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FoxI1e activates ectoderm formation and controls cell position in the *Xenopus* blastula

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The segregation of the vertebrate embryo into three primary germ layers is one of the earliest developmental decisions. In *Xenopus*, where the process is best understood, the endoderm is specified by a vegetally localized transcription factor, VegT, which releases nodal signals that specify the adjacent marginal zone of the blastula to become mesoderm. However, little is known about how the ectoderm becomes specified. In this paper, we show that the forkhead protein FoxI1e (also known as Xema) is required at the blastula stage for normal formation of both the central nervous system and epidermis, the two early derivatives of the ectoderm. In addition, FoxI1e is required to maintain the regional identity of the animal cells of the blastula, the cells that are precursors of ectodermal structures. In its absence, they lose contact with the animal cap, mix with cells of other germ layers and differentiate according to their new positions. Because *FoxI1e* is initially expressed in the animal region of the embryo and is rapidly downregulated in the neural plate, its role in neural and epidermal gene expression must precede the division of the ectoderm into neural and epidermal. The work also shows that FoxI1e plays a role in the embryo in the poorly understood process of differential adhesion, which limits cell mixing as primary germ layers become specified.

KEY WORDS: FoxI1e, Xema, *Xenopus*, Ectoderm

INTRODUCTION

Specification of the three primary germ layers in the *Xenopus* embryo occurs during the blastula stage (Heasman et al., 1984). Animal cells are specified to become ectoderm, while equatorial and vegetal cells are specified to form mesoderm and endoderm, respectively. The molecular mechanisms of these events have been best studied in vegetal and equatorial cells. Vegetal cells become specified to become endoderm by the vegetally localized maternal T-box transcription factor VegT (Zhang et al., 1998). These cells release nodal-related factors, which act upon equatorial cells to induce mesoderm. By contrast, little is known about how animal cells are specified to become ectoderm. Ectoderm has long been considered a default pathway of embryonic development, with cells not under the influence of the VegT pathway adopting an ectodermal program of gene expression and development. Indeed, in embryos that have been depleted of VegT, cells normally fated to become endoderm and mesoderm instead become ectoderm (Zhang et al., 1998).

Previous work has shown that the process of ectoderm specification begins during the blastula stage. Dissociated cells from different regions of the blastula that are mixed and allowed to reaggregate separate according to their original regional identities (Turner et al., 1989). The molecular mechanisms of this differential adhesion are not yet known. However, it has been shown that the ability of animal and vegetal cells to sort from one another is lost when VegT is depleted (Houston and Wylie, 2003). Further, single-cell transplantation experiments have shown that animal cells, which are able to contribute to all three germ layers during the early and mid-blastula stages, become gradually restricted to ectodermal fates from the late blastula to gastrula stages (Snape et al., 1987). Thus, specification of animal cells begins during the late blastula stage.

Little is known about the initial events of ectoderm specification during the blastula stage. The maternally and zygotically encoded Smad4 ubiquitin ligase ectodermin (Dupont et al., 2005) and the zygotic forkhead-box transcription factor Xema (Suri et al., 2005), have both been found to be important in this process. Both molecules have been reported to be repressors of mesoderm formation in the ectoderm domain, rather than activators of ectodermal gene expression. During the gastrula stage, the cells of the animal region begin to subdivide into different ectodermal subpopulations, with cells on the ventral side of the embryo giving rise to epidermis in response to high levels of Bmp signaling, and those on the dorsal side giving rise to the central nervous system (CNS) upon exposure to Bmp antagonists released from the organizer. The cells at the border of the CNS and the epidermis contribute to the neural crest population. It is clear that all of the cells of the animal cap have the potential to contribute to either neural structures (reviewed by De Robertis and Kuroda, 2004; Stern, 2005) or epidermal structures (Luo et al., 2002; Tao et al., 2005). However, not much is known about genes that act upstream of the decision to become neural or epidermal, nor which signaling molecules and transcription factors, present at the blastula stage, actively initiate ectodermal specification.

To identify candidate genes that may address these problems, we carried out Affymetrix chip analyses to identify mRNAs that are upregulated both in animal caps relative to vegetal masses of dissected blastulae, and in VegT-depleted embryos (in which the marginal and vegetal regions form ectoderm) relative to wild-type embryos. This identified several candidate genes, expressed from the mid-blastula stage onward, at high levels in the cells destined to form both epidermal and neural structures.

The gene most highly overexpressed in VegT-depleted blastulae was a forkhead-type transcription factor previously identified as Xema (Suri et al., 2005). Sequence similarity places *Xema* in the *FoxI1* class of genes, and so we suggest the more systematic name

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FoxI1e, which we will use throughout this paper. The forkhead transcription factor family includes a large number of genes involved in a variety of developmental processes. Currently, over ten subclasses and 20 forkhead genes have been identified in *Xenopus*. The *FoxI1*-class genes in *Xenopus* are reportedly involved in ventral head specification (*FoxI1a)* (Matsuo-Takasaki et al., 2005), mesoderm formation (*FoxI1b*) and eye development (*FoxI1c*) (Pohl and Knochel, 2005). In a previous study, *FoxI1e* was found to be expressed only in the animal hemisphere of the embryo, starting at the blastula stage, and when overexpressed, it inhibited the formation of mesoderm and endoderm. It was therefore proposed to maintain the expression domain of ectodermal genes by inhibiting the formation of other germ layers (Suri et al., 2005).

In this work, we first confirm that *FoxI1e* is expressed in the animal half of the embryo at the blastula stage, but curiously, not in all of the cells, and that it is switched off in the CNS at the early neurula stage. We show that it remains on only in non-ciliated cells in the epidermis. We then show that *FoxI1e* expression is confined to the animal half of the blastula via inhibition by nodal signaling, downstream of *VegT*, in the vegetal half of the embryo. If this inhibition is overridden by injection of *FoxI1e* mRNA into vegetal cells, they adopt characteristics of ectodermal development, and express genes normally expressed in both the CNS and epidermis. This shows that *FoxI1e* is sufficient, when overexpressed ectopically, for activation of genes in both branches of ectodermal differentiation. This suggests that *FoxI1e* may be an activator of ectodermal differentiation, in addition to its previously identified role as an inhibitor of the other germ layers, and may act upstream of the separation of neural and epidermal cell fates in the ectoderm. To test this, we used a splice-blocking morpholino antisense oligodeoxynucleotide (SBMO) to block *FoxI1e* mRNA maturation, and hence expression of its gene product, in the early embryo. This caused defects in both epidermis and nervous system development, and the downregulation of both epidermal and neural mRNAs. Lastly, we show that *FoxI1e* is required for animal:animal cell adhesion at the mid-gastrula stage. In its absence, animal cells lose contact with the animal region of the embryo and fall into the blastocoel, where they mix with, and differentiate into, other lineages. These results show that *FoxI1e* is involved in the active initiation of ectodermal gene transcription, and probably plays a role before separation into epidermal and neural pathways.

MATERIALS AND METHODS

Oocytes and embryos

VegT-depleted embryos were generated as previously described (Zhang et al., 1998) using manually defolliculated oocytes injected with 7.5 ng *VegT* antisense oligo before being fertilized via the host transfer technique. Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Dissections were performed at either stage 7 or stage 9 on agarose-coated dishes in 1×MMR and then cultured in OCM. For the animal cap versus vegetal mass microarray experiment, vegetal masses were explanted at stage 11. Total RNA for Affymetrix experiments was isolated either by proteinase-K treatment followed by phenol:chloroform extraction, or by Trizol (Gibco) followed by purification through RNeasy columns (Qiagen). Each Affymetrix experiment was performed once, and then confirmed by RT-PCR.

RT-PCR

Total RNA was extracted as previously described (Zhang et al., 1998). Unless otherwise indicated, input was two whole embryos, ten animal caps or five vegetal masses per sample. cDNA was synthesized using oligo dT primers, and semi-quantitative RT-PCR was carried out using the LightCycler system as described by Kofron et al. (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. Sequences for marker-specific primers are listed in Houston and Wylie (Houston and Wylie, 2003). New primers were generated for *NRP-1* (U: 5′-TGATCCGCTCTG-CACTCTTT-3′, D: 5′-TGCTTTGAATTTGCAGTACATTG-3′), *AP-2* (U: 5′-GCAGCCACCAACTCTTCTCT-3′, D: 5′-CGTAGCTCCATTGCCT-GTTC-3′) and *Sox-2* (U: 5′-TCTGCACATGAAGGAGCATC-3′, D: 5′-CGTTCATGTGGGCATAAGTG-3′).

Immunostaining and whole-mount in situ hybridization

α-Tubulin staining was performed with 1:500 DMEM antibody (Sigma), followed by HRP- (Jackson ImmunoResearch), Cy5- (Jackson), or Alexa-488-conjugated (Molecular Probes) anti-mouse secondary antibody. Blocking solution was 20% fetal calf serum and 4% bovine serum albumin in PBS + 0.1% Tween-20. For co-staining with *FoxI1e* antisense-mRNA probe, embryos were first processed by the whole-mount in situ protocol modified from Harland (Harland, 1991) and then stained for α-tubulin. The proteinase-K step was omitted to ensure survival of the reactive epitope.

mRNA, morpholino oligos and injections

A coding-sequence-only *FoxI1e* clone was generated with primers that included restriction sites for *Cla*I and *Not*I (U: 5′-TCGAATCG-ATATGAGTGCATTTGATCCACA-3′, D: 5′-TCGAGCGGCCGCACC-CATGTTAAACCCCACAG-3′), and then cloned into the corresponding sites in pCS107. mRNA was synthesized using the SP6 mMessage mMachine kit (Ambion). Three morpholinos targeting *FoxI1e* mRNA were synthesized and rejected: two were designed to inhibit translation: 5′-GCTTGTGGATCAAATGCACTCATAG-3′ (toxic) and 5′-CTAGCA-GGTCACAAATACACCTGTA-3′ (ineffective), and one was designed against the splice donor site: 5′-TGAATGGTTATTACCTGGATCATCT-3′ (only partially effective). A splice acceptor-blocking oligo (SBMO) was found to be effective and non-toxic: 5′-ATAATTGCCCTTGC-CTGTAAAGAAAA-3′. Intron junction sequence was determined by designing primers against *X. tropicalis* genomic sequence. PCR was performed using *X. laevis* genomic DNA as a template, and the product was sequenced. Two pseudoalleles were identified, and the morpholino was designed to target both. Intron-spanning primers were designed to detect mature and immature forms of *FoxI1e* mRNA (U: 5′-CATGGA-GCCCCAGATAAAAG-3′, D: 5′-TTGGGTCCAAGGTCCAATAA-3′). For rescue experiments, mRNA and SBMO, along with different lineage tracers, were injected sequentially rather than mixed together in one injectate.

Lineage analysis

Four hundred picograms of mRNA encoding enhanced green fluorescent protein (GFP) was injected to trace the descendents of individual D-tier cells at the 32-cell stage. For tracing injected animal cells, rhodamine-lysine dextran (RLDX) or fluorescein-lysine dextran (FLDX) were co-injected: 15 ng was used for eight-cell stage injections, and 5 ng was used for 32-cell stage injections. For histology, embryos were fixed, dehydrated, cleared in xylenes, embedded in paraplast and sectioned before imaging.

RESULTS

FoxI1e mRNA is upregulated in *VegT*-depleted embryos and in animal caps

Gene expression changes were compared in two different Affymetrix microarray experiments. First, expression in control and VegT-depleted early gastrula stage (stage 10) (Nieuwkoop and Faber, 1967) embryos was compared. Second, expression was compared between animal caps and vegetal masses at stage 11. We then looked for candidates that were upregulated in *VegT*-depleted vegetal masses relative to control vegetal masses and in wild-type animal caps relative to wild-type vegetal masses. Normalized expression levels were compared and fold changes were calculated. *FoxI1e* was upregulated approximately 40-fold in isolated VegT-depleted vegetal masses,

Table 1. Affymetrix microarray results for *FoxI1e* (*Xema*)

	Normalized expression	Fold change
Uninjected base	0.0496	12.26
VegT–base	2.4000	
Uninjected whole embryos	1.0026	41.13
VegT–whole embryos	12.2900	
Base	0.0485	128.52
Cap	6.2330	

compared with wild-type vegetal masses. In wild-type animal caps, *FoxI1e* mRNA was overexpressed over 128-fold relative to wild-type vegetal masses (Table 1). These microarray results were confirmed by semi-quantitative RT-PCR. VegT-depleted whole embryos, marginal zones and vegetal masses were compared with the corresponding regions of control embryos at stage 10 to show that *FoxI1e* mRNA is upregulated in the absence of VegT (Fig. 1A). We then dissected normal embryos at the late blastula stage (stage 9) into animal caps, equators and vegetal masses, representing prospective ectoderm, mesoderm and endoderm, respectively, and cultured them until the mid-gastrula stage (stage 11). Comparative analysis of gene expression revealed that *FoxI1e* is expressed primarily in the ectoderm, with lower levels in the mesoderm (Fig. 1B). This result confirms the previously reported expression pattern, which shows expression beginning after the mid-blastula transition in the prospective ectoderm (Suri et al., 2005). Additionally, in situ hybridization for *FoxI1e* shows that although it is expressed in the animal cap of the blastula, it is not present in all cells (Fig. 2A), but instead, is present in a salt and pepper arrangement. This pattern of expression is maintained throughout early development. At later stages, expression is lost from the developing nervous system, and maintained only in the epidermis. The salt and pepper expression pattern confirms those shown in an in situ screen of random cDNAs, by the Japanese National Institute of Basic Biology (http://xenopus.nibb.ac.jp, clone XL016g24), but not previously published.

The salt and pepper pattern of expression in the epidermis is similar to that of α-tubulin, which is expressed only in cells destined to develop cilia on their surfaces (Deblandre et al., 1999). To test the possibility that *FoxI1e* is expressed specifically in ciliated cells, we co-stained tailbud embryos (stage 30) for α-tubulin protein and *FoxI1e* mRNA by both immunochemistry and in situ hybridization, respectively. We found that, in fact, the reverse was the case (Fig. 2B,C). Analysis of multiple sections showed that these two markers were not expressed in the same cells (Fig. 2D).

Expression of *FoxI1e* only in the animal half of the blastula could be due to either localized activation, or to a combination of global activation and localized inhibition in the vegetal cells. It is known from previous work that *VegT* activates expression of nodal-class ligands in vegetal cells, which, in turn, induce mesoderm expression in the marginal zone. As *FoxI1e* is upregulated in the absence of VegT in both the prospective mesoderm and endoderm, it is possible that its expression is normally inhibited in the vegetal half of the embryo, either by VegT directly (in the vegetal mass), or by nodal signaling downstream of VegT (in the marginal zone), or both. To test this hypothesis, embryos were injected vegetally at the two-cell stage with 1.5 ng of mRNA encoding CerS, a soluble nodal inhibitor (Agius et al., 2000), and cultured to the mid-gastrula (stage 11) and late neurula (stage 18) stages. Embryos injected with *CerS* mRNA had increased levels of *FoxI1e* mRNA, as shown by RT-PCR, relative to controls (Fig. 1C). Therefore, *FoxI1e* is an ectodermal gene with expression that is normally suppressed by nodal signaling downstream of *VegT*.

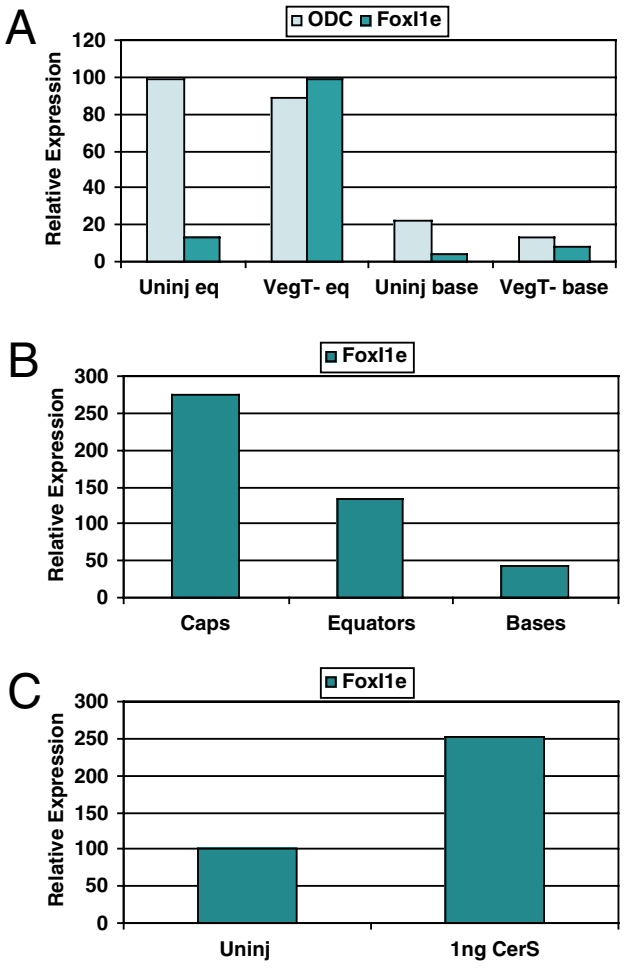

Fig. 1. *FoxI1e* mRNA is animally localized and upregulated in the absence of VegT. RT-PCR analysis of *FoxI1e* expression. (**A**) *FoxI1e* is upregulated in VegT-depleted (VegT–) equatorial (eq) and vegetal (base) explants compared with control explants. (**B**) Comparison of isolated animal caps, equators and bases dissected from wild-type embryos shows enrichment of *FoxI1e* expression in prospective ectoderm. Data are normalized to ODC. (**C**) Loss of Nodal signaling by injection of 1 ng *CerS* mRNA at the two-cell stage increases expression of *FoxI1e* in whole embryos.

FoxI1e is an activator of ectoderm differentiation

FoxI1e has been reported to be an inhibitor of mesoderm differentiation (Suri et al., 2005). Its overexpression in the marginal zone leads to a downregulation of several mesodermal genes and upregulation of the epidermal marker epidermal keratin. To test more extensively what properties *FoxI1e* confers on cells that do not normally express it, we injected vegetal cells with *FoxI1e* mRNA and tested their fates in two ways. First, we excised vegetal masses at the late blastula stage and assayed them for expression of ectodermal markers. As *FoxI1e* is normally expressed in the animal cap, in cells that will form both epidermis and CNS, we tested for markers of both of those tissues. Two-cell embryos were injected with 300 or 600 pg of *FoxI1e* mRNA, dissected at the late blastula stage (stage 9), and vegetal explants were cultured until the mid-gastrula stage (stage 11). As determined by RT-PCR, *FoxI1e* upregulated mRNAs encoding the early pan-ectodermal marker *E-cadherin*, the epidermal markers *epidermal cytokeratin* and *AP-2*,

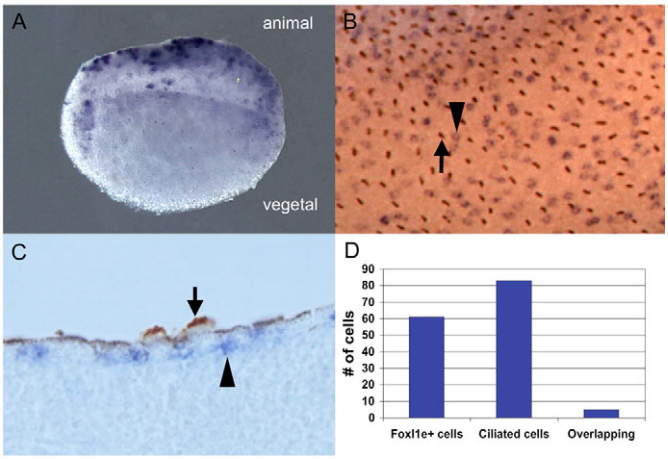

Fig. 2. *Foxl1e* is not expressed in all ectodermal cells and does not co-localize with epidermal cilia. (**A**) In situ hybridization for *Foxl1e* at stage 10 showing salt and pepper expression. (**B**,**C**) Co-immunostaining for α-tubulin (brown, arrows) and in situ hybridization for *Foxl1e* (purple, arrowheads) demonstrates that these two markers are expressed in different cell populations. Staining was done in whole mount (B), and stained embryos were then embedded and sectioned. A high-power picture is shown in C. (**D**) Twenty high-power fields were analyzed for expression of the two markers. Sixty-one *Foxl1e*-positive cells and 83 ciliated cells were counted. There were five instances of overlapping expression.

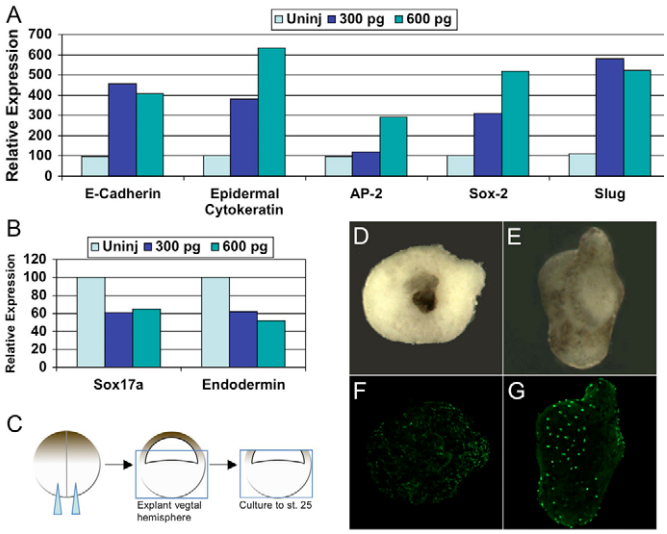

Fig. 3. *Foxl1e* expression is sufficient for ectoderm formation. RT-PCR analysis of vegetal explants injected with 300-600 pg *Foxl1e* mRNA and cultured to stage 11. (**A**) Expression of ectoderm-specific markers is increased, including *E-cadherin*, epidermal markers *epidermal cytokeratin* and *AP-2*, the neural marker *Sox-2* and the neural crest marker *slug*. (**B**) Endodermal markers *Sox17α* and *endodermin* were reduced. (**C**) Schematic of experimental design to determine behavior of vegetal hemispheres ectopically expressing *Foxl1e*. (**D**) In control vegetal hemispheres, pigmented mesodermal cells formed aggregates that extended from bases, whereas in *Foxl1e*-positive hemispheres, pigmented mesodermal cells formed a layer around the explant (**E**). Immunostaining for α-tubulin to mark cilia was negative in control embryos (**F**), but demonstrated surface ciliation on *Foxl1e*-positive hemispheres (**G**).

the neural marker *Sox-2* and the neural crest marker *Slug* (Fig. 3A). Additionally, mRNAs encoding the endodermal markers *Xsox17α* and *endodermin* were decreased (Fig. 3B). To determine if *FoxI1e* could further increase expression of these markers in the ectoderm, where they are already expressed, we injected *FoxI1e* mRNA into the animal cytoplasm at the two-cell stage and then examined gene expression in animal caps at stage 11. Interestingly, high doses of *FoxI1e* mRNA (600 pg) caused animal caps to dissociate, and lower doses had no effect on ectodermal gene expression (data not shown).

As *FoxI1e* is not normally expressed in the endoderm and mesoderm, we wanted to know whether the cells misexpressing it behave as normal endoderm and mesoderm or if they adopt characteristics of developing ectoderm. Cells of the different germ layers normally exhibit specific behaviors during early stages of development. Ectodermal cells undergo epiboly during gastrulation, while mesodermal cells undergo a complex series of movements, including ingression and convergent extension. Additionally, all cells display differential adhesive properties that limit cell mixing between the germ layers as they become specified. Vegetal hemispheres were separated from animal hemispheres at the late blastula stage (stage 9) in embryos that had been injected at the two-cell stage with 600 pg of *FoxI1e* mRNA. The vegetal hemispheres included all of the prospective endoderm and part of the prospective mesoderm, as indicated by the presence of a row of pigmented cells, but excluded the animal cells that would normally give rise to ectoderm (illustrated in Fig. 3C). In control vegetal hemispheres cultured to sibling early tailbud stages, the pigmented mesodermal cells formed aggregates on the surface of the vegetal masses (100%, n=18) and in some cases extended away from the mass (44%, n=18, Fig. 3D). However, pigmented cells in *FoxI1e*-injected vegetal hemispheres partially enveloped the vegetal masses in a manner resembling epiboly (80%, n=20, Fig. 3E). Further, immunostaining

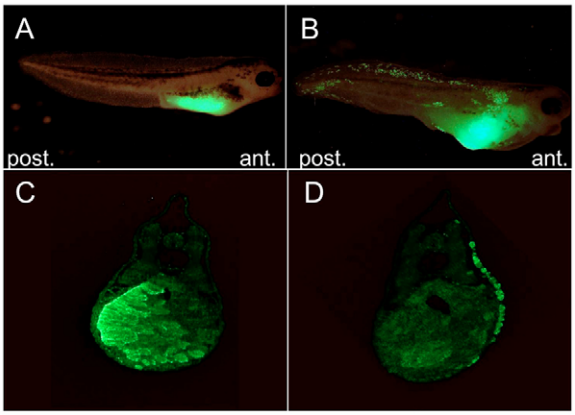

Fig. 4. Injection of *Foxl1e* mRNA in D-tier cells at the 32-cell stage causes their descendents to enter other germ layers. (**A**,**C**) Whole-mount and transverse section of embryos injected with GFP alone in D-tier cells. GFP signal is restricted to the endoderm. (**B**,**D**) Co-injection of 10-30 pg *Foxl1e* mRNA causes cells to enter other germ layers as shown in whole mount (B). The section shown in D shows GFP/ *Foxl1e*-positive cells located in the epidermis.

for cilia, a specialization of *Xenopus* epidermis, with an antibody against α-tubulin revealed the presence of cilia on the surfaces of the explants, which were absent from controls (Fig. 3F,G). We conclude

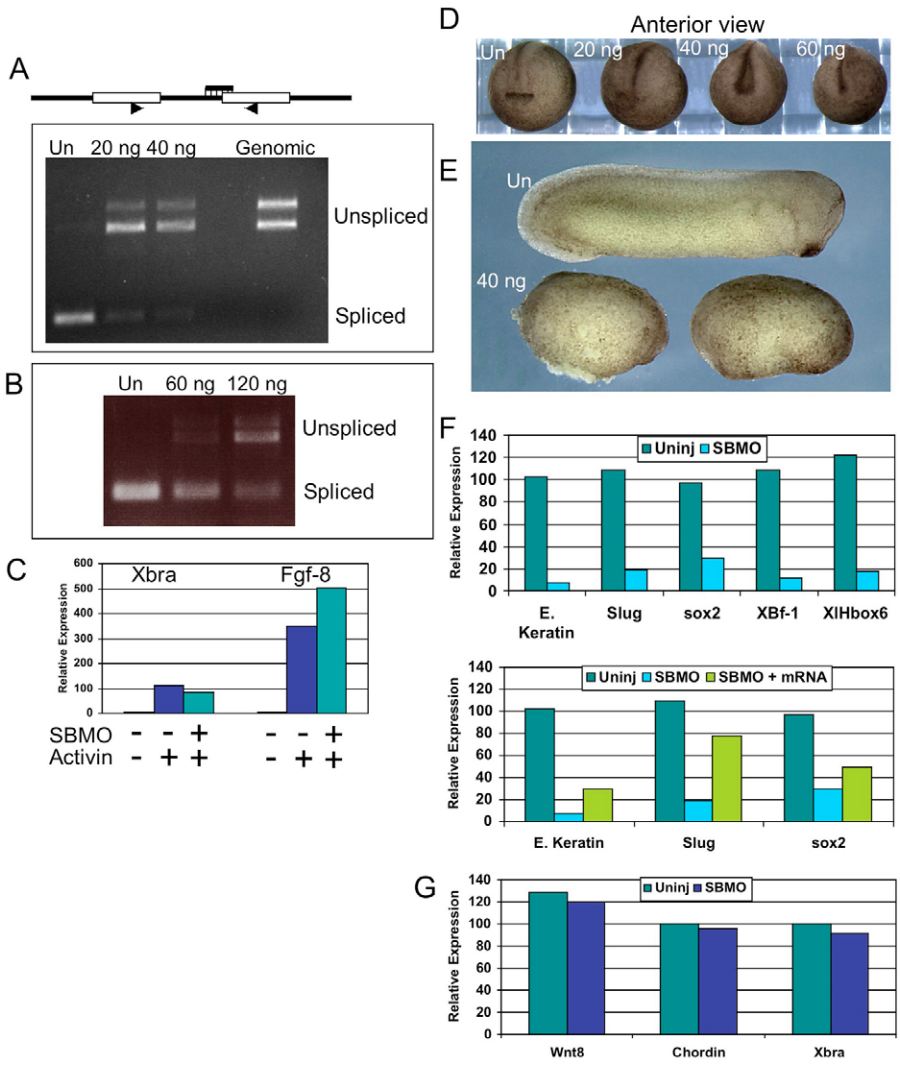

Fig. 5. An SBMO prevents mRNA maturation and causes developmental abnormalities in embryos. (**A**) Schematic showing oligo binding site and RT-PCR demonstrating inhibition of mRNA maturation by the SBMO. Both pseudoalleles are affected. Genomic DNA template was used as a positive control for unspliced product. (**B**) An alternative morpholino, targeting the splice donor site, does not efficiently inhibit mRNA maturation. (**C**) RT-PCR analysis of *XBra* and *FGF8* expression in animal caps depleted of FoxI1e and treated with 1 μg/ml Activin A. SBMO-injected animal caps showed levels of mesoderm induction similar to uninjected caps. (**D**,**E**) Embryos injected animally at the two-cell stage with a dose range of SBMO. There were dose-dependent abnormalities in development, shown at the neurula stage (stage 19, D) and in tailbud stage embryos (stage 27, E). (**F**) *Epidermal cytokeratin, slug, sox-2, FoxG1* (*BF-1*) *and Xlhbox6* are all reduced at stage 23. *E. keratin, slug* and *sox-2* are partially rescued by 50 pg *FoxI1e* mRNA. (**G**) *Xbra, Xwnt-8* and *chordin* are unaffected by SBMO injection at stage 11.5.

that *FoxI1e* overexpression in vegetal cells leads to behavior and gene expression characteristic of ectoderm.

Next, we sought to determine whether *FoxI1e* also controls cell localization in the embryo. We injected 10-30 pg of *FoxI1e* mRNA into one vegetal cell at the 32-cell stage (normally fated to contribute to endoderm), together with mRNA encoding the lineage tracer GFP. The descendants of vegetal blastomeres injected with GFP mRNA only were restricted to the developing gut, as expected from the fate map (Fig. 4A,C). Co-injection of *FoxI1e* mRNA, however, caused many of the descendants of these single vegetal cells to leave the vegetally derived region of the embryo and move into tissues derived from more animally localized blastomeres (Fig. 4B,D). GFP-positive cells were observed in both mesodermal and ectodermal tissues in tailbud embryos in 89% of embryos injected with *FoxI1e* and GFP (n=109), but only in 5% of embryos injected with GFP alone (n=92) (Fig. 4). This suggests that downstream targets of *FoxI1e* include molecules that mediate adhesion and/or migration.

As shown above, *FoxI1e* can activate ectoderm, in addition to its already known function of inhibiting mesoderm and endoderm formation. To test the hypothesis that activation of ectoderm differentiation is a normal function of *FoxI1e*, and if so, whether it is required for both neural and epidermal differentiation, we carried out a loss-of-function analysis. We designed a splice-blocking morpholino oligo (SBMO) that spans the splice-acceptor site of the gene. RT-PCR analysis of random hexamer primed cDNA from stage-14 embryos previously injected with the SBMO confirmed that it inhibits *FoxI1e* mRNA maturation (Fig. 5A). It has been previously reported that the use of a morpholino that targets the translational start site results in embryonic death by the gastrula stage (Suri et al., 2005). To determine if *FoxI1e* mRNA depletion using a splice-blocking morpholino is toxic, we performed an animal cap assay to see if embryos depleted of FoxI1e respond normally to mesoderm induction by activin. Embryos were injected at the two-cell stage with 40 ng SBMO and animal caps excised at the late blastula stage (stage 9) were treated with activin. Animal caps depleted of FoxI1e showed the same level of mesoderm induction as control animal caps (Fig. 5C). Therefore, loss of FoxI1e is not inherently toxic to embryonic cells.

To determine the role of *FoxI1e* in embryonic development, we first examined the effects of global loss of *FoxI1e* function on early development, by injecting whole embryos at the two-cell stage with 20, 40 or 60 ng of the SBMO, into the animal region of each cell. FoxI1e-deficient embryos displayed dose-dependent abnormalities beginning at the gastrula stage. All injected embryos were delayed during gastrulation, but gastrulated completely by the time sibling controls reached mid-neurula stage (stage 16). Cement gland and neural-fold formation was also delayed dose-dependently (Fig. 5D). During early tailbud stages, FoxI1e-deficient embryos failed to

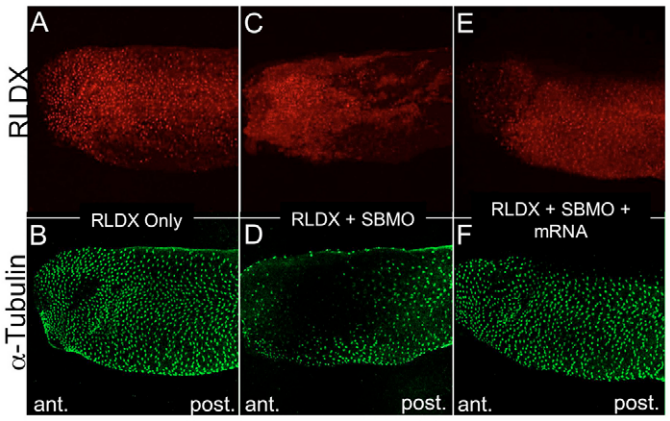

Fig. 6. Loss of FoxI1e causes a rescuable loss of epidermal cilia. Control embryos (**A**,**B**) injected with RLDX (red) in an animal, ventral cell at the eight-cell stage and stained for α-tubulin (green) have a normal cilia pattern in injected cells. (**C**,**D**) Co-injection of SBMO resulted in loss of cilia in cells that received the morpholino. (**E**,**F**) SBMO-resistant *FoxI1e* mRNA was injected after the SBMO, restoring normal cilia formation.

elongate properly along the anteroposterior axis, had reduced head structures and began to develop gaps in their epidermis (Fig. 5E). The epidermal lesions in embryos injected with the highest does of the SBMO resulted in death of the embryos by osmotic lysis by the early tailbud stage. A morpholino oligonucleotide designed against the splice donor site of the *FoxI1e* mRNA caused similar but less severe effects (data not shown). RT-PCR analysis of embryos injected with this morpholino shows that *FoxI1e* mRNA maturation is not efficiently inhibited (Fig. 5B). We therefore used the splice acceptor site-targeting oligo for the rest of our experiments. Analysis of gene expression at stage 23 by RT-PCR in embryos injected with *FoxI1e* mRNA shows a downregulation of both neural and epidermal genes. mRNAs encoding the pan-epidermal genes such as *E-cadherin* and *epidermal cytokeratin* were reduced, as were those encoding pan-neural genes such as *Sox-2*, and the neural crest gene *slug* (Fig. 5F).

To test whether *FoxI1e* plays a role in patterning, rather than a general role in ectodermal differentiation, we assayed the expression levels of anterior neural (*FoxG1/BF-1*) and posterior neural (*Xlhbox6/HoxB9*) genes. Both were consistently and equally reduced, showing that the effect was pan-neural, rather than regional. To test the effect on mesodermal patterning, embryos at the gastrula stage (stage 11.5) were assayed for expression of *Xbra* (pan-mesodermal), *Xwnt8* (lateral and ventral mesoderm) and *chordin* (dorsal mesoderm). *Xbra* and *Xwnt8* were unaffected by depletion of FoxI1e in five repeats of the experiment, while *chordin* showed no consistent change (Fig. 5G). Reintroduction of morpholino-resistent *FoxI1e* mRNA produces a modest but reproducible rescue of expression of *epidermal cytokeratin*, *E-Cadherin* and *slug* (Fig. 5F).

From the tailbud stage (stage 22) onward, the *Xenopus* epidermis contains cilia, the beating of which causes gliding movements of the larva over the floor of the culture dish. We noticed that FoxI1e-deficient embryos, unlike control embryos, failed to do this. To determine if this was due to lack of cilia, we dissected out the role of *FoxI1e* in the epidermis using the *Xenopus* fate map. We injected 10 ng SBMO, together with RLDX as a lineage tracer, into one animal, ventral cell at the eight-cell stage. In a normal embryo, this cell contributes to most of the epidermis on one side of the embryo (Dale and Slack, 1987). Embryos injected with RLDX alone had large clones of fluorescent cells in the epidermis, which showed normal patterns of cilia on their surfaces, as revealed by immunostaining for α-tubulin (0% abnormal, n=52). Embryos injected with RLDX, together with the SBMO, also had clones of labeled cells in the epidermis. However, these had dramatically decreased numbers of cilia, compared with cells derived from non-injected cells (non-fluorescent clones) in the same embryo (67% abnormal, n=66). To show that this effect is specific, we followed the injection of SBMO and RLDX with another injection of *FoxI1e* mRNA with GFP mRNA as a lineage label. Reintroduction of morpholino-resistant mRNA rescues the loss of cilia (6% abnormal, n=68) (Fig. 6A-F).

We next wanted to determine if FoxI1e is necessary for the activation of ectoderm in the early embryo. To do this, we injected 60 ng SBMO into two-cell stage embryos and dissected the animal caps at stage 7, before the onset of zygotic transcription. Removal of the animal cap before the mid-blastula transition ensures that the cells of the animal cap are never exposed to mesoderm-inducing factors. If FoxI1e works only by repressing endoderm and mesoderm in the animal cap, levels of early ectodermal marker expression would be unchanged. If, however, there were a reduction in those markers, we would conclude that FoxI1e actively promotes ectoderm formation. Animal caps cultured alone from stage 7 would be expected to form only epidermal markers, as they are not exposed to Bmp inhibitors from the organizer, which cause neural specification. In this experiment, we therefore compared caps cultured alone, to analyze their ability to form epidermis in the presence and absence of FoxI1e, and injected with Bmp inhibitors, to analyze their ability to express neural markers in the presence and absence of FoxI1e. In uninjected animal caps, *E-cadherin* and *epidermal cytokeratin* mRNAs are expressed, but not those of the neural markers *Sox-2*, *NRP-1* or *NCAM*. Injection of mRNA encoding cmBmp7, which inhibits all Bmp signaling, causes a reduction in epidermal markers and an increase in neural markers (Hawley et al., 1995). In this experiment, embryos were injected with the SBMO at the two-cell stage, and some were further injected with *cmBmp7* mRNA at the four-cell stage. Animal caps were excised at stage 7 and cultured until stage 14. We found by RT-PCR that animal caps depleted of FoxI1e expressed reduced levels of epidermal markers relative to uninjected animal caps, and that those injected with *cmBmp7* mRNA and depleted of FoxI1e expressed reduced levels of neural genes relative to those injected with *cmBmp7* mRNA alone (Fig. 7A-E). As expected in caps excised at stage 7, mesodermal gene transcripts were almost undetectable (Fig. 7F). From these data, we conclude that FoxI1e actively promotes ectodermal fates, both neural and epidermal, in animal cap cells.

FoxI1e controls the localization of ectodermal cells within the embryo

One of the gain-of-function effects of FoxI1e was that vegetal cells expressing it moved to the ectoderm rather than the endoderm. This raises the possibility that one of the functions of FoxI1e might be to control positions of cells expressing it in the embryo. It is known that regional cell localization is strictly controlled in the blastula. There is limited cell mixing, as indicated by the existence of a fate map, and cells from different regions that are mixed will rapidly sort out again (Turner et al., 1989). However, very little is known about the molecular mechanisms of this process, or whether it is part of the ectodermal specification that takes place during the blastula stage. If FoxI1e were required for maintaining animal cells in the animal

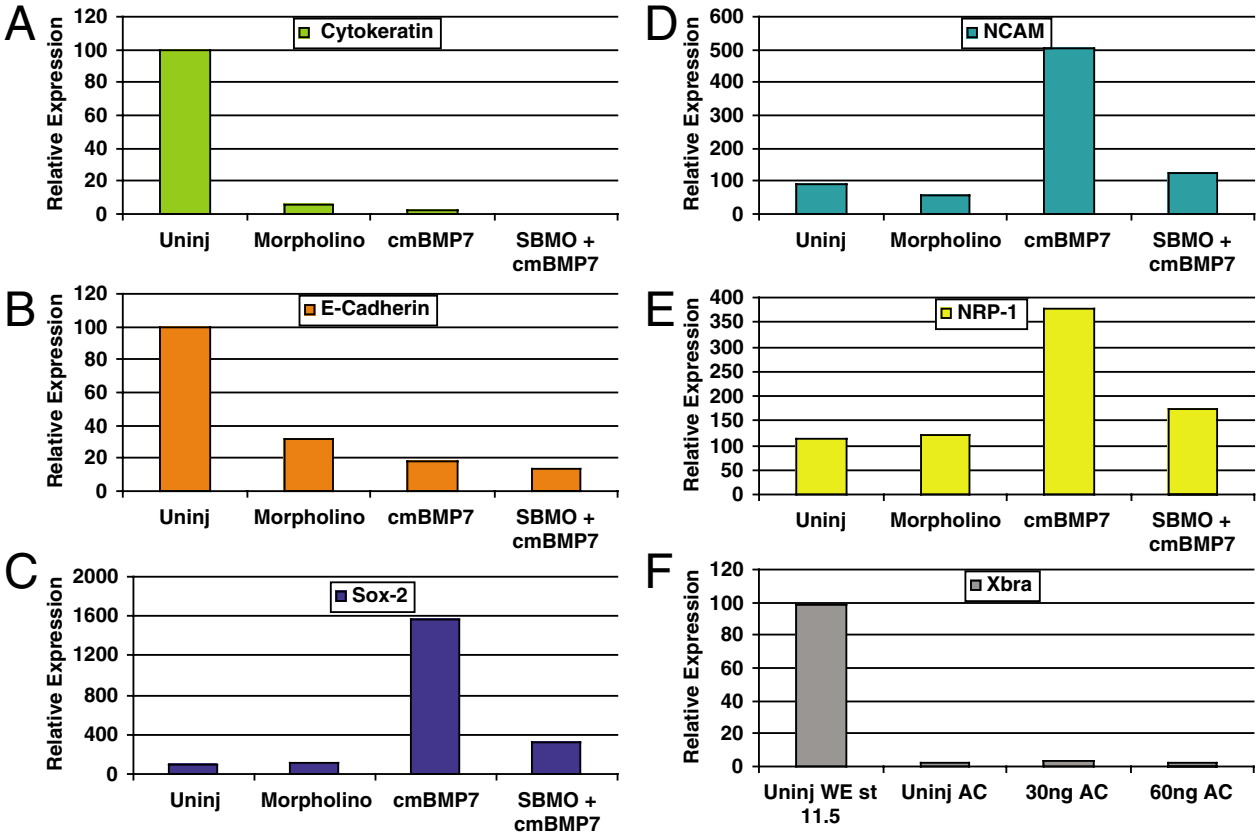

Fig. 7. FoxI1e actively promotes ectoderm formation. Embryos were injected at the two-cell stage with SBMO and then at the four-cell stage with cmBMP7. Animal caps were excised at stage 7 and cultured to stage 14. (**A**,**B**) *Epidermal cytokeratin* and *E-cadherin* are expressed in uninjected (epidermal) animal caps. Loss of FoxI1e downregulates expression of these markers. cmBMP7 neuralizes these caps, so the epidermal markers are reduced. (**C-E**) The neural markers *Sox-2, NCAM* and *NRP-1* are induced relative to controls in cmBMP7-injected (neural) caps. This induction requires the presence of *FoxI1e*. Therefore, *FoxI1e* is necessary for expression of both epidermal and neural genes. (**F**) Expression of the mesodermal marker *Xbra* at stage 11 in caps excised at stage 7. Whole embryo expression is shown as a reference.

hemisphere, then removing it would cause them to relocate to other regions of the embryo. To test this hypothesis, we injected RLDX alone or RLDX + 15 ng SBMO into a single ventral animal cell at the 32-cell stage (cell A4). Embryos were fixed at the tailbud stage (stage 40) and stained for cilia with α-tubulin antibody. The descendents of this cell are normally restricted to the epidermis and are therefore located at the surface of the tailbud stage embryo. This was the case in embryos that were injected with RLDX alone (92%, *n*=60, Fig. 8A). Of the remaining 8%, the most frequent location of extra-epidermal cells was the CNS. However, even in these embryos, the vast majority of signal is in the epidermis, with only occasional cells elsewhere. In embryos co-injected with 15 ng SBMO, there was a dramatic decrease in the amount of RLDX signal at the surface of the embryos, indicating fewer descendents of the injected cell had reached their normal position within the embryo (Fig. 8B). Instead, there was a collection of RLDX-positive cells in the abdomen of the embryo (72%, *n*=60). We performed similar experiments for cells that normally contribute to the nervous system. We injected RLDX alone or RLDX + 15 ng SBMO into a single dorsal animal cell at the 32-cell stage (cell A1) (Dale and Slack, 1987) and cultured the embryos to the tailbud stage (stage 35-37). This cell would normally give rise to descendents in the nervous system. Eighty-seven percent of RLDX-injected embryos had signal restricted to neural structures (*n*=45). As with the ventral injection, there were a small number of embryos that had cells in other structures, primarily the epidermis. In embryos co-injected with SBMO, signal was found both in the nervous system and in the gut (25%, *n*=32).

There are three possibilities regarding the status of cells that have a location affected by loss of *FoxI1e*. They may eventually die, they may migrate to inappropriate locations but retain their identities as CNS or epidermis, or they may change their fate completely to one appropriate to their new locations. To determine which of these possibilities was the case, we allowed embryos treated as above to develop to the stage at which feeding had started, and the gut epithelium had differentiated (stage 47) so that we could determine the identities of ectopic cells. In embryos injected in A4 with RLDX only, cells were located in the skin or in the skin and nervous system. The structure of the cells was clearly visible by DIC (Fig. 8C). In agreement with quantitative fate map studies (Dale and Slack, 1987), some cells were found scattered in other germ layers (data not shown). In embryos co-injected with the splice-blocking morpholino, large groups of RLDX-positive cells were clearly visible along loops of the intestinal epithelium and contributed to the brush border along with RLDX-negative cells (Fig. 8D). Experiments in which we injected the A1 blastomere (which gives rise predominantly to the CNS), rather than A4, gave corresponding

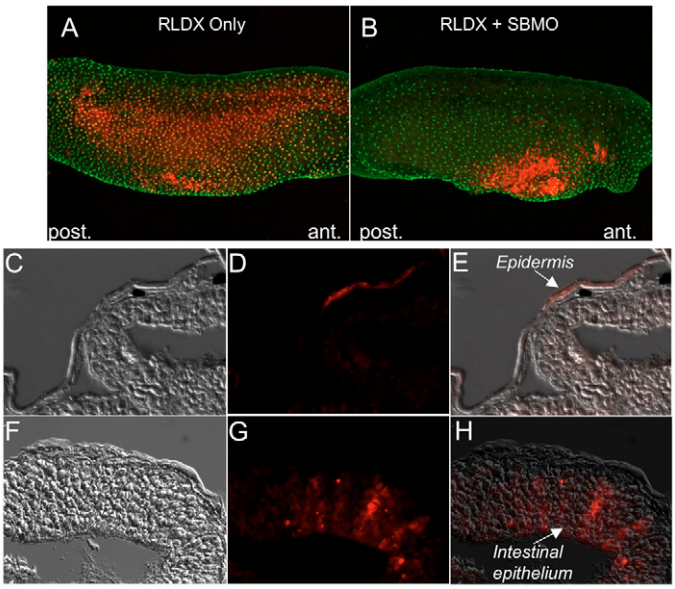

Fig. 8. FoxI1e controls regional position of ectodermal cells. (**A**,**B**) Stage 25 embryos injected at the 32-cell stage in the A4 blastomere, a precursor of epidermis, with RLDX (red) alone (A) or with RLDX + SBMO (B) and then stained for α-tubulin (green). With RLDX alone, injected cells co-localize with cilia (A). With SBMO, the cells are located in the interior of the embryo (B). At stage 47, embryos were sectioned and embedded. (**C-E**) DIC and fluorescent images of a control embryo injected with RLDX in A4. RLDX is localized to the epidermis. (**F-H**) Co-injection with SBMO causes cells to localize to the gut. These cells give rise to morphologically normal endodermal structures, including the intestinal epithelium.

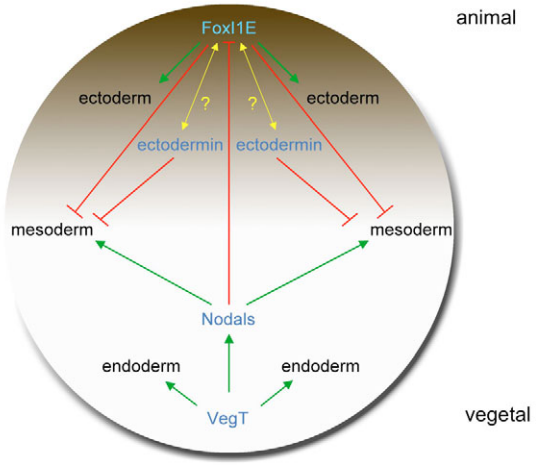

Fig. 10. Model of germ-layer formation.

results. In RLDX-only embryos, the majority of cells contributed to the CNS, but when co-injected with SBMO, they also contributed to normal gut epithelium (data not shown).

To test when the descendents of animal blastomeres lacking *FoxI1e* first leave the ectoderm and enter other germ layers, we took embryos that had been injected with a lineage marker, Fluorescein-dextran (FLDX), with or without the SBMO as described above in the A4 cell, and fixed and bisected them at stages 9, 10, 11 and 18. At stages 9 and 10, the SBMO-co-injected embryos were identical to FLDX-only embryos, with a small patch of fluorescence in the ventral ectoderm (data not shown). However, at stage 11, there was reduced signal in the ectoderm on the surface of the gastrula. Instead, when the embryos were bisected, a group of FLDX-positive cells were found dissociated on the floor of the blastocoel, which had lost adhesion to the animal cap (94%, n=65, Fig. 9A-D). This effect was rescued by injection of 5-10 pg of the *FoxI1e* mRNA into the animal cytoplasm at the two-cell stage, followed by injection of the morpholino at the 32-cell stage as before (Fig. 9E-G). These data suggest that *FoxI1e* regulates the adhesion of the animal cells of the gastrula. In its absence, the affected cells lose contact with one another and end up in other locations in the embryo.

DISCUSSION

These data identify FoxI1e as playing a major role in ectoderm differentiation. Gain-of-function experiments show that it activates ectoderm gene expression in cells that do not normally express it, and that its absence from cells that do normally

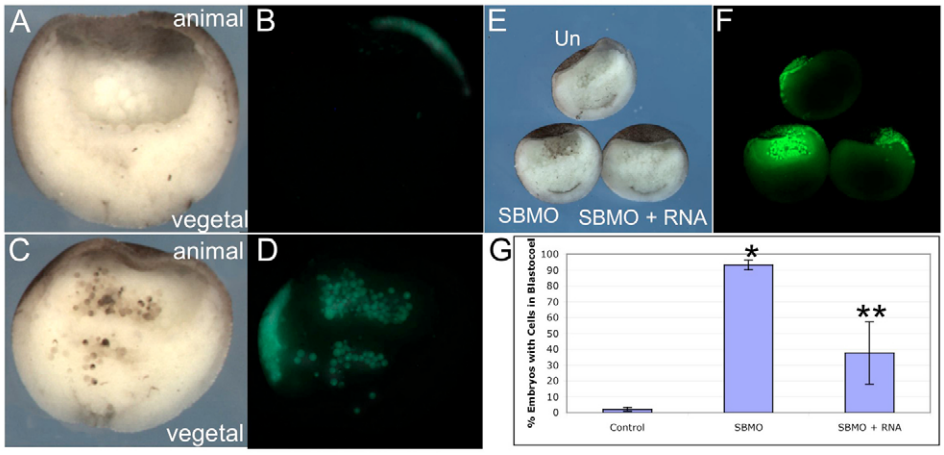

Fig. 9. FoxI1e is required for normal ectodermal cell adhesion in the gastrula. (**A**,**B**) Brightfield and fluorescent images of a hemisected control stage 11 embryo injected with FLDX alone into A4 at the 32-cell stage. (**C**,**D**) Co-injection of SBMO causes the cells to disaggregate and fall into the blastocoel. (**E-G**) This effect is rescued by morpholino-resistant mRNA injected at the two-cell stage. *P=8.8×10^{-5}, **P=0.03.

express it causes downregulation of the expression of ectodermal genes. In addition, the fact that both epidermal and neural markers are downregulated after depletion of FoxI1e suggests that it plays a role in both epidermal and neural differentiation. After gastrulation, FoxI1e is rapidly lost from cells forming the neural plate, which suggests that it plays a role before the division of the ectoderm into epidermal and neural progenitors.

The data also show that FoxI1e controls cell position in the early embryo. It has been known for many years that there is a good fate map in the early *Xenopus* embryo. In fact, this is one of the attractive features of using *Xenopus* as a model for early vertebrate development. However, a fate map requires limited cell mixing during early development, a fact that has also been shown by blastomere sorting experiments (Turner et al., 1989) and tissue recombination experiments (Nieuwkoop, 1973; Smith and Slack, 1983). Although it is generally understood that as germ-layer specification occurs genes are expressed that somehow control differential adhesion in the embryo, and maintain the separation of these nascent germ layers in the blastula, its mechanism is not known. The data presented here show that molecules that control regional identity of animal cells are controlled by FoxI1e. This represents the first developmental system in which it has been shown that a forkhead-class transcription factor controls cell position in the embryo.

It is interesting to note that animal cells lacking FoxI1e that lose contact with the rest of the animal cell population do not die or differentiate as ectopic ectoderm, but instead become specified according to their new positions. This suggests that the normal function of FoxI1e is to switch animal cells from a pluripotent state to a more specified state, and in its absence, animal cells remain pluripotent.

The expression pattern of *FoxI1e* is interesting because in situ hybridizations do not show expression in every cell of the tissues in which it is expressed. In fact, this information is present on a website that documents the results of an expression screen (http://xenopus.nibb.ac.jp), but has not been previously published. This variegated pattern of expression could be due to cell cycle control of expression, in which case it would be expressed in all cells, but not at the same time, once cell divisions have become asynchronous. Alternatively, intercellular signaling could restrict expression, by mutual inhibition mechanisms, to one cell surrounded by non-expressing cells. The mechanisms underlying this expression pattern, as well as its function, require further analysis.

A previous functional analysis of *FoxI1e* (Suri et al., 2005) suggested that its primary role was to act as an inhibitor of mesoderm specification. This work used an antisense morpholino oligo that caused the embryos to die at the gastrula stage, so that analysis past this point could not be carried out. Before the publication of the Suri et al. paper, we had started a functional analysis using a sequence in the same region of the mRNA. However, its toxicity made us look for alternative sequences that we could use. After screening a number of sequences, we chose a morpholino oligo that blocks maturation of the mRNA, by binding to a splice acceptor site. This oligo did not cause any general toxicity of the embryo, and thus allowed a more detailed functional analysis of FoxI1e, which revealed the fact that it plays essential roles in the activation of ectodermal genes as well as regional identity of the animal cells.

The identification of the roles of FoxI1e allows a more complete model to be proposed of the cell interactions that lead to the division of the blastula into three cell populations (Fig. 10). The maternal activators of *FoxI1e* expression must be present throughout the early embryo, because, in the absence of VegT, molecular markers of both neural and epidermal lineages appear in marginal and vegetal cells (Xanthos et al., 2001; Zhang et al., 1998). Alternatively, in the absence of VegT, the influence of ectoderm-determining molecules could reach further vegetally due to a reduction in ectoderm-inhibitory factors. In areas of high levels of nodal expression, downstream of VegT, *FoxI1e* expression is inhibited. In the most animal region, where nodal expression is lowest, *FoxI1e* expression is activated. Once *FoxI1e* is expressed, a mutual inhibition is set up that generates a progressively more precise boundary between the ectoderm and the other two germ layers. Cell mixing across this boundary is prevented by expression of surface proteins that control cell position by differential adhesion. Many elements of this model require further testing, including the functional relationship between FoxI1e and other factors known to be important in ectoderm differentiation such as ectodermin (Dupont et al., 2005) Xoom (Hasegawa et al., 2001) and Xlim5 (Houston and Wylie, 2003), as well as the epidermal proteins AP-2 (Luo et al., 2002) and grainyhead-like1 (Tao et al., 2005), and the many early CNS factors including Sox2 and Sox3. It is also likely that there will be signaling pathways in addition to nodals that control the spatial and temporal expression of FoxI1e, and we know little detail concerning its downstream targets. However, the outline of a global mechanism of germ-layer formation is beginning to emerge.

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