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Maternal Oct1/2 is required for Nodal and Vg1/Univin expression during dorsal–ventral axis specification in the sea urchin embryo

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ABSTRACT

The TGFβ family member Nodal is expressed early in the presumptive ventral ectoderm of the early sea urchin embryo and its activity is crucial for dorsal–ventral (D/V) axis specification. Analysis of the *nodal* promoter identified a number of critical binding sites for transcription factors of different families including Sox, Oct, TCF and bZIP, but in most cases the specific factors that regulate *nodal* expression are not known. In this study, we report that the maternal factor Oct1/2 functions as a positive regulator of *nodal* and that its activity is essential for the initiation of *nodal* expression. Inhibition of Oct1/2 mRNA translation produced embryos with severe axial defects similar to those observed following inhibition of Nodal function. We show that perturbing Oct1/2 function specifically disrupted specification of the ventral and dorsal ectodermal regions and that these effects were caused by the failure of *nodal* to be expressed early in development. Furthermore, we identified the key gene *vg1/univin*, which is also necessary for *nodal* expression, as an additional factor that was completely dependent on Oct1/2 for its zygotic expression. These data demonstrate that the maternal Oct1/2 protein plays an early and essential role in D/V axis specification by initiating the expression of *nodal* and *vg1/univin*, two genes that act at the top of the D/V ectoderm gene regulatory network.

Introduction

A combination of maternally localized factors and inductive interactions establish the dorsal/ventral (D/V) axis in many deuterostomes from sea urchins to vertebrates. In anamniote vertebrate embryos, maternal factors are used to initiate the expression of a conserved gene regulatory network (GRN) in the dorsal region, creating the "organizer" that patterns the D/V axis (Kimelman, 2006). For example, in *Xenopus* embryos, the initial polarization of the D/V axis relies on translocation of a maternal *wnt11* mRNA towards the dorsal region of the embryo where the corresponding protein will activate canonical Wnt (cWnt) signaling, resulting in nuclear localization of β-catenin (Tao et al., 2005). Then, at the onset of zygotic transcription this nuclear β-catenin overlaps in the dorsal-vegetal blastomeres with the vegetally produced maternal transcription factor VegT and the TGFβ signaling molecule Vg1. This combination of maternal VegT, Vg1 signaling, and cWnt signaling acts synergistically to trigger expression of high levels of Nodal-related signaling molecules (Xnrs), resulting in activation of the zygotic organizer GRN (Agius et al., 2000; Birsoy et al., 2006; Zhang and King, 1996; Zhang et al., 1998). Even in chicken and mice embryos where it is thought that axis specification relies entirely on zygotic factors, interactions between the Vg1, Nodal, and cWnt signaling pathways are essential for organizer specification (Andersson et al., 2006; Bertocchini et al., 2004; Chen et al., 2006; Cheng et al., 2003; Skromne and Stern, 2001, 2002). Taken together, these data suggest a conserved role for the synergistic activity of Nodal, Vg1, and cWnt signaling pathways in specifying the D/V organizer in vertebrates.

Sea urchins belong to a phylum that diverged early in the deuterostome lineage. Although the adult body plan of echinoderms is radial this feature is considered a recent modification of a bilateral body plan and sea urchin embryos, like embryos of other bilaterians, posses a larval D/V axis (also known as the oral–aboral axis). Unlike the animal–vegetal axis, the specification of the sea urchin D/V axis is highly regulative and does not strongly rely on spatial anisotropies in the egg cytoplasm. The fact that each blastomere at the 4-cell stage can respecify a D/V axis after dissociation illustrates this trait (Horstadius, 1973). Therefore, D/V axis specification relies on cell interactions rather than on inheritance of localized determinants in the sea urchin. Genes encoding TGFβs of the Nodal and Vg1 families play crucial roles in these cell interactions. Nodal is, so far, the earliest factor expressed asymmetrically along the D/V axis and its function is essential for establishment of the D/V polarity. Expression of *nodal* is strictly zygotic with *nodal* transcripts being first detected in the embryo at the 32/60 cell-stage. Examination of *nodal* expression at these early stages revealed that this gene is first expressed broadly in most cells of the embryo then rapidly restricted to a smaller region of the ectoderm that corresponds to the presumptive ventral ectoderm. When the function of Nodal is blocked, specification of both the ventral and the dorsal ectoderm fails and the embryos develop with a strongly radialized phenotype. Consistent with these observations,

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Nodal activates in the ventral ectoderm the expression of key transcription factors and signaling molecules such as Gsc, FoxA, Chordin, Lefty, and BMP2/4, that are essential for cell fate specification along the D/V axis (Duboc et al., 2004; Lapraz et al., 2009; Saudemont et al., 2010). Furthermore, Nodal and BMP2/4 regulate the expression of signaling molecules and transcription factors in the endoderm and mesoderm (Duboc et al., 2010), thus imposing D/V polarity on all three germ layers.

In the sea urchin, like in *Xenopus* and zebrafish, several maternal factors are necessary for the initiation of *nodal* expression and D/V axis specification. One of these factors, Vg1/Univin, is the sea urchin orthologue of Vg1/GDF1, which likely functions as a heterodimerization partner for Nodal (Tanaka et al., 2007). Vg1/Univin is first expressed uniformly and maternally, then zygotic Vg1/Univin transcripts accumulate in a belt of ectodermal cells surrounding the equatorial region of the embryo. Thus, both maternal and zygotic expression of *vg1/univin* overlaps with early *nodal* expression. Strikingly, blocking translation of the *vg1/univin* mRNA abolishes *nodal* expression and mimics the Nodal loss-of-function phenotype. Therefore, in the sea urchin, Vg1/Univin and Nodal play equally important roles during D/V axis formation. Another maternal factor required for *nodal* expression and D/V axis formation is TCF, the effector of the cWnt signaling pathway. When the function of TCF is blocked, *nodal* expression is abrogated (Duboc et al., 2004). Therefore, both cWnt and Vg1 signaling work together to activate and/or maintain the levels of *nodal* expression necessary to specify and pattern the D/V axis of sea urchin embryos, which is remarkably similar to what is observed in vertebrate embryos.

Although D/V axis formation is thought to rely on cell interactions rather than on localized determinants of cell fates, several intriguing results suggest that an oxidative gradient in the early urchin embryo may influence D/V axis formation. Many years ago, experiments showed that respiratory inhibitors could bias D/V axis orientation (Child, 1941; Coffman and Davidson, 2001; Coffman and Denegre, 2007; Czihak, 1963; Pease, 1941). More recently, it was shown that asymmetrically localized mitochondria in the egg might prefigure the ventral side of the embryo and localized microinjection of purified mitochondria on one side of the embryo caused that side to tend toward a ventral fate (Coffman et al., 2004). Furthermore, by simultaneously examining the spatial distribution of mitochondria in the early embryo and the pattern of pSmad2/3 by immunostaining, a modest but significant correlation between Nodal signaling activity and mitochondrial distribution was found (Coffman et al., 2009). The higher mitochondrial gradient presumably makes one side of the embryo more oxidative and produces reactive oxygen species (ROS) that may act as signaling effectors. Indeed, Coffman et al. showed that overexpression of a mitochondrially targeted catalase, which decreases H_2O_2 levels, into one blastomere at the 2-cell stage biased orientation of the dorsal–ventral axis, with the progeny of the injected blastomere becoming preferentially dorsal. However, surprisingly, overexpression into one blastomere of a mitochondrially targeted superoxide dismutase that increased the level of mitochondrial H_2O_2, had no effect on the orientation of the D/V axis, indicating that a local increase of ROS signaling might not be sufficient to polarize *nodal* expression. Finally, inhibition of p38 MAP kinase signaling prevents the initial expression of *nodal* in the sea urchin, suggesting that the activation of *nodal* expression requires a phosphorylation event catalyzed by this maternal protein (Bradham and McClay, 2006). Interestingly, the p38 MAP kinase signaling pathway is responsive to ROS in the sea urchin embryo (Coffman et al., 2009). Thus, p38 signaling may be part of the mechanism that links the redox state of the embryo to the activation of *nodal* expression. However, the complete molecular mechanism(s) that translates the redox bias into localized expression of *nodal* is still not established (Agca et al., 2009; Coffman et al., 2009).

In order to identify the maternal factors responsible for the initiation of *nodal* expression, we performed an in-depth cis-regulatory analysis on the sea urchin *nodal* promoter (Range et al., 2007). This analysis allowed us to delineate a proximal regulatory module called the R module, within the 5′ regulatory region of *nodal* and to identify several conserved binding sites for transcription factors necessary for *nodal* transcriptional activation. Intriguingly, one of these predicted binding sites was for a transcription factor of the Oct family that has several members known to be responsive to oxidative stress and to p38 MAP kinase signaling (Kang et al., 2009b; Saxe et al., 2009). The sea urchin genome encodes a single *oct* gene that is most closely related to the Oct1/Oct2 class of Oct factors (Oct1/2). Previous studies using antisense DNA oligonucleotides suggested that Oct1/2 function is required during cleavage for transcription of early histone genes (Char et al., 1994). In this study, we reinvestigated the function of Oct1/2 during early development of the sea urchin embryo using antisense morpholino oligonucleotides. We report that Oct1/2 plays an important role in D/V axis formation by regulating the zygotic expression of *nodal* and *vg1/univin*, two key genes that initiate the D/V gene regulatory network.

Material and methods

Animals, embryos, and treatments

Paracentrotus lividus sea urchins were collected in the Bay of Villefranche. Gametes and embryos were handled as described previously (Lepage and Gache, 1989, 1990).

Cloning of PlOct1/2 cDNA

P. lividus oct1/2 was isolated from as a *P. lividus* EST clone after a search of the EST database (Marine genomics). The clone was fully sequenced and it contained both the 5′UTR and the 3′ stop site. The GenBank accession number for *P. lividus* Oct1/2 is GU270855.

Morpholino and mRNA injections

The *P. lividus* Oct EST sequence was used to generate two separate antisense morpholino oligonucleotides and was produced by GeneTools LLC (Eugene, OR). The sequences for the morpholino oligonucleotides are:

Oct 1: 5′-TCATGATGAGGACACCGTTTGATCT-3′.

Oct 2: 5′-GTGTGATTTCGACAGTCTTCCGATG-3′. The Oct1 and Oct2 morpholinos were injected at 0.6–0.7 mM and 1.8 mM, respectively. The efficiency of these morpholinos was tested by showing that they effectively block translation of a synthetic mRNA made of the Venus open reading frame fused to the 5′UTR of Oct1/2 (see Supplementary Fig. 3a).

pCS2 and pCS2-Venus constructs containing the coding sequence and the 5′UTR of *P. lividus* Oct1/2 were generated by standard molecular biology techniques. The clones were verified by sequence analysis. The 5′UTR sequence of Oct1/2 used to test the efficacy of the Oct1/2 morpholino 1 was: 5′-TGAAGATCAAACGGTGTCCTCATCATGACC-3′. Capped mRNA was synthesized from Not1 linearized templates using mMessage Machine kit (Ambion). Embryos were co-injected with morpholino-oligonucleotides (0.6–1.8 mM) and rhodamine dextran (5 mg/mL). *P. lividus* Oct1/2 mRNA was injected up to 4 μg/μL. In rescue experiments, 2 μg/μL of Oct1/2 mRNA was co-injected with 0.7 mM of Oct1/2 morpholino 1. Venus–Oct1/2 mRNA was injected from 0.1 to 1 μg/μL. All microinjected embryos were fertilized and raised at 18 °C.

Expression analysis

The whole-mount in situ hybridization probes for *P. lividus nodal, vg1, gcm, tbx2/3, foxA, chordin, pax2/5/8 and hnf6* were previously described (Duboc et al., 2010 #12691; Duboc et al., 2004; Range et al., 2007; Rottinger et al., 2008). The probe for *P. lividus* Oct1/2 corresponds to the full-length cDNA sequence. The hybridization was carried out as described inDuboc et al.(2004).

Results

Identification of the P. lividus Oct1/2 transcription factor

During our recent characterization of the *nodal* promoter, we showed that at least six transcription factors necessary for *nodal* activation and/or autoregulation likely bind to the 5′ proximal region of the promoter (Fig. 1). In this study, we focused on a site similar to the Octamer transcription factor binding site that caused a 70% decrease in reporter gene activity when mutated (Range et al., 2007). The only known factors that bind this site belong to the Pou domain family, including class 2 (Oct1, Oct2), 3 (Brn-1, Brn-2, Brn-4), and 5 (Oct4) Pou domain proteins (Kang et al., 2009b). The sea urchin encodes one class 2 homolog, Oct1/2, and one class 3 homolog, Brn1/2/4, but not a class 5 ortholog (Howard-Ashby et al., 2006). Of the two homologs, we focused on Oct1/2 because it is abundantly expressed in a spatiotemporal expression profile consistent with a role as an activator of *nodal* expression, whereas Brn1/2/4 is expressed during cleavage at levels below the limits of detection by in situ hybridization, according to QPCR and in situ data from this and other studies (Cole and Arnone, 2009; Howard-Ashby et al., 2006; Yuh et al., 2005). Furthermore, *Strongylocentrotus purpuratus* Oct1/2 binds to a site similar to the Oct1/2 consensus site in the *nodal* promoter (Char et al., 1994) (Fig. 1). Therefore, we searched a *P. lividus* EST database and isolated a Oct1/2 cDNA with a 2406 bp open reading frame encoding a protein of 802 amino acids. The predicted *P. lividus* Oct1/2 protein sequence shares 80% identical residues with the *S. purpuratus* Oct1/2 protein sequence and contains the two regions characteristic of Pou family of transcription factors, the Pou and Pou-specific homeodomain DNA binding domains. The predicted protein sequence of these domains shows conservation of 91% and 69% with the vertebrate Pou and homeodomain, respectively.

Oct1/2 is a maternal factor with a dynamic expression pattern during development

P. lividus embryos ubiquitously expressed Oct1/2 transcripts before fertilization and this expression pattern persisted through the cleavage stages (Fig. 2A-C). During early blastula stages, the expression of *oct1/2* dropped precipitously throughout the embryo so that very little to no expression can be observed during the hatched blastula and mesenchyme blastula stages (Fig. 2D-G). Zygotic expression of *oct1/2* began during the gastrula stages and was restricted to the invaginating endoderm and excluded from the delaminating non-skeletogenic mesoderm (NSM) (Fig. 2H). At prism stages, *oct1/2* expression persisted in the gut, but was also expressed in what appeared to be the oral ectoderm and/or ciliary band of the embryo (Fig. 2I,J). These data are consistent with QPCR data collected in *S. purpuratus* that show a high maternal load of *oct1/2* drops below the threshold detectable by in situ hybridization by the mesenchyme blastula stage and that embryos initiate zygotic expression around the early gastrula stage (Howard-Ashby et al., 2006). The early spatial and temporal expression pattern of maternal *oct1/2* is the most relevant to this study. This pattern suggests that Oct1/2 is a candidate for the initiation of *nodal* transcription at the 32–60 cell stage, which is first activated broadly in the early embryo, then subsequently restricted to the ventral ectoderm.

Loss of Oct1/2 function severely perturbs dorsal–ventral axis formation

The sea urchin larval D/V axis is first observed phenotypically during the early gastrula stage when the ventral side of the embryo flattens out compared to the dorsal side and the skeletogenic mesenchyme cells migrate to form two ventral–lateral clusters that initiate spiculogenesis (Fig. 3C). In addition, the archenteron bends towards the ventral side during the late gastrula stage, eventually fusing with the stomodeum to form the larval mouth. In Nodal loss-of-function embryos, the ectoderm and the skeletogenic mesenchyme cells are radialized, the gut remains vertical and the embryo never forms a mouth (Duboc et al., 2004). Because our cis-regulatory analysis suggested that an Oct factor may be necessary for *nodal*'s expression, we hypothesized that embryos injected with morpholinos directed against Oct1/2 would have a similar phenotype to the Nodal loss-of-function phenotype. Oct1/2 morphants developed normally through the cleavage stages until the beginning of gastrulation, which is marked by skeletogenic mesenchyme cell ingression into the blastocoel (Fig. 3A,D). Soon after this stage, the archenteron of control embryos invaginated and elongated into the blastocoel. In contrast, Oct1/2 morpholino-injected embryos remained at the late mesenchyme blastula stage, suggesting a possible role for Oct1/2 during gastrulation. After a delay of up to 6–7 h, normal gastrulation began in Oct morphants and these embryos showed defects in axial patterning, including a radialized ectoderm, skeletogenic mesenchymal cells that do not form ventral–lateral clusters, and a vertical gut during gastrula and prism stages (Compare Fig. 3C-E with 3H-J). These Oct1/2 loss-of-function phenotypes are remarkably similar to Nodal loss-of-function phenotypes, suggesting that Oct1/2 may play a role in early *nodal* expression.

Zygotic expression of nodal and vg1/univin requires maternal Oct1/2

Since the Nodal and Oct1/2 loss-of-function phenotypes were similar, we examined *nodal* expression in embryos injected with Oct1/2 morpholinos. Indeed, we found that there was a drastic reduction of

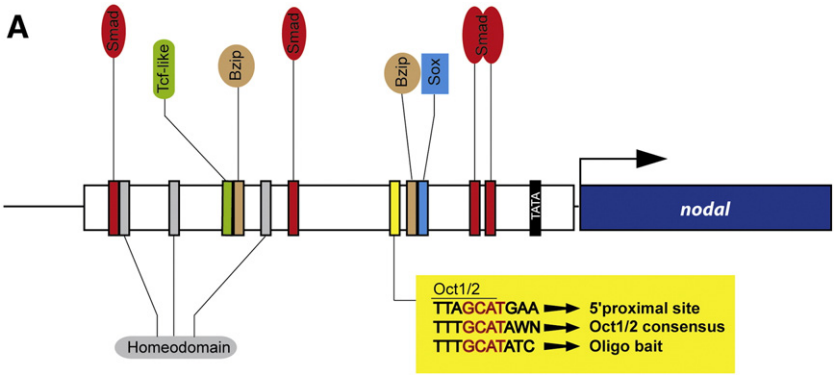

Fig. 1. Identification of *Paracentrotus lividus* Oct1/2. (A) Diagram showing the presumptive binding sites for activators of *nodal* expression in the 350 base pair *nodal* 5′ proximal cis-regulatory module. The position of the Oct1/2 binding site is in yellow. Predicted binding site sequences are shown in the yellow box (Oligo bait refers to the octamer site used to bind Oct1/2 in *S. purpuratus*).

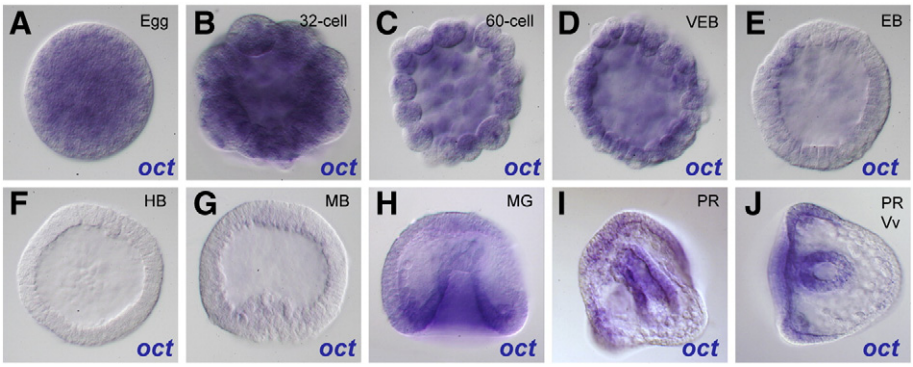

Fig. 2. Spatio-temporal expression pattern of *oct1/2* mRNA during development. Stages of development are shown in the upper right corner of each image. (A-E) Maternal *oct1/2 mRNA* is expressed ubiquitously from fertilization until the early blastula stage. (F,G) *oct1/2* mRNA is not expressed or is at levels below the detection of in situ analysis. (H-J) Zygotic expression of *oct1/2* during gastrula and prism stages. The embryo in I is shown from a dorsal view and the embryo in J is shown from a vegetal view (Vv). VEB, very early blastula; EB, early blastula; HB, hatched blastula; MB, mesenchyme blastula; MG, mid-gastrula; and PR, prism.

nodal expression in embryos injected with either of two Oct1/2 morpholinos at the hatched blastula stage (the developmental stage with highest *nodal* expression) (Fig. 4Ab,c). We used Oct1/2 morpholino 1 (Oct1/2 MO1) for most of the following experiments because it was more efficient than Oct1/2 morpholino 2 (Oct1/2 MO2) at quenching *nodal* expression (Fig. 4Ad).

At the 32- to 60-cell stage, *nodal* is activated in a broad expression domain and then it is progressively restricted to the ventral ectoderm during blastula stages (Fig. 4Bf-j). Soon after its initial activation, Nodal signaling activates its own expression and this autoregulation is critical for the maintenance of *nodal* expression (Nam et al., 2007; Range et al., 2007; Yaguchi et al., 2008). To test whether it is initiation and/or autoregulation of *nodal* that requires Oct1/2 we injected embryos with Oct1/2 morpholino and analyzed the temporal and spatial expression of *nodal* by in situ hybridization. Strikingly, *nodal* expression was completely prevented in Oct1/2 loss of function embryos at the 32- to 60-cell stage and remained downregulated throughout cleavage stages to the beginning of gastrulation (Fig. 4Bk-o). Interestingly, the early expression of *oct1/2* was downregulated by the early blastula stage at a time when the auto-regulation is critical for robust expression of *nodal* (Fig. 4Bc,e), suggesting that Oct1/2 is primarily involved in the initial activation of *nodal*.

In a previous study, we showed that a morpholino directed against translation of both maternal and zygotic *vg1/univin* transcripts prevents early *nodal* expression at the 32- to 60-cell stage until prism stages (Range et al., 2007). We concluded that that an input from early, ubiquitous maternal Vg1/Univin signaling is necessary to activate *nodal* expression. We also hypothesized that zygotic Vg1/Univin, which is expressed in a ring of ectoderm cells overlapping *nodal* expression (Fig. 4Ad), is necessary to maintain *nodal* expression levels during later stages (Range et al., 2007). One possibility to explain the loss of *nodal* expression in Oct1/2 morphants is that Oct1/2 is necessary for zygotic *vg1/univin* transcription. Indeed, we found that zygotic *vg1/univin* expression was severely downregulated in Oct1/2 loss-of-function embryos (Fig. 4Ae,f). Taken together, these data suggest that Oct1/2 is necessary for the initiation and maintenance of *nodal* expression either directly by activating *nodal* expression, or indirectly through the regulation of zygotic *vg1/univin* expression.

Loss of Oct1/2 disrupts the Nodal-dependent D/V ectoderm gene regulatory network

To confirm that Nodal signaling was lost in Oct1/2 morphants, we monitored the expression of early Nodal-dependent genes in the DV GRN, including *chordin* and *goosecoid (gsc)*. In addition, we looked at *tbx2/3*, which encodes an ectoderm factor necessary for dorsal specification whose expression is activated by the diffusion of BMP2/4 secreted from the ventral ectoderm (Lapraz et al., 2009). Expression of

Fig. 3. Loss of Oct1/2 severely perturbs the formation of the dorsal–ventral axis. (A-E) Top row shows early developmental time course of control embryos. (A) mesenchyme blastula stage embryo. (B, C) late gastrula stage embryos from a lateral and vegetal view (Vv), respectively. Notice the flat ventral side with ventral–lateral clusters of PMCs in C. (D, E) prism stage embryo from a dorsal and vegetal view (42 h post-fertilization), respectively. Notice the elongated ventral and dorsal skeletal arms. Blue arrows indicated the ventral (V) and dorsal (D) sides of the embryo. (F-J) Bottom row shows characteristic phenotypes of embryos injected with Oct morpholino. (F) Mesenchyme blastula stage embryo injected with Oct1/2 MO1 is at the same developmental time point as A. (G, H) Late gastrula stage embryos injected with Oct1/2 MO1 from a lateral and vegetal view, respectively. These embryos were 6–7 h delayed from the embryos shown in B and C. Also note that these embryos were radialized, lacking the flat ventral side and organized ventral-lateral PMC clusters. (I, J) Oct1/2 MO1 injected prism stage embryos from a lateral and vegetal view, respectively. These embryos were still clearly radialized. Blue circle indicate radialized embryos.

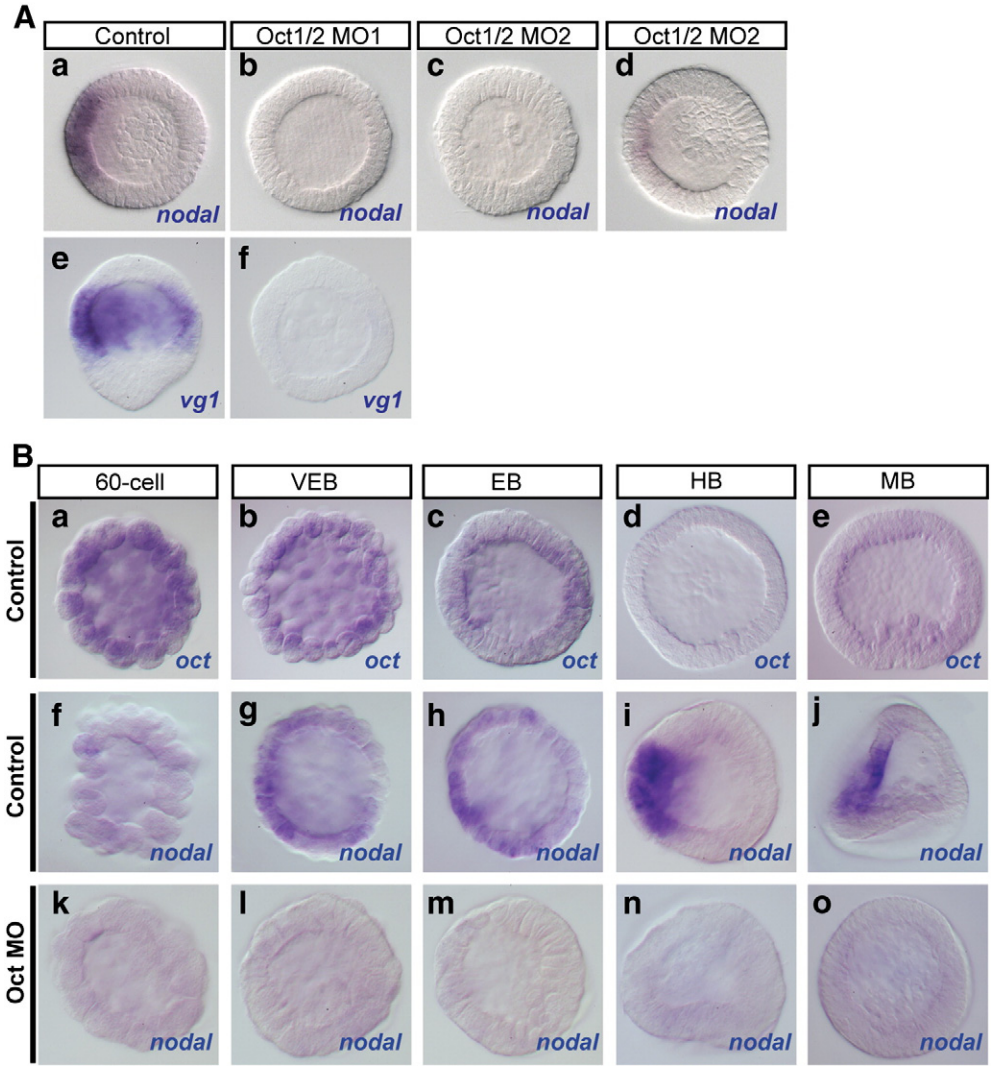

Fig. 4. Zygotic expression of *nodal* and *vg1/univin* requires maternal Oct1/2. (Aa-d) Effects of Oct1/2 MO1 and Oct1/2 MO2 on the expression of endogenous *nodal* in hatched blastula embryos. Approximately 25% of embryos injected with Oct MO2 showed faint *nodal* expression (Ad). (Ae, Af) Zygotic expression of *vg1* required Oct1/2 in hatched blastula embryos. (Ba-Bo) The initiation of *nodal* expression during the early cleavage stages to mesenchyme blastula stages required input from maternal Oct1/2.

all of these Nodal-dependent genes was lost in Oct1/2 loss-of-function embryos (Fig. 5Aa-f). Furthermore, the expression of two Nodal-dependent ventral ectoderm genes, *chordin* and *foxA*, was strongly downregulated in the ectoderm of Oct1/2 morphants at mesenchyme blastula stages, when the D/V ectoderm GRN is well established in control embryos (Fig. 5Ba,b,e,f), consistent with the absence of *nodal* expression in these embryos (Fig. 5Ca,c).

In addition, we examined the expression of two ciliary band genes: *hnf6*, which is expressed within the presumptive ciliary band (Fig. 5Bc, Cb) (Otim et al., 2004; Poustka et al., 2004), and *pax2/5/8* (Fig. 5Bd) (Rottinger et al., 2008), which is activated by FGFA signaling in the ventro–lateral regions of ciliary band. These ectoderm factors are not activated by Nodal signaling, but their expression is blocked by Nodal and BMP2/4 signaling in the ventral and dorsal ectoderm, respectively. Neither *hnf6* nor *pax2/5/8*was downregulated in loss-of-function Oct1/2 embryos (Fig. 5Bg,h, Cd). However, the expression of *hnf6* and *pax2/5/8* was expanded throughout the ectoderm instead of being restricted to the presumptive ciliary band in Oct1/2 morphants, consistent with the characteristic derepression of ciliary band marker genes in the Nodal loss-of-function embryos (Fig. 5Bg,h, Cd) (Lapraz et al., 2009; Saudemont et al., 2010).

Whereas ectoderm patterning appeared severely affected in the absence of Oct1/2, the more general Nodal-independent expression of *pax2/5/8* and *hnf6* was not disturbed (Fig. 5Bg,h, Cd). Moreover, the endoderm appeared to be specified correctly as judged by the normal levels of *foxA* transcripts in the endodermal precursors at either the hatching blastula or mesenchyme blastula stage (Fig. 5Bb,f, Da,d). Similarly, both the skeletogenic and non-skeletogenic mesodermal precursors were also specified, as evidenced by the expression of *msp130* (Fig. 5Db,e) (Anstrom et al., 1987), and of *gcm* (Fig. 5Dc,f), respectively (Ransick et al., 2002). Not surprisingly however, the non-skeletogenic mesodermal precursors were not patterned correctly in the absence of Oct1/2 since *gcm* expression remained radial (Fig. 5Dc,f), consistent with the known role of Nodal signaling in the downregulation of *gcm* in the ventral non-skeletogenic mesoderm territory (Duboc et al., 2010).

These results indicate that Oct1/2 is essential for the specification and patterning of the D/V axis. Interfering with Oct1/2 expression specifically disrupted the Nodal-dependent D/V GRN, suggesting that early Oct1/2 does not act as a general activator in the specification of the three primary germ layers.

Oct1/2 expression rescues Oct1/2 morphants but is not sufficient to cause ectopic expression of nodal

In previous studies, upregulating Nodal signaling caused ectopic expression of *nodal* expression and radialized embryos by converting

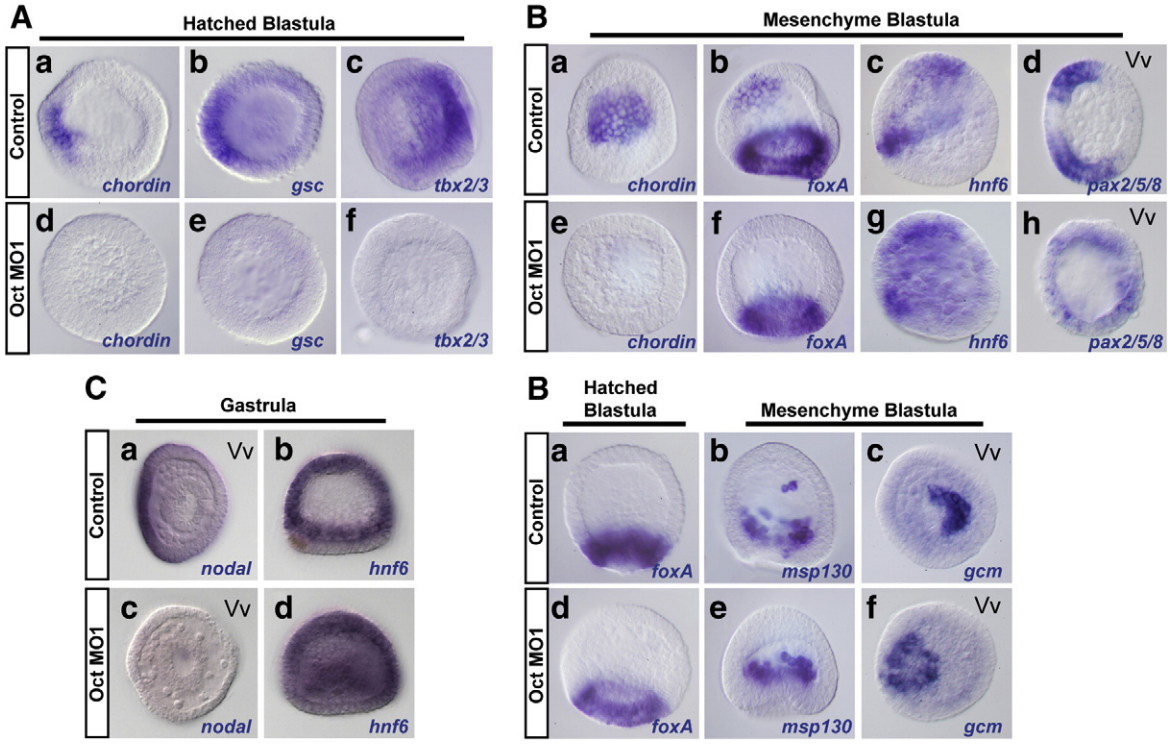

Fig. 5. Loss of Oct1/2 disrupts Nodal dependent ectoderm specification and patterning. (Aa-Af) Loss of Oct1/2 perturbed the expression of D/V regulatory genes that depend on Nodal signaling at hatched blastula stage. (Ba-Bh) At the mesenchyme blastula stage, loss of Oct1/2 caused the same ectodermal expression pattern defects of key D/V regulatory factors as in Nodal loss-of-function embryos. Vv, vegetal view. (Ca-d) *nodal* remained downregulated and *hnf6* was broadly expressed in gastrula stage Oct1/2 morphants. (Da-Df) Zygotic expression of endoderm (a,d), skeletogenic mesoderm (b,e), and non skeletogenic mesoderm (c,f) genes was not abolished in the absence of Oct1/2. However, the expression of the non skeletogenic mesodermal gene *gcm* was radialized (f).

the entire lateral and dorsal ectoderm to a ventral fate. Therefore, we were surprised that embryos injected with high doses of Oct1/2 mRNA developed normally and showed a normally restricted expression of *nodal* (Fig. 6Aa,b). Moreover, rescued embryos co-injected with an Oct1/2 morpholino directed against a region upstream of the ATG and a synthetic Oct1/2 mRNA immune to the morpholino developed normally and showed normally localized *nodal* expression in a large majority of embryos (~78%) (Fig. 6Ba-d). These data suggest that spatially restricted expression of Oct1/2 is not required to initiate restricted *nodal* expression. Consistent with this idea, endogenous *oct1/2* transcripts are expressed ubiquitously and embryos injected with low levels of mRNA encoding a fusion protein Venus–Oct1/2 (0.1 μg/μL) displayed a uniform nuclear staining in all blastomeres (Supplementary Fig. 1A).

Taken together, these results suggest that the ability of Oct1/2 to positively regulate expression of *nodal* may depend on spatial anisotropies in the early embryo that may modify its activity. Alternatively, the ability of Oct1/2 to activate Nodal expression may depend on cooperation with a spatially restricted co-activator or repressor, although, to date, no factor asymmetrically localized along the D/V axis and acting upstream of *nodal's* activation has been identified.

Discussion

One of the key unresolved issues in developmental biology is to determine the exact molecular mechanisms underlying the specification of the secondary axes in deuterostome embryos. In the sea urchin, *nodal* is the earliest gene asymmetrically expressed along the D/V axis and it is critical for specification and patterning along this axis. However, the mechanism responsible for the activation of *nodal* is incompletely understood. Our previous cis-regulatory analysis designed to uncover the maternal GRN necessary for *nodal's* expression suggests that an Oct factor is necessary for the activation of *nodal*. Here, we characterized the maternally supplied factor Oct1/2 and showed that it is critical for secondary axis formation. We found that Oct1/2 function is required for the initiation and maintenance of *nodal* and *vg1/univin*, two genes that act at the top of the D/V gene regulatory network.

Maternal control of D/V axis specification in the sea urchin embryo

Previous studies show that *nodal* is first expressed broadly throughout the ectoderm and that its expression is progressively down regulated in the presumptive dorsal ectoderm until it is only expressed in the presumptive ventral ectoderm. Here, we showed that Oct1/2, a ubiquitously expressed transcription factor, was essential for the initiation of *nodal* expression. We have shown in a previous study that at least two other ubiquitous maternal factors, Vg1/Univin and SoxB1, are necessary for the expression of *nodal* (Range et al., 2007). This leads us to propose a model for the initiation of *nodal* transcription by ubiquitously expressed factors. According to this model, a broadly expressed group of maternal factors, which includes Vg1/Univin, SoxB1 and Oct1/2 are required and may work synergistically in order to initiate Nodal expression throughout the early embryo during early cleavage stages. Then, redox sensitive transcription factors such as bZip factors and Oct1/2, may read the bias in the embryonic redox gradient and/or p38 MAPK signaling, leading to an up regulation of *nodal* expression on one side of the embryo relative to the other side (Fig. 7B). As soon as a slight asymmetry of *nodal* expression is established, it is amplified on the presumptive ventral side of the embryo by Nodal auto-regulation that reinforces the maternal bias provided by Oct1/2 and bZip factors (Fig. 7B). Finally, Nodal signaling activates the expression of Lefty, a

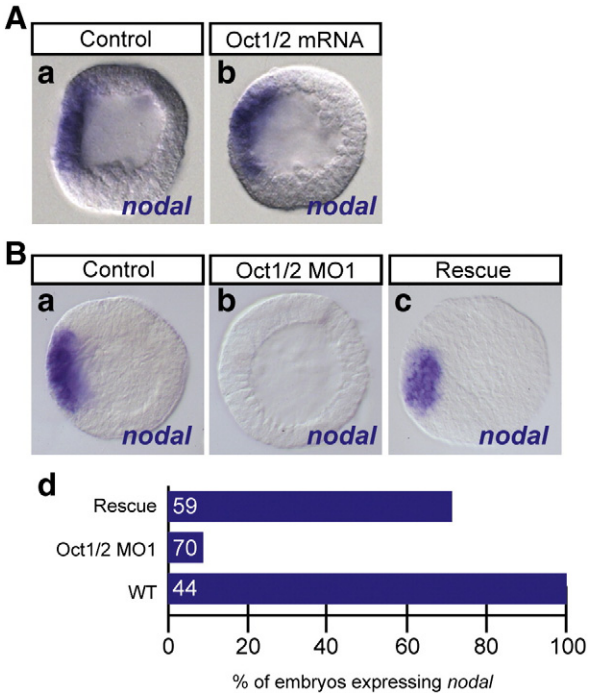

Fig. 6. Oct1/2 expression rescues Oct1/2 morphants but is not sufficient to cause ectopic *nodal* expression. (Aa, Ab) Embryos injected with Oct1/2 mRNA showed normal, localized *nodal* expression. (Ba-d) Rescue experiment showing embryos co-injected with Oct1/2 MO1 and Oct1/2 mRNA had localized expression of *nodal*. (Bd) Graph shows that a large majority (~75%) of rescued embryos expressed *nodal*. Numbers within each bar represent the number of embryos scored from 3 separate experiments.

Nodal signaling pathway antagonist, which diffuses farther than the Nodal ligand (Duboc et al., 2008), establishing lateral inhibition that results in a sharp restriction of Nodal to the ventral side of the embryo (Fig. 7C).

The presence of a potential Oct1/2 binding site in the Nodal promoter and the absence of early *nodal* expression in the Oct1/2 morphants strongly suggest that Oct1/2 regulates *nodal* expression directly by binding to its cis-regulatory region. However, the regulation of *nodal* expression by Oct1/2 may also be indirect. This hypothesis is based on our previous data showing that the initiation of *nodal* expression at the 32/60-cell stage also depends on Vg1/Univin signaling (Range et al., 2007). Therefore, it is possible that Oct1/2 is involved in the activation of zygotic *vg1/univin* during early cleavage stages. The presence in the *vg1/univin* promoter of a potential Oct1/2 binding site identical to the site present in the *nodal* promoter (ATGCTAA) and conserved between *S. purpuratus* and *P. lividus*, and our finding that zygotic *vg1/univin* expression was also absent from the Oct1/2 morphants support this alternative possibility for the mechanism by which Oct1/2 regulates *nodal* expression.

It is possible that Oct1/2 is not required for the maintenance of *nodal* expression past the very early blastula stages because only extremely low levels of Oct1/2 transcripts were detected during the blastula stages when maintenance of *nodal* transcription is required (Nam et al., 2007; Range et al., 2007). The fact that inhibition of Oct1/2 function prevented expression of *nodal* during a long period, extending from cleavage up to late gastrula stages, suggests instead that Oct1/2 activates expression of another factor required for later *nodal* expression. Vg1/Univin is an excellent candidate for such a factor because we previously showed that Vg1/Univin function is required for the maintenance of *nodal* expression. Zygotic *vg1/univin* is expressed in a pattern overlapping that of *nodal* and is regulated similarly, since it is down regulated in the absence of SoxB1 and Oct1/2 (Range et al., 2007). Thus, the reason that Oct1/2 morpholinos had such a strong effect on *nodal's* expression throughout blastula and gastrula stages, may have been due, at least in part, to the fact that zygotic Vg1/Univin protein was no longer expressed in those embryos.

Although our cis-regulatory analysis shows that we still have more work to identify the other factors necessary for Nodal's expression, these functional data on Oct1/2, combined with our previous functional data on SoxB1, and Vg1/Univin signaling, strongly argue that these factors are key components of the early maternal regulatory circuit driving Nodal expression and initiating D/V axis specification the sea urchin embryo. It is remarkable that individually blocking the function of any one of these positive regulators so severely downregulates *nodal* expression. One might expect that the other factors would compensate for the missing factor to some degree, yet this is clearly not the case. The results imply an interaction among these genes at the level of forming a functional transcriptional activating complex on the *nodal* promoter. Supporting this idea is the fact that predicted binding sites for Sox, Oct, and Smad (the downstream effector of Vg1/Univin signaling) factors are juxtaposed together close to the TATA box on the Nodal promoter. In addition, the genes encoding these factors could also interact by positively reinforcing each other's transcription to ensure that each factor is maintained above threshold concentrations for complex formation. Support for this possibility is that both Oct1/2 and SoxB1 are required for *univin/vg1* as well as *nodal* expression. Future in-depth promoter based studies of these interactions will likely uncover how this 'AND logic' is interpreted at the level of the promoter (Istrail and Davidson, 2005).

Comparison with previous studies on Oct1/2 function in the sea urchin embryo

This study is not the first to look at the function of Oct1/2 in early sea urchin development. In a previous study in *S. purpuratus*, Char et al. used antisense DNA oligonucleotides directed against different regions of the Oct1/2 transcript to decrease endogenous transcript levels and thus perturb Oct1/2 function. They showed that embryos injected with these antisense oligonucleotides but not with control sense or antisense oligonucleotides, failed to cleave at the first or second cleavage, and that injection of SpOct1/2 mRNA reduced this cleavage block by 2-fold. Moreover, they found that early protein synthesis was strongly reduced in these embryos (Char et al., 1994). Since transcription is not necessary for protein accumulation during the early cleavage stages, they suggested that Oct1/2 might have a novel non-transcriptional role in protein accumulation during the early cleavage period. Thus, Char et al. hypothesized that proteins necessary for cell division are down regulated in Oct1/2 antisense-treated embryos (Char et al., 1994). The notable differences between our results and those presented in this study may be due to the different techniques used to perturb the function of Oct1/2. Single stranded DNA oligonucleotides are notoriously toxic when injected into embryos (Heasman, 2002; Heasman et al., 1991), therefore the cleavage arrest observed in the study of Char et al. may result from non-specific effects of the injected oligonucleotides. However, another possible explanation for the different results observed in the two studies may be linked to the fact that the Oct1/2 transcripts exist in at least four different isoforms (Char et al., 1993). Consistent with this finding, we identified 3 classes of ESTs generated by alternative use of transcription start sites and differing at the level of their 5′UTR. Although more than 85% of the Oct1/2 transcripts do contain the sequence targeted by our morpholinos, one low abundance class of Oct1/2 transcript appears to lack the sequence recognized by the morpholinos used in this study (data not shown). It is therefore possible that the phenotypes we describe for the Oct1/2 morphants reflect a partial loss of function for Oct1/2 and that a complete inhibition of Oct may result in a cleavage arrest as described in the study of Char et al. However, experiments using a combination of morpholinos to knock down all the Oct1/2 isoforms suggest that this is probably not the case (data not shown). Future studies should clarify this issue.

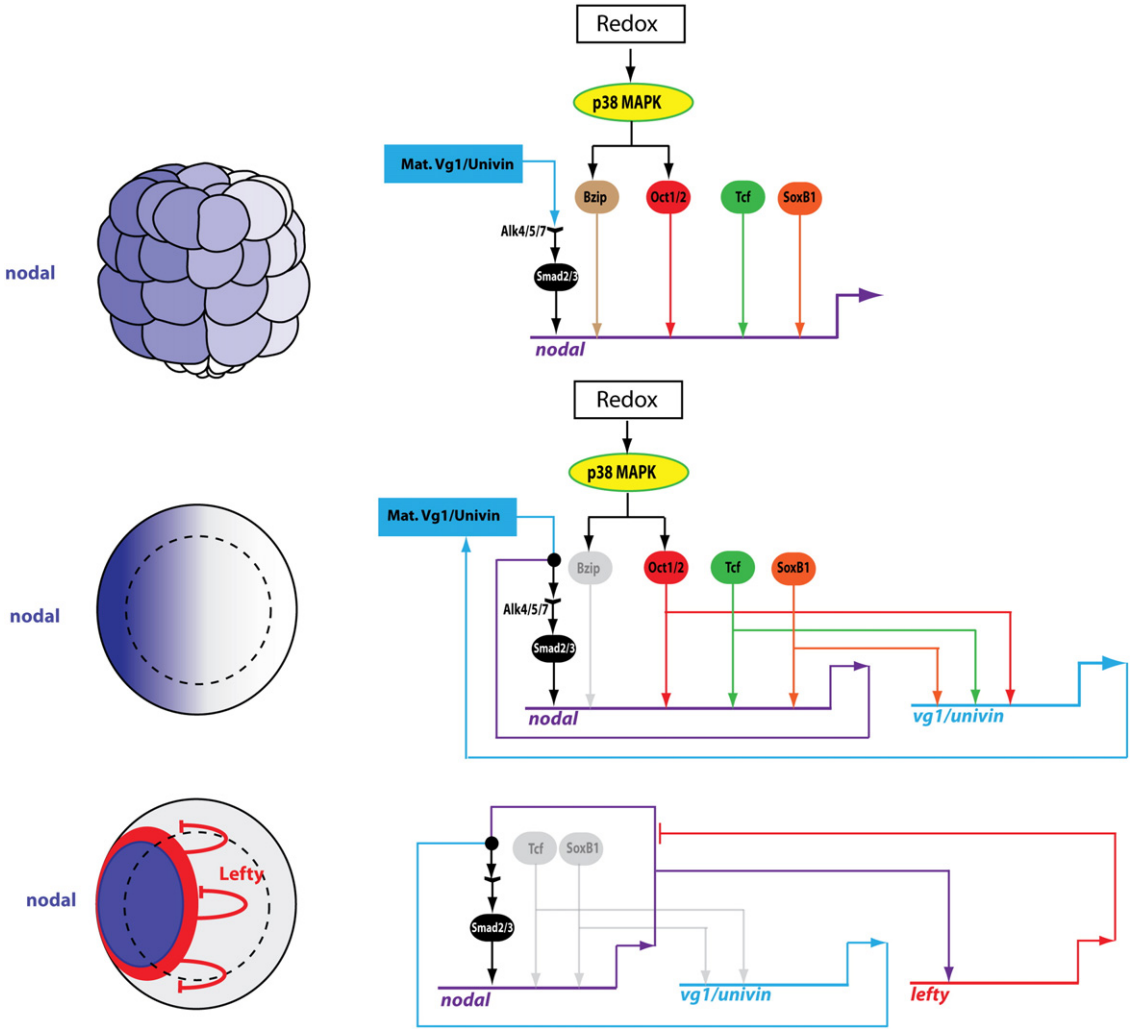

Fig. 7. Model for the initiation and maintenance of *nodal* in the ventral ectoderm of the sea urchin embryo. (A) Beginning at the 32–64 cell stage, combinatorial maternal inputs from Univin signaling, SoxB1 and Tcf, initiate *nodal* expression throughout most of the presumptive ectoderm (purple embryo on left). A redox gradient in the early embryo influences the activity of maternal p38 MAP kinase signaling, as well as the activity of the redox sensitive bZIP and maternal Oct1/2 transcription factors, causing *nodal* to be expressed at a slightly higher level on the presumptive ventral side of the embryo. (B) Starting during the early blastula stage, the asymmetric localization of *nodal* expression is more pronounced in the presumptive oral ectoderm (purple in embryo on left). This spatial restriction in the expression of *nodal* is due to the Nodal autoregulation loop reinforcing the lingering influence of the redox gradient on p38, bZIP, and Oct1/2 factors. In addition, maternal Oct1/2, Tcf, and SoxB1 are required for the activation of zygotic *vg1/univin*, whose expression is necessary to maintain *nodal*'s ventral expression. (C) During the blastula stages and beyond, Nodal auto-regulation and Vg1/Univin signaling maintain robust *nodal* expression. In addition, secreted Lefty (red) diffuses further than Nodal (purple), establishing a reaction diffusion system that results in the sharp restriction of *nodal* expression to the ventral territory.

Oct1/2 is a potential link between redox signaling and D/V axis specification

According to current models, the apparent redox gradient, possibly working through H_2O_2, regulates the active phosphorylated state of the stress sensor p38 MAPK, which is in turn necessary for the activation of *nodal* (Coffman et al., 2009; Nam et al., 2007; Range et al., 2007). However, neither overproduction of ROS nor over activation of the p38 pathway has been shown to be sufficient to cause ectopic *nodal* expression (Bradham and McClay, 2006; Coffman et al., 2009). Although these studies are informative, it is still unclear what molecular mechanisms are used to read the apparent redox gradient and to regulate *nodal* transcription in the presumptive ventral ectoderm. Therefore, it is important to identify the transcription factors that are regulated by redox gradients and p38 signaling and that are required to activate *nodal* expression. Interestingly, several lines of evidence link the Oct1/2 transcription factor to cellular stress. For example, studies in mouse have shown that transcription of the H2B gene depends on binding of Oct1 and of a protein complex named OCA-S to the H2B promoter, a process that is regulated by the NAD+/NADH redox status of the cell (Dai et al., 2008; Zheng et al., 2003). Similarly, Oct1 interacts with several factors depending on the cellular context, and many of these factors, such as BRCA1, are involved in the response to genotoxic stress (Fan et al., 2002; Nie et al., 1998). Moreover, Oct1−/− mouse embryonic fibroblast cells are hypersensitive to oxidative as well as genotoxic stress and, furthermore, microarray analysis showed that Oct1 controls the expression of many genes involved in the cellular stress response (Tantin et al., 2005). An interesting hypothesis that emerged from these and other studies is that Oct factors maintain genes in an inactive, as opposed to repressed, state that can be quickly activated in response to intracellular stimuli (Kang et al., 2009b). However, until recently little is known about how Oct factors might be induced to regulate gene expression. It is now evident that Oct1 factors can be post-translationally modified by phosphorylation at multiple positions in response to oxidative stress signals, resulting in a modulation of Oct1 DNA binding specificity which in turn controls target gene expression (Kang et al., 2009a). The kinases that phosphorylate Oct factors have not been fully characterized, but p38 MAP kinase has recently been implicated in this process through its activity on PKA, which is known to phosphorylate Oct factors (Saxe et al.,

2009). Interestingly, two critical human Oct1 phosphoserines (S335 and S385) that alter its sequence selectivity are conserved in sea urchin Oct1/2 and, furthermore, we have identified 2 MAPK sites just outside of the Pou domains, which suggests that sea urchin Oct1/2 may be similarly controlled by post-translational phosphorylation events (Kang et al., 2009a) (Supplementary Fig. 1B). We have not shown in this study that post-translational activation of Oct1/2 is necessary to activate *nodal's* expression. However, this hypothesis may help explain our finding that over-expression of Oct1/2 had no effect on either the D/V axis or on the spatial expression pattern of *nodal* as well as the fact that the rescue of Oct1/2 morphants caused normally localized expression of *nodal*. Taken together, these data support future avenues of study that will attempt to show that Oct1/2 is a link between redox signaling and Nodal transcription in the sea urchin.

The identity of the pathways that control the post-translational modifications of Oct factors, and how these changes regulate gene expression are still enigmatic. Recent studies suggest that the redox state of ESCs is involved in differentiation of these cells into particular fates (Ateghang et al., 2006; Buggisch et al., 2007; Ding et al., 2008). In light of these studies, and considering the fact that Oct factors are regulated by the oxidative state, future studies on sea urchin Oct1/2 are warranted. It will be important to determine whether or not the phosphorylation state of Oct1/2 influences its activity and to definitively connect redox dependent MAPK signaling to modulations in Oct1/2 function. Moreover, identifying the proteins that interact with Oct1/2, and dissecting how these factors interact at the *nodal* promoter, will provide insight into how the early sea urchin embryo integrates maternal information to establish the D/V axis. These studies will also likely inform studies on Oct factors in the higher deuterostome model systems as well as provide insight into how Oct factors and their interacting partners have evolved during the course of evolution.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.07.005.

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