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Expression of an NK2 homeodomain gene in the apical ectoderm defines a new territory in the early sea urchin embryo

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Abstract

We have identified an NK2 family homeodomain transcription factor, *SpNK2.1*, in the sea urchin *Strongylocentrotus purpuratus* whose transcripts are initially detected within the apical plate ectoderm of the hatching blastula and are confined to the apical organ at least through 2 weeks of development. Protein localization studies demonstrate that SpNK2.1 is restricted to the apical plate epithelium, but is excluded from the nucleus of serotonergic neurons. The expression profile of *SpNK2.1* is dictated via two separate regulatory systems. Initially, *SpNK2.1* is restricted to the apical pole domain by β-catenin-dependent processes operating along the animal–vegetal axis, as evidenced by an expansion of *SpNK2.1* expression upon cadherin overexpression. Starting at gastrulation, expression in the apical plate is maintained by *SpDri*, the sea urchin orthologue of *dead ringer*. Abrogation of SpDri results in the downregulation of *SpNK2.1* after gastrulation, but SpDri is not necessary for the initial activation of *SpNK2.1*. Loss of function experiments using SpNK2.1-specific morpholino antisense oligonucleotides and SpNK2.1 overexpression experiments do not disrupt embryonic development and have no effect upon the development of neuronal components of the apical organ. Nonetheless, *SpNK2.1* defines a new early territory of the sea urchin embryo.

Keywords: NK2; Sea urchin; Territory

Introduction

The presence of an apical organ is a shared feature among a wide range of marine invertebrate larvae (Lacalli, 1994; Nielsen, 2001). This structure develops within a thickened epithelium at the animal pole of the larval body, termed the apical plate, and is often associated with a bundle of elongated cilia, the apical tuft. The apical organ of the sea urchin is composed of two clusters of nerve cells, whose axons spread laterally across the anterodorsal edge of the oral hood, innervating the ciliated band (Bisgrove and Burke, 1986, 1987). The apical organs of many invertebrate larvae seem to play a role in sensory integration (Marois and Carew, 1997), and it has been suggested that in the sea urchin the apical organ coordinates larval feeding, locomotion, and metamorphosis (Burke, 1983a; Yaguchi and Katow, 2003). Like most marine invertebrates, including mollusks (Marois and Carew, 1997), the apical organ along with most of the larval nervous system is lost at metamorphosis (Chia and Burke, 1978).

The specification processes directing apical organ development remain unknown. A few regulatory genes have been identified that are expressed in the apical organ of the hemichordate, *Ptychodera flava*. The Sox family genes, *Pf-SoxB1* and *Pf-SoxB2,* are both expressed in the apical organ of the 4-day tornaria larva as well as in the ciliated bands and oral hood region (Taguchi et al., 2002). The hemichordate orthologue of the mammalian forebrain-specific gene *Tbrain-1* is expressed in several apical ectoderm cells of the gastrula embryo, eventually becoming restricted to the apical organ (Tagawa et al., 2000). Finally, transcripts of *PfNK2.1*, an NK2 family homeodomain regulatory gene, are initially detected within the apical

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ectoderm of mid-gastrula embryos and become localized to cells of the apical organ, as well as to cells of the ciliated band, and in large cells outlining the mouth of the 7-day-old larva (Takacs et al., 2002). Based on this expression pattern, *PfNK2.1* was interpreted as playing a neurogenic role in the tornaria larva, consistent with the function of *NK2.1* in direct-developing bilaterians (Harfe and Fire, 1998; Sussel et al., 1999; Zaffran et al., 2000). However, experimental limitations in *P. flava* have precluded functional analyses.

Here, we characterize the *NK2.1* orthologue in the sea urchin *Strongylocentrotus purpuratus*. *SpNK2.1* transcripts are initially detected within apical ectoderm cells of the hatching blastula. This expression is governed by early animal–vegetal patterning processes, which are dependent indirectly upon β-catenin activity. Later, starting around gastrulation, the maintenance of *SpNK2.1* within the apical plate is governed by a different set of regulatory inputs including *SpDri*, the sea urchin orthologue of *dead ringer* (Amore et al., 2003), that are implemented within committed oral ectoderm. Although abrogation of SpNK2.1 does not result in detectable developmental defects, the expression profile of *SpNK2.1* is unique and defines a new transcriptional territory called the apical domain in the early sea urchin embryo.

Materials and methods

Embryos

Adult *S. purpuratus* animals were obtained from Charles Hollahan (Santa Barbara, CA). Nickel-treated and lithium-treated embryos were incubated in 0.1 mM $NiCl_2$ or 120 mM LiCl, respectively, in artificial seawater from fertilization onward until the desired stages were reached.

Isolation of the SpNK2.1 and SpNK2.2 orthologues in S. purpuratus

To isolate the *SpNK2.1* gene, touchdown style polymerase chain reaction (PCR) (Don et al., 1991) was employed against the conserved homeobox region using the degenerate primers of Holland et al. (1998) with *S. purpuratus* genomic DNA isolated from sperm nuclei as the template. The amplification conditions were as described in Takacs et al. (2002). Phylogenetic analysis of the isolated fragments suggested that we cloned orthologues of *NK2.1*, *NK2.2*, and *NK2.5*. The *NK2.1* DNA fragment was radiolabelled using standard techniques and then used to screen a 20-h arrayed cDNA library using standard hybridization and washing conditions (http://sugp.caltech.edu:7000/resources/methods). The screening yielded four *SpNK2.1* clones, the largest of which was shown by sequence analysis to possess the entire coding region of *SpNK2.1*. The same screening produced six clones of *SpNK2.2*, although none of which contained the 3′ end of the coding sequence. Clones of *SpNK2.5* were not found in this library.

To screen a high-density genomic BAC library (Cameron et al., 2000), a 643-bp probe was digested from one of the pSPORT1 (Stratagene, CA) clones obtained from the arrayed library using *Eco*RV and *Xba*1. The same hybridization and washing was carried out as above. Six positive clones were isolated from screening approximately 4.5 genomes, and one of these clones (131N15) contained an approximately 40-kb fragment in which the *NK2.1* coding sequence was centered in the recombinant fragment. This clone was sequenced at the Institute for System Biology (Seattle WA), and the sequence is available at http://sugp.caltech.edu/.

To identify the transcriptional start site of *SpNK2.1*, 5′ RACE was employed against 20 h RNA according to the manufacturer's instructions (Marathon cDNA Amplification Kit, Clontech, CA) with the following primers; 5′-TCTCTCTTTCTCTCTCCCACTCTCAAAGTG-3′ and 5′-CTCGATGAGAGGGAAAGACAAGGACTCTCT-3′. Of six clones that were sequenced, the two clones possessing the most 5′ stretch sequence began at the same nucleotide position as two of the original clones isolated from the arrayed library.

Because none of the clones isolated from the arrayed library had a poly-A tail, we attempted to identify the 3′ end of the *SpNK2.1* mRNA by screening an approximately 50 h lambda cDNA library (ZAP Express cDNA Synthesis kit, Stratagene) with the 643-bp probe using the same hybridization and washing conditions as above. Screening of approximately 6×10^5 plaques yielded six positive clones, but sequence analysis revealed that none possessed a poly-A tail.

Genomic Southern blot analysis

Ten micrograms of sperm DNA extracted from three individual sea urchins was digested separately with three restriction enzymes: *Eco*RI, *Bam*H1, and *Hin*dIII. After organic extraction and gel electrophoresis, digested DNA was transferred to a Hybond N+ filter (Amersham, England). The 643-bp probe from above was employed for Southern hybridization using standard hybridization and washing conditions.

RNA blot analysis

Total RNA was extracted with RNAzol (Leedo Medical Laboratories, TX). Denaturing agarose gel electrophoresis was performed for 20 μg of RNA per sample followed by transfer onto Hybond N+ filters (Amersham). Hybridization and washing used the same conditions and probe as above.

Fixation and whole mount in situ hybridization

Whole mount in situ hybridization (WMISH) followed the protocols outlined in Arenas-Mena et al. (2000) except

that the embryos were fixed in 4% paraformaldehyde, 32.5% filtered ASW, 32.5% MOPS (pH 7) overnight at 4°C.

SpDri and *Spec1* probes were from Amore et al. (2003). For *SpNK2.1*, a 570-nucleotide antisense riboprobe directed at the 5′ end of the *SpNK2.1* coding region (+45 to +615) was generated from one of the original clones isolated from the arrayed library, which contained the 5′ UTR and 615 bp of the coding region cloned into pSPORT1 (Stratagene). This construct was linearized by *Eco*RV and used as a template for riboprobe synthesis by T7 polymerase according to the manufacturer's instructions (DIG RNA labelling kit, Roche, IN). Hybridization signal was apparent between 3 and 5 h after start of staining reaction. For *SpNK2.2*, a 1400-nucleotide antisense riboprobe was generated by linearizing the pSPORT1 construct with *Eco*RI and using SP6 for riboprobe synthesis.

Polyclonal antibody production

For antibody production, PCR primers were designed to amplify from bp 502 to 969 of the cDNA (Forward 5′-CGAAAGCGCAGGGTGCTGTT-3′ and Reverse 5′-GTGGTGGTTTGCAGCGGTCTG-3′). This fragment was cloned in frame with a C-terminal His-tag in the pET21d expression vector (Novagen, Germany). The bacterial strain BL21 (DE3) pLysS (Novagen) was used as expression host and recombinant protein was expressed, denatured, and purified. SDS-PAGE and immunoblots with anti-PentaHis antibody (QIAGEN, CA) were used to identify and confirm the purity of the protein before immunization. Immunization procedures followed Harlow and Lane (1988). Recombinant SpNK2.1 (400 μg) mixed with 500 μl of Freund's adjuvant was injected subcutaneously into a New Zealand White rabbit. After an initial immunization, the rabbit was boosted three times at 2-week intervals. Titer of the antisera from test bleed was assessed by immunocytochemistry and Western blot analysis with recombinant and native proteins (data not shown). For immunocytochemical assessments, the antigen was expressed in chick primary culture cells and mouse 3T3 cells by subcloning into the pCMS-eGFP vector with an in frame myc tag. Cells were transfected with Fugene6 (Roche). After 24 h, cells were fixed and double labelled with SpNK2.1 antisera and anti-myc (9E10). Immune sera that had the same nuclear co-localization as the myc tag were used (data not shown).

Indirect immunofluorescence

Embryos were fixed in 4% paraformaldehyde for 10–30 min at room temperature then rinsed and stored in filtered sea water containing 0.01 M sodium azide (Burke, 1983a,b). In some preparations, embryos were postfixed for 2 min in −20°C methanol. Fixed embryos were rinsed in phosphate-buffered saline (PBS) then incubated in PBS containing either 5% of 10% goat serum and 0.3% Triton X-100 or 0.1% Tween 20 for 30 min at room temperature to reduce nonspecific binding and increase permeability to antibodies. Specimens were incubated in primary antibody for 16 h at 4°C. Primary antibodies used were rabbit anti-SpNK2.1 (1: 800), rat anti-serotonin (Chemicon, CA) (1: 200), and 1E11 (1: 800) (Nakajima et al., 2004) Embryos were rinsed in PBS and incubated in FITC-conjugated goat anti-rabbit IgG diluted 1/16 in PBS for 1 h at room temperature or goat anti-rabbit Alexa 488 (1: 800) and goat anti-rat Alexa 568 (1: 1500) (Molecular Probes). Preparations were examined and photographed using a Zeiss Universal epifluorescence microscope (Carl Zeiss Vision, Germany) or a Zeiss 410 confocal microscope. Images were adjusted using Adobe Photoshop (v. 6.0).

Injection of morpholino oligonucleotides and mRNAs

Eggs were prepared for injection as described (McMahon et al., 1985). For each experiment, 5 mg/ml lysyl-rhodamine-dextran (Molecular Probes, OR) was included in the injection solution. The *SpNK2.1* morpholino antisense-substituted oligonucleotide (MASO) and a control MASO were obtained from Gene Tools (Corvalis, OR) and dissolved in RNAse-free H_2O at a concentration of 2 mM. This stock solution was diluted to 300 μM in 120 mM KCl for injection. The sequence of the *SpNK2.1* MASO (5′-GGGCTATATGACAT CTCTTGGAGA-3′) was complementary to nucleotides −10 to +14 with respect to the translation start site of SpNK2.1 which is underlined in the MASO sequence above.

To test the effectiveness of the *SpNK2.1* MASO, a SpNK2.1-GFP fusion construct was assembled by cloning a fragment of the *SpNK2.1* cDNA (nucleotides −157 to +134) upstream and in frame with the green fluorescent protein (GFP) coding sequence used by Arnone et al. (1997). Primers for the amplification were morphF (5′-GGTACC CCAG ATAGAGAGAGTCCTTG-3′) and morphR (5′-GACGTC CATAGACCGTCTGAAG CTC-3′). The fragment was cloned using *Kpn*I and *Aat*II to create insertion sites (underlined sequences). The pMAR1-GFP fusion plasmid was from Oliveri et al. (2002).

The ΔLv-Cadherin plasmid was from Logan et al. (1999). The in vitro mRNAs for injection were transcribed from 1 μg of linearized plasmid by using the mMessage-mMachine Kit (Ambion, TX) as instructed by the manufacturer, purified by organic extraction and precipitation, verified by gel electrophoresis and spectrophotometry, and injected at a concentration of 80–100 ng/μl in a 120 mM KCl solution.

RNA isolation, cDNA preparation, and QPCR assay

RNA isolation, cDNA preparation, and QPCR assay used the protocols outlined in Amore et al. (2003). The *SpNK2.1* primers used in the QPCR reactions were forward 5′-CGTGAGAGCTTCCCTACCTG-3′ and reverse 5′-GAAGCTCCCTAGCTCGATGA-3′.

Results

Characterization of SpNK2.1

The cDNA sequence of *SpNK2.1* contains an open reading frame that is 412 amino acids long (GenBank accession number AAM94862). This sequence includes a TN domain, a repressive domain (Smith and Jaynes, 1996), a homeodomain (containing the conserved nuclear localization signal), and an NK-2-specific domain (for review of NK2 genes, see Harvey, 1996). Phylogenetic analysis supports the inclusion of *SpNK2.1* with other *NK2.1* genes (Takacs et al., 2002). Organization of the *SpNK2.1* gene is shown in Fig. 1A. The coding sequence is interrupted by a single intron that is 3303 base pairs long. Using 5′ RACE, we determined the start of transcription to be −602, which corresponded to the beginning of two of the clones from a 20 h arrayed cDNA library. We did not isolate any clone containing the complete 3′ end, but a consensus polyadenylation site (Zarudnaya et al., 2003) is located at +7729.

To determine *SpNK2.1* gene copy number, we searched the Sea Urchin Genome Project "Trace Gene Matches" (http://issola.caltech.edu/~kevinb/wangp/tmp/). Only one copy of each of the three *NK2* genes was found. To confirm this preliminary genomic search, we also performed genomic DNA hybridization analysis. Of the three restriction enzymes used, all produced 1–3 fragments that reacted with the *SpNK2.1* probe in all three individuals, suggesting that *SpNK2.1* exists as a single copy gene (data not shown). This is consistent with the BAC library screen in which 4.5 total sea urchin genomes (73,728 clones screened; library recombinant fragments averaging 60 kb in length) gave only six positive clones.

SpNK2.1 expression during embryogenesis

To address the expression profile of *SpNK2.1*, we used QPCR to quantitate the number of transcripts per embryo from egg (0 h) through 72 h of development (Fig. 1B). *SpNK2.1* expression is not detectable in the unfertilized egg or during early cleavage (0–10 h pf). Transcripts were first evident at the hatching blastula stage (approximately 15 h pf) as assayed by QPCR, and increase to more than 500 transcripts/embryo at 36 h of develop-

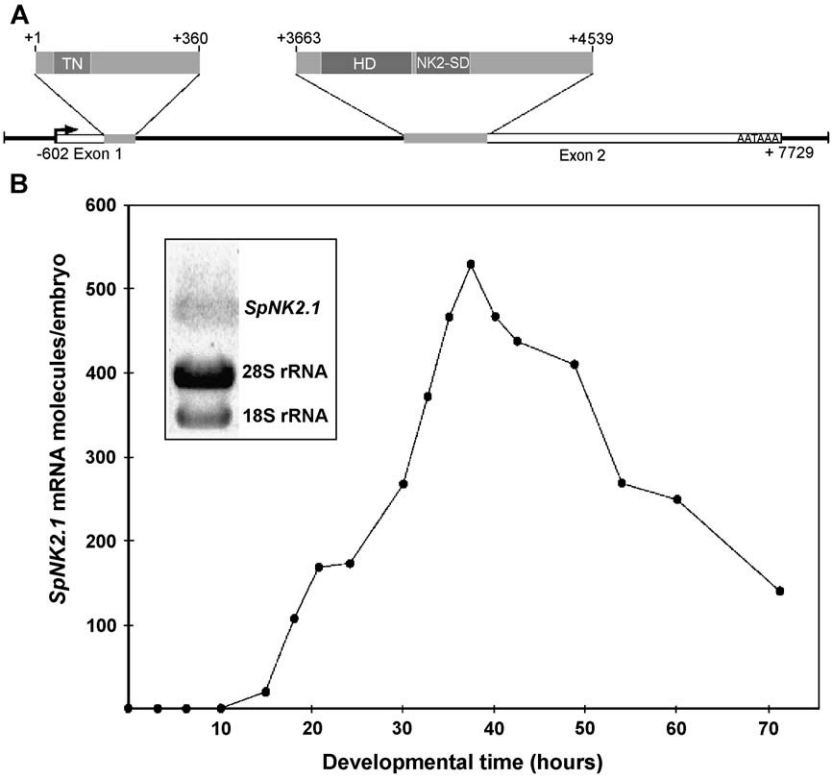

Fig. 1. (A) Genomic structure of the *SpNK2.1* gene. The coding region is divided into two separate exons. The untranslated portions of the exons are shown as open boxes; the translated portions are shown in gray and are magnified above with the major NK2 domains indicated. The numbering is with respect to the "A" of the start codon. The putative transcription start site is indicated with an arrow at −602. The putative 3′ polyadenylation signal sequence is shown (AATAAA). (B) *SpNK2.1* transcripts per embryo, measured by QPCR. The measurements of *SpNK2.1* transcript prevalence at each stage were converted to molecules per embryo by reference to an internal standard (see Amore et al., 2003 for details). Inset shows the expression of *SpNK2.1* as revealed by RNA blot hybridization. Total RNA was prepared from 18 h pf embryos, and the lane was loaded with 10 μg of total RNA. The blot was hybridized against a radiolabelled *SpNK2.1* probe and washed under high stringency conditions. Note the relatively high molecular weight of *SpNK2.1* as compared to 28 S rRNA, and that only a single band is detected.

ment, and then gradually decrease to approximately 150 transcripts/embryo at the pluteus stage (approximately 72 h pf). Using RNA blot analysis, only a single band was detected, and this band is considerably larger than the 28 S rRNA band, consistent with the size estimate above (Fig. 1B, inset).

To address the spatial localization of *SpNK2.1*, we used whole mount in situ hybridization (WMISH). In the hatching blastula, expression is restricted to a group of ectodermal cells at the apical pole of the embryo (Fig. 2A). These cells contribute to the apical plate, a thickened epithelium from which components of the larval nervous system differentiate, including ciliated band elements that mark the border between oral and aboral ectodermal domains (Cameron et al., 1993; Davidson et al., 1998). During gastrulation, expression is confined to the oral territory (Figs. 2B and C). We often observed what appeared to be staining within the foregut (Fig. 2C, arrow). However, as shown in Figs. 2D and E, this perceived endodermal signal is due to overlying ectodermal staining. In contrast to embryos examined from the oral side (Fig. 2D), embryos examined from the anal side never display foregut staining (Fig. 2E) at this stage of development. In the early pluteus larva, as the apical plate becomes incorporated into the circumoral ciliated band (Burke, 1983b), the gap between the zone of *SpNK2.1* expression and the mouth (Fig. 2F, asterisk) is gradually lost (Fig. 2G). Expression in the circumoral ciliated band continues at least through the 2-week pluteus (Fig. 2G).

SpNK2.1 protein localization recapitulated the pattern observed for *SpNK2.1* mRNA (Fig. 3). The protein begins to accumulate at the mesenchyme blastula stage within the apical domain (Fig. 3A) and persists through gastrulation (Figs. 3B and C). The protein was detected in a few cells in the foregut after the mouth opened (Fig. 3C), but not before (Fig. 3B), consistent with the WMISH results discussed

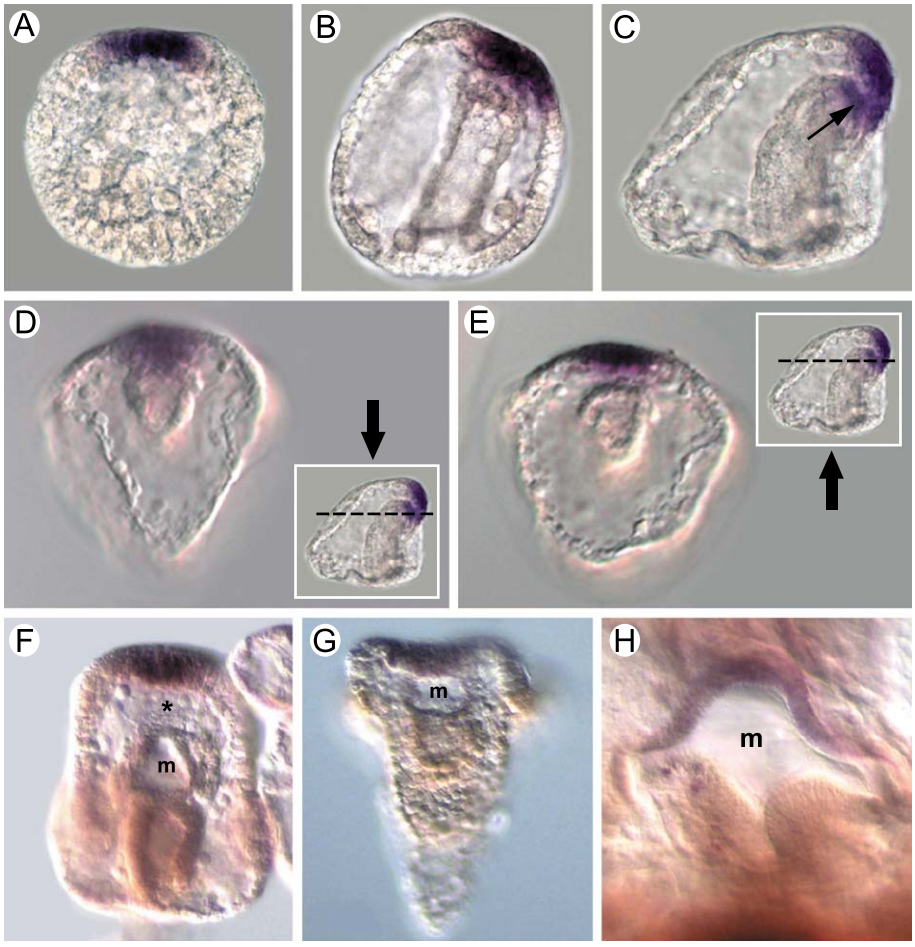

Fig. 2. *SpNK2.1* mRNA expression during embryogenesis as revealed by WMISH. (A) Lateral view of a hatched blastula (20 h pf). Transcripts are restricted to the apical plate. (B) Lateral view of mid-gastrula stage (28 h pf) embryo. (C) Lateral view of late gastrula stage (44 h pf) embryo. Note that the top of the foregut (arrow) lies beneath the ectodermal zone of expression. (D–E) Oral view (D) and anal view (E) of 44-h embryo. The deceptive endodermal signal is due to overlying positively stained oral ectoderm as no signal is observed when viewed from the anal side. (F) Oral view of an early pluteus larva (72 h pf). (G) Oral view at 96 h pf. By this stage, the apical plate has become incorporated into the circumoral ciliated band, closing the gap between expression locus and mouth (m) (asterisk in F). (H) Oral view of a 2-week larva. Transcripts continue to be detected in the circumoral ciliated band.

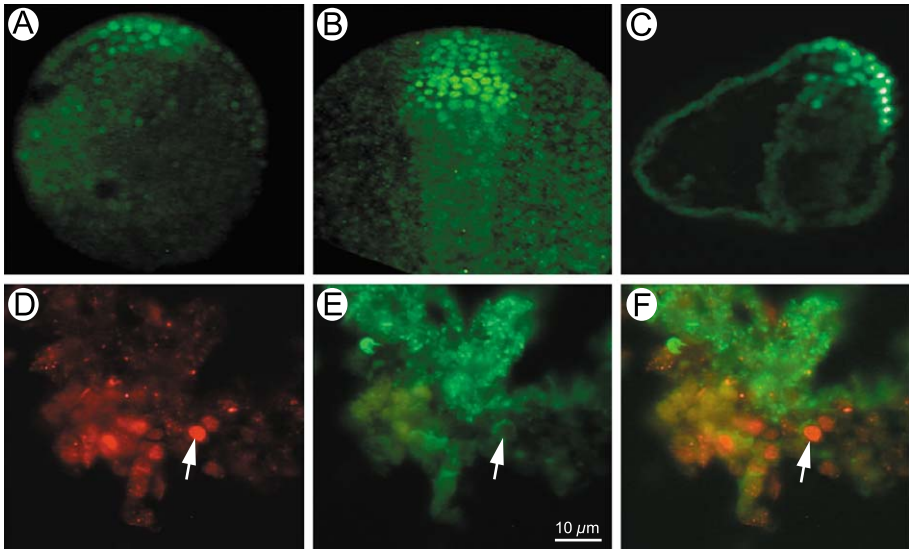

Fig. 3. SpNK2.1 protein localization as revealed by indirect immunofluorescence. (A) Epifluorescence image of a 36-h embryo (oral view). (B) Epifluorescence image of oral view of 49-h embryo. (C) Confocal optical section of the lateral view of 52-h prism stage embryo. (D–F) SpNK2.1 is detected in radial nerve cells of adult. Epifluorescent image of a cryostat section through an interface between neuronal cells bodies and the neuropil. The neuronal cell bodies occupy the lower part of the image and with anti-SpNK2.1 (D, red). The neuropil (upper right) and cell bodies react with 1E11 and are green (E). (F) Merged image of D and E. The arrow indicates a 1E11 immunoreactive cell that has SpNK2.1 in the nucleus.

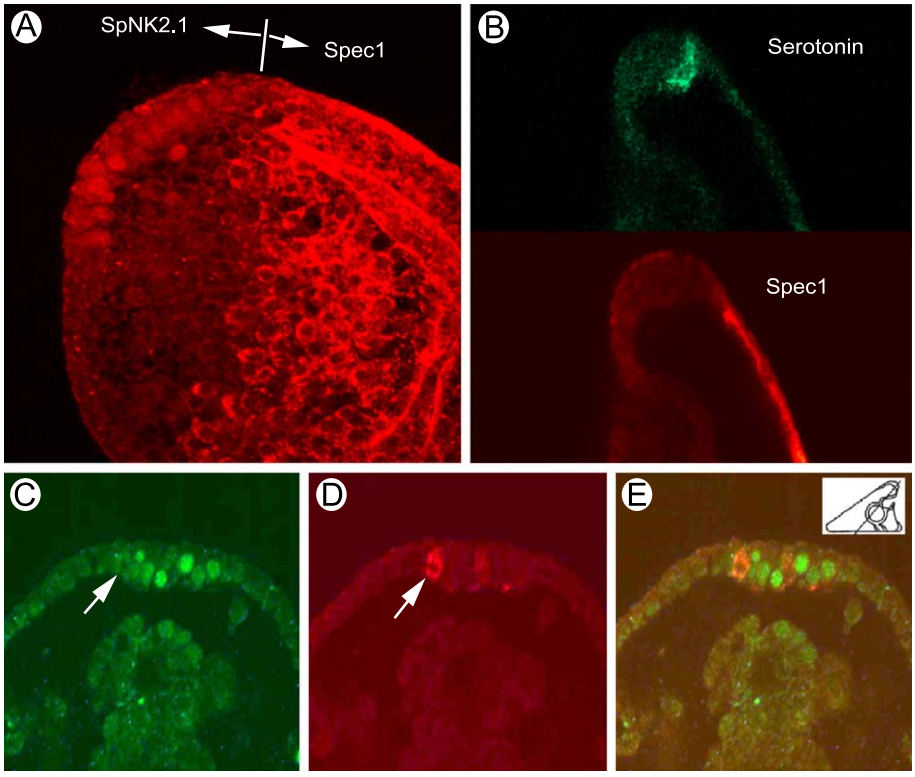

Fig. 4. SpNK2.1 is restricted to oral ectoderm. (A) Confocal image of a 56-h embryo viewed laterally. This is a projection of a through focus series. SpNK2.1 and Spec1 do not co-localize to the same cells; Spec1 is detected throughout the cytoplasm in squamous cells, and SpNK2.1 is in the nuclei of more cuboidal cells. (B) Confocal images of a 56-h embryo viewed laterally. Anti-serotonin (green, top) and anti-Spec (red, bottom) do not co-localize. (C–E) SpNK2.1 protein does not localize to the nuclei of serotonergic cells. Confocal optical section of an oral view of a 65-h pf embryos prepared for indirect immunofluorescence (see inset for view). (C) Anti-SpNK2.1 staining (green). (D) Anti-serotonin staining (red). (E) Merged image of C and D. Serotonergic cells lie directly against field of SpNK2.1-expressing cells. The arrow denotes a serotonergic cell that lacks nuclear SpNK2.1 protein.

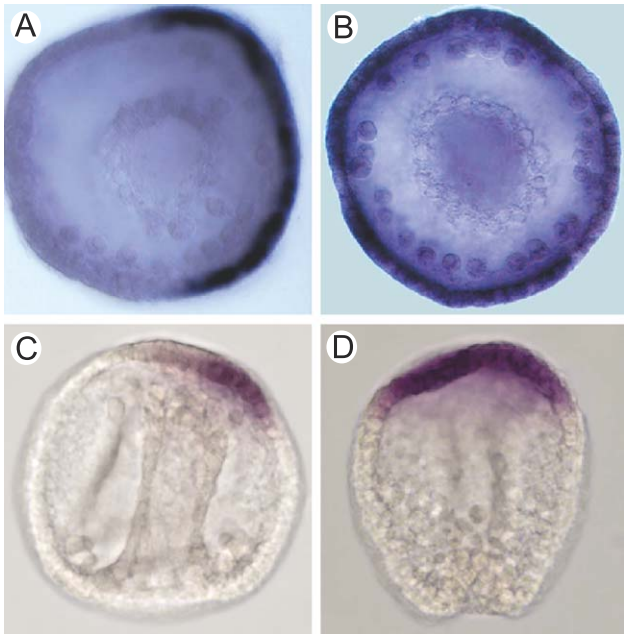

Fig. 5. Treatment with nickel results in an expansion of both *SpDri* (A, B) and *SpNK2.1* (C, D) expression as assayed by WMISH on mid-gastrula embryos. A and B are vegetal views; C and D are lateral views. (A, C) Untreated control embryos. (B, D) $NiCl_2$-treated embryos.

above (Figs. 2C–E). We also examined protein expression in newly metamorphosed animals (Figs. 3D–F). The protein was detected in juvenile radial nerve cells (Fig. 3D) which also expressed the pan-neural marker 1E11 (Figs. 3E and F).

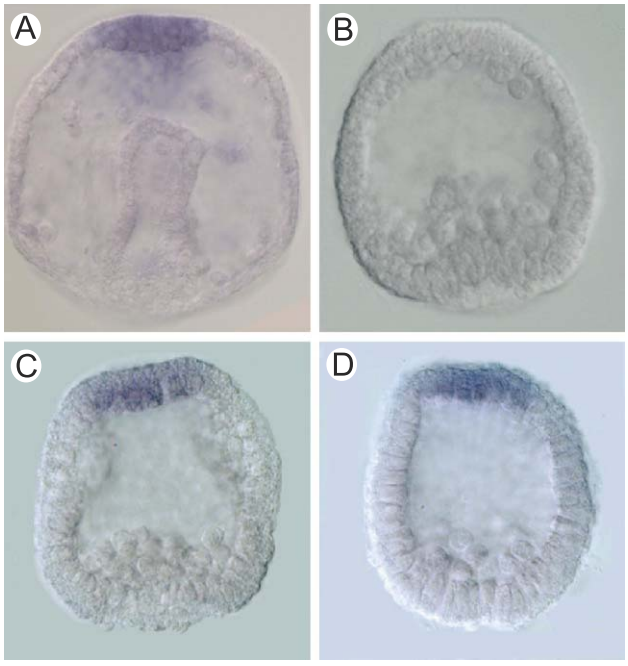

Fig. 6. Knockdown of SpDri abrogates the expression of *SpNK2.1* in mid-gastrula embryos (A, B), but does not affect the initial activation of *SpNK2.1* in hatching-blastula embryos (C, D), as assayed by WMISH. (A, C) Lateral views of control MASO-injected embryos. (B, D) Lateral views of *SpDri* MASO-injected embryos.

To better address the lineage relationships between the expression domain of *SpNK2.1* and oral–aboral ectodermal territories, we examined the protein expression profile of *SpNK2.1* and *Spec1*, an aboral ectodermal marker (Hardin et al., 1985; Tomlinson and Klein, 1990), as well as serotonin which labels the serotonergic components of the apical plate (Fig. 4). SpNK2.1 and Spec1 do not co-localize; although both antibodies are rabbit polyclonal, they each have a distinctive labelling pattern. Spec1 was detected throughout the cytoplasm in the aboral ectodermal squamous cells, whereas SpNK2.1 was detected in the nuclei of more cuboidal cells (Fig. 4A). In addition, although serotonergic cells have previously been reported as forming on the aboral side of the apical plate (Yaguchi et al., 2000; Nakajima et al., 2004), Nakajima et al. (2004) have shown that serotonergic cells are in fact oral, as they do not co-localize with Spec1-

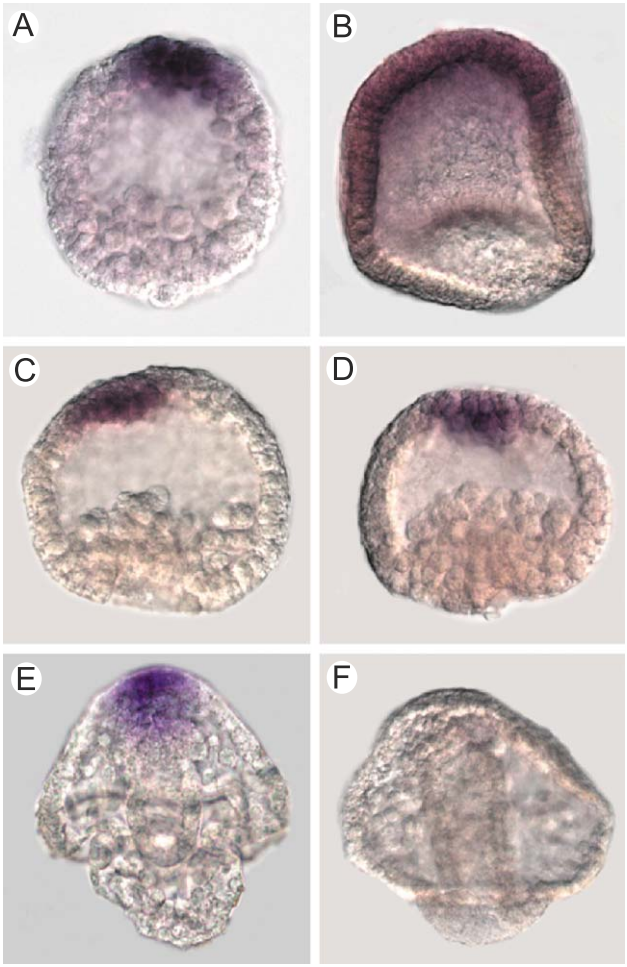

Fig. 7. *SpNK2.1* expression is expanded in cadherin-injected embryos (A, B), but is not affected by lithium (C, D) in hatching-blastula embryos, as assayed by WMISH. (A) Lateral view of a control embryo injected with GFP mRNA. (B) Lateral view of a cadherin-injected embryo. (C) Lateral view of a control embryo. (D) Lateral view of a lithium-treated embryo. (E, F) Lithium treatment greatly attenuates expression of *SpNK2.1*, as assayed by WMISH. (E) Oral view (tilted towards the anal surface) of a control embryo several hours after placement into stain. (F) Oral view of a lithium-treated embryo 24 h after placement into stain.

positive cells (Fig. 4B). Finally, the serotonergic-positive cells have SpNK2.1 protein in their cytoplasm, but none of those examined possessed nuclear SpNK2.1 (Fig. 4C, arrow). This is in sharp contrast to adjacent serotonin-minus cells, which showed strong staining of SpNK2.1 within the nuclei (Figs. 4D and E). These data suggest that expression of SpNK2.1 is restricted from its initial activation to future oral ectoderm, and that the serotonergic component of the apical plate is partitioned from the most aboral region of the SpNK2.1-positive domain.

Oralization of embryo results in expansion of SpNK2.1 expression

To test whether *SpNK2.1* is susceptible to perturbations in O/A specification, we examined embryos treated with $NiCl_2$. Nickel affects O/A patterning by oralizing the affected embryo at the onset of gastrulation (Hardin et al., 1992). Consistent with this hypothesis, *SpDri*, the sea urchin orthologue of *dead ringer* (Amore et al., 2003), is expressed throughout the entire ectoderm (Figs. 5A and B). Exposure to nickel also resulted in an expansion of *SpNK2.1* message, with expression radialized around the apical pole of the embryo (Figs. 5C and D).

β-catenin functions early to restrict SpNK2.1 expression to the apical pole domain

Amore et al. (2003) demonstrated that starting around gastrulation, the expression of *SpNK2.1* is dependent upon *SpDri*, which is upregulated within the oral ectoderm approximately 27 h pf, and is required for maintaining the oral ectoderm specification state. Injection of a *SpDri* MASO abrogates *SpNK2.1* expression starting at approximately 30 h pf (Figs. 6A and B). However, *SpNK2.1* transcripts are detected before *SpDri* ectodermal expression begins, suggesting that *SpDri* is not the initial activator of *SpNK2.1*. Consistent with this observation, SpDri inhibition does not affect the early accumulation of *SpNK2.1* message at 24 h pf as analyzed by QPCR (Amore et al.) and WMISH (Figs. 6C and D).

We next investigated whether the upregulation of *SpNK2.1* is influenced by animal–vegetal (A/V) patterning processes. One of the first steps in the elaboration of the A/V axis is the nuclearization of β-catenin in vegetal blastomeres during cleavage (Angerer and Angerer, 2003; Logan et al., 1999). This process can be abrogated by injection of mRNA encoding the cytoplasmic tail of cadherin, which serves to sequester β-catenin and prevent its entry into the

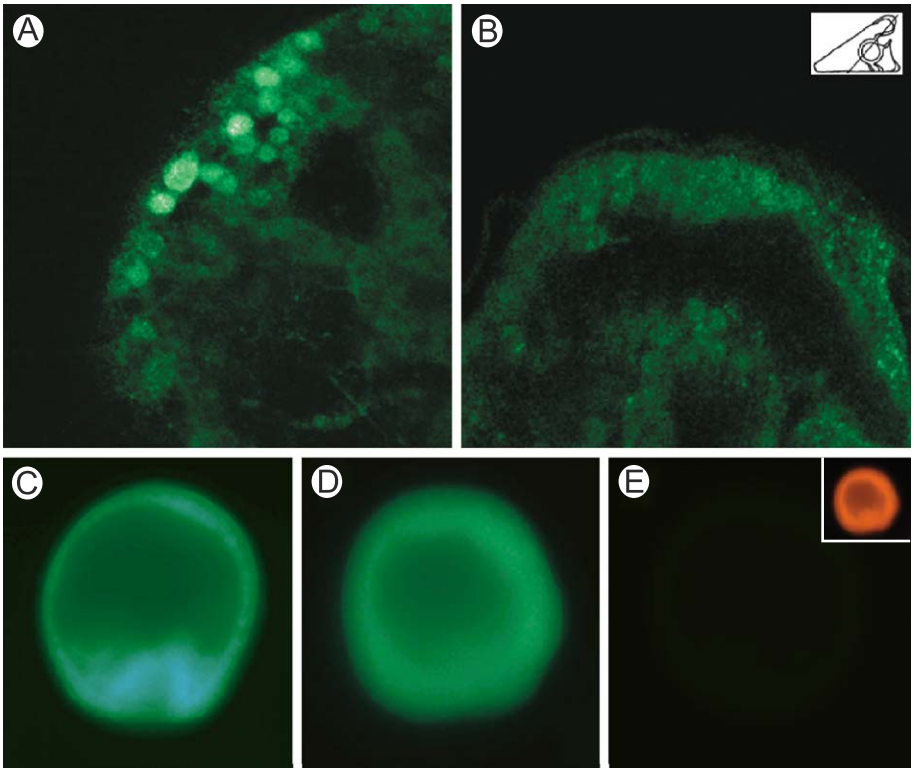

Fig. 8. SpNK2.1 protein levels are dramatically reduced in *SpNK2.1* MASO-injected embryos. (A, B) Oral view (see inset) of oral hood stained with anti-SpNK2.1 antibody. Embryos were fixed for indirect immunofluorescence at 65 h pf. (A) Control MASO-injected embryo. (B) *SpNK2.1* MASO-injected embryo. Signal was not detected above background levels in *SpNK2.1* knockdown embryos. (C–E) Lateral views of the control experiments observed at 22 h pf, demonstrating that the SpNK2.1 MASO inhibits translation in a sequence-specific manner. (C) Co-injection of a randomized control MASO with a fusion GFP mRNA that includes the *SpNK2.1* leader sequence to which the *SpNK2.1* MASO is targeted. (D) Co-injection of *SpNK2.1* MASO with fusion GFP mRNA that contains the leader sequence of the *pmar1* gene. (E) Co-injection of *SpNK2.1* MASO with *SpNK2.1*-GFP fusion mRNA. The *SpNK2.1* MASO abolishes the translation of *SpNK2.1*-GFP mRNA. All embryos were successfully injected, as assayed by the presence of rhodamine (inset).

nucleus (Heasman et al., 1994). Cadherin-injected embryos fail to generate vegetal cell types and form epithelial balls of uncommitted ectoderm (Logan et al., 1999; Wikramanayake et al., 1998). Cadherin overexpression resulted in a dramatic increase in *NK2.1* expression (Figs. 7A and B), suggesting that β-catenin activity serves to repress *SpNK2.1*. However, the addition of lithium does not affect *SpNK2.1* expression at 22 h pf (Fig. 7D). Curiously, if embryos were incubated in the presence of lithium until gastrulation, then expression of *SpNK2.1* was greatly attenuated (Figs. 7E and F). Stain was eventually detected in the proper spatial domain in a few embryos, but not for at least 24 h after stain was clearly detected in batch-control embryos.

SpNK2.1 is not involved in apical organ neurogenesis

We used a *SpNK2.1* MASO to ascertain *SpNK2.1* function. As shown in Figs. 8A–B, SpNK2.1 protein levels are dramatically reduced in *SpNK2.1* MASO-injected embryos when compared to control embryos, and quantitative analysis confirms that fluorescence intensity was reduced to background levels in knockdown embryos (data not shown). This reduction in activity is due to the specific sequence of *SpNK2.1* targeted by the MASO (Figs. 8C–E). A synthetic mRNA containing the 5′ *SpNK2.1* leader sequence inserted upstream of green fluorescent protein (NK2.1-GFP) produces a strong fluorescent signal in the absence of *SpNK2.1* MASO. However, upon co-injection of the *SpNK2.1* MASO, the signal was completely abolished (Fig. 8E). The randomized control MASO had no effect on GFP synthesis (Fig. 8C), nor did co-injection of *SpNK2.1* MASO with GFP message that contained the pmar1 (Oliveri et al., 2002) leader sequence (Fig. 8D).

SpNK2.1 knockdown embryos appeared normal throughout development and showed no obvious defects in swimming ability. In addition, both SpNK2.1 knockdown and control embryos consistently generated two to four correctly positioned serotonergic cells (Fig. 9A), and immunocytochemical staining of the pan-neural marker 1E11 revealed nerve cells successfully undergoing axonogenesis (Fig. 9B). Overexpression of SpNK2.1 via mRNA injection also did not result in any morphological defect, nor did it affect neuronal development as assayed by serotonin and 1E11 markers (data not shown).

We further investigated the possible regulatory function(s) of *SpNK2.1* by examining transcript abundances of other early oral regulatory genes upon SpNK2.1 knockdown. Introduction of the SpNK2.1 MASO did not affect the expression of *SpDri* (Amore et al., 2003), *SpGsc* (Angerer et al., 2001), *SpNK1* (T. Minokawa and E. Davidson, unpublished), *SpOtp* (Di Bernardo et al., 2000), or *SpLim* (P. Oliveri and E. Davidson, unpublished). We also examined whether *SpNK2.1* served to regulate its own expression. QPCR (data not shown) and WMISH (Figs. 9C and D) analysis of *SpNK2.1* expression in late gastrula MASO-injected embryos revealed no observable difference

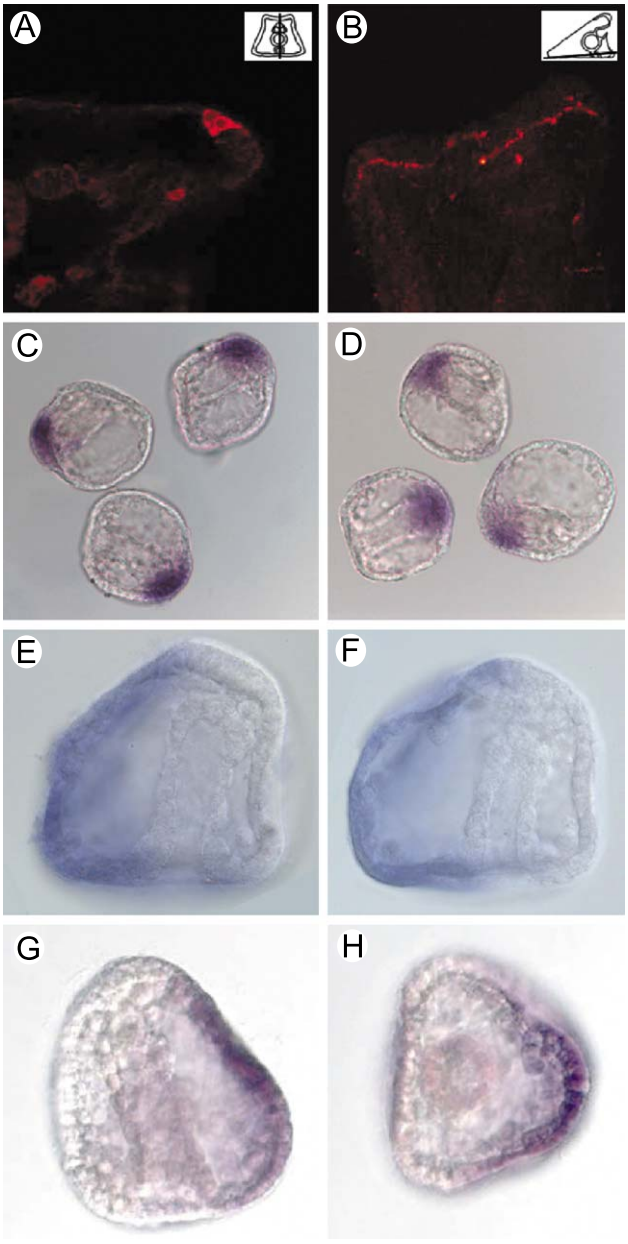

Fig. 9. SpNK2.1 knockdown does not affect apical organ development. (A, B) *SpNK2.1* MASO-injected embryos were prepared for indirect immunofluorescence at 65 h pf. Insets on each panel indicate the view. (A) Confocal optical section, right lateral view showing two serotonergic cells. Anti-serotonin labelling reveals the correct number of serotonergic neurons that develop in the correct position in *SpNK2.1* MASO-injected embryos. (B) MASO-injected embryo prepared with 1E11. Confocal optical section through the developing ventral transverse ciliary band showing neurons extending immunoreactive processes. The number and location of neurons did not differ from embryos injected with control MASO. (C–F) WMISH analysis of *SpNK2.1* (C, D) and *Spec1* (E, F) expression in SpNK2.1 knockdown embryos. (C, E) Control MASO-injected embryos. (D, F) *SpNK2.1* MASO-injected embryos. No observable difference could be seen between the controls and experimentals. (G, H) The absence of a role for *SpNK2.1* in apical organ development is not due to redundancy. WMISH analysis of *SpNK2.2* at mid-gastrula stage. (G) Lateral view. (H) Cross-section. Transcripts are restricted to the aboral ectoderm and are never detected in the apical plate. A fuller description of *SpNK2.2* expression and function will be published elsewhere (Poustka et al., in preparation).

from control embryos. We also looked at the aboral gene *Spec1* (Hardin et al., 1985; Tomlinson and Klein, 1990). Quantitative PCR (data not shown) and WMISH expression analysis (Figs. 9E and F) of *Spec1* also showed no effect, suggesting that the oral–aboral boundary at the apical pole is correctly specified in knockdown embryos.

To address whether there exists redundancy among *NK2* genes within the apical plate, we isolated *SpNK2.2*, and a fragment of the sea urchin orthologue of *NK2.5*. *NK2.5* was not found in the 20 h arrayed cDNA library. Transcripts of *SpNK2.2* (GenBank accession AY549449) were restricted to the aboral ectoderm and were not detected in the apical plate region (Figs. 9G and H).

Discussion

We characterized herein the expression of an NK2-family homeodomain transcription factor in the sea urchin *S. purpuratus*. *SpNK2.1* is the first transcription factor whose expression is restricted to the apical domain of the developing embryo, and as such defines a new early territory of the sea urchin embryo. The expression of *SpNK2.1* is directed via two separate regulatory phases. Initially, expression is upregulated in the blastula by determinants situated along the animal–vegetal axis. Although the expression of *SpNK2.1* is sensitive to perturbations affecting the nuclearization of β-catenin, it is not affected by the addition of lithium to the system, and no known early regulator or combination of regulators can account for this expression pattern. However, the maintenance of expression after the start of gastrulation seems to be under the control of *SpDri*, a key activator of the oral ectoderm gene regulatory network (GRN). The juxtaposition of these two phases results in a seamless and contiguous expression pattern that chronicles the transfer of regulatory control from broad A/V regulators to a genetic regulatory network running within the definitive oral ectoderm territory.

Early expression of SpNK2.1 is dictated by A/V patterning processes

Recent experimental investigations into the early development of the sea urchin have revealed that two maternally inherited systems set up the animal–vegetal axis (reviewed in Angerer and Angerer, 2000; Angerer and Angerer, 2003; Brandhorst and Klein, 2002): an animalizing set of transcription factors (called ATFs) including *Sox* proteins, and the vegetal transcriptional complex of β-catenin-Lef1/TCF. Upon nuclearization of β-catenin, three zones of specification are defined: the micromeres (which cause the skeletogenic mesenchyme) at the most vegetal end, the ectoderm at the animal end, and in between, a zone of overlap which gives rise to endoderm, secondary mesenchyme, and some ectoderm.

The work described here supports the existence of a new territory at the most animal end of the embryo, which we term the apical domain. Although equivalent to the "animal pole domain" of Angerer and Angerer (2003), we prefer "apical" to "animal" because in cnidarians and ctenophores, the polar bodies are extruded at the blastoporal pole, and thus the "animal–vegetal" axis is reversed between diploblasts and triploblasts (Nielsen, 2001). Given the possible homology of the apical organ (and apical–blastoporal axis) between diploblasts and triploblasts (Nielsen, 2001), and expression of the NK2.1 orthologue in the peduncle and lower end of the body column in *Hydra* embryo (Grens et al., 1996), we prefer the more applicable and pan-metazoan label "apical" rather than "animal."

Until now, aside from its unique morphology, the only indication of an apical domain was the lack of transcript accumulation of several genes including *SoxB2* (Kenny et al., 2003; reviewed in Angerer and Angerer, 2003). *SpNK2.1* is the first gene that is positively regulated in the apical domain and thus sets the apical domain as a distinct transcriptional territory from generalized ectoderm. Curiously, despite the amount of effort put in to understanding A-V patterning, no known regulator or combination of regulators can adequately account for this expression profile. All that is known is that blockage of β-catenin results in an expansion of *SpNK2.1* expression (Fig. 7), suggesting that β-catenin indirectly functions to antagonize the inherent activation potential resident at the top of the embryo. Nonetheless, upregulation of nuclear β-catenin via lithium incubation (Emily-Fenouil et al., 1998; Klein and Melton, 1996) has no effect upon *SpNK2.1* expression at 22 h pf (Fig. 7). The protein localization of ATFs such as SoxB1 in the apical plate is also resistant to vegetalizing perturbations (Howard et al., 2001), and both observations attest to the refractory nature of the apical domain to enhanced vegetal signaling (Angerer and Angerer, 2003). Furthermore, the specification of both ciliated band and serotonergic precursors appears to be autonomous, as differentiation occurs in animal halves (Wikramanayake and Klein, 1997) which are thought to lack β-catenin-dependent signaling. We hypothesize that the apical domain is specified autonomously via global ATF activation of *NK2.1*, but whose vegetal extent is determined ultimately by unknown β-catenin-dependent mechanisms.

Postgastrular expression of SpNK2.1 is dependent upon oral ectodermal regulators

Starting around gastrulation, *SpNK2.1* expression becomes dependent upon a different set of regulatory players that serve to specify oral ectoderm fate. As with A/V patterning, the elaboration of the oral–aboral (O/A) axis requires β-catenin (Angerer et al., 2001; Logan et al., 1999; Wikramanayake and Klein, 1997). A key event in the specification of oral ectoderm is the upregulation of *goosecoid* in presumptive oral at the hatching blastula stage

(Angerer et al., 2001). Upregulation of *SpGsc* is dependent upon the β-catenin system as cadherin-injected embryos do not express *Gsc* and the ectoderm is specified towards aboral fate (Angerer et al., 2001). *Goosecoid* appears to promote oral differentiation, in part, via the repression of aboral-specific genes, one of which is an unknown repressor of *SpDri*, another key oral regulatory gene (Amore et al., 2003). Once established, both genes act to positively regulate each other's expression, establishing a cross-regulatory stabilization loop, upon which the oral ectodermal specification state is maintained (Amore et al., 2003).

Interestingly, Amore et al. (2003) have shown that SpDri abrogation results in the loss of *SpNK2.1* expression (Fig. 6), demonstrating that *SpNK2.1* is linked into the oral ectoderm gene regulatory network (GRN), and whose continued expression is dependent upon this input (Fig. 10). Herein we demonstrate that, despite this dependence, *SpNK2.1* expression is not initially regulated by the oral ectoderm GRN. Our conclusions are based on two observations. First, *SpNK2.1* is activated in cadherin-injected embryos (Fig. 7), which never express *SpGsc* (and hence do not express *SpDri*) and fail to generate O/A polarity (Angerer et al., 2001). Secondly, SpDri abrogation does not affect early *SpNK2.1* expression in blastula stage embryos, as demonstrated by quantitative PCR (Amore et al., 2003) and WMISH (Fig. 6). Thus, the oral GRN serves to maintain, but not to initiate, *SpNK2.1* expression within committed oral ectoderm. Finally, this maintenance also requires an input which can be greatly attenuated by the presence of lithium, although this input is not necessary for proper spatial localization.

A preliminary model of the regulatory "wiring" responsible for directing *SpNK2.1* expression is proposed in Fig. 10. As argued above, *SpNK2.1* expression is dictated by separate early and late regulatory phases, both of which are influenced by β-catenin. The initial activation of *SpNK2.1* within the apical domain constitutes the first phase, which begins around the hatching blastula stage. We hypothesize that ATFs serve to activate expression of *SpNK2.1*, and β-catenin-dependent processes serve to repress *SpNK2.1*, thus defining a unique zone of expression at the apical pole of the embryo. The second phase is implemented at gastrulation and is characterized by the transfer of control to *SpDri*. Our data suggest that SpDri serves a positive regulatory role, maintaining *SpNK2.1* expression within the oral region of the apical plate, but does not participate in the initial activation of *SpNK2.1*; whether the attenuation of *SpNK2.1* by the presence of lithium after approximately 30 h pf (Figs. 7E and F) is mediated via *SpDri* or another input remains unknown and is not indicated in Fig. 10. Therefore, although the apical plate comes to lie within definitive oral ectoderm, its demarcation as assayed by expression of *SpNK2.1* is independent of known oral-specification transcription factors, suggesting that the apical domain is initially a distinct territory separate from oral ectoderm.

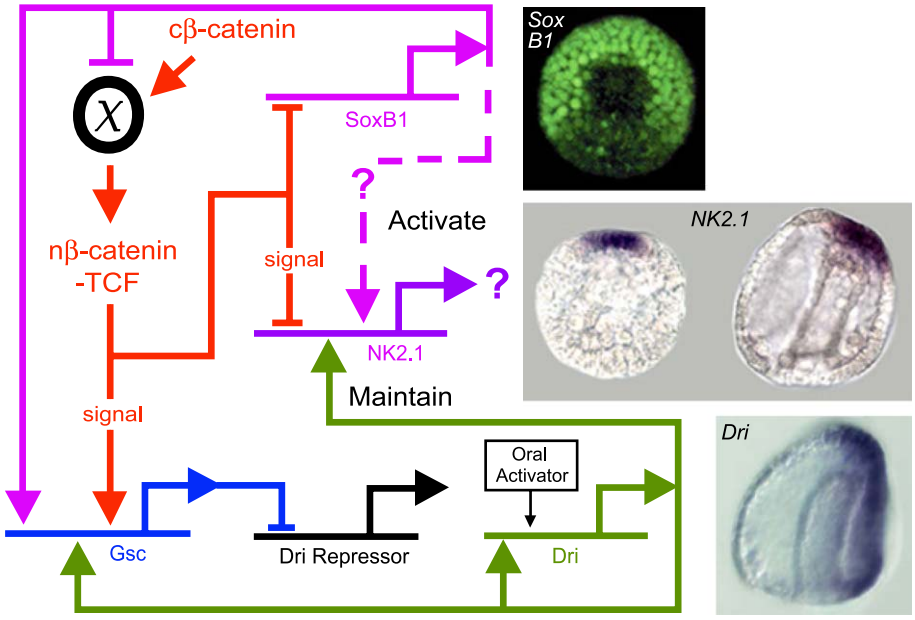

Fig. 10. A preliminary model for the regulation of *SpNK2.1*. Expression can be separated into two distinct phases. The initial activation of *SpNK2.1* is most likely dependent upon the zygotic upregulation of ATFs (e.g., *SoxB1*) in the nonvegetal domain (reviewed in Angerer and Angerer, 2003); however, it is not known whether there is a direct interaction between ATFs such as SoxB1 and *NK2.1* (dashed line). The vegetal extent of *SpNK2.1* expression is dictated by β-catenin-dependent processes acting indirectly upon a set of unknown factors. During gastrulation, maintenance of *NK2.1* is dependent upon positive input from *Dri,* which is an integral component of the oral ectoderm GRN (Amore et al., 2003). This maintenance is also ultimately dependent upon β-catenin which is responsible for implementing a downstream signal which activates *Gsc* in presumptive oral ectoderm (Angerer et al., 2001). *Goosecoid*, an obligate repressor, is necessary for the activation of *Dri* in the oral domain by inhibiting an unknown repressor of *Dri* (Amore et al., 2003). For details of the β-catenin GRN, see Davidson et al. (2002); for the oral ectoderm GRN and *SpDri* expression pattern, see Amore et al. (2003). SoxB1 expression pattern from Kenny et al. (1999).

SpNK2.1 function

The developmental role that *SpNK2.1* plays during embryogenesis remains unclear. The apical plate gives rise to a diversity of cell types, including ciliated band components which form at the interface between the oral and aboral territories (Cameron et al., 1993; Davidson et al., 1998). Serotonergic neurons also develop within this region, with immunoreactive cells appearing by the end of gastrulation (Bisgrove and Burke, 1986). These cells form a neuropile within the oral hood of the prism embryo, running axons into the ciliated band. Nakajima et al., (2004) have shown using Spec1 expression as an aboral marker that serotonergic neurons arise within the oral ectoderm at the interface with aboral ectoderm. We demonstrate here that the serotonergic cells of the apical ganglion arise within the SpNK2.1 domain of expression along its aboral ectodermal margin. In gastrulae, when neural markers first appear, the serotonergic cells contain SpNK2.1 protein, but it is restricted to the cytoplasm, whereas SpNK2.1 localizes strongly to nuclei in all other apical cells. This difference suggests that the specification of serotonergic neurons may involve regulation of SpNK2.1 localization.

Our study does not reveal a role for *SpNK2.1* during embryogenesis (Fig. 9). SpNK2.1 knockdown embryos and *SpNK2.1* overexpression embryos appear to develop normal nervous systems, as assayed with antibodies specific to serotonin and the pan-neural marker, 1E11. This may not be surprising given that throughout the differentiation of the larval nervous system, cells expressing the neuronal marker 1E11 do not express SpNK2.1, although there are cells in the adult radial nerves that do. Moreover, there is no evidence for any redundancy, either with other copies of *NK2.1* or with other *NK2* genes (Figs. 9G and H), although it remains possible that SpNK2.1 represses *SpNK2.2* function, and thus removal of SpNK2.1 results in the expansion of *SpNK2.2* expression into the apical domain where its product regulates SpNK2.1 target genes. Nonetheless, we feel that the most likely explanation is that perturbation of *SpNK2.1* activity affects apical organ function during either the larval or metamorphic period, possibly affecting the animal's ability to respond to bacterial cues for the initiation of settlement.

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