for *FoxI1e* expression in embryos depleted of VegT using a morpholino oligo injected into the oocyte. The increase in *FoxI1e* expression in VegT-depleted embryos was found to be much more dramatic in the animal than in the vegetal hemisphere (Fig. 3A), supporting the hypothesis that a major role of VegT in the embryo is to repress the level of *FoxI1e* in its normal expression domain, rather than inhibit expression elsewhere in the embryo.

This role for VegT must be non-cell-autonomous, since its transcripts are vegetally localized. It has been shown previously that Nodal signaling is downstream of VegT, and activates mesoderm gene expression in the adjacent marginal zone. In addition, Nodal signaling has previously been shown to repress *FoxI1e* expression, as indicated by an upregulation in embryos injected with the Nodal inhibiting construct CerS (Agius et al., 2000). We therefore analyzed this effect more carefully by dissecting vegetal masses which had all potential mesodermal contamination removed, from embryos injected with 1 ng of CerS mRNA at the 2-cell stage. Real-time RT–PCR analysis at stage 10 confirmed that although there was an upregulation of *FoxI1e* in the vegetal mass relative to controls, a much greater increase was found outside the vegetal mass (Fig. 3B). In situ hybridizations for *FoxI1e* mirrored the pattern of upregulation caused by loss of VegT, with a very large increase of expression in the animal cap, but little in the vegetal mass. Interestingly, the increased expression in the animal cap did not include the outer layer of cells (Fig. 3A). These results indicate that the VegT–Nodal pathway regulates the level of *FoxI1e* expression within its normal expression domain, rather than determining the expression domain itself.

Since VegT and Nodal signaling do not seem to be the main factors involved in repression of *FoxI1e* in the vegetal mass, we next analyzed the role of the maternally encoded, vegetally localized TGF-β ligand, Vg1. Vg1 has recently been shown to function as an essential endogenous mesoderm inducing signal in *Xenopus* (Birsoy et al., 2006), and so we wanted to know if it also controls *FoxI1e* expression. Vg1-depleted embryos were generated by injecting 4 ng of Vg1-specific thioate-modified DNA oligo (Vg1c) into oocytes as previously described, and

Fig. 2. Wnt-dependent dorsal axis formation controls late, but not early, FoxI1e expression. Embryos injected with 40 ng β-Cat MO had reduced levels of direct targets Siamois and Xnr3 at stage 10 (A), and embryos injected with 50 pg β-Cat mRNA had increased levels (B). In control explants, the level of FoxI1e was higher in dorsal halves than ventral halves (C). The total level and distribution of FoxI1e was unchanged by β-Cat MO or mRNA (D–F). (G) Levels of FoxI1e mRNA at stages 9.5, 10, and 14 (compared to ODC mRNA levels at each stage) in embryos injected with either 100 pg BMP4 mRNA (upper panels), or 10, 40, or 160 pg of noggin mRNA (lower panels). Neither BMP4 overexpression, nor inhibition using Noggin, consistently affected the level of FoxI1e expression at the late blastula stage (stage 9.5). By the early gastrula stage (stage 10), BMP4 overexpression increased FoxI1e expression, but Noggin still had little effect. Expression of FoxI1e in the early neurula (stage 14) was increased by BMP4 overexpression and completely ablated by Noggin. This indicates that the early expression of FoxI1e is BMP-independent, but that the restriction of FoxI1e to the epidermis at neurulation is BMP-dependent. (H) Overall level of FoxI1e expression at stage 14 is unaffected by β-Cat MO. Embryos injected with 40 ng β-Cat MO at the 2-cell stage were injected with β-Gal at the 32-cell stage in the A1 blastomere. Red-gal staining, and FoxI1e in situ hybridization were carried out at stage 14. The anterior (A), posterior (P), dorsal (D), and ventral (V) regions of the embryo are marked in the control embryo, with the derivatives of the dorsal animal blastomere (A1), marked by the yellow arrowhead in the neural plate. Embryos lacking β-Cat (right panel) lack axes altogether. The red-gal positive cells (arrowed) mark the derivatives of the A1 blastomere. FoxI1e expression in β-Cat-depleted embryos persists in that clone of cells, indicating the β-Cat dependence of restriction of FoxI1e from the prospective CNS. The reddish cast toward the posterior end of the uninjected embryo is residual maternal pigment, not affected by bleaching. Scale bars represent 200 μm.
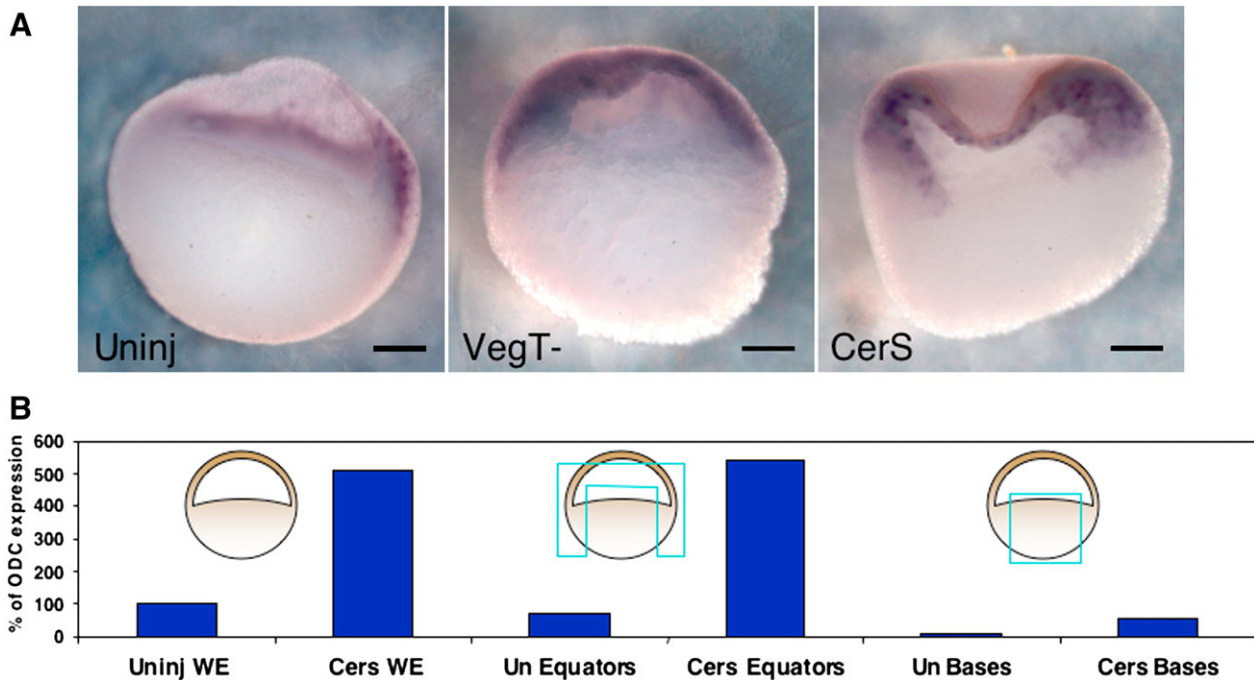
Fig. 3. VegT and Nodal signaling act at long-range to affect FoxI1e expression in the animal cap. (A) In situ hybridization in VegT-depleted and CerS-injected embryos shows FoxI1e is most upregulated in the animal cap rather than the vegetal mass at stage 10. Dorsal is the to the right in the bisected uninjected embryo. The VegT and CerS embryos did not dorsal axes. (B) Vegetal masses stripped of all mesoderm contamination dissected from control and CerS-injected embryos confirm that the vast majority of increase in FoxI1e expression is derived from non-endodermal tissue. Scale bars represent 200 μm.

then fertilizing them by the host transfer procedure. Vegetal masses were dissected from blastula-stage embryos, and frozen at stage 10. Real-time RT–PCR analysis showed a dramatic upregulation of *FoxI1e* expression in Vg1-depleted whole embryos compared to controls, again, with a disproportionate upregulation in the animal hemisphere (Fig. 4A). In situ hybridization reveals a massive upregulation of *FoxI1e* within the inner layer of the animal cap, with all cells in the inner layer expressing *FoxI1e* in the most strongly affected embryos (Fig. 4B, 20%, *n*=55).

To address the possibility that Vg1 and VegT cooperate to repress *FoxI1e* in the vegetal mass, embryos lacking both Vg1 and VegT were generated. Depletion of each mRNA alone caused a modest increase in *FoxI1e* expression in cultured vegetal masses. Depletion of both mRNAs did not show an increased effect (Fig. 4C).

Taken together, these data indicate that VegT and TGF-β signaling from the vegetal mass act in a long-range, non-cell-autonomous manner to control *FoxI1e* expression in the animal cap. They also suggest the presence in the early embryo of either animally localized activators of *FoxI1e*, or vegetally localized repressors.

Notch represses FoxI1e expression

To determine if the Notch signaling pathway is responsible for the mosaic expression of *FoxI1e*, we designed antisense DNA oligonucleotides against the *Xenopus* Notch homolog (*Xotch*) coding sequence. These were tested for efficiency by injection into full-grown oocytes. After 48 h in culture, the level of depletion was analyzed by real-time RT–PCR. Of the oligos tested, oligo 14 was most effective at reducing endogenous levels of *Xotch* mRNA. To stabilize the oligo, we used a phosphorothioate-modified version, which was able to reduce *Xotch* mRNA to 15–25% of wild type. *Xotch*-depleted embryos were generated using the host transfer method, and Fig. 5A shows that the mRNA remained depleted through the gastrula stage.

At the late blastula stage in *Xotch*-depleted embryos, there was a reproducible increase in *FoxI1e* mRNA expression (Fig. 5B). We further analyzed these embryos by in situ hybridization, and found that whereas in the controls, *FoxI1e* expression was restricted to a small number of cells on the dorsal side of the embryo, in *Xotch*-depleted embryos, expression of *FoxI1e* extended further across the animal cap towards the ventral side (Fig. 5C, 90%, *n*=46).

To test whether an increase in Notch signaling would have the reciprocal effect, we injected oocytes with 500 pg of synthetic mRNA encoding the intracellular domain of Notch (N^{ICD}), which is constitutively translocated to the nucleus and activates Notch signaling (Coffman et al., 1993; Deblandre et al., 1999). At the late blastula and early gastrula stages, *FoxI1e* mRNA expression was dramatically decreased in the N^{ICD}-injected embryos compared to uninjected controls (Fig. 5D). The Notch target; enhancer of split-related-1 (*ESR-1*); was dramatically upregulated in these embryos, indicating the expected activity of the construct (Fig. 5F). These results show that Notch signaling is responsible for the restriction of *FoxI1e*
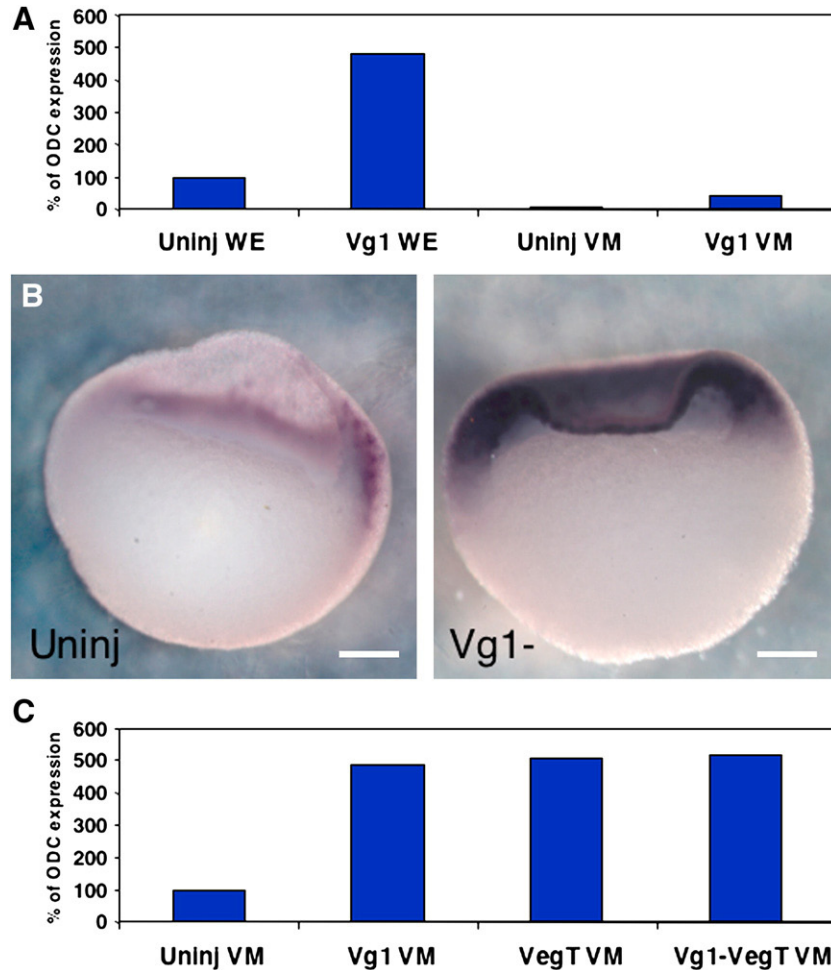
Fig. 4. Vg1 is a long-range inhibitor of FoxI1e expression. (A, B) Depletion of Vg1 results in a 5-fold increase in FoxI1e expression at stage 10, resulting largely from an increase in expression in non-endodermal tissues. (C) Co-depletion of VegT and Vg1 does not increase the expression of FoxI1e in the vegetal mass over either one alone, indicating the presence of an unidentified inhibitor in the vegetal mass, or the absence of an activator. Dorsal is to the right in the bisected embryos shown. Scale bars represent 200 μm.

expression to its mosaic pattern in the animal hemisphere, and for its initial dorsal expression.

We next wanted to determine if the core Notch signaling pathway was involved in regulating *FoxI1e* expression. In *Xenopus*, the core pathway is mediated by the transcription factor Suppressor of Hairless [Su(H)]. We injected oocytes with 500 pg of mRNA encoding a mutated version of Su(H) that is missing the DNA binding domain [Su(H)-DBM], and acts in a dominant negative manner (Deblandre et al., 1999). Su(H)-DBM-injected oocytes were fertilized by the host transfer method, as above, and analyzed by RT–PCR and in situ hybridization. Real-time RT–PCR analysis revealed a reproducible upregulation of *FoxI1e* (Fig. 5E). *ESR-1* expression was downregulated in Su (H)-DBM-injected embryos, confirming the inhibition of Notch signaling (Fig. 5F). In situ hybridization showed expression of *FoxI1e* in all cells of the inner layer of the ectoderm (Fig. 5G, 85%, $n=52$), rather than the dorsal, mosaic pattern seen in control embryos. The results are similar to the *Xotch* knock-down, confirming the requirement for Notch signaling to restrict *FoxI1e* to a salt-and-pepper expression pattern.

Maternal Vg1 activates Notch signaling

Depletions of Vg1 and Notch have similar effects of *FoxI1e* expression. This could be because they act in parallel, or because they act in series in a single pathway to control *FoxI1e* expression. It has been suggested previously that Activin can activate Notch signaling in the blastula (Abe et al., 2004). To test the possibility that Vg1 controls Notch signaling in the animal hemisphere, cultured oocytes were depleted of Vg1 mRNA by injection of 4 ng Vg1c oligo, and fertilized. They were then injected at the 2-cell stage with 50 to 500 pg N^{ICD} mRNA. Embryos were harvested during the late blastula and early gastrula stages for real-time RT–PCR analysis, and during the early gastrula stage for in situ hybridization. Introduction of the activated Notch construct rescued *FoxI1e* expression levels in the Vg1-depleted embryos (Figs. 6A, B). This suggests that maternal Vg1 activates Notch signaling in the blastula. To confirm this, we showed that expression of the Notch target gene *ESR-1* is reduced by depletion of Vg1 in the early embryo (Fig. 6C), and that Vg1 overexpression could not rescue the
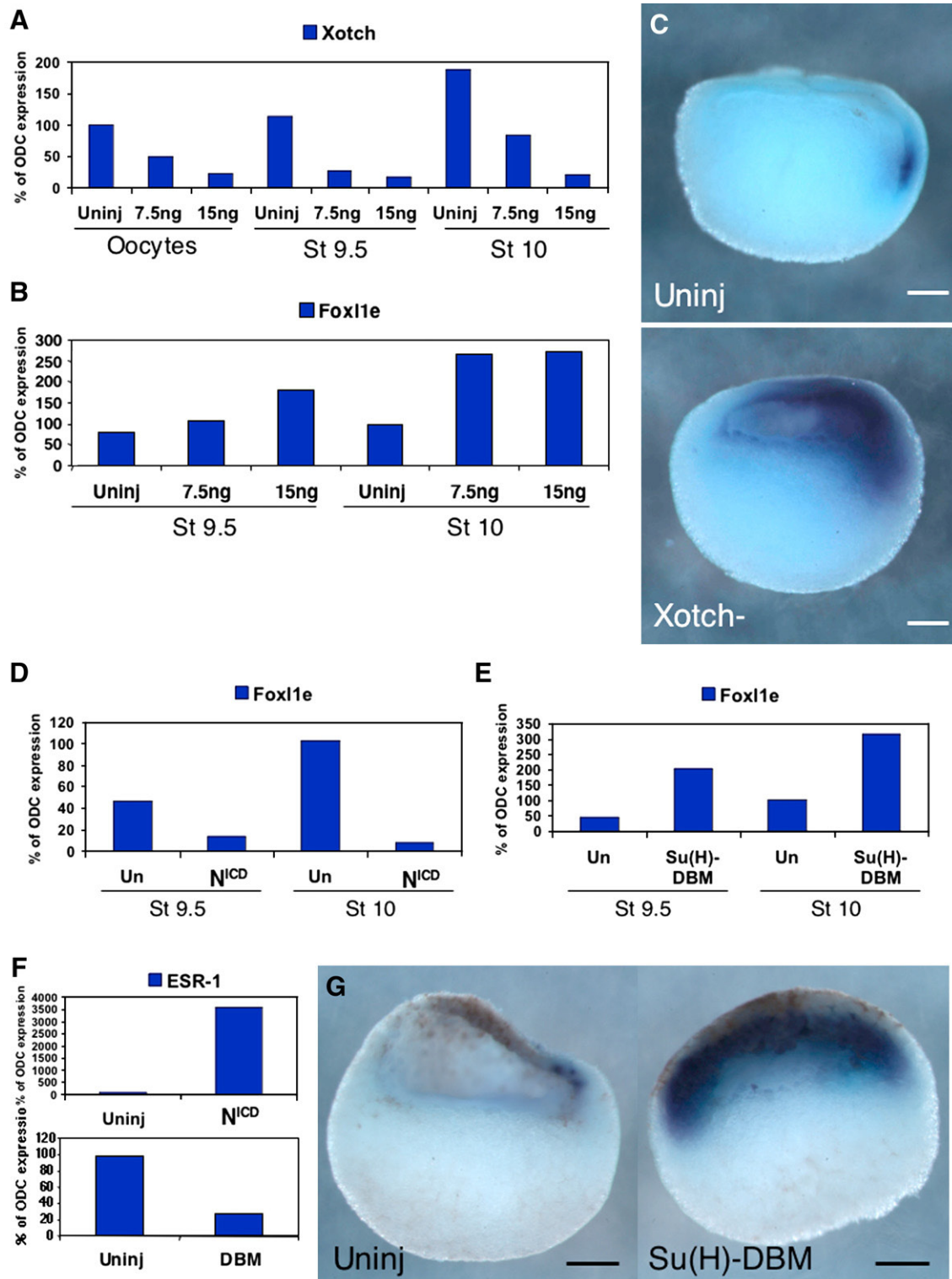
Fig. 5. Notch signaling is responsible for the initial dorsal restriction and mosaic expression of FoxI1e. (A) Maternal *Xotch* mRNA is depleted to 20–25% with 15 ng of thioate-modified DNA oligo. The level of *Xotch* remains low in the blastula and gastrula. (B) FoxI1e expression is 2- to 3-fold upregulated by depletion of *Xotch*. In situ hybridization shows an expansion of FoxI1e expression (C). (D) Injection of the constitutively active Notch Intracellular Domain (N[ICD]) causes downregulation of FoxI1e relative to controls, and injection of the dominant negative construct Su(H)-DBM causes an upregulation of FoxI1e (E). (F) N[ICD] upregulates the Notch target ESR-1, and Su(H)-DBM downregulates it. (G) In situ hybridization for FoxI1e comparing control and Su(H)-DBM injected embryos at stage 9.5 indicates that more, and in the most severe cases, all of the sensorial-layer animal cap cells express FoxI1e. Dorsal is to the right in all embryos shown. Scale bars represent 200 μm.
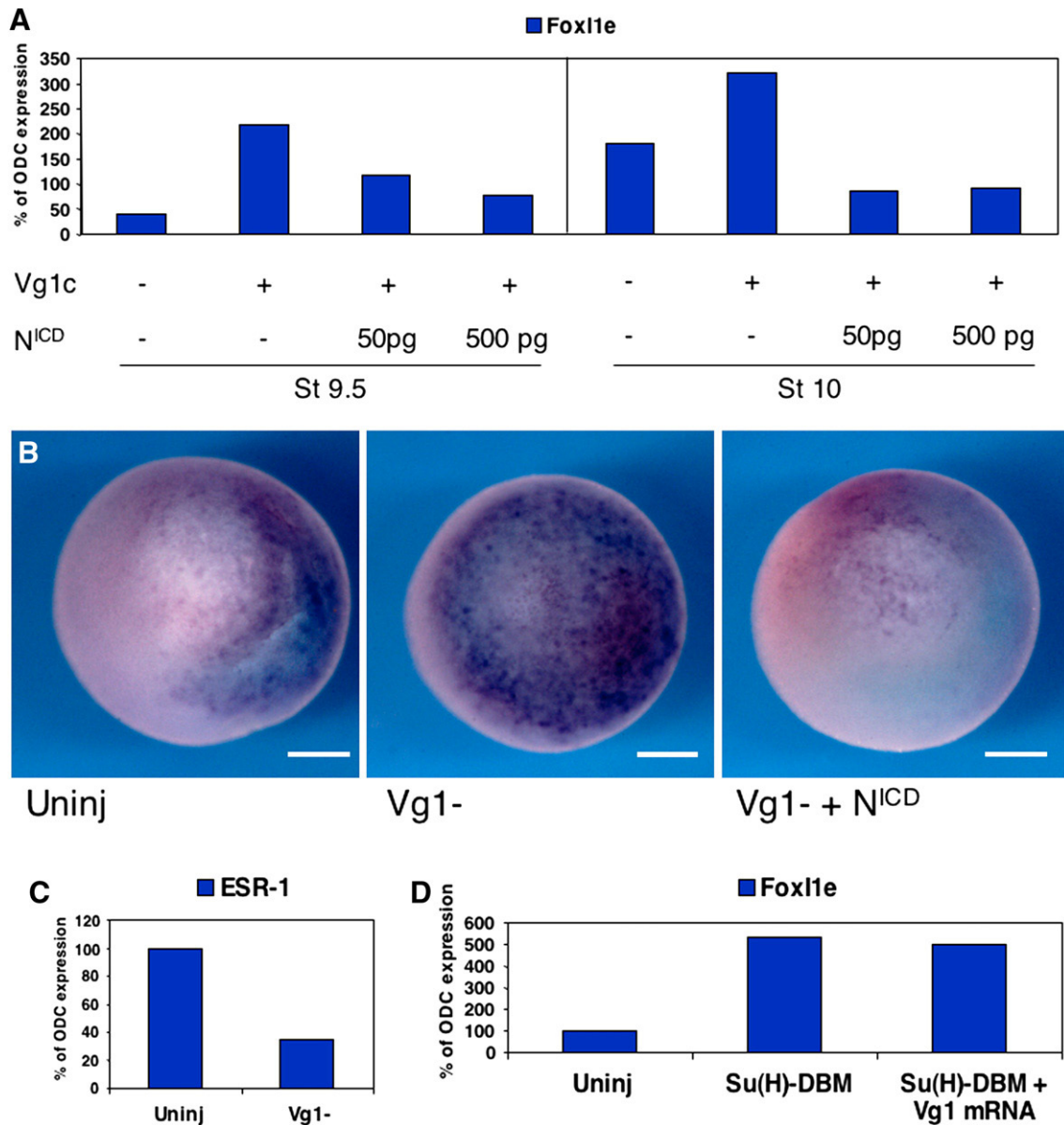
Fig. 6. Maternal Vg1 activates Notch signaling in the blastula to control FoxI1e expression. (A) Vg1-depleted embryos were injected with 50 or 500 pg of N^{ICD} mRNA at the 2-cell stage. N^{ICD} rescued the increase in FoxI1e expression caused by Vg1 depletion. (B) These results were confirmed by in situ hybridization for FoxI1e at stage 10, which shows an upregulation of FoxI1e in Vg1-depleted embryos, and a reversal of this upregulation by subsequent injection with N^{ICD}. The control embryo is oriented with dorsal to the right. Depletion of Vg1 results in a delay of gastrulation, and so the orientations of both the Vg1-depleted and the N^{ICD}-rescued embryos are indeterminate. Scale bars represent 200 μm. (C) Real-time PCR at stage 10 shows that the Notch target ESR-1 is downregulated in Vg1-depleted embryos relative to controls, indicating that Notch signaling depends on Vg1 at this stage. (D) 200 pg of Vg1 mRNA was unable to rescue the increase in FoxI1e expression induced by loss of Notch signaling by injection of 500 pg Su(H)-DBM mRNA.

FoxI1e overexpression caused by reduction of Notch signaling caused by injection of 500 pg Su(H)-DBM mRNA (Fig. 6D).

Discussion

In this work, we have shown that expression of *FoxI1e*, a gene expressed in the animal hemisphere, which controls ectoderm formation, is subject to multiple levels of control. First is the surprising observation that it is expressed in a mosaic fashion throughout its whole period of expression in the embryo. This is particularly interesting because no other genes have been shown to be expressed in such a pattern at this early stage in

development. In a previous paper, we showed that expression of genes in both neural and epidermal branches of ectodermal differentiation are downregulated in *FoxI1e*-depleted embryos. These target genes are not expressed in mosaic fashions, indicating that *FoxI1e*-expressing cells in the blastula and gastrula are probably controlling expression of downstream targets in a non-cell-autonomous manner.

The mosaic expression of *FoxI1e* could be accounted for in 3 possible ways. First, it could be cell cycle-dependent, so that at any given time, only a subset of cells at a particular point in the cell cycle express it. The second possibility is that *FoxI1e*-expressing cells could originate from a few cells and then

disperse by migration across the animal cap. Finally, it could be activated or repressed in a mosaic pattern by intercellular signaling. We show in this paper that blockade of Notch signaling abrogates the mosaic expression of *FoxI1e*, suggesting that the third possibility is correct. This does not preclude the first two. However, it is unlikely that *FoxI1e* mRNA is turned over during part of each cell cycle, and lineage analysis excludes that possibility that there is large-scale migration of inner animal cells from the dorsal to the ventral side of the embryo. This work also confirms that Notch signaling is active in the blastula, a fact that was previously underappreciated.

Second, we show that the normal expression domain of *FoxI1e* extends across the whole animal cap, but excludes the most superficial layer of cells throughout its expression period (Fig. 7). Cells in this expression domain turn on *FoxI1e* in a temporal sequence, from the dorsal to the ventral side, so that at the gastrula stage, cells all across the expression domain are expressing *FoxI1e*. Such a progression could be controlled by local factors in the animal cap, or at longer range by factors that control dorsal/ventral patterning in the rest of the embryo. We show that Notch, and thus short-range signaling, is involved. However, we find that longer-range signaling, originating in vegetal cells, also controls the expression pattern of *FoxI1e*. We show that the shifting expression of *FoxI1e* within its expression domain results from different signaling pathways acting at different times. For example, *FoxI1e* expression in the blastula and gastrula is unaffected by manipulation of the Wnt signaling that establishes the dorsal axis. However, the later downregulation of *FoxI1e* in the neural plate requires the inhibition of BMP signaling, which is downstream of Wnt signaling. In the late blastula and early gastrula ectoderm, vegetal pathways initiated by VegT and Vg1 influence the spatial pattern of *FoxI1e* expression. In the absence of either Vg1, VegT, or nodal signaling, early expression of *FoxI1e* is initiated all across the animal cap, instead of gradually spreading from dorsal to ventral.

Finally, we have shown that Vg1 activates the Notch signaling pathway to restrict *FoxI1e* expression to a mosaic pat-
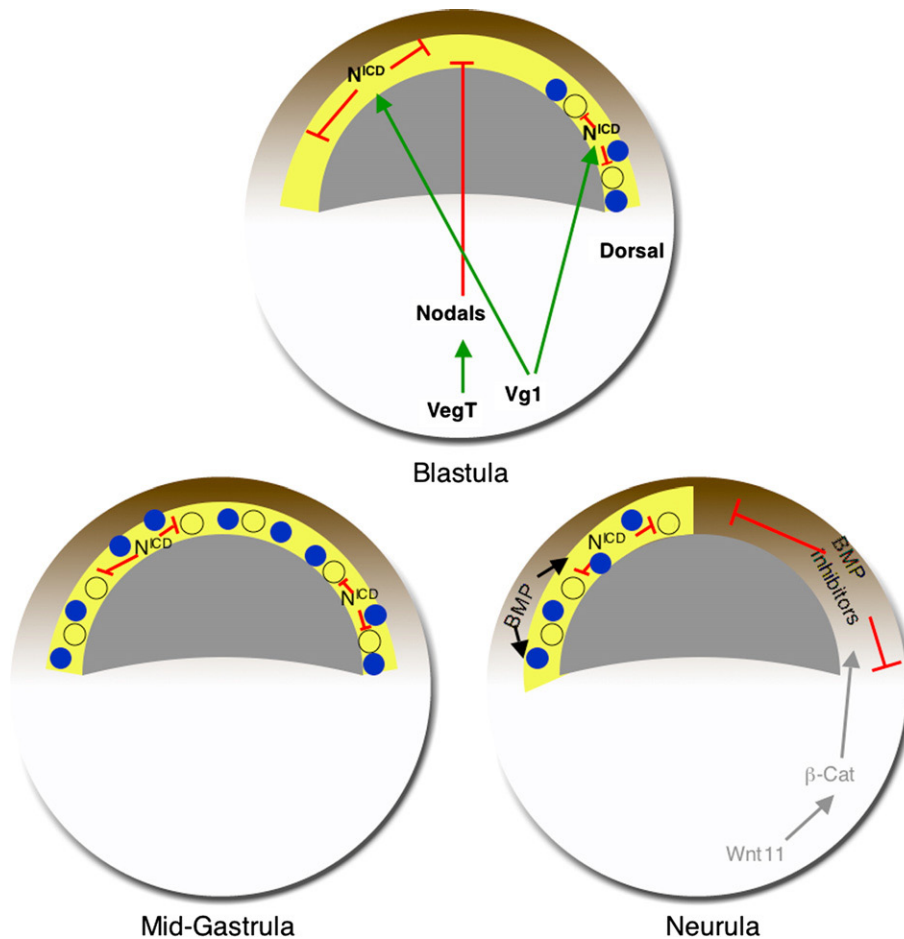

Fig. 7. Model of control of FoxI1e expression in the early embryo. (A) At the blastula stage, signals from the vegetal hemisphere restrict the expression of FoxI1e to the dorsal side of the embryo within the subset of prospective ectodermal cells that are competent to express FoxI1e. Vg1 activates Notch signaling to maintain mosaic expression. The competent tissue, in yellow, is adjacent to the blastocoel in the sensorial layer of the animal cap. The molecular nature of this region that permits FoxI1e expression is unknown. It could be the inheritance of an intrinsic transcription factor, or signaling from other tissues. (B) As signaling from the vegetal hemisphere weakens, FoxI1e expression spreads to the ventral side of the embryo. The appearance and spread of FoxI1e expression coincides temporally with the restriction of animal cells to ectodermal lineages. The inner layer of cells is still competent to express FoxI1e. (C) At the neurula stage, FoxI1e expression is BMP-dependent. Activation of dorsal axis formation earlier in development leads to the release of BMP inhibitors from the dorsal mesoderm, resulting in a restriction of FoxI1e expression potential to the ventral ectoderm.

tern. The mechanism by which Vg1 activates Notch signaling remains unclear, but the most likely possibilities are through interaction of phospho-smad2 with Notch or by upregulating transcription of zygotic components of Notch signaling. Our attempts to identify these components have been unsuccessful, thus far. Though it has been shown that activin can induce Delta-1 and Delta-2 in animal caps (Abe et al., 2004), our analysis of their expression in Vg1-depleted embryos has not confirmed this relationship. Previously, Vg1 has been shown to be a maternal inducer of mesoderm (Birsoy et al., 2006). The effect of Vg1 on Notch could be downstream of mesoderm induction, or through release of signaling molecules into the blastocoel fluid, which would then act on the inner surface of the animal cap. The mechanisms by which vegetal pathways influence animal patterning require further study.

Previous studies have shown that *FoxI1e* is upregulated in VegT-depleted and in CerS-injected embryos. However, it has not, until now, been fully appreciated that the increase in expression of *FoxI1e* and other ectodermal genes in the vegetal mass is minor compared to the increase in the animal half of the embryo. This strengthens the hypothesis that there is an animally localized maternal activator of ectoderm formation. Additionally, there must be either an activator(s) specific to the deep layer of the ectoderm, or a repressor(s) of *FoxI1e* in the superficial layer. Indeed, a number of differentially expressed transcription factors have been identified, and the candidate may be among them (Chalmers et al., 2006).

This work represents the first description of regulation of animally expressed zygotic genes. Previous work has focused primarily on the exclusion of mesodermal gene expression from the ectoderm. *FoxI1e* has been shown to inhibit FGF-mediated mesoderm induction (Suri et al., 2005), and the Smad4 ubiquitin ligase ectodermin attenuates mesoderm induction in the animal cap by inhibiting all TGF-β signaling, both activin-type and BMP-type (Dupont et al., 2005). Additionally, the MADS box transcription factor SRF disrupts the interaction of Smad2 and FoxH1, thereby preventing activin-type TGF-β signaling (Yun et al., 2007). In the absence of ectodermin, SRF, or *FoxI1e*, mesodermal gene expression expands animally. It is clear from these studies that the inhibition of mesoderm induction in the animal cap keeps the stage clear for ectoderm specification, and offers a mechanism to control its boundaries. However, they also provide evidence that signals originating from vegetal hemisphere can reach the ectoderm. Although we have not shown that the Vg1 pathway or nodal signaling directly affects *FoxI1e* expression, these data do allow for this possibility.

FoxI1e is not the first zygotic gene identified that is expressed in the entire early ectoderm. The transcription factors *Xlim5* (Toyama et al., 1995; Houston and Wylie, 2003) and *AP-2* (Luo et al., 2002) are restricted to the CNS and epidermis, respectively, late during the gastrula stage, but are both broadly expressed throughout the ectoderm before this restriction. However, this early expression is generally ignored. In an unpublished study, we have shown that both of these genes are upregulated in VegT-depleted embryos, indicating overlapping regulation. It will be important to analyze the expression of these genes when testing animally localized maternal factors for their role in ectoderm specification.

We have begun to assemble a pathway from maternal control, to intercellular signaling, to ectoderm patterning, but the question of why *FoxI1e* is expressed in a mosaic pattern remains. Although it is not expressed in every ectodermal cell, and later becomes confined to the epidermis, *FoxI1e* is important for the formation of the ectoderm germ layer, before it divides into epidermis and CNS. There must be signaling events downstream of *FoxI1e* that allow it to activate gene expression in a non-cell-autonomous fashion. It has been shown that Notch signaling prolongs mesodermal competence in the animal cap (Abe et al., 2004, 2005; Coffman et al., 1993). It has also been shown that ectoderm determination is a gradual process that begins during the late blastula stage and continues through gastrulation (Heasman et al., 1984; Snape et al., 1987). It is possible that Notch signaling represses *FoxI1e* expression in the animal cap in the mid-late blastula, but as animal Notch signaling weakens, *FoxI1e*+ cells begin to appear in the ectoderm, forcing the cells around them to activate other ectodermal genes. The gradual activation of *FoxI1e* coincides with the restriction of animal cap cells to ectodermal fates. This represents a model integrating Vg1, Notch, VegT, Nodals, and *FoxI1e* into the specification and patterning of the early ectoderm.

Acknowledgments

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