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There was an error published in *Development* **136**, 1179-1189.

On page 1181, it was incorrectly stated that ‘We confirmed previous studies showing that *Six3* is expressed in a dynamic pattern during development of *Paracentrotus lividus* (Poustka et al., 2007), a species closely related to *Strongylocentrotus purpuratus* used in this study.’

It should have stated ‘We confirmed previous studies showing that *Six3* is expressed in a dynamic pattern during development of *Strongylocentrotus purpuratus* (Poustka et al., 2007).’

The authors apologise to readers for this mistake.

The sea urchin animal pole domain is a Six3-dependent neurogenic patterning center

Zheng Wei, Junko Yaguchi*, Shunsuke Yaguchi*, Robert C. Angerer and Lynne M. Angerer†

Two major signaling centers have been shown to control patterning of sea urchin embryos. Canonical Wnt signaling in vegetal blastomeres and Nodal signaling in presumptive oral ectoderm are necessary and sufficient to initiate patterning along the primary and secondary axes, respectively. Here we define and characterize a third patterning center, the animal pole domain (APD), which contains neurogenic ectoderm, and can oppose Wnt and Nodal signaling. The regulatory influence of the APD is normally restricted to the animal pole region, but can operate in most cells of the embryo because, in the absence of Wnt and Nodal, the APD expands throughout the embryo. We have identified many constituent APD regulatory genes expressed in the early blastula and have shown that expression of most of them requires Six3 function. Furthermore, Six3 is necessary for the differentiation of diverse cell types in the APD, including the neurogenic animal plate and immediately flanking ectoderm, indicating that it functions at or near the top of several APD gene regulatory networks. Remarkably, it is also sufficient to respecify the fates of cells in the rest of the embryo, generating an embryo consisting of a greatly expanded, but correctly patterned, APD. A fraction of the large group of Six3-dependent regulatory proteins are orthologous to those expressed in the vertebrate forebrain, suggesting that they controlled formation of the early neurogenic domain in the common deuterostome ancestor of echinoderms and vertebrates.

KEY WORDS: Microarray, Nodal, BMP2/4, Primary axis, Secondary axis, Canonical Wnt signaling, β -catenin, Gene expression profiling, Neural development, Transcription factor

INTRODUCTION

The earliest neurogenic domains of vertebrate embryos form in their anterior regions and derive from ectoderm that is protected from TGF- β and Wnt signals (reviewed by Wilson and Edlund, 2001; Levine and Brivanlou, 2007; Wilson and Houart, 2004). This early ectoderm is inherently biased toward neural fate, as suggested by the observations that single cells derived from *Xenopus* animal caps (Grunz and Tacke, 1989; Sato and Sargent, 1989) or mouse embryonic stem (ES) cells, cultured in serum-free media (Smith et al., 2008; Smukler et al., 2006; Watanabe et al., 2005) express neural rather than epidermal markers and will maintain this state in the absence of signals. This early neural-biased region is subsequently shaped into a definitive neurogenic domain by several processes. One involves the localized activities of the TGF- β cytokines, Nodal (Camus et al., 2006) and BMP, which promote epidermal fates, and of their antagonists, which inhibit these fates. Additional signaling pathways, such as FGF and Wnt, regulate BMP signaling activities by regulating the stability of the downstream effector, Smad1/5/8 [(Fuentelba et al., 2007) and references cited therein], but may have additional roles in regulating neural versus non-neural ectodermal decisions. Another process is the activation of the cell-autonomous neural gene regulatory program that converts cells from an early neural bias to a pre-neural gene regulatory state. These steps include not only the production of new neural regulatory proteins but also the activation of mechanisms that exclude signaling and allow the autonomous gene regulatory program to proceed.

The transitional state of pre-neural ectoderm is not well understood, but, based on molecular marker expression, it appears to be forebrain-like (Ang et al., 1994; Ang and Rossant, 1993; Yang and Klingensmith, 2006) (reviewed by Foley et al., 2000). In embryos lacking BMP and Nodal receptors, nearly all of the ectoderm becomes anterior neural tissue (reviewed by Levine and Brivanlou, 2007), which expresses both general neural markers and those normally restricted to forebrain, such as Six3, Dlx5 and Hes1 (Camus et al., 2006). Similarly, mouse ES cells cultured in serum-free conditions in the presence of BMP and Nodal antagonists preferentially express several forebrain markers (Ikeda et al., 2005). However, there are relatively few known regulatory proteins, and their regulatory connections are not yet understood.

The sea urchin embryo, which shares a common ancestor with the vertebrates, is an excellent system to identify regulatory activities constituting the gene regulatory state of early pre-neural ectoderm. This region, localized at the animal pole, is specified by the early mesenchyme blastula stage (Burke et al., 2006) and gives rise to the animal plate, a disk of 40-60 cells in the ciliated band of the 3-day pluteus larva that contains serotonergic neurons on its aboral side and non-serotonergic neurons on all sides (Nakajima et al., 2004a), as well as cells bearing long, immotile cilia. Gene expression patterns suggest that the animal pole domain (APD) also includes immediately flanking epithelial ectoderm cells (Burke et al., 2006; Howard-Ashby et al., 2006a; Howard-Ashby et al., 2006b) (see also Results).

Ectoderm patterning in the sea urchin embryo is regulated by a series of signaling events, beginning with a wave of canonical Wnt signaling that originates in the most vegetal blastomeres of the 16-32-cell embryo, passes upwards through vegetal tiers of blastomeres and specifies endomesodermal tissues (Davidson et al., 2002; Logan et al., 1999) (reviewed by Angerer and Angerer, 2003). Canonical Wnt signaling is also required for Nodal-dependent patterning along the secondary axis by removing a repressor of *nodal* expression, FoxQ2, from the lateral ectoderm

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(Yaguchi et al., 2008). Nodal is required for production of BMP2/4 (Duboc et al., 2004), which activates the gene regulatory network for aboral ectoderm differentiation (Angerer et al., 2000), and Chordin (Bradham et al., 2009), which inhibits BMP2/4 function. These events occur during blastula stages and direct the lateral ectoderm to oral and aboral epidermal fates, while the APD persists, marked first by the expression of *foxq2* (Tu et al., 2006; Yaguchi et al., 2008) followed by *homeobrain* (*hbn*), *achaete-scute* (*ac-sc*) and *retinal anterior homeobox* (*rx*) (Burke et al., 2006; Howard-Ashby et al., 2006a; Howard-Ashby et al., 2006b). This region cannot be converted to squamous epidermal fates by misexpression of Nodal or BMP2/4, unless FoxQ2 is removed (Yaguchi et al., 2008). By contrast, when Nodal-BMP2/4 and canonical Wnt signals are eliminated through Δ cadherin mRNA injection (Logan et al., 1999; Wikramanayake et al., 1998), then the animal plate expands, as shown by a greatly increased number of serotonergic neurons distributed radially throughout a large portion of the embryo (Yaguchi et al., 2006).

The ability to greatly enlarge the early neurogenic ectoderm of the sea urchin embryo experimentally offers a unique opportunity to identify regulatory proteins that specify this region of the embryo. Many genes expressed specifically in this territory are expected to be strongly upregulated in Δ cadherin-misexpressing embryos in comparison to normal embryos, as has been shown for *foxq2* (Yaguchi et al., 2008) and *nk2.1* (Takacs et al., 2004). Here we use microarray analyses to identify such genes and sort them according to their onset of expression (Wei et al., 2006). In this paper, we focus on *six3* because it is one of the first of these genes to be activated after fertilization and expressed in the APD at blastula stage. We show that this factor is required for development of all neurons, for antagonizing signals that inhibit neural differentiation and for the expression of the large majority of regulatory genes expressed early in the APD. Furthermore, misexpressed *Six3* can convert nearly all cells of the embryo to form an appropriately patterned APD. Consequently, *six3* operates at, or near the top of, the gene regulatory networks that control specification of cell fates in the APD, and the *Six3*-dependent properties of the APD suggest that this domain functions as a patterning center.

MATERIALS AND METHODS

Embryo culture

Adult sea urchins, *Strongylocentrotus purpuratus*, were obtained from The Cultured Abalone, Goleta, CA and maintained in seawater at 10°C. Embryos were cultured by standard methods with artificial seawater (ASW) at 15°C.

Microinjection of morpholino antisense oligonucleotides (MO) and mRNAs

Eggs were de-jellied by six passages through 74 μ m Nitex and arrayed in rows on a plastic culture dish coated with 1% protamine sulfate. After insemination in ASW with 3-amino-1,2,4-triazole (Sigma, St Louis, MO), approximately 2 μ l of solution containing 22.5% glycerol and morpholinos (Gene-Tools, Eugene, OR) or synthetic mRNAs at the following concentrations were injected: Δ cadherin mRNA (0.3 μ g/ μ l), *Six3* mRNA (0.2 μ g/ μ l), *Six3*-MO1 (0.75 mM), *Six3*-MO2 (1.5 mM), *FoxQ2*-MO (0.8 mM), Nodal-MO (0.6 mM). The morpholino sequences were: *Six3*-MO1, 5'-GGGCCGCTCTCATGGCGCCCGGTC-3'; *Six3*-MO2, 5'-CCCCGGTCGCTGGGCGATGTTTCTG-3', 4 nt upstream of AUG; *FoxQ2*-MO, 5'-GGTTGTCAATGCTGAATAAAGTCAT-3' (Yaguchi et al., 2008); Nodal-MO: 5'-GATGTCTCAGCTCTCTGAAATGTAG-3', 56 nt upstream of AUG.

mRNAs were synthesized using mMACHINE Kit (Ambion, Austin, TX), according to the manufacturer's protocol, except that the RNA was precipitated with three volumes of ethanol after addition of LiCl and glycogen.

Microarray methods

Microarrays and data processing were described previously (Wei et al., 2006). RNA from 800-1000 embryos was purified using Nucleospin columns (Macherey-Nagel, Bethlehem, PA) and amplified with the MessageAmp aRNA Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The amplified antisense RNA (aRNA) was further converted to double-stranded cDNA as follows: for first-strand cDNA synthesis, 5 μ g of aRNA was mixed with random primers (1.25 μ g), incubated at 70°C for 5 minutes, transferred to ice and then reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) in the presence of 1 mM dNTP and 1.3 U/ μ l RNase Out at 50°C for 2 hours. Template aRNA was removed with 5 units of RNaseH at 37°C for 30 minutes and the cDNA was purified with a QIAquick PCR purification column and eluted with 30 μ l water. The cDNA was mixed with oligo dT (2 μ M final concentration), incubated for 5 minutes at 70°C and second-strand cDNA was synthesized with DNA polymerase I (*E. coli*) (0.4 U/ μ l) in the presence of 1 mM dNTP at 16°C for 2 hours. The double-stranded cDNAs were purified by QIAquick chromatography and labeled, hybridized and scanned by Nimblegen microarray services.

Quantitative PCR

Quantitative PCR (QPCR) was performed as described previously (Ransick, 2004) with some modifications. Total RNA from 300 to 1000 injected embryos was purified using NucleoSpin columns (Macherey-Nagel, Bethlehem, PA) and reverse transcription was performed using Superscript III (Invitrogen, Carlsbad, CA). iQ SYBR Green Supermix (Bio-Rad, <http://www.biorad.com>) was used for PCR reactions carried out with an iQ thermal cycler (Bio-Rad). Details of the primer sets used for QPCR are available upon request. Relative concentrations of mRNA were normalized to mitochondrial 12sRNA C_t values.

Whole-mount in situ hybridization

Embryos were fixed in 4% paraformaldehyde in ASW for 1 hour at RT and washed four times in MOPS buffer (0.1 M MOPS, pH 7.0, 0.5 M NaCl, 0.1% Tween-20). Pre-hybridization, hybridization and staining were as described previously (Minokawa et al., 2004). Two-color fluorescent in situ hybridization was carried out as described previously (Yaguchi et al., 2008). The *foxq2* probe was labeled with FITC and detected with Cy3-TSA and the *Hbn* probe was labeled with digoxigenin and detected with FITC-TSA.

Immunohistochemistry

Primary antibodies were incubated overnight at 4°C using the following dilutions: serotonin (1:1000, Sigma, St Louis, MO), *Nk2.1* (1:800), Gsc [1:600 (Kenny et al., 2003)], *Spec1* [1:80 (Wikramanayake and Klein, 1997)], *synaptotagmin* [1e11, 1:800 (Nakajima et al., 2004b)]. Bound primary antibodies were detected by incubation with Alexa-coupled secondary antibodies for 1 hour. The embryos were observed using a Zeiss microscope (Axiovert 200M). Optical sections were obtained with an ApoTome unit (Zeiss, Thornwood, NY) and stacked images were prepared using Adobe Photoshop.

RESULTS

Identification of candidate animal pole domain (APD) regulatory genes

To define the early regulatory state of the APD in the sea urchin embryo, we simultaneously used both bioinformatics and experimental approaches to identify genes encoding regulatory proteins that are expressed in this territory before gastrulation. The bioinformatics approach exploited the genome sequence annotation efforts from our laboratory (Burke et al., 2006) and from others (Howard-Ashby et al., 2006a; Howard-Ashby et al., 2006b; Materna et al., 2006; Tu et al., 2006), which documented APD expression patterns of many genes encoding transcription factors and/or that were orthologs of factors known to function in neural tissue in embryos of other species. The experimental approach compared representation of RNAs in Δ cadherin mRNA-injected versus normal embryos at the hatching blastula stage. Embryos lacking nuclear β -

catenin consist almost exclusively of animal pole ectoderm, as shown by the expression patterns of *foxq2* and *hbn*. In normal embryos, these mRNAs are restricted to the animal pole beginning at blastula stages (Burke et al., 2006; Tu et al., 2006; Yaguchi et al., 2008) and the domains of their expression at mesenchyme blastula stage overlap at the animal pole (Fig. 1B-D). As development proceeds through gastrulation, the domain of *hbn*-expressing cells forms a ring that surrounds cells expressing *foxq2* (Fig. 1F). When canonical Wnt, Nodal and BMP signaling are all eliminated by Δ cadherin mRNA injection, the animal half of the embryo expresses *foxq2* while most of the remainder of the embryo expresses *hbn* (Fig. 1H). These patterns indicate that embryos lacking canonical Wnt and Nodal-BMP2/4 signaling consist almost entirely of a dramatically expanded APD, the outer borders of which are defined by *hbn* expression. *Foxq2* and *hbn* transcripts appear in the APD during late cleavage/early blastula stages, indicating that specification of the APD occurs at least by this time.

To identify early APD regulatory genes, we compared the relative concentrations of individual mRNAs in Δ cadherin mRNA- versus glycerol-injected embryos at the hatching blastula stage using a microarray representing all gene predictions found in the sea urchin genome sequence (Wei et al., 2006). This stage marks the transition between cleavage and the onset of morphogenesis and is shortly after the time when the earliest markers for the APD have been detected (Burke et al., 2006; Howard-Ashby et al., 2006a; Howard-Ashby et al., 2006b; Tu et al., 2006; Yaguchi et al., 2008). A set of genes encoding transcription factors and components of signaling pathways with an average of at least 3-fold higher expression in two batches of Δ cadherin mRNA-injected embryos was identified. These partially overlapped with the set of candidate genes identified by the bioinformatics approach. The combined sets contain 27 genes and constitute a provisional early APD regulatory gene set (E-APD) (Table 1).

In order to identify the earliest expressed genes within the E-APD set, we examined microarray-generated temporal expression profiles as described previously (Wei et al., 2006). Patterns were verified by comparison with published data (Burke et al., 2006; Croce et al., 2006; Howard-Ashby et al., 2006a; Howard-Ashby et al., 2006b; Materna et al., 2006; Sweet et al., 2002; Takacs et al., 2004; Tu et al., 2006; Yaguchi et al., 2008) and by our whole-mount in situ hybridizations (our unpublished observations). The genes were sorted into three groups: those represented maximally in the maternal RNA population (Fig. 2A), those strongly upregulated during cleavage stages (Fig. 2B,C) and those upregulated between late cleavage and early gastrula stages (Fig. 2D). We first focused on members of the early embryonic expression group. As shown below,

using both loss- and gain-of-function assays, we identified one, *Sp-Six3* (hereafter *Six3*), which operates at or near the top of the APD gene regulatory hierarchy.

Six3 is expressed early in the APD

The identification of sea urchin *six3* is unambiguous because its sequence is very highly conserved in the homeodomain (98% identical to HsSix3), the six domain (91%) and the groucho interaction domain (71%) (see Fig. S1 in the supplementary material). We confirmed previous studies showing that *Six3* is expressed in a dynamic pattern during development of *Paracentrotus lividus* (Poustka et al., 2007), a species closely related to *Strongylocentrotus purpuratus* used in this study. The most important features are that *Six3* transcripts are expressed in the animal hemisphere during late cleavage (Fig. 3A), in the APD by the hatched blastula stage (Fig. 3B) and then in two rings (Fig. 3G,H), one near the periphery of the APD (Fig. 3C-F,H) and the other in the endomesoderm (Fig. 3C-G), during mesenchyme blastula stages. During gastrulation, *Six3* is expressed in some secondary mesenchyme cells scattered throughout the blastocoel and at the tip of the archenteron (Fig. 3I, arrow), as reported by Howard-Ashby et al. (Howard-Ashby et al., 2006b), in cells in the animal pole (Fig. 3I,J) and oral ectoderm (Fig. 3J,K). At pluteus stage, *Six3* RNA is detected in two clusters of cells flanking the mouth (Fig. 3K, arrows) and in the ciliated band (Fig. 3K).

Six mRNA overall levels at early mesenchyme blastula stage do not significantly change upon Δ cadherin mRNA injection (Table 1). In situ hybridizations are consistent with this result and show that the distribution differs in Δ cadherin mRNA-injected embryos, being absent from vegetal cells and retaining the broad animal hemisphere expression that is established before restriction by processes dependent on canonical Wnt (see Fig. S2 in the supplementary material).

Six3 function is required for APD formation and differentiation of neurons

To test the function of *Six3*, we injected into fertilized eggs two different *Six3*-translation-blocking morpholinos, each of which elicited the same developmental defects. Embryos at 3 days assumed a rounded morphology (Fig. 4A,B) and spicules were either reduced or absent. The animal pole ectoderm lacked the thickened epithelial morphology characteristic of the animal plate (Fig. 4, compare A,B with C, brackets). In some embryo batches, gastrulation occurred normally, although it was delayed, and the position of the archenteron showed that oral/aboral polarity was established (Fig. 4A). In other batches, most of the embryos exogastrulated. In

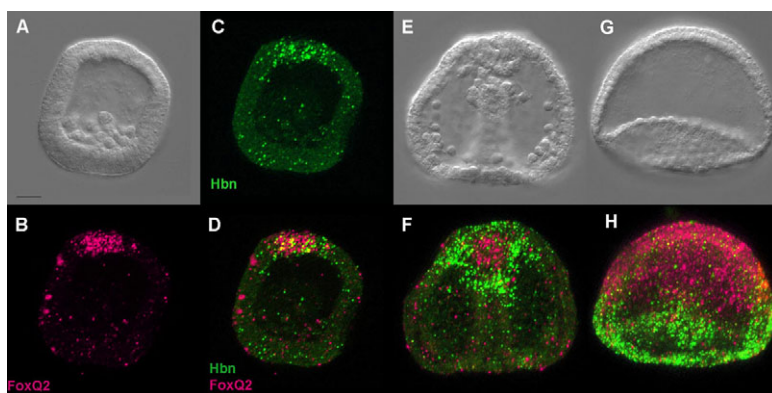


Fig. 1. Δ cadherin-misexpressing embryos consist of animal pole domain (APD) tissues defined by expression of *foxq2* and *hbn*. Whole-mount in situ hybridizations to embryos at mesenchyme blastula (A-D) and gastrulae (E-H) stages. (E,F) Glycerol-injected control. (G,H) Δ cadherin-mRNA injected. (A,E,G) DIC; (B-D,F,H) Two-color fluorescence, *hbn* (green) and *foxq2* (magenta) RNAs. Scale bar: 20 μ m.

Table 1. Sensitivity of genes in the E-APD set to loss of nuclear β -catenin

Gene ID	Gene name	\log_2 change signal intensity versus glycerol-injected*	
		Embryo 1	Embryo 2
SPU_027369	Sp-Crim1-like	3.4	4.8
SPU_000757	Sp-Nkx2-1	4.1	4.6
SPU_028148	Sp-Ac-sc	2.1	4.4
SPU_027491	Sp-fez	6.3	4.1
SPU_013047	Sp-Nkx3-2	5.7	3.8
SPU_012506	Sp-Dkk-3	4.7	3.8
SPU_011271	Sp-sFRP1/5	4	3.7
SPU_022916	Sp-Frizzled 5/8	2.5	3.6
SPU_023177	Sp-Hbn	3.9	3.2
SPU_028583	Sp-zic2	2.9	2.9
SPU_020680	Sp-Fgfr-like	1.7	2.7
SPU_022841	Sp-z142	1.3	2.7
SPU_014289	Sp-rx	3.6	2.5
SPU_002603	Sp-SoxC	1.3	2.4
SPU_026958	Sp-Hox only	1.5	2.2
SPU_023727	Sp-z188	1.5	1.8
SPU_006814	Sp-Hairy 2/4	1.5	1.6
SPU_009250	Sp-Fjx1	2.2	1.5
SPU_004702	Sp-EBF3	2.4	4.5
SPU_020459	Sp-dispatched	1.3	1.6
SPU_022242	Sp-zfh1	0.4	1.3
SPU_019002	Sp-FoxQ2	0.6	0.9
SPU_002677	Sp-Ptf1a	0	0.8
SPU_004414	Sp-Hlf	0.8	0.8
SPU_027969	Sp-FoxJ1	0.2	0.7
SPU_016128	Sp-delta	-2.1	0.2
SPU_018908	Sp-Six3	0.5	-0.3

*Average values in bold indicate that the gene is significantly upregulated in embryos lacking nuclear β -catenin function.

embryos lacking Six3, neural differentiation was strongly inhibited, as assayed by immunostaining for neural markers normally expressed during late gastrula and pluteus stages (Fig. 4, compare A,B with C). The majority (2/3) of embryos contained no serotonergic neurons and the remainder had a reduced number (Fig. 4D) compared with the normal number at this stage (3-5). The same was true for all other neurons, assayed by the pan-neural marker synaptotagmin (1e11) (Fig. 4A,B), which are found within the APD and ciliated band of normal 3-day embryos (Fig. 4C). Significantly, the expression of *hbn* was reduced to a level undetectable by in situ hybridization (Fig. 4F versus Fig. 4E). QPCR measurements confirmed this observation and showed that the levels of both *hbn* and *foxq2* mRNAs, which mark the outer boundaries and inner portions of the APD, respectively, were reduced 8- to 10-fold in Six3 morphants at the mesenchyme blastula stage (Fig. 5).

Six3 is required for expression of most genes in the E-APD set

The striking phenotype produced by loss of Six3, as well as its early expression in the embryo, suggested that it might function near the top of the APD gene regulatory network. To examine this possibility further, we used microarray analysis to search for Six3-dependent genes at 27 hours, the time when genes in the E-APD set approach maximum expression levels. To eliminate the endomesodermal functions of Six3, we carried out this screen in Δ cadherin-injected embryos. As shown in Fig. 5A, loss of Six3 in these embryos completely eliminated development of all of the excess neurons found in Δ cadherin-injected embryos and no thickened epithelium formed, again demonstrating an important role for Six3 in APD

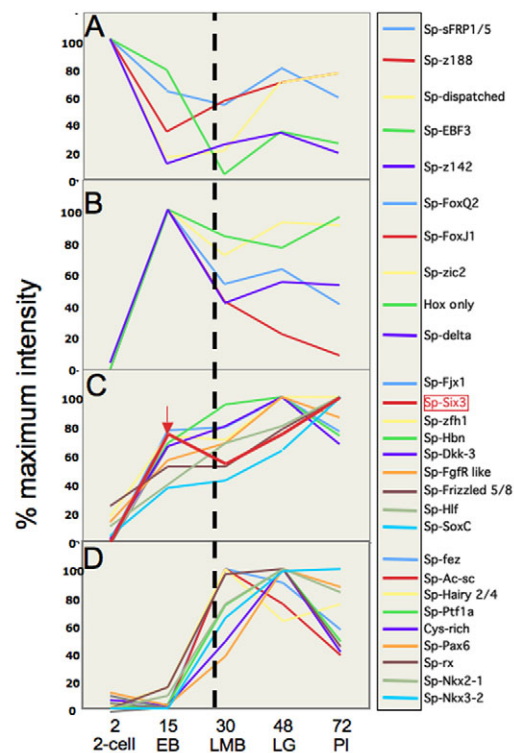


Fig. 2. Temporal expression profiles of genes in the provisional early APD set. Profiling methods were as described by Wei et al. (Wei et al., 2006). Values at different hours post-fertilization from 2 to 72 are shown, as a percentage of the maximum signal intensity, for each gene at 2-cell (maternal RNA), 15-hour early blastula (EB), 30-hour late mesenchyme blastula (LMB), 48-hour late gastrula (LG) and 72-hour pluteus larva (PL). Profiles are grouped according to time of earliest detectable expression: (A) maternal; (B,C) early blastula; (D) early blastula to mesenchyme blastula. The position of the dashed line represents the time of assay in Six3 morphants. Data for the *six3* gene are highlighted with a red arrow.

development. The microarray data identified a surprisingly large number of gene predictions (682) that were strongly downregulated (at least 4-fold) in embryos doubly injected with Δ cadherin mRNA and Six3-MO, compared with embryos injected with Δ cadherin mRNA alone. Furthermore, more than 60% of all genes in the previous microarray screen that were upregulated at least 3-fold in Δ cadherin mRNA-injected embryos, and therefore likely to be expressed in the APD, were significantly downregulated by loss of Six3. Importantly, microarray data indicated that the majority of the E-APD genes were sensitive to Six3 (Fig. 5B, yellow). In good agreement with this, QPCR measurements in two other batches of embryos showed that only 5 E-APD genes did not depend significantly on Six3 (Fig. 5B, red, blue).

Six3 overexpression is sufficient to expand the APD

These results strongly support the idea that Six3 functions early in APD gene regulatory networks, and raise the possibility that it might be sufficient to induce other cells in the embryo to adopt APD fates. Misexpression of Six3 resulted in embryos displaying an extraordinary change in morphology. A horseshoe-shaped band of densely packed cells extended in the vegetal direction from the animal pole (Fig. 6, compare B,C with A). Serotonergic neurons,

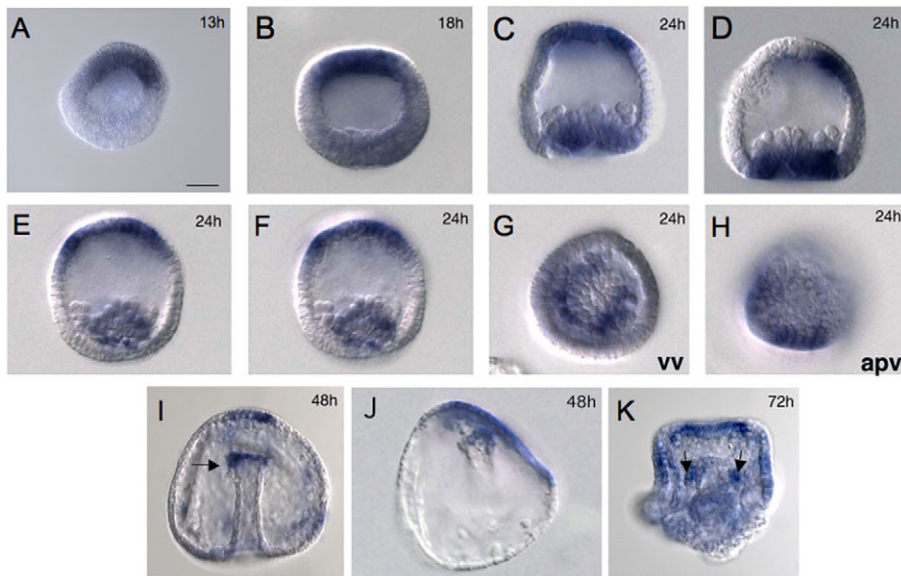


Fig. 3. Whole-mount in situ hybridization for *six3* mRNA during development. Times as hours post-fertilization are shown in the upper right corner of each image. (A) Very early blastula. (B) Hatching blastula. (C-H) Mesenchyme blastula. (I, J) Late gastrula. (K) Pluteus. All embryos are shown in lateral view, except in G and H, which illustrate vegetal pole (vv), and animal pole view (apv), respectively. Arrows in I and K mark positions of secondary mesenchyme cells and cells flanking the mouth, respectively. Scale bar: 20 μ m.

normally restricted to the animal plate (Fig. 6D), increased 4-fold in number and were distributed along the dense band (Fig. 6G). Furthermore, the columnar shapes and arrangement of neural projections in synaptotagmin-containing cells (1e11) are similar to those in the animal plate of control embryos (Fig. 6E, white dashed box versus Fig. 6H). All of these cells contain in their nuclei NK2.1 (Fig. 6J-M, green), a transcription factor normally expressed in the animal plate and adjacent supra-oral ectoderm (Fig. 6I, I'; green circles in Fig. 6U), as well as a few cells in the foregut (Takacs et al., 2004). A chain of 1e11-positive neural cells bisects the band (Fig. 6K, red) and cells on the inner side of the NK2.1-positive band also express Gsc (Fig. 6L, N, red). The combination of NK2.1 and Gsc uniquely marks the supra-oral facial epithelium at the oral edge of the animal plate (Fig. 6I, I', yellow cells and Fig. 6U, yellow circles).

The densely packed columnar cells of the expanded band surround more flattened epithelial cells that express NK2.1, but not Gsc (Fig. 6M, N), as do cells of the upper regions of the mouth of normal embryos (Fig. 6I, mouth, m). Further evidence that these cells are similar to those near the mouth of normal embryos is that they express *hbn* mRNA, which, in normal 3-day embryos, accumulates around the margin of the animal plate and extends into the supra-oral ectoderm (Fig. 1, Fig. 6O). In Six3-misexpressing embryos, *hbn*-positive cells are concentrated in the thin epithelium at the vegetal pole and thus located in the same position relative to the expanded animal plate and the NK2.1-positive cells in the upper foregut as in normal embryos (Fig. 6P, Q). The side opposite the oral region differentiates as a thin squamous epithelium, expresses the aboral ectoderm marker, *Spec1* (Fig. 6R-T) and may correspond to the *hbn*-positive strip of aboral ectoderm adjacent to the animal plate. Collectively, these gene expression patterns lead to the remarkable conclusion that Six3 is sufficient to re-specify the fates of cells in most of the rest of the embryo, generating a 3-day embryo consisting of a greatly expanded, but correctly patterned, animal pole domain with oral/aboral polarity. The APD in normal and Six3-misexpressing embryos is marked by the blue circles in Fig. 6U.

Because misexpressed Six3 can expand the entire APD and promote development of ectopic neurons, we asked which genes in the E-APD gene set were upregulated, using microarray and QPCR measurements. Table 2 lists 10 such genes whose mRNA levels are elevated at least 3-fold in Six3 mRNA-injected embryos.

Six3 can suppress TGF- β signaling but does not eliminate oral/aboral polarity

Six3 misexpression does not eliminate oral/aboral polarity because oral and aboral ectoderm markers are expressed on opposite sides of the embryo and neurons normally found in the APD are confined to an intervening band. However, misexpressed Six3 does reduce expression of *nodal* as well that of *lefty* and *chordin*, in mesenchyme blastulae (Table 3, left), perhaps because Six3 provides positive input into expression of FoxQ2, which has been shown to repress *nodal* expression (Yaguchi et al., 2008). Surprisingly, Six3 does not suppress *bmp2/4* mRNA accumulation as much as it reduces *nodal*, although *bmp2/4* expression requires *nodal* function in normal embryos (Duboc et al., 2004). This apparent paradox may

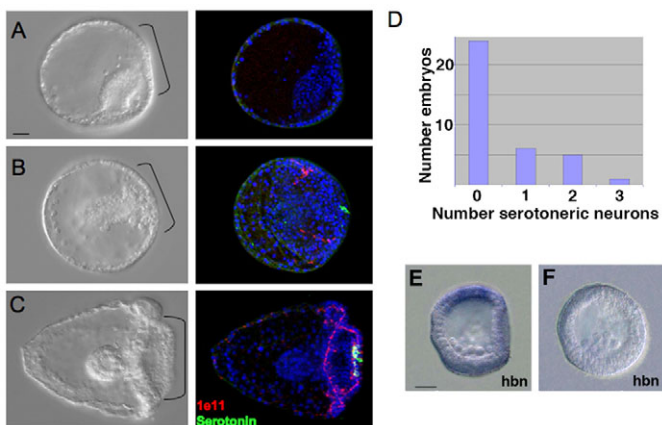


Fig. 4. Loss of Six3 results in loss of neurons and the thickened epithelium characteristic of the APD. (A, B) Three-day embryos injected with Six3-MO2 at the one-cell stage, which are typical of stronger and weaker phenotypes, respectively. (C) Normal 3-day embryo. APD in A-C indicated by brackets; 1e11 (pan-neural, magenta), serotonin (green), DAPI (nuclei, blue). (D) Numbers of embryos containing either 0, 1, 2 or 3 serotonergic neuron per embryo in Six3 morphants. At this stage, normal embryos have from 3 to 5 serotonergic neurons. (E, F) *hbn* mRNA in normal mesenchyme blastulae (E) or in Six3 morphants (F). Scale bar: 20 μ m.

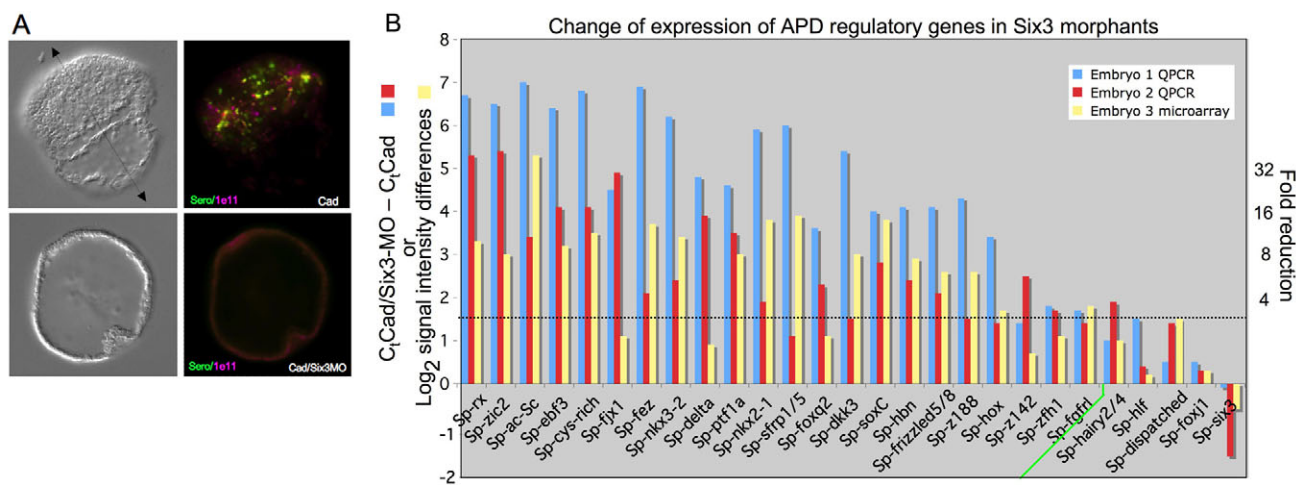


Fig. 5. The sensitivity of early APD regulatory genes to loss of Six3. (A) Three-day embryos injected with Δ cadherin mRNA (top) or with Δ cadherin mRNA and Six3-MO (bottom). The arrow indicates the orientation of the animal-vegetal axis. Embryos are immunostained with anti-serotonin and 1e11, a pan-neural marker. (B) QPCR cycle changes (blue and red bars) or \log_2 signal intensity differences (yellow bars) (y-axis, left) or fold changes (y-axis, right) in the levels of individual mRNAs in two batches (red and blue bars) of Six3 morphant and control 27-hour embryos, both containing Δ cadherin. Genes whose expression is changed at least 3-fold are to the left of the green line.

result from a combination of reduced Chordin-mediated inhibition of BMP2/4 signaling coupled with diffusion of BMP2/4 and subsequent autoactivation, as has been demonstrated in other systems (Biehs et al., 1996; Jones et al., 1992).

Canonical Wnt, not TGF- β , signals prevent APD expansion into the lateral ectoderm

The signals that delimit the APD depend on canonical Wnt signaling, as its elimination allows the APD to encompass nearly all of the embryo (Yaguchi et al., 2006) (Fig. 1). Because Nodal and BMP depend on canonical Wnt signals, we eliminated both TGF- β ligands with a Nodal-MO (Yaguchi et al., 2008) to test whether they were responsible for the Wnt-dependent restriction of the APD. Fig. 7B,F shows that this is not the case because serotonergic neurons remain restricted to the APD in these embryos. However, when Nodal morphants are provided with exogenous Six3, then serotonergic neurons increase greatly in number and appear throughout the animal half of the embryo (Fig. 7D), as is observed in Δ cadherin-misexpressing embryos (Yaguchi et al., 2006), which lack canonical Wnt signaling. Therefore, ectopic expression of Six3 can override the other signals, presumably Wnt, that restrict these neurons to the APD of normal embryos.

Although serotonergic neurons do not form in the lateral ectoderm in Nodal morphants, some non-serotonergic neurons do (Fig. 7B). This results primarily, if not exclusively, from loss of BMP2/4 signaling, as the same result is obtained in BMP2/4-MO-injected embryos (S.Y., J.Y., L.M.A., R.C.A and R. D. Burke, unpublished). Because development of all neurons depends on Six3 in the normal embryo, we asked whether the ectopic neurons in Nodal morphants also depend on Six3 by co-injecting Nodal-MO and Six3-MO. As expected, the serotonergic neurons present at the animal pole in Nodal morphants were lost in the double morphants (Fig. 7G,H). These embryos do not contain differentiated non-serotonergic neurons with axonal processes, although some 1e11-immunoreactive spots were observed. These might indicate the presence of incompletely differentiated neurons or reflect an initial neural bias of ectoderm cells that is normally overridden by TGF- β signaling.

Six3 can antagonize Wnt signaling

The facts that the APD does not expand in Nodal morphants but does in Δ cadherin-injected embryos and that Six3 can overcome canonical Wnt-dependent effects in the lateral ectoderm raise the possibility that Six3 represses Wnt signaling. In support of this hypothesis, we find that Six3 misexpression downregulates most of the genes encoding Wnt ligands that are expressed during early development (Table 3, left) (Fig. 8). One of these is Wnt8, a crucial vegetal signal required for normal endomesoderm development (Wikramanayake et al., 2004), a result that is consistent with the lack of vegetal development in Six3-misexpressing embryos. Collectively, these results suggest that the borders of the APD are determined by Six3/Wnt antagonism.

Six3 is not sufficient to repress expression of *wnt* and *nodal* in the APD

The ability of Six3 to strongly downregulate (directly or indirectly) genes encoding Wnt ligands, as well as *nodal*, *lefty* and *chordin*, raises the possibility that it normally prevents expression of these genes in the APD. To evaluate this, we first examined the effects of Six3 on gene expression in Δ cadherin-injected embryos in order to eliminate possible counteracting effects of Six3 function in the endomesoderm. Both microarray and QPCR data show that, in embryos consisting mostly of the APD, Six3 suppresses expression of the Wnt genes, and *nodal*, *lefty* and *chordin* (Table 3; Fig. 8). However, in normal embryos (glycerol-injected), Six3 repression of these genes cannot be detected (Table 3; Fig. 8), indicating that additional mechanisms protect the APD from Wnt and TGF- β expression in the normal embryo.

Six3 regulation of other signaling pathways

Six3 also positively regulates (either directly or indirectly) genes encoding proteins that function in other signaling pathways (Table 3). These include *delta*, the Notch ligand that mediates lateral inhibition, a crucial process in neural development, as well as other potential regulators of neurogenesis. The latter include *fgf9/16/20*, *fgfr-like*, a membrane-bound receptor lacking the tyrosine kinase domain, and *frizzled 5/8*, a Wnt receptor that may

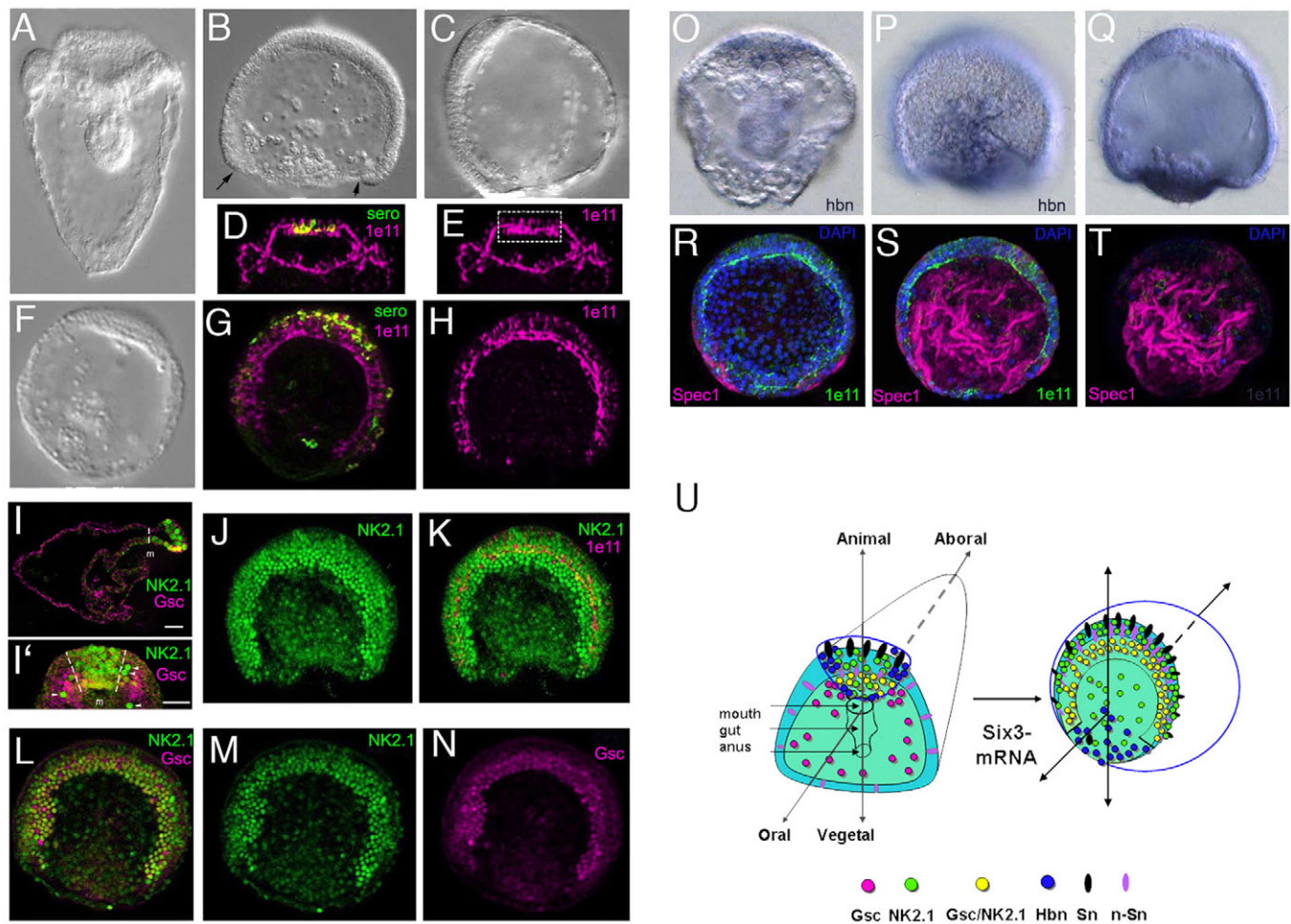


Fig. 6. Misexpression of *Six3* converts the embryo to an expanded APD. All embryos are 3 days old. (A) Normal embryo, DIC; blastopore view, oral up. (B,C) *six3* mRNA-misexpressing embryos, DIC; (B) oral view, (C) lateral view; arrows in B indicate the border between the expanded animal plate and the more vegetal thin epithelium. (D,E) Serotonergic (*sero*, green) and all (*1e11*, magenta) neurons in normal embryos. (F-H) DIC and immunostains, as in D and E of *six3* mRNA-misexpressing embryos; oral view, animal pole up. (I,I') Immunostains of normal 3-day embryos for NK2.1 (green) and *Gsc* (magenta); (I) lateral view; cells in the APD to the right of the white dashed line; (I') oral view, cells in the APD are between the dashed white lines. NK2.1-positive cells marked with arrowheads are in the blastocoel and not part of the APD; m, mouth. (J-T) *six3*-mRNA-misexpressing embryos. (J,K) NK2.1 (green); *1e11* (magenta). (L-N) NK2.1 (green); *Gsc* (magenta). (O-Q) DIC images of *hbn* whole-mount in situ hybridizations to control (O) and *six3* mRNA-injected embryos (P,Q); animal pole up; white circle in O marks the mouth. (R-T) A through-focal series of an embryo stained with DAPI, and for *1e11* (green) and *Spec1* (magenta) epitopes; *Spec1* labels the thin, expanded aboral epidermis which collapses and wrinkles in preparation. (U) Diagram illustrating distribution of APD cell types in normal and *Six3*-mRNA-misexpressing embryos. The colored dots show the distribution of cells expressing the indicated proteins or mRNAs and black and purple ovals indicate serotonergic (Sn) and non-serotonergic neurons (n-Sn), respectively. Green and blue shadings identify facial epithelium and ciliated band, respectively. *Six3* mRNA injection (arrow) results in a spherical 3-day embryo (right; see also B,C) consisting largely of the region outlined in blue in the normal embryo (left). In the *Six3*-mis-expressing embryo, the number of neurons and NK2.1/*gsc*-positive (yellow; supraoral) cells greatly expands and the *hbn*-positive cells (blue), which normally flank the animal plate on the oral side at this stage, are found at the vegetal pole. The arrows indicate the positions of the oral-aboral and animal-vegetal axes in both normal and *Six3*-misexpressing embryos. Scale bars: 20 μ m.

transduce non-canonical signals, but whose function in the APD is unknown (Croce et al., 2006). Future studies will examine how the activities of these signaling pathways and early *Six3*-dependent transcription factors interact in the APD gene regulatory network.

DISCUSSION

This study reports the finding that *Six3* is both necessary and sufficient for the specification and differentiation of a variety of cell types within the animal pole domain of the sea urchin embryo. This gene regulatory domain specifies the animal plate, the initial neurogenic territory of this embryo, and small regions of flanking

oral and aboral ectoderm. We show that *Six3* is required for the normal expression of most of the regulatory genes expressed in the APD of the blastula, for the specification of the thickened epithelium of the animal plate and for the development of both serotonergic and non-serotonergic neurons. Furthermore its misexpression is sufficient to convert the entire embryo to one consisting almost entirely of correctly patterned animal pole ectoderm containing the known cell types found in this region of the 3-day embryo. We conclude that the gene regulatory network (GRN) that specifies the APD depends on *Six3* and that the APD serves as an anterior patterning center governing the development of many different cell types in the animal pole ectoderm.

Table 2. Genes upregulated in Six3-misexpressing embryos

Gene	Urchin 1 QPCR ΔCt	Urchin 2 QPCR ΔCt	QPCR Average	Microarray log ₂ signal
dkk3	3.7; 2.4	0.9	2.3	0.9
fez	2.2; 2.4; 1.8	2.3; 2.3	2.2	1.4
FoxQ2	0.4; 2.9; 2.0	1.6; 0.9	1.5	0.7
Fzl5/8	2.3	2.2	2.3	1.7
hbn	3.3	2.6	3	2.2
nk2.1	3.4; 3.9	4.1	3.8	2.2
Nk3.2	2.7	1.5	2.1	2.4
rx	2.3	1.8	2.1	2
sfrp1/5	2.8; 2.2	0.8; 2.7	2.1	0.6
zic2	3.2; 2.6	1.7; 1.4	2.2	0

Values for QPCR cycle differences that are separated by semicolons indicate multiple measurements on samples from the same batch of embryos.

The known functions of sea urchin Six3-dependent genes or their orthologs in other embryos are consistent with its requirement for specification of the multiple cell types that differentiate in animal pole ectoderm. For example, NK2.1 and its regulator, FoxQ2 (Yaguchi et al., 2008), are necessary in cells that produce the long, immotile apical tuft cilia (Dunn et al., 2007). FoxQ2 also supports neural differentiation (Yaguchi et al., 2008) and several other Six3-dependent genes are subject to lateral inhibition through Notch signaling (our unpublished data), which is a cardinal feature of neurogenic genes (reviewed by Lewis, 1996). *Sp-Rx* is likely to function specifically in serotonergic neurons because it is exclusively co-expressed with serotonin during the early stages of differentiation (see Fig. S3 in the supplementary material). Orthologs of *Sp-Zic2* and *Sp-Ac-Sc* function in neural development in other embryos (Andreazzoli et al., 2003; Grinberg and Millen, 2005; Kageyama et al., 1995) and *hbn* is expressed in the anterior ectoderm of *Drosophila* (Walldorf et al., 2000) and polychaete annelids (Frobios and Seaver, 2006). Thus, Six3 is necessary for the differentiation of the non-neural cells producing long specialized cilia, serotonergic and non-serotonergic neurons and cells in the flanking ectoderm that express *hbn*. The large number of Six3-dependent regulatory genes reinforces the conclusion that Six3 functions at or near the top of the APD gene regulatory networks that specify animal ectoderm cell types.

The establishment of the APD depends on Six3, whereas specification and patterning of all other regions of the embryo ultimately depends on canonical Wnt signaling, initially at the opposite pole in the most vegetal blastomeres. Our results show that

canonical Wnt-dependent suppression of APD fates in the lateral ectoderm does not work through TGF- β signaling because the animal plate does not expand when Nodal and BMP2/4 are eliminated but Wnt signaling is maintained. However, the fact that it does expand in a TGF- β -deficient environment when Six3 is misexpressed (Fig. 7D) suggests that Six3 can antagonize Wnt-dependent processes. It can strongly repress genes encoding Wnt ligands, as is observed in both Six3-misexpressing and Δ cadherin-injected embryos, and it is required for APD-specific expression of *sFRP1/5* (Fig. 5; our unpublished observations), which may restrict Wnt signaling from the APD if it behaves as in the mouse embryonic forebrain (Houart et al., 2002; Lagutin et al., 2003). These observations suggest that the border between the APD and lateral ectoderm depends, at least in part, on interactions between Six3 and canonical Wnt-dependent processes.

Our data indicate that a primary role of TGF- β signals is to promote the differentiation of epidermal oral and aboral epithelia in the lateral ectoderm by suppressing the innate neural bias of early ectoderm. If Nodal/BMP signaling is knocked down, then neural cells can begin to differentiate in the lateral ectoderm. When these signals are present, then lateral ectoderm cells become epidermal except at the oral/aboral border, where the ciliated band forms and where non-serotonergic neurons develop. Where and when these ciliated band neurons are specified is not clear, but they do require the function of Six3. An interesting question for future experiments will be to determine whether they are originally specified throughout the lateral ectoderm but continue to differentiate only in the ciliated band, a site of late *six3* expression, or whether they are originally specified in the APD and then migrate to this site.

The Six3 misexpression phenotype is striking. Expression of marker genes indicates that Six3 is sufficient to convert nearly the entire embryo into animal pole ectoderm that is specified by the APD gene regulatory state during blastula stages and is molecularly and morphologically patterned into the cell types found in the 3-day pluteus larva. Furthermore, expression of most, if not all, of these genes requires Six3 in the normal embryo. These observations support the view that Six3 is not only upstream in neural GRNs, but also in those that specify other tissues in animal pole ectoderm. Collectively our findings strongly support a model in which the APD is a third patterning center, along with those operating in vegetal and oral blastomeres. Although the APD regulome controls the development of a discrete region of the embryo, its activity combines with that of the Nodal-dependent oral patterning center to

Table 3. Six3 regulation of signaling pathway components

Gene	Six3-mRNA/glycerol		Six3-MO/ Δ cadherin		Six3-MO/glycerol	
	Array log ₂ signal	QPCR ΔCt	Array log ₂ signal	QPCR ΔCt	Array log ₂ signal	QPCR ΔCt
Nodal	-1.3	-2.1	2.3	3	-0.2	-0.1
Lefty	-1.6	-2.7	2.4	2.2	-1.6	-2
Chordin	-2	-2.4	2.5	2.7	-2	-2
BMP	-0.72	-0.1	-0.5	-2	-1.1	-0.8
Wnt1	-2.1	-5.4	2.6	2.1	0.6	0.2
Wnt4	-4.1	-5.1	4	5.9	0.7	0.6
Wnt8	-1.5	-3.5	2.4	1.8	0.5	0.8
Wnt16	-2.5	-5.5	1.6	1.4	0.9	0.5
delta*			-0.9	-3.9		
fgf*			-2	-2.4		
fgfr1*			-1.8	-1.5		
Frizzled5/8*			-2.6	-2.1		

*Measured in cadherin-misexpressing embryos to eliminate Six3 effects in endomesoderm.

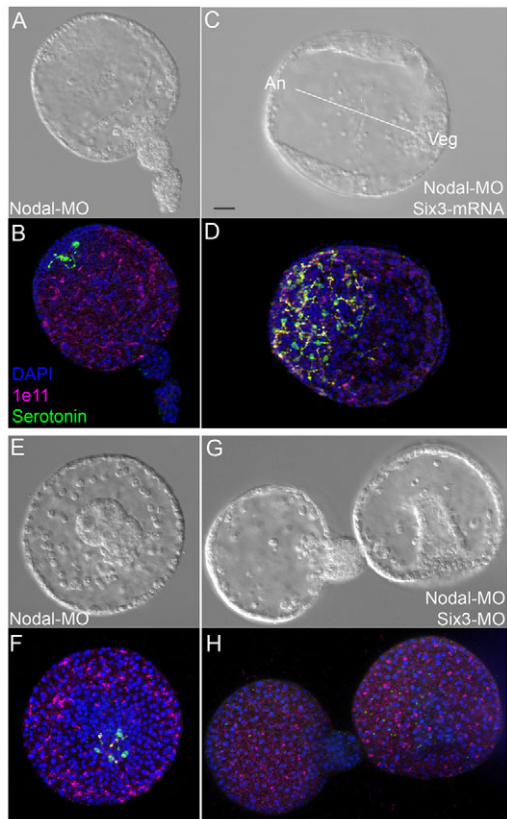


Fig. 7. Canonical Wnt-dependent APD restriction does not depend on Nodal and can be overridden by Six3 misexpression. (A,B,E,F) Nodal-MO only. (C,D) Nodal-MO and Six3 mRNA. (G,H) Nodal-MO and Six3-MO. (A,C,E,G) DIC; (B,D,F,H) immunostaining for serotonergic neurons (green) or all neurons (1e11, red). An, animal pole; Veg, vegetal pole. Scale bar: 20 μ m.

specify the supra-oral ectoderm defined by the combination of *gsc* (Nodal-dependent) and *nk2.1* (Six3-dependent) expression. Similarly, we anticipate the existence of Six3/BMP combinatorial control in the aboral portion of the APD.

An attractive hypothesis to explain the broad effects of Six3 in the APD is that it creates and preserves a territory resistant to the signals of the other patterning centers. This idea requires that, by the end of cleavage, Six3 reach levels in the APD that are sufficient to antagonize them. During cleavage, a Wnt-dependent process dominates *six3* and *foxq2* in the lateral ectoderm, restricting their expression to the APD. As a consequence, Nodal and BMP signaling are upregulated and oral/aboral patterning commences (Duboc et al., 2004; Yaguchi et al., 2008). We propose that as Nodal/BMP signaling increases during blastula stages, Six3 and FoxQ2 concentrations continue to rise in APD cells, creating a TGF- β - and canonical Wnt-free zone necessary for APD patterning and neurogenesis.

Here we focused on the role of Six3 in the APD, but it is also expressed in endomesoderm cells starting at mesenchyme blastula stage. Its role in this context is not known, but undoubtedly is controlled by the vegetal regulatory state.

The anterior neuroectoderm of vertebrate embryos and the APD of sea urchin embryos contain the initial neurogenic domains and the development of each requires Six3. Consequently, these embryos

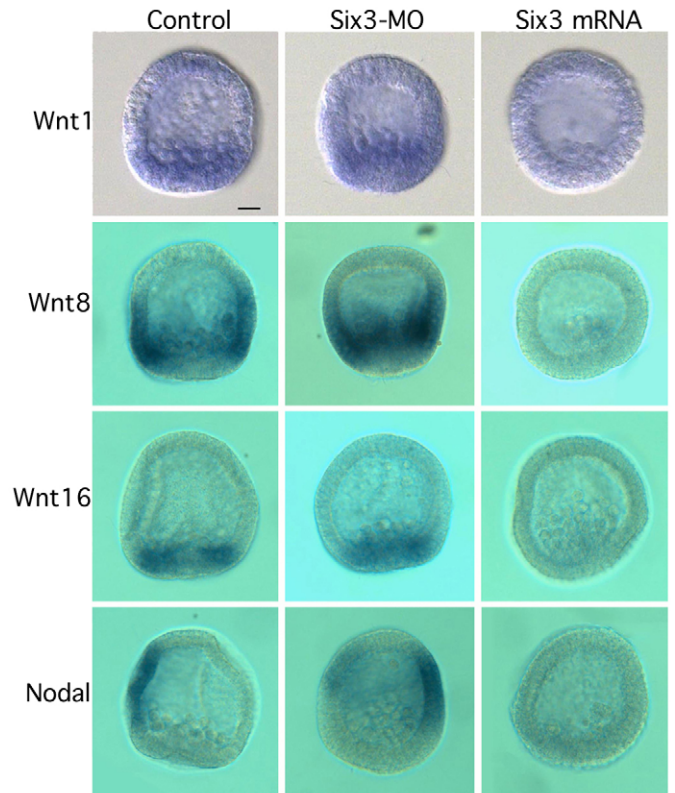


Fig. 8. Misexpression of Six3 suppresses expression of genes encoding Wnt ligands and Nodal, but loss of Six3 is not sufficient to allow their expression in the APD. Whole-mount in situ hybridizations detecting mRNAs encoding Wnt1, Wnt8, Wnt16 and Nodal in 26-hour embryos injected with either Six3-MO or Six3 mRNA. Embryos in the top row were photographed using DIC illumination on a Zeiss Axiomat microscope; all others were photographed using phase contrast illumination on a Leica microscope. Scale bar: 20 μ m

may share significant portions of the same gene regulatory network. In support of this, a significant number of the sea urchin Six3-dependent early-APD regulatory genes have orthologs expressed in either the forebrain or eye field, or both. These include *zic2*, *rx*, *achaete-scute*, *nkx2.1*, *fez*, *dkk3* and *sFRP1/5* (Andreazzoli et al., 2003; Diep et al., 2004; Elms et al., 2004; Ferreiro et al., 1993; Guillemot and Joyner, 1993; Houart et al., 2002; Jeong et al., 2007; van den Akker et al., 2008). It is likely that Six3-dependent regulatory proteins function in the core of an ancient GRN specifying the forebrain neural identity, which suggests that other newly identified sea urchin APD regulatory genes may also function in vertebrate early forebrain development. Consequently, the relatively advanced state of GRN analysis in the sea urchin embryo (Oliveri et al., 2008) and the discovery of a large set of Six3-dependent APD genes offers a potential blueprint for elucidating the control circuits that guide specification of neurogenic domains in other embryos and will provide a foundation for understanding how they evolved.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/7/1179/DC1>

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