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RESEARCH ARTICLE

Neurogenesis in sea urchin embryos and the diversity of deuterostome neurogenic mechanisms

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ABSTRACT

A single origin to the diverse mechanisms of metazoan neurogenesis is suggested by the involvement of common signaling components and similar classes of transcription factors. However, in many forms we lack details of where neurons arise, patterns of cell division, and specific differentiation pathway components. The sea urchin larval nervous system is composed of an apical organ, which develops from neuroepithelium and functions as a central nervous system, and peripheral neurons, which differentiate in the ciliary band and project axons to the apical organ. To reveal developmental mechanisms of neurogenesis in this basal deuterostome, we developed antibodies to SoxC, SoxB2, ELAV and Brn1/2/4 and used neurons that develop at specific locations to establish a timeline for neurogenesis. Neural progenitors express, in turn, SoxB2, SoxC, and Brn1/2/4, before projecting neurites and expressing ELAV and SynB. Using pulse-chase labeling of cells with a thymidine analog to identify cells in S-phase, we establish that neurons identified by location are in their last mitotic cycle at the time of hatching, and S-phase is coincident with expression of SoxC. The number of cells expressing SoxC and differentiating as neurons is reduced in embryos injected with antisense morpholino oligonucleotides to *SoxC*, *SoxB2* or *Six3*. Injection of RNA encoding SoxC into eggs does not enhance neurogenesis. In addition, inhibition of FGF receptors (SU5402) or a morpholino to *FGFR1* reduces expression of SoxC. These data indicate that there are common features of neurogenesis in deuterostomes, and that sea urchins employ developmental mechanisms that are distinct from other ambulacraria.

KEY WORDS: Neurogenesis, Neural specification, SoxC, SoxB2, Brn1/2/4, ELAV, FGF, Neural progenitors, Neural precursors, Deuterostomes, Evolution, *Strongylocentrotus purpuratus*

INTRODUCTION

Diverse developmental mechanisms mediate the specification and differentiation of metazoan nervous systems. There are similarities suggesting homology, but we lack a broad understanding of neural development mechanisms in metazoans. In some organisms neurogenic potential is spread throughout the ectoderm and neural precursors arise and differentiate to form a nerve network (Richards and Rentzsch, 2014; Cunningham and Casey, 2014). In many bilaterians, neurogenesis is restricted to specific domains of ectoderm by early patterning (Bier and De Robertis, 2015). A key aspect of neurogenesis is the cell division that marks the transition of pre-mitotic neural progenitors to post-mitotic neural precursors committed to differentiation (Hartenstein and Stollewerk, 2015). In the best-studied models there are various arrangements of asymmetric, self-renewing mitoses within populations of dedicated neural progenitors (Goodman and Doe, 1993; Götz and Huttner, 2005). There are common components and shared gene families that appear to have key roles in the specification and differentiation of neurons (Wei et al., 2009; Sinigaglia et al., 2013; Marlow et al., 2014). In addition, Notch/Delta lateral inhibition of neurogenesis is a common feature, but the details of how it is employed vary widely (Zhong, 2003; Yoon and Gaiano, 2005). It is not clear whether these similarities result from common origin or are features that arose independently because of functional constraints. Overall, we lack details of neurogenic mechanisms for many taxa that would enable a broader understanding of neural evolution (Hartenstein and Stollewerk, 2015).

Bilaterians have central and peripheral components to their nervous system, which are commonly derived from different regions of ectoderm. Typically, the central nervous system (CNS) is derived from a neuroepithelium and peripheral neurons arise in ectoderm and subsequently connect to the CNS (Hartenstein and Stollewerk, 2015). In sea urchin embryos, the animal pole domain (APD) is a specialized region of ectoderm with features of a neuroepithelium (Angerer et al., 2011). The genes expressed in the APD are similar to those expressed in the vertebrate forebrain (Wei et al., 2009) and the APD expands in the absence of Wnt signaling (Yaguchi et al., 2006; Cui et al., 2014). The APD gives rise to the apical organ, the most prominent ganglion of the larval nervous system. The larval nervous system also includes an array of neurons, with features of sensory cells and interneurons, in the ciliary band. Descriptions of the development of the larval nervous system indicate that these cells differentiate in the ciliary band, and neurons extend axons in tracts at the base of the ciliated cells to the apical organ (Burke et al., 2014). The nervous system is thought to function by controlling the direction of ciliary beat by regulating depolarization of ciliated cells in the ciliary band (Mackie et al., 1969; Satterlie and Cameron, 1985; Strathmann, 2007). Thus, a conventional interpretation of the organization of the larval nervous system is that the apical organ constitutes a central, integrating component, and there are peripheral neurons in the ciliary band that respond to stimuli and locally, or coordinately, control ciliary activity.

The apical organ neurons appear to be derived from cells in the APD that differentiate as neurons, but it is not clear which regions of ectoderm give rise to the neurons of the ciliary band. Markers for neural differentiation are expressed in gastrulae by cells within the ciliary band or APD (Bisgrove and Burke, 1986; Nakajima et al., 2004). Thus, the ciliary band has been suggested to be neurogenic (Poustka et al., 2004; Wei et al., 2009; Saudemont et al., 2010). Based on the pattern of expression of Six3 and its requirement for neurogenesis, Wei et al. (2009) suggested that neurons might be specified in the APD or lateral ectoderm and only differentiate in the ciliary band or migrate from the APD to the ciliary band. The

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number of neurons increases throughout development of the larva, which indicates that neurogenesis is not restricted to embryonic stages and that there must be sites of continuous neurogenesis (Bisgrove and Burke, 1986; Beer et al., 2001). However, the origin of neurons remains unknown because we lack molecular markers for neurogenesis and studies of neurogenesis tracking individual cells.

Here we describe the expression in sea urchin of several proteins implicated in neurogenesis. We have developed antibodies to putative markers for neural progenitors to determine the sequence in which they are expressed. We restricted our analysis to the first neurons that differentiate, as the time and location of their differentiation are predictable. We used a series of pulse-chase experiments with EdU, a thymidine analog (Chehrehasa et al., 2009), to demonstrate that the last mitotic division of neural progenitors for these neurons coincides with the expression of SoxC. Neurogenesis appears to require the expression of several key neurogenic transcription factors and is also blocked by interfering with FGF signaling or blocking expression of an FGF receptor. These data indicate that, in addition to the neuroepithelium of the APD, neurons arise in restricted regions of ectoderm adjacent to the ciliary bands. For some neurons there is an invariant pattern of appearance of neural progenitors, but most of the ciliary band neurons arise asynchronously and unpredictably. Neural progenitors appear to express SoxC during their last cell cycle and divide once before a single neural precursor expresses Brn1/2/4 and subsequently differentiates. This model of neurogenesis has features that are shared with chordates and is distinct from neurogenesis in other ambulacrarians.

RESULTS

SoxC and neural progenitors

In situ hybridization data suggest that SoxC is expressed by neural progenitors (Howard-Ashby et al., 2006; Poustka et al., 2007). To determine if this is so, we prepared polyclonal rat antiserum and a mouse monoclonal antibody to *Strongylocentrotus purpuratus* SoxC (Fig. S1). SoxC is expressed initially during cleavage in vegetal lineages (Fig. S2). In the APD, expression of SoxC protein was first detected prior to hatching (blastula, 20 h, 14°C) at the same time that NK2.1 is first detected (Fig. 1, Fig. 2A-I). Initially all cells of the APD express SoxC and levels of fluorescence vary, but by 24 h (hatching) the number of cells expressing SoxC is reduced to one or two cells (Fig. 2A-I). The cells are at the periphery of the APD along the dorsal margin. The number of cells in the APD expressing SoxC increases to eight to ten cells dispersed around the periphery of the apical organ of early plutei (Fig. 2J-N).

Cells in the oral ectoderm also express SoxC. At about 28 h there are two pairs of cells in the positions where postoral neurons differentiate that express SoxC (Fig. 2K). The postoral neurons are identified by their distinctive position at the base of the postoral arms (Fig. 1) (Burke et al., 2014). Cells in these positions continue to express SoxC until late gastrula stages. During gastrulation (36-48 h), cells adjacent to the presumptive ciliary band express SoxC (Fig. 2L-Q). There are 15-25 cells, often in pairs, along the oral or the aboral edge of the ciliary band in prism and pluteus stages that express SoxC (Fig. 2M-Q). In addition, there are scattered cells surrounding the presumptive mouth that express SoxC (Fig. 2M,N,P). Some cells of the ciliary band, identified by expression of Hnf6 (Poustka et al., 2004; Otim et al., 2004) also express SoxC (Fig. 2N-Q). With differential interference contrast or transmitted light imaging, the cells expressing SoxC are morphologically indistinguishable from adjacent cells. Cells expressing SoxC do not co-express synaptotagmin B (SynB) (Fig. 2M). Thus, SoxC is expressed transiently in endomesoderm, in individual cells in the APD, and restricted regions of ectoderm beginning in late blastulae.

To determine if the ectodermal cells expressing SoxC are neural progenitors, we developed antibodies to ELAV and Brn1/2/4 (Figs S1 and S3). ELAV is an RNA-binding protein used as a marker for undifferentiated neurons in several organisms (Robinow and White, 1988, 1991; Good, 1995). ELAV protein is expressed in blastulae (24 h) in primary mesenchyme cells and throughout gastrulation in pigment cells (Fig. S3). Differentiated neurons within the ciliary band and APD also express ELAV (Fig. 3B). ELAV protein localizes throughout the cytoplasm and in patches in nuclei of neurons (Fig. 3D-F). Brn1/2/4, a POU family transcription factor with neural specification roles in other organisms (Ryan and Rosenfeld, 1997; Wegner et al., 1993; Brombin et al., 2011), is also expressed by differentiated neurons within the ciliary band, where it localizes to nuclei (Fig. 3A-F). Thus, ELAV and Brn1/2/4 serve as additional markers for neurons and neural progenitors, although ELAV expression in mesodermal lineages limits its utility. Brn1/2/4 is initially expressed at about 48 h in cells in the position of postoral neurons and in the position of the serotonergic neurons along the dorsal margin of the APD (Fig. 1, Fig. 3G-I). Cells in the position of postoral neurons also express SoxC and, in doubly labeled preparations, it is clear that SoxC and Brn1/2/4 are co-expressed

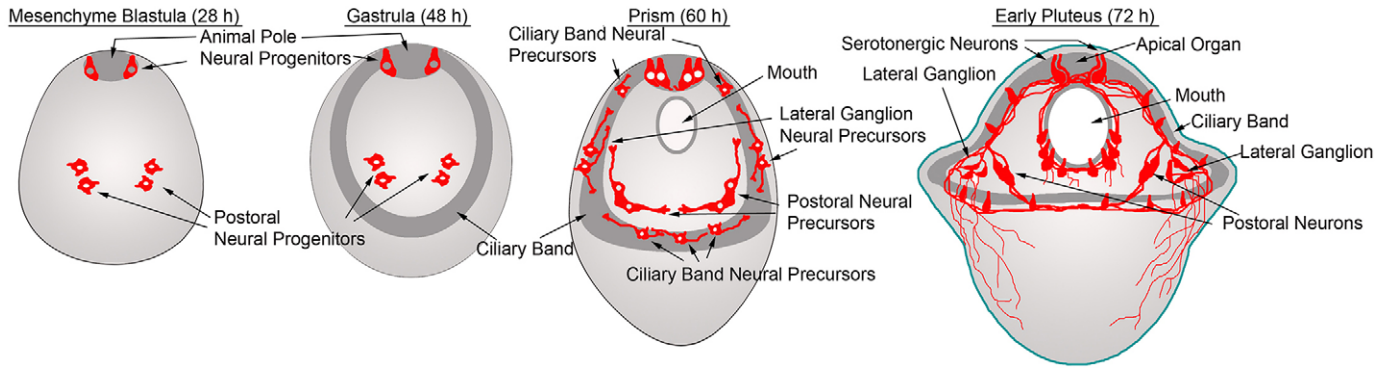

Fig. 1. Stages of neurogenesis in sea urchin embryos. In blastulae SoxC is expressed in neural progenitors of the animal pole domain (APD) and in cells where the postoral neurons will form. Neural precursors for postoral neurons and apical organ neurons can be identified by position and expression of Brn1/2/4 during gastrulation (48 h). Subsequently, lateral ganglion precursors and precursors for ciliary band neurons arise in the ciliary band or the oral and aboral ectoderm adjacent to the ciliary band. The neural precursors associated with the larval mouth arise in early plutei. The diagrams present a ventral view with the apical organ at the top. Not to scale.

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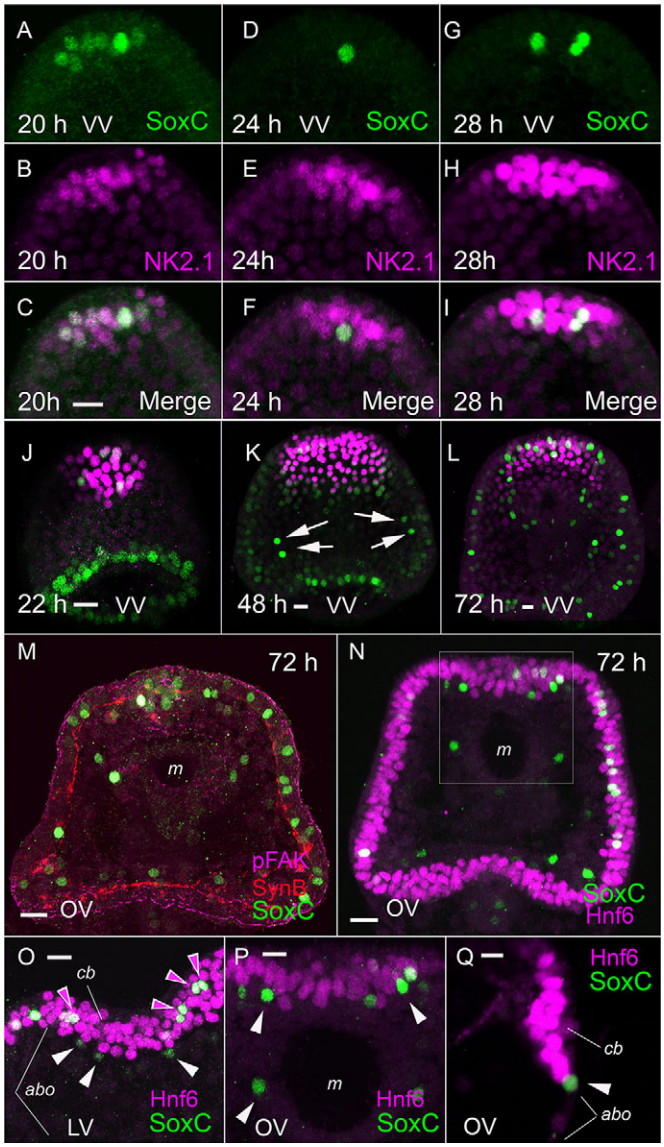

Fig. 2. SoxC is expressed in the APD and ectodermal cells throughout gastrulation and in early larval stages. Maximum intensity projections of through-focus stacks (VV, ventral view; LV, lateral view; OV, oral view). (A-I) Initial expression in the APD (dorsal view) begins in blastulae, prior to hatching (20-28 h, 14°C). (A-C) SoxC and NK2.1 expression begins at about 20 h and all cells co-express SoxC and NK2.1. (D-F) At the time of hatching (24 h, 14°C), one to three cells along the dorsal margin of the APD are the only cells expressing SoxC. (G-I) By 28 h there are two to four cells along the dorsal margin expressing SoxC. (J-L) In hatched blastulae, SoxC expression is in the APD and a band of cells surrounding the blastopore. The vegetal expression gradually decays, and throughout gastrulation and in early plutei SoxC is expressed in nuclei of scattered ectodermal cells. At 28 h, cells in the position of the postoral neurons express SoxC (K, arrows) and, after 48 h, expression is no longer detectable (L). (M) In early plutei (72 h), SoxC is expressed in scattered ectodermal cells and SynB is expressed in differentiated neurons. pFAK serves as a marker for ciliary band cells. (N-Q) SoxC is expressed by cells in the oral and aboral ectoderm and in Hnf6-expressing cells of the ciliary band. The boxed region in N is magnified in P. (O) In ciliary band, cells expressing Hnf6 (magenta arrowheads) and cells in the adjacent aboral ectoderm (arrowheads) express SoxC. (P) Cells in the oral ectoderm adjacent to the ciliary band and the mouth express SoxC (arrowheads). (Q) In an optical section, a cell adjacent to the ciliary band (Hnf6^{+}) in the aboral ectoderm expresses SoxC (arrowheads). abo, aboral ectoderm; cb, ciliary band; m, mouth. Scale bars: 10 µm.

by these cells (Fig. 3G-I, insets). Thus, Brn1/2/4 and SoxC appear to be expressed by partially overlapping sets of ectodermal cells. Initially, SoxC is expressed, subsequently SoxC and Brn1/2/4 are co-expressed, then Brn1/2/4 alone is expressed by cells in the position of postoral neurons.

SoxB2 is expressed in all cells beginning in early cleavage. SoxB2 is an HMG-box-containing transcription factor thought to be important in neuronal differentiation (Richards and Rentzsch, 2014; Taguchi et al., 2002). At hatching, SoxB2 is downregulated at the vegetal pole (Fig. 4A,B, inset). In gastrulae it is apparent that the endomesodermal lineages do not express SoxB2 (Fig. 4C). In addition, SoxB2 expression is downregulated in the animal pole; however, all other ectoderm continues to express SoxB2 (Fig. 4C). In embryos prepared with SoxB2 and SoxC antibodies, cells in the APD expressing SoxC are adjacent to the zone in which SoxB2 is cleared. Thus, SoxC and SoxB2 are co-expressed in cells at the periphery of the APD (Fig. 4D). Similarly, cells in the ectoderm adjacent to presumptive ciliary band that express SoxC also express SoxB2 (Fig. 4E-G). Differentiated neurons do not express SoxB2.

Post-mitotic neural precursors

As neurons are post-mitotic, we have used pulse-chase experiments with labeled nucleoside to determine the time at which the neurons identified by position are in S-phase of their final cell cycle. Embryos were labeled with EdU for intervals beginning in late cleavage stages, and then prepared for immunofluorescence with anti-SynB as early plutei (72 h, Fig. 5). Nuclei that had incorporated EdU during the labeling interval were distinguishable from unlabeled nuclei (Fig. 5A,B). Differentiated neurons were not labeled with EdU when pulsed at 48 h or later, yet almost all neurons were labeled with EdU when pulsed between 16 h and 20 h (Fig. 5C). The interval during which the postoral neurons could be labeled was between 16 h and 32 h, with the majority of the postoral neurons labeled between 16 h and 28 h (Fig. 5D). Similarly, the first differentiated serotonergic neurons in the apical organ could be EdU labeled between 16 h and 28 h, with the majority being labeled between 16 h and 24 h (Fig. 5E). As cells expressing SoxC are occasionally observed in mitosis (Fig. 5F-H), we attempted to determine whether SoxC-expressing cells are mitotically active by pulsing with EdU for 2 h, then looking for cells labeled with EdU that are expressing SoxC. A small proportion of cells expressing SoxC at the time of fixation were labeled with EdU (Fig. 5I-K). These experiments indicate that neural specification is likely to begin during late cleavage. In addition, SoxC expression begins at almost the same time as neural progenitors, identified by position, are in S-phase of their last cell cycle. These data suggest that neural progenitors express SoxC before completing their last cell cycle and dividing to become neural precursors.

Neurogenic pathway components

To determine if SoxC is essential for neurogenesis, we used morpholino antisense oligonucleotides (MASO) to interfere with the expression of SoxC (Figs 6 and 7). In embryos injected with the SoxC MASO, fewer cells were detected with anti-SoxC, indicating that any of the three overlapping MASOs that we have used blocks expression of SoxC protein (Fig. 6A-K, Fig. 7A). In 28 h mesenchyme blastulae, the expression of SoxC is completely eliminated in vegetal and APD cells, without any effect on the expression of SoxB2 (Fig. 6A-D, Fig. 7A). In 48 h gastrulae, MASO-injected embryos have fewer cells expressing SoxC (Fig. 6E-G, Fig. 7A). The number of neurons that differentiate by 72 h (SynB expression) in embryos injected with the SoxC MASO

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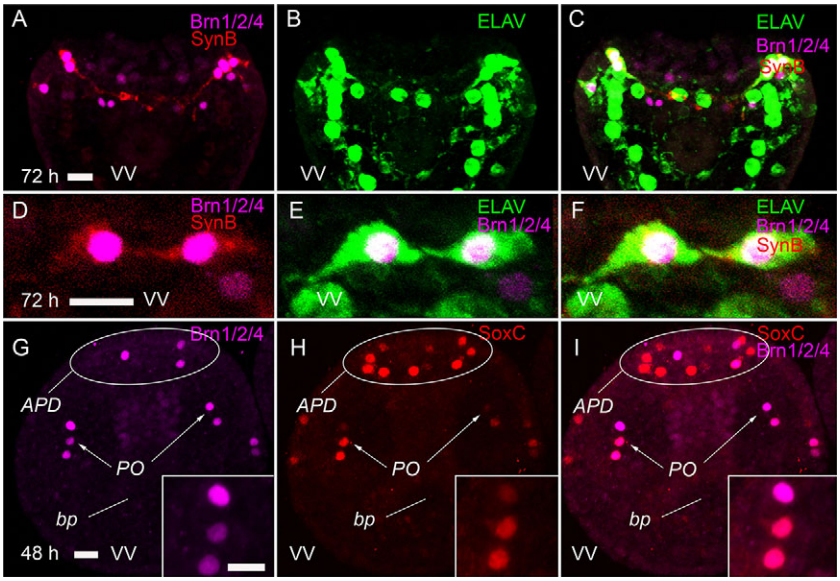

Fig. 3. Differentiated neurons express Brn1/2/4, SynB and ELAV. Maximum intensity projections of through-focus stacks; all in ventral view (VV). (A-C) In early plutei (72 h), neurons identified by expression of SynB also express Brn1/2/4, which localizes to the nuclei. ELAV, which is expressed in the mesoderm (Fig. S3), is expressed by neurons. (D-F) A detailed confocal optical section of the same specimen as A-C that shows expression of SynB, Brn1/2/4 and ELAV in the same neurons. (G-I) In late gastrulae, SoxC and Brn1/2/4 are expressed in oral ectoderm and APD cells. In embryos at this stage, cells in the position of postoral neurons co-express SoxC and Brn1/2/4 (insets). At earlier stages, SoxC-expressing cells are located in these positions and, at later stages, cells expressing Brn1/2/4 are in these positions. Similarly, the APD contains cells along the dorsal margin expressing SoxC or Brn1/24. Some cells express both at this stage, but at earlier stages SoxC only is expressed. bp, blastopore; PO, postoral neurons; Scale bars: 10 µm.

is reduced and most of the neurons are abnormal in morphology (Fig. 6H,I, Fig. 7A). Most commonly there are multiple short axons that are not organized into tracts. We concluded that expression of SoxC is necessary for neurogenesis.

Several studies of neurogenesis indicate that there are common features in components mediating embryonic neurogenesis in a wide range of metazoans (Hartenstein and Stollewerk, 2015). In some metazoans, expression of SoxB2 is a necessary condition for neurogenesis (Richards and Rentzsch, 2014). When we suppressed the expression of SoxB2 with a MASO, there was a reduction in the number of cells expressing SoxC (Fig. 6L, Fig. 7A). It is apparent that Six3 is near the top of a regulatory network mediating neurogenesis in sea urchins (Wei et al., 2009). To determine if SoxC expression is dependent on activation of the Six3 gene regulatory network we interfered with expression of Six3 with a MASO. The number of cells expressing SoxC in Six3 MASO-injected embryos was reduced compared with controls (Fig. 6M, Fig. 7A), indicating that SoxC expression is dependent on expression of Six3.

There are also indications from studies of neurogenesis in other metazoans that FGF and FGF receptors are necessary for embryonic neurogenesis (Rentzsch et al., 2008; Altmann and Brivanlou, 2001; Lemaire et al., 2002; Kengaku and Okamoto, 1993). FGF and its role in ectodermal signaling, specifically to pattern mesoderm, have been examined in sea urchins, and there is evidence that FGF is expressed in ectodermal cells adjacent to postoral neural progenitors and the APD (Lapraz et al., 2006; Rottinger et al., 2008; Adomako and Ettensohn, 2013). In addition, FGF receptor 1 (FGFR1) is expressed in cells of the oral half of the embryo, including the APD, presumptive ciliary band, oral ectoderm, and what will become the aboral ectoderm adjacent to the ciliary band (Lapraz et al., 2006;

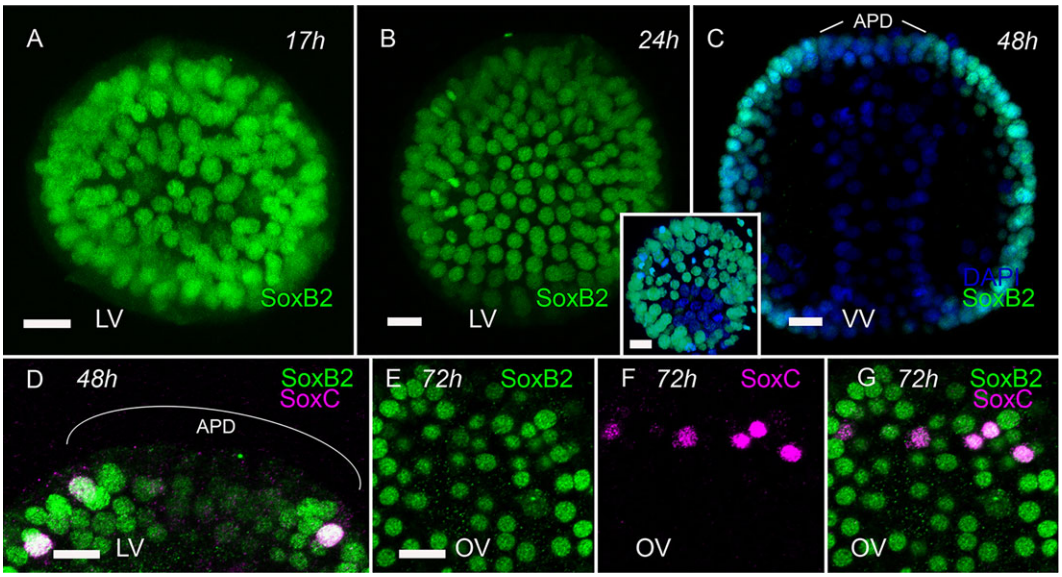

Fig. 4. SoxB2 is expressed throughout the ectoderm. Maximum intensity projections of through-focus stacks (LV, lateral view; OV, oral view). (A-C) In cleavage stages through hatching (24 h, 14°C), SoxB2 localizes to the nuclei of all cells. As vegetal lineages differentiate, SoxB2 is downregulated (inset) and, in gastrulae, it is apparent that endoderm and mesoderm do not express SoxB2, and SoxB2 is also downregulated in the APD. (D) In gastrulae, cells in the central region of the APD downregulate SoxB2, but cells along the periphery expressing SoxC co-express SoxB2. (E-G) Cells on the edge of the oral ectoderm of early plutei express SoxC and SoxB2. Scale bars: 10 µm.

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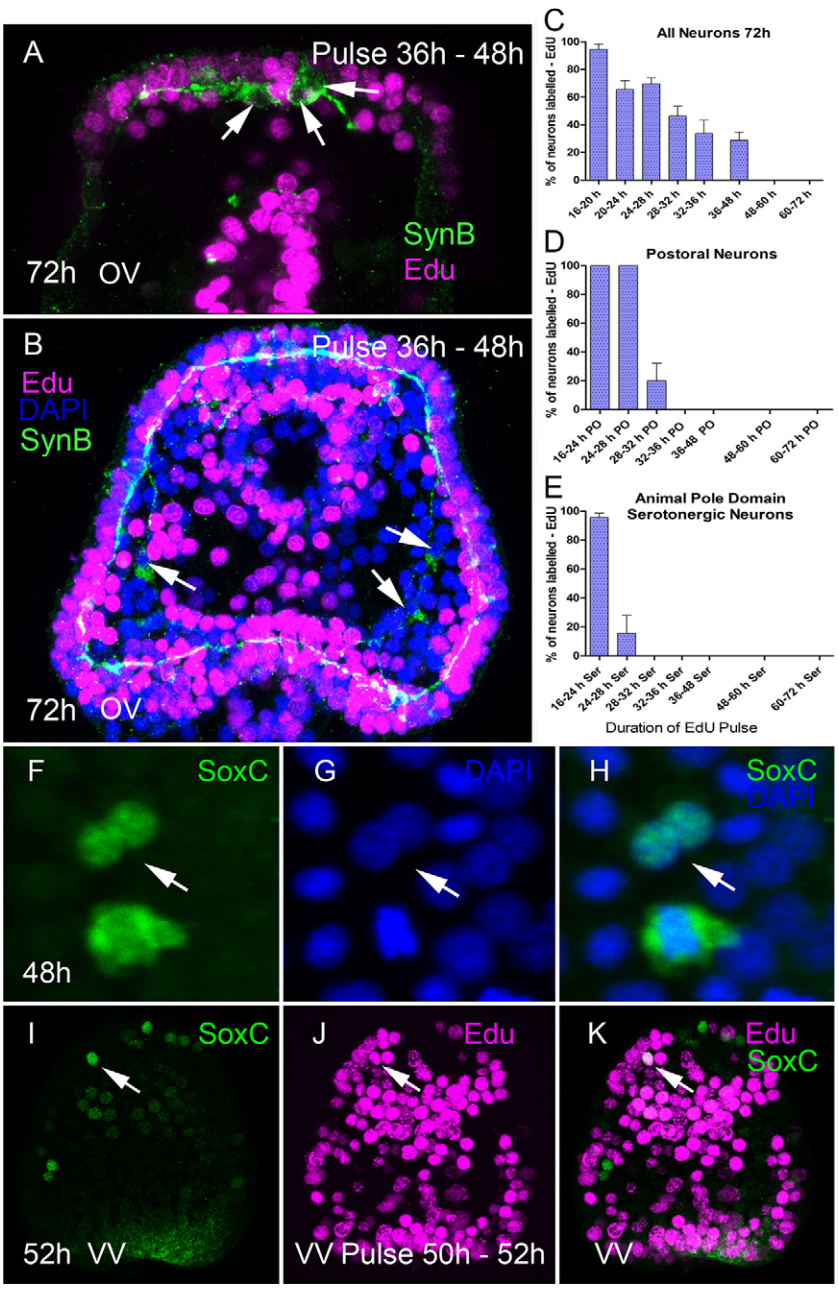

Fig. 5. Birthdating neurons of the 72 h pluteus. To determine when neurons are in their last mitotic S-phase, embryos were incubated in EdU. Embryos that had been pulsed with EdU were then examined as early plutei (72 h) to determine if nuclei of individual neurons had taken up EdU during the labeling interval. (A,B) Examples of embryos labeled between 36 and 48 h with EdU (magenta), fixed at 72 h, and immunostained for the neural differentiation marker SynB. Arrows indicate neurons in the APD (A) or the postoral neurons (B), which were not in S-phase throughout the labeling period. (C-E) Quantitative analysis of the times at which neurons were labeled. For each labeling interval, five embryos were optically sectioned and labeled neuronal nuclei were counted manually by two observers. Error bars indicate s.e. (C) All of the neurons expressing SynB at 72 h (15 neurons) complete their last S-phase during gastrulation (36-48 h). (D) The postoral neurons, identified by their distinctive position at the base of the postoral arms, are labeled at 32 h before the initiation of archenteron formation. (E) The serotonergic neurons of the APD were identified with anti-serotonin. (F-H) Cells expressing SoxC are occasionally found in mitosis. A cell expressing SoxC in metaphase is shown (intense DAPI staining of aligned chromosomes). In addition, SoxC-expressing cells are often found as adjacent pairs of cells, suggesting recent mitosis (arrows). (I-K) To determine if cells expressing SoxC are mitotically active, 48 h embryos were labeled with a 2 h pulse of EdU and immediately fixed and examined for EdU-labeled cells expressing SoxC. A small number of cells are colabeled (arrow), indicating that SoxC-expressing cells can divide. OV, oral view; VV, ventral view. Scale bars: 10 µm.

Saudemont et al., 2010). To determine if FGF signaling is required for expression of SoxC we used a selective inhibitor of FGF receptor activation (SU5402) (Mohammadi et al., 1997) and MASO knockdown of FGFR1. There was a decrease in the number of cells expressing SoxC after treatment with SU5402 (Fig. 7B). Treatment with 5 µM SU5402 reduced the number of cells expressing SoxC significantly (Fig. 6U, Fig. 7B). Similarly, interfering with the expression of FGFR1 with a MASO eliminated expression of SoxC (Fig. 6S, Fig. 7B). When we injected embryos with a MASO that interferes with Brn1/2/4 expression, the number of neurons that differentiate by 72 h is reduced (Fig. 6O-Q, Fig. 7A). However, the number of cells expressing SoxC at 48 h and 72 h was unaffected by interfering with Brn1/2/4 expression (Fig. 6E-G,O-Q, Fig. 7A). These experiments indicate that expression of FGFR1, SoxB2, Six3, and FGF receptor activation are necessary for the expression of SoxC and neurogenesis. However, interfering with Brn1/2/4 expression reduces the number of neurons produced, but has no effect on the expression of SoxC, consistent with Brn1/2/4 being downstream. We conclude that these proteins are components of a neurogenic pathway.

To determine if SoxC expression is sufficient to initiate neurogenesis, we injected mRNA encoding full-length SoxC (SoxC FL) or full-length SoxC fused to GFP (SoxC:GFP) into eggs. When SoxC:GFP mRNA is injected, there is a clear GFP localization to nuclei in 24 h blastulae (Fig. 8A). In addition, SoxC:GFP- or SoxC FL-injected blastulae prepared with anti-SoxC indicate that the SoxC protein is expressed and that it localizes to all nuclei, without altering the pattern of expression of SoxB2 (Fig. 8B-D). Beginning at 30 h, the nuclear localization of SoxC:GFP begins to decline and by 48 h SoxC:GFP is no longer detectable (Fig. 8E-G). Similarly, preparations with anti-SoxC at 48 h or 72 h indicate normal control levels of SoxC and SynB expression, with normal patterning when embryos are injected with

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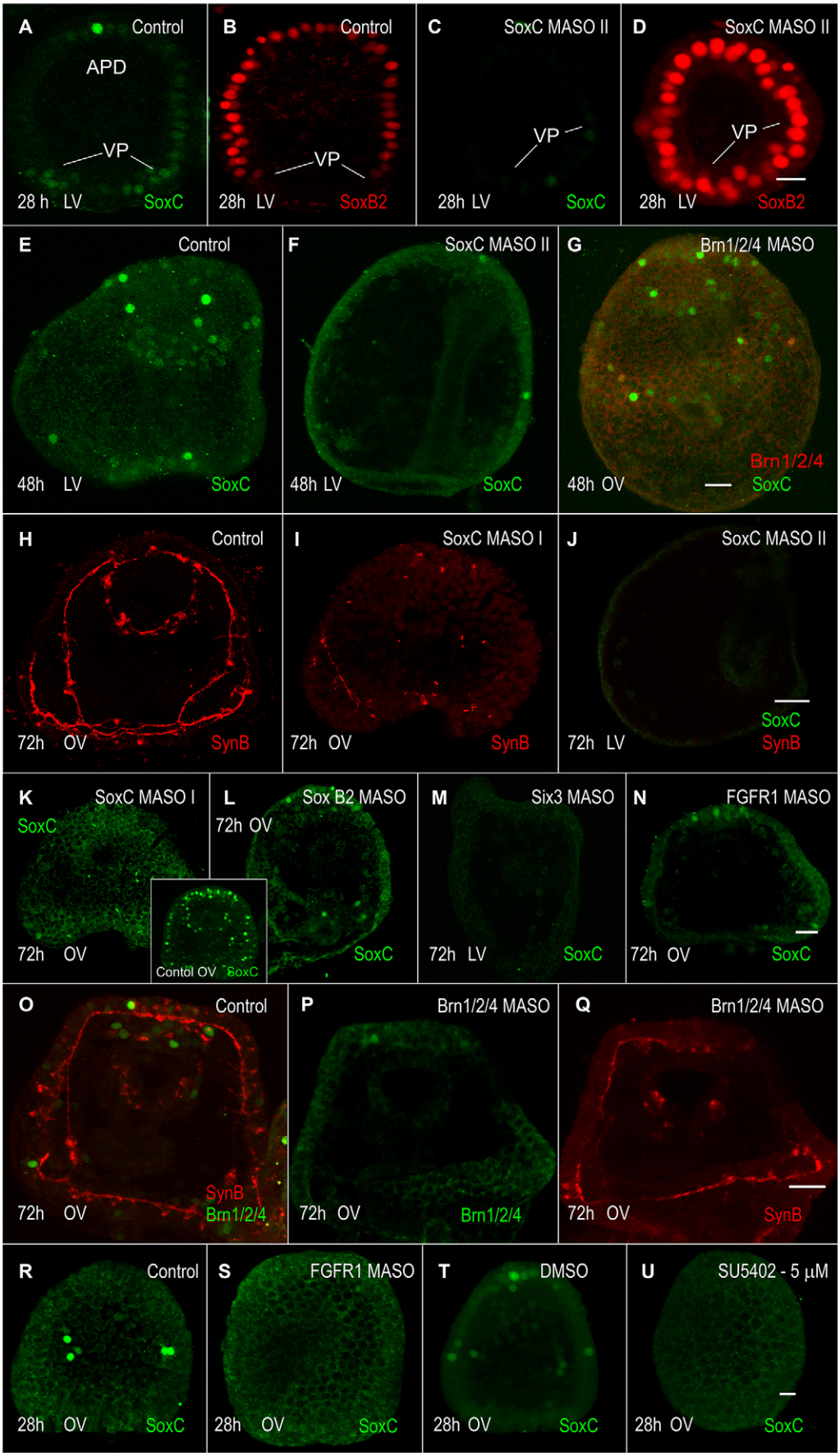

Fig. 6. Effects of morpholino antisense oligonucleotides on the expression of SoxC or SynB. Maximum intensity projections of through-focus stacks (LV, lateral view; OV, oral view). (A-D) Control and SoxC morpholino antisense oligonucleotide (MASO) II injected embryos (28 h) immunostained with anti-SoxC and anti-SoxB2. The MASO eliminates expression of SoxC without affecting the expression of SoxB2. (D) A lateral view of an embryo, in which the stack includes a tangential section of the vegetal plate. (E-G) At 48 h SoxC MASO II substantially reduces the expression of SoxC, whereas the Brn1/2/4 MASO does not interfere with expression of SoxC in late gastrula stage embryos. (H-K) Embryos injected with SoxC MASO I or II fail to express SoxC and do not differentiate neurons in early plutei. In oral view or lateral view, the number and distribution of neurons are abnormal and, in some instances, expression of SoxC and neurons is eliminated. (L-N) MASOs for SoxB2, Six3 and FGFR1 all reduce the number of cells expressing SoxC in 72 h embryos (inset shows a 72 h embryo prepared with SoxC oral view). (O-Q) Embryos injected with the Brn1/2/4 MASO show no expression of Brn1/2/4 and reduced numbers of mispatterned neurons. (R,S) Embryos injected with FGFR1 MASO fail to express SoxC in cells in the position of postoral cells in mesenchyme blastulae. (T,U) Treatment with an inhibitor of FGF signaling (SU5402) interferes with expression of SoxC in mesenchyme blastulae, as compared with controls treated with DMSO alone. VP, vegetal plate. Scale bars: 10 µm.

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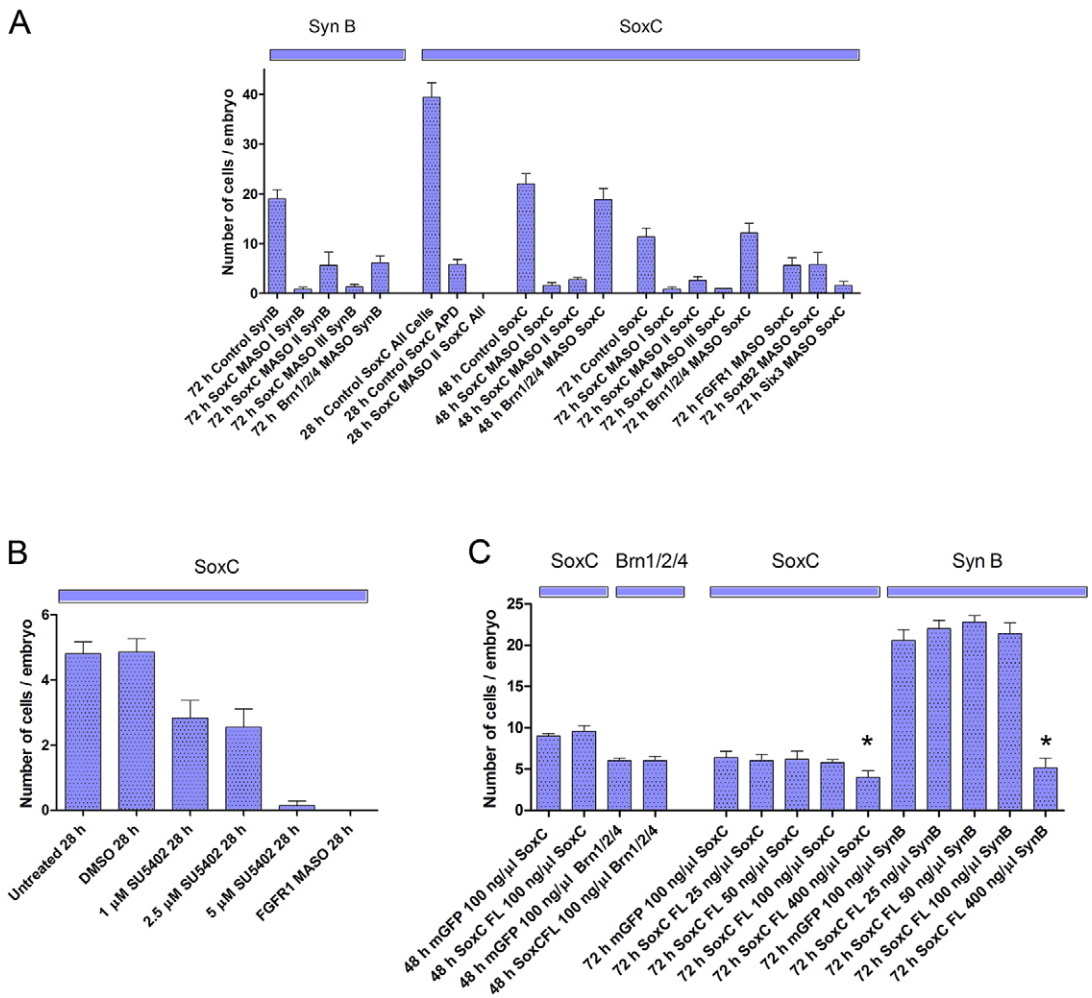

Fig. 7. Quantitative analysis of the effects of MASOs on the number of cells expressing putative pro-neural and neural proteins. (A) Embryos were injected with a MASO and the number of cells expressing SynB or SoxC per embryo was determined. (B) Embryos were treated with various concentrations of an inhibitor of FGF signaling (SU5402) and the number of cells per embryo expressing SoxC in mesenchyme blastulae was determined. In one set of experiments, the number of cells expressing SoxC in mesenchyme blastulae was determined after injection of FGFR1 MASO. (C) Embryos were injected with a range of doses of mRNA encoding full-length SoxC (FL) or membrane-targeted GFP (mGFP) and the number of cells per embryo expressing SoxC, Brn1/2/4 or SynB was determined in late gastrulae (48 h) or early plutei (72 h). (A-C) Mean±s.e.m., *n*=5 embryos. Asterisk indicates treatments with low survivorship and showing abnormal embryos.

up to 0.5 pg RNA (Fig. 8I-N, Fig. 7C). Embryos injected with higher doses (2 pg) had low survival and exhibited a range of developmental abnormalities. Expression of SoxB2 and of Brn1/2/4 were unaffected by SoxC mRNA injection (Fig. 8G,J,L). Control embryos expressing equivalent doses of GFP have high levels of fluorescence through at least 96 h with no effect on the expression of SoxC or SynB (Fig. 8H,O,P, Fig. 7C). We conclude from these experiments that overexpression of SoxC protein up to 30 h is not sufficient to initiate neurogenesis.

DISCUSSION

The model of neurogenesis in the sea urchin

In sea urchin embryos, the first neurons to develop are in specific locations and they appear in a predictable sequence (Burke et al., 2014). We have made use of serotonergic neurons that appear along the dorsal margin of the APD and the neurons at the base of the postoral arms, which are predictable in the location and time at which they form. Without having a direct marker for cellular lineage, this positional information allows us to interpret data collected from successive stages in several embryos as representative of stages taken from the same embryo. The model has two to four cells in the APD beginning to express SoxC at the time of hatching. Subsequently, there is an interval during gastrulation in which these SoxC-expressing cells co-express Brn1/2/4 and in late gastrulae they differentiate as neurons, co-expressing ELAV, SynB and serotonin (Fig. 9). The distinctive location of postoral cells enables us to identify two to four cells in the oral ectoderm that express SoxC. In gastrulae these cells co-express Brn1/2/4 before downregulating SoxC, and in prism stage embryos there are cells in this location co-expressing ELAV and SynB. Each of the markers is expressed for an interval before subsequent markers are co-expressed. Thus, our model has neural progenitors expressing, in turn, SoxB2, SoxC, and Brn1/2/4, before projecting neurites and expressing ELAV and SynB (Fig. 9). This model is testable, and genetically based lineage-tracing data would provide a continuous record of changes of successive stages of individual cells.

SoxC is expressed in ectodermal cells that are in the location of postoral neurons and in animal pole cells in the location of serotonergic neurons at approximately the same time that neural progenitors are in S-phase of their last cell cycle. In most of the animal pole neural

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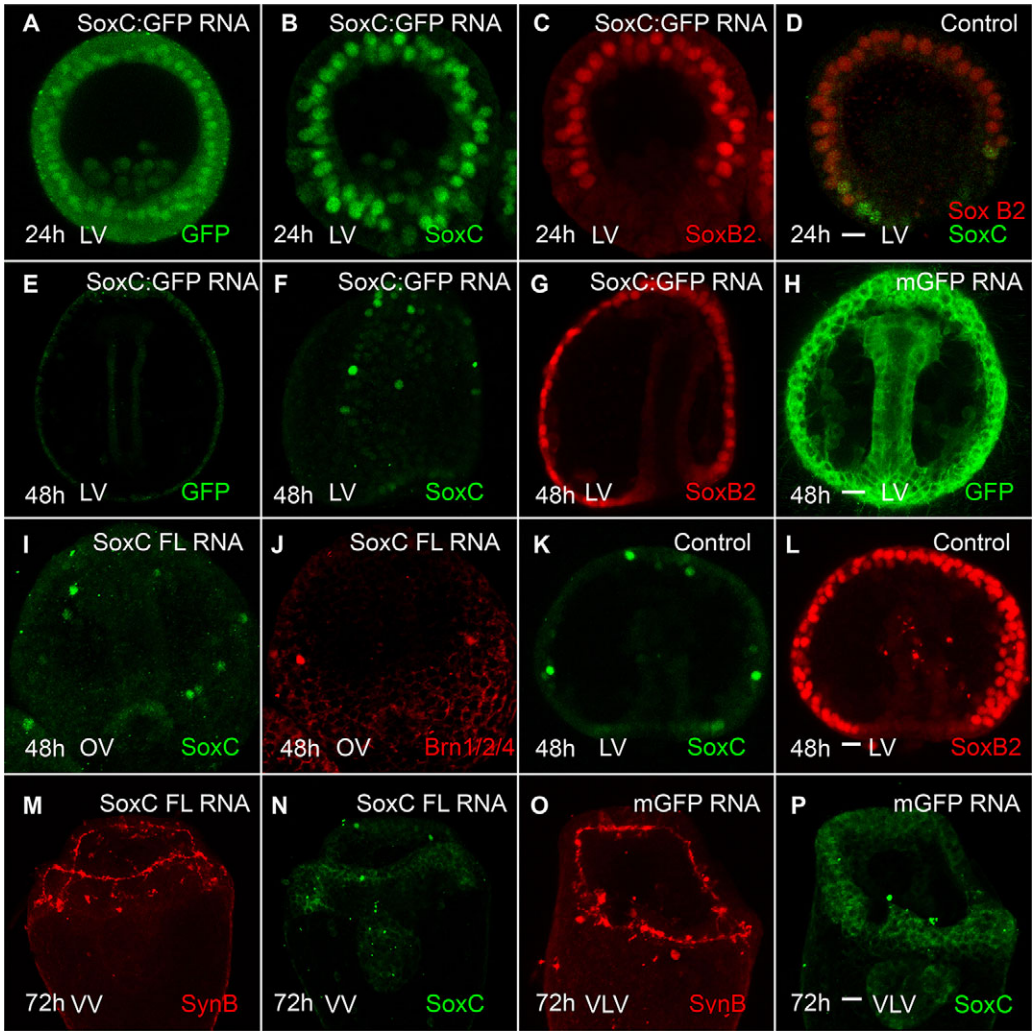

Fig. 8. Injection of RNA encoding SoxC does not increase the number of neurons produced. Maximum intensity projections of through-focus stacks (LV, lateral view; OV, oral view; VV, ventral view; VLV, ventrolateral view). (A) Image of a living embryo from an egg injected with RNA encoding full-length SoxC fused to GFP (SoxC:GFP). Nuclear localization of GFP is seen throughout the blastoderm. (B) Embryo prepared as in A, except that it was fixed and probed with anti-SoxC. SoxC protein is expressed and localizes to nuclei. (C) The same embryo as in B, but showing that the distribution of SoxB2 protein is similar to that of control embryos. (D) An uninjected control embryo showing the normal distribution of SoxB2 and SoxC. (E-G) Preparations of 48 h embryos expressing SoxC:GFP. (E) Image of a live embryo indicating that SoxC:GFP protein has been cleared from embryonic cells. (F,G) An embryo fixed and prepared with anti-SoxC (F) or SoxB (G) showing that SoxC is expressed in only a few cells, similar to control preparations, and that the SoxB2 distribution remains normal. (H) Image of a living control embryo injected with membrane-targeted GFP, showing that expression of GFP alone persists (a similar preparation had robust GFP expression at 96 h). (I,J) 48 h embryos expressing a full-length SoxC construct. As with the embryos in E-G, there is no indication that SoxC is expressed throughout the embryo, as SoxC localizes to scattered cells, similar to control preparations, and Brn1/2/4 expression is normal. (K,L) Control preparation showing the normal distribution of SoxC and SoxB2. (M,N) Embryos derived from eggs injected with *SoxC* full-length RNA showing normal numbers of SynB-expressing neurons and SoxC-expressing cells. (O,P) Control preparations of eggs injected with SoxC:mGFP showing the normal number and distribution of SynB-containing neurons (O) and SoxC-expressing cells (P). Scale bars: 10 µm.

progenitors, the last S-phase occurs between 16 and 24 h and SoxC protein is first expressed at 20 h. In most postoral cells S-phase occurs between 16 and 28 h and SoxC protein is expressed at about 28 h. The fact we see division of cells expressing SoxC and that we are able to label cells expressing SoxC with a pulse of EdU indicates that cells expressing SoxC are not post-mitotic. A parsimonious interpretation of these observations is that SoxC is expressed coincident with neural progenitors initiating a final mitotic division.

Cells expressing SoxC are frequently observed as pairs of adjacent cells. It is reported that SoxC cells co-express Delta (Wei et al., 2016) and that treatment with DAPT, an inhibitor of Notch signaling, or a Delta morpholino results in the formation of clusters of neurons (Materna and Davidson, 2012; Wei et al., 2009; Yaguchi et al., 2011). This suggests that Notch signaling occurs subsequent to the final cell division and that the division is asymmetric, at least in the distribution of molecular components. A model that is consistent with these observations is that SoxC is expressed at the time of the transition of cells from mitotically active neural progenitors to undifferentiated post-mitotic neural precursors. Notch signaling then mediates a process of lateral inhibition in which one cell differentiates as a neuron and the other does not. A key question is the fate of the cell that does not differentiate into a neuron – it could remain as a self-renewing persistent neural progenitor, or it could differentiate to another type of cell. Cells expressing SoxC do not accompany differentiating neurons and there is no consistent patterning of cells expressing SoxC, suggesting that SoxC expression does not persist in the cell presumed to experience elevated Notch signaling. In addition,

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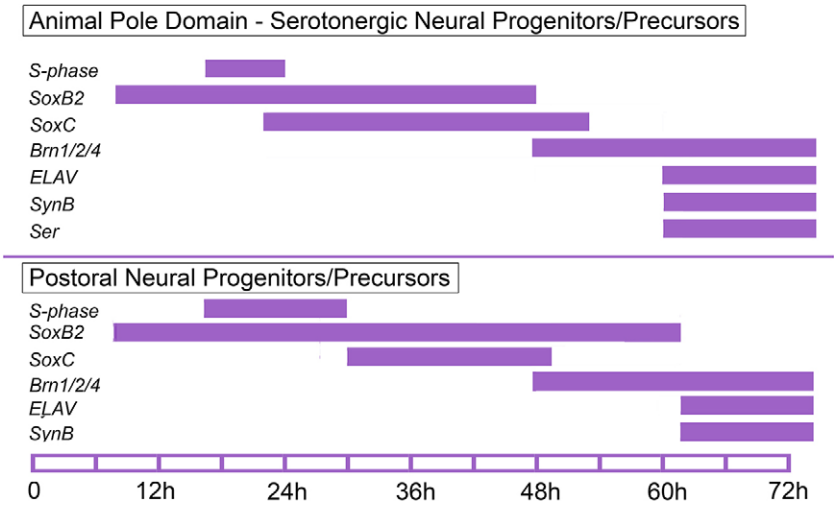

Fig. 9. A timeline of expression of proteins associated with neurogenesis or of S-phase of the last cell cycle in neural progenitors identified by location. For the APD (top), serotonergic neurons provide an example of the differentiation of neurons in the neuroepithelium that gives rise to a central nervous component. The postoral neurons (bottom) serve as an example of the differentiation of peripheral neurons derived from oral ectoderm.

SoxC-expressing cells that arise within the ciliary band co-express Hnf6, a transcription factor associated with the differentiation of ciliated cells of the ciliary band. This suggests that early larval stages continue to recruit neural progenitors from adjacent ectoderm and ciliary band.

A neurogenic pathway

As the suppression of SoxC results in a marked reduction in the number of neurons and in some embryos no neurons form at all, we concluded that neurogenesis requires SoxC and that all neurons express SoxC during neurogenesis. However, it is not possible to conclude from our studies that all cells that express SoxC form neurons. The initial expression of SoxC throughout the APD before becoming restricted to a small number of cells suggests that cells expressing SoxC are not immediately committed to a neural precursor fate. Although the proteins we have examined have found use in other organisms as neuron-specific markers, none is expressed in that manner in sea urchins. SoxC is initially expressed in vegetal lineages and is downregulated as the cells differentiate. ELAV is expressed robustly in mesoderm-derived cells throughout early development. Brn1/2/4 protein can be detected in foregut endoderm as previously described (Cole and Arnone, 2009) and SoxB2 is broadly expressed throughout ectoderm. Thus, there are limitations to the use of these proteins as exclusive markers for neural progenitors.

Embryos that develop from eggs injected with full-length *SoxC* RNA, either fused to GFP or not, express the protein, which then accumulates in all nuclei. The protein can be detected until about 30 h, when it is reduced to background levels in all cells, except those that normally express SoxC. As neural precursors normally form in the APD and the oral ectoderm as early as 24 h, ectopic expression of SoxC by RNA injection has the potential to reveal whether it is sufficient to commit cells to neural precursors. As there is no increase in the number of cells differentiating as neurons through a range of doses that does not cause random abnormalities, we conclude that SoxC is necessary but not sufficient for the formation of neural precursors. The mechanism by which SoxC protein, but not control GFP, is dramatically reduced in early gastrulation merits further investigation.

Neurogenesis and ectodermal patterning

Our data indicate that peripheral neurons arise in oral ectoderm, ciliary band and adjacent aboral ectoderm and that specification of the first neurons to differentiate is likely to occur in late cleavage. Previous studies have established that ectoderm differentiates in distinct domains in which TGFβ signaling initiates gene regulatory networks that establish cellular identity (Duboc et al., 2004, 2008; Duboc and Lepage, 2008). Nodal signaling during cleavage specifies oral ectoderm and initiates BMP signaling that establishes aboral ectoderm (Duboc et al., 2004; Saudemont et al., 2010; Ben-Tabou de-Leon et al., 2013). The ciliary band appears to form as a consequence of being protected from Nodal and BMP signaling by the antagonists Lefty and Chordin (Duboc et al., 2008; Bradham et al., 2009; Lapraz et al., 2009; Saudemont et al., 2010; Yaguchi et al., 2010). Oral and aboral ectoderm are fields of cells with bilateral subdomains in which distinct regulatory programs appear to operate (Chen et al., 2011; Li et al., 2012; Barsi et al., 2015). Our data indicate that neurons arise within the ciliary bands and adjacent regions of oral and aboral ectoderm. This is consistent with data that indicate Nodal and BMP signaling produce ectoderm that does not support neurogenesis (Yaguchi et al., 2010; Bradham et al., 2009) and suggests that the ciliary band is not the only region of ectoderm that is protected, at least in part, from TGFβ signaling.

None of the models of specification of oral, aboral or ciliary band ectoderm predicts the appearance of individual cells expressing a different regulatory program within these domains (Saudemont et al., 2010; Chen et al., 2011; Li et al., 2012; Barsi et al., 2015). Yet our data suggest that a gene regulatory network that specifies a neural fate controls the differentiation of individual neural progenitors within specific domains of ectoderm. FGF signaling and FGFR1 are necessary for the differentiation of neurons and are expressed such that they potentially initiate neurogenesis. A component of our hypothesis is that FGFR1 signaling diverts cells from oral ectoderm, ciliary band or aboral ectodermal fates by inducing expression of Six3, which initiates a neurogenic regulatory network. FGFR1 is expressed throughout the tissues that appear to give rise to neurons beginning in late cleavage stages (Lapraz et al., 2006). We envision a localized effect modulated by other factors, as all these cells do not become neural. FGFA is expressed in restricted regions of the blastoderm (Rottinger et al., 2008) and FGFR2 is expressed in mesodermal lineages. To date, analysis of FGF function in sea urchin embryos has focused on roles in patterning mesoderm (Lapraz et al., 2006; Rottinger et al., 2008; Adomako and Ettensohn, 2013). However, a role for FGF in initiating neurogenesis in other metazoans suggests that this is a conserved function (Rentzsch et al., 2008; Sinigaglia et al., 2013). In ascidians,

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Table 1. Comparison of general features of neurogenesis within the deuterostomes

	Hemichordates/asteroids	Echinoids	Urochordates/cephalochordates/vertebrates
Neurogenic potential	Throughout ectoderm	Restricted	Restricted
Patterning of neural progenitors	Variable	Invariant and variable	Invariant and variable
Division of progenitors	?	Asymmetric	Asymmetric and symmetric
Movement of neural precursors	Extensive	Minor	Extensive

Features and their state within taxa other than echinoids are derived from the literature (Nakajima et al., 2004; Yankura et al., 2013; Cunningham and Casey, 2014; Hartenstein and Stollewerk, 2015).

FGF is essential for the specification of peripheral neurons that are arrayed laterally along the tail, a process that is initiated during cleavage (Ohtsuka et al., 2014). In vertebrates, FGF has an essential role in the specification of sensory placodes (Ariza-Cosano et al., 2015; Kengaku and Okamoto, 1993).

Deuterostome neurogenesis

Deuterostomes share close kinship at a molecular level, yet body plans and neural organization are bafflingly diverse. Chordates restrict most neurogenesis to one region of the embryo and employ dedicated neural progenitors extensively in the generation of neurons. The other extreme appears in hemichordates, where neurogenesis occurs throughout the embryonic ectoderm and gives rise to a subepidermal network of nerves (Cunningham and Casey, 2014). Similarly, in asteroids, neural precursors arise throughout the ectoderm and migrate extensively before differentiating in the ciliary bands (Yankura et al., 2013; Nakajima et al., 2004). Broad comparisons within the deuterostomes, when echinoid neurogenesis is considered, indicate that the differences between ambulacrarians and chordates are less extreme than initial comparisons suggest, and that echinoid neurogenesis is distinct from that of hemichordates and asteroids (Table 1). Apparently, deuterostomes have in common the potential for restricting neurogenesis to specific domains of ectoderm, the ability to pattern neural progenitors to produce invariant positioning of neurons, and asymmetric division of neural progenitors. Detailed analyses of the neurogenic mechanisms of a complete range of taxa have the potential to distinguish features shared by common origin from features derived independently because of functional limits.

MATERIALS AND METHODS

Embryo culture and injection

Gametes were collected from *S. purpuratus* induced to spawn by shaking. Sperm was diluted 1:1000 in sea water prior to fertilization and embryos were grown at 12-14°C. Eggs were prepared for microinjection as described previously (Krupke et al., 2014). Injection solutions contained water, 120 mM KCl, MASO or RNA and 1 µg/ml Rhodamine Green or Rhodamine B. Some injection preparations included 22% glycerol. MASOs were obtained from GeneTools, diluted to a suitable stock concentration (1 mM) and further diluted and filtered before filling injection needles. MASO sequences (5′-3′) and concentrations used: SoxC I, TTTTGAGGAACCATCTTGAAGTCAG (400 µM); SoxC II, CTTGAAGTCAGCATTCACTTTCGTG (1 mM); SoxC III, GAACCATCTTGAAGTCAGCATTCAC (400 nM); Six3-MO2, CCCCGGTCGCTGGGCGATGTTTCTG (1 mM) (Wei et al., 2009); FGFR1, TCCTCGGACAACGCGGCAGACTCAT (400 µM); SoxB2, GATATGACGGTCTCCATCTTACTCC (500 µM); Brn1/2/4, TCAATGAATCCTGGATCCCGAACAT (100 µM); StdControl1, CCTCTTACCTCAGTTACAATTTATA (300 µM); and Control2, TTTCAACCGTTTCCAAAGAACCAGG (300 µM). Knockdowns were confirmed with depletion of antibody binding where applicable (Fig. S1). Ectopic expression of SoxC was achieved using synthetic, capped mRNAs derived from the full-length *S. purpuratus SoxC* cDNA, or a construct in which the *SoxC* cDNA lacks a stop codon and is fused in frame to eGFP, cloned into a pCS2+ vector and transcribed using the SP6 mMessage mMachine Kit (Ambion). FGF inhibitor was diluted in DMSO and this stock further diluted into embryo cultures immediately after fertilization, and embryos were treated for the duration of the experiment. All experiments had at least three replicates.

Antibody production

Antibody production was as previously described (Krupke and Burke, 2014). Antigens were made using a pET28b(+) plasmid (Novagen) for expression of 6×His-tagged proteins. SoxC, SoxB2, ELAV and Brn1/2/4 constructs, encoding partial or full-length proteins, were produced with high-fidelity PCR from cDNA isolated from *S. purpuratus* embryos and cloned using the pGEM-T Easy system (Promega) (Fig. S1). Protein expression was induced in *E. coli* (BL21). Bacterial lysate was prepared and protein was solubilized in binding buffer (6 M guanidine HCl, 0.5 M NaCl, 100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 10 mM imidazole, 10 mM Tris, 1 mM 2-mercaptoethanol, pH 8.0) prior to affinity purification by immobilized metal ion affinity chromatography (IMAC) using Chelex 100 resin (Bio-Rad). Following IMAC, proteins were further purified by size exclusion on Hi Load 16/60 Superdex 75 prep grade column (GE Healthcare) using the ÄKTA Prime Plus chromatography system (GE Healthcare). Purified protein in PBS was mixed 1:1 with Freund's complete adjuvant for immunization or with Freund's incomplete adjuvant for booster injections. Rats, rabbits or mice were immunized by subcutaneous injection of 100 µg antigen in 250 µl adjuvant, and booster injections were performed 21 days and 42 days after the initial immunization. Terminal bleed via cardiac puncture was performed after 52-56 days. Blood was incubated at 37°C for 45 min and then 4°C overnight. Samples were centrifuged at 1000 ***g*** and the serum collected.

Immunofluorescence

Embryos were collected and fixed for 15 min in PEM fixative (Krupke et al., 2014). To determine the stages at which specific markers were expressed, embryos from a batch of eggs were fixed at intervals. Initially, intervals were 12 to 24 h, but to determine times at which expression began or ended, intervals of 2 h between fixations were used. Embryos were rinsed with PBS, blocked for 1 h in SuperBlock (Thermo) before incubation overnight in primary antibody diluted in SuperBlock. Embryos were rinsed three times with PBS and then Alexa Fluor-conjugated fluorescent secondary antibodies (Invitrogen) were used to visualize antibody labeling on a Zeiss LSM 700 confocal microscope. Imaging and analysis were conducted using ZEN software (Zeiss). Adobe Photoshop was used to prepare figures and adjust image contrast and brightness. Cell counts were made by sequential examination of individual images from stacks that focused through the entire thickness of the specimen. Statistics were calculated using Sigma Plot version 5.0 (Systat Software). At least three biological replicates of all experiments were performed. For details of primary and secondary antibodies used, see Table S1.

EdU labeling

A 2 ml aliquot of embryos that were at the desired time to initiate labeling was incubated with 0.2 µl 10 mM stock solution of ethynyl deoxyuridine (EdU). After the interval of labeling, embryos were rinsed twice with sea water and incubated until 72 h, at which time they were fixed. Click-iT reaction mixture (Click-iT EdU Alexa 647, Life Technologies) was added directly to the primary antibody labeling solution and embryos were prepared as described above. For each labeling interval, five embryos were optically sectioned and labeled neuronal nuclei were counted manually by two observers. There are 15-20 neurons expressing SynB in early plutei (72 h).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
G.B., I.Z., M.K., D.M. and V.T. developed reagents, performed experiments, analyzed data and revised the manuscript. R.D.B. and S.G. developed concepts and approaches, performed experiments, analyzed data and prepared the manuscript.

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References

Adomako-Ankomah, A. and Ettensohn, C. A. (2013). Growth factor-mediated mesodermal cell guidance and skeletogenesis during sea urchin gastrulation. *Development* **140**, 4214-4225.
Altmann, C. R. and Brivanlou, A. H. (2001). Neural patterning in the vertebrate embryo. *Int. Rev. Cytol.* **203**, 447-482.
Angerer, L. M., Yaguchi, S., Angerer, R. C. and Burke, R. D. (2011). The evolution of nervous system patterning: insights from sea urchin development. *Development* **138**, 3613-3623.
Ariza-Cosano, A., Bensimon-Brito, A., Gómez-Skarmeta, J. L. and Bessa, J. (2015). sox21a directs lateral line patterning by modulating FGF signaling. *Dev. Neurobiol.* **75**, 80-92.
Barsi, J. C., Li, E. and Davidson, E. H. (2015). Geometric control of ciliated band regulatory states in the sea urchin embryo. *Development* **142**, 953-961.
Beer, A.-J., Moss, C. and Thorndyke, M. (2001). Development of serotonin-like and SALMFamide-like immunoreactivity in the nervous system of the sea urchin Psammechinus miliaris. *Biol. Bull.* **200**, 268-280.
Ben-Tabou de-Leon, S. B.-T., Su, Y.-H., Lin, K.-T., Li, E. and Davidson, E. H. (2013). Gene regulatory control in the sea urchin aboral ectoderm: spatial initiation, signaling inputs, and cell fate lockdown. *Dev. Biol.* **374**, 245-254.
Bier, E. and De Robertis, E. M. (2015). BMP gradients: a paradigm for morphogen-mediated developmental patterning. *Science* **348**, aaa5838.
Bisgrove, B. W. and Burke, R. D. (1986). Development of serotonergic neurons in embryos of the sea urchin, Strongylocentrotus Purpuratus. *Dev. Growth Differ.* **28**, 569-574.
Bradham, C. A., Oikonomou, C., Kühn, A., Core, A. B., Modell, J. W., McClay, D. R. and Poustka, A. J. (2009). Chordin is required for neural but not axial development in sea urchin embryos. *Dev. Biol.* **328**, 221-233.
Brombin, A., Grossier, J.-P., Heuzé, A., Radev, Z., Bourrat, F., Joly, J.-S. and Jamen, F. (2011). Genome-wide analysis of the POU genes in medaka, focusing on expression in the optic tectum. *Dev. Dyn.* **240**, 2354-2363.
Burke, R. D., Moller, D. J., Krupke, O. A. and Taylor, V. J. (2014). Sea urchin neural development and the metazoan paradigm of neurogenesis. *Genesis* **52**, 208-221.
Chehrehasa, F., Meedeniya, A. C. B., Dwyer, P., Abrahamsen, G. and Mackay-Sim, A. (2009). EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. *J. Neurosci. Methods* **177**, 122-130.
Chen, J.-H., Luo, Y.-J. and Su, Y.-H. (2011). The dynamic gene expression patterns of transcription factors constituting the sea urchin aboral ectoderm gene regulatory network. *Dev. Dyn.* **240**, 250-260.
Cole, A. G. and Arnone, M. L. (2009). Fluorescent in situ hybridization reveals multiple expression domains for SpBrn1/2/4 and identifies a unique ectodermal cell type that co-expresses the ParaHox gene SpLox. *Gene Expr. Patterns* **9**, 324-328.
Cunningham, D. and Casey, E. S. (2014). Spatiotemporal development of the embryonic nervous system of Saccoglossus kowalevskii. *Dev. Biol.* **386**, 252-263.
Cui, M., Siriwon, N., Li, E., Davidson, E. H. and Peter, I. S. (2014). Specific functions of the Wnt signaling system in gene regulatory networks throughout the early sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **111**, E5029-E5038.
Duboc, V. and Lepage, T. (2008). A conserved role for the nodal signaling pathway in the establishment of dorso-ventral and left-right axes in deuterostomes. *J. Exp. Zool. B Mol. Dev. Evol.* **310B**, 41-53.
Duboc, V., Röttinger, E., Besnardeau, L. and Lepage, T. (2004). Nodal and BMP2/4 signaling organizes the oral-aboral axis of the sea urchin embryo. *Dev. Cell* **6**, 397-410.
Duboc, V., Lapraz, F., Besnardeau, L. and Lepage, T. (2008). Lefty acts as an essential modulator of Nodal activity during sea urchin oral-aboral axis formation. *Dev. Biol.* **320**, 49-59.
Good, P. J. (1995). A conserved family of Elav-like genes in vertebrates. *Proc. Natl. Acad. Sci. USA* **92**, 4557-4561.
Goodman, C. S. and Doe, C. Q. (1993). *Embryonic Development of the Drosophila Central Nervous System*. Plainview, NY, USA: Cold Spring Harbor Laboratory Press.
Götz, M. and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* **6**, 777-788.
Hartenstein, V. and Stollewerk, A. (2015). The evolution of early neurogenesis. *Dev. Cell* **32**, 390-407.
Howard-Ashby, M., Materna, S. C., Brown, C. T., Chen, L., Cameron, R. A. and Davidson, E. H. (2006). Identification and characterization of homeobox transcription factor genes in Strongylocentrotus purpuratus, and their expression in embryonic development. *Dev. Biol.* **300**, 74-89.
Kengaku, M. and Okamoto, H. (1993). Basic fibroblast growth factor induces differentiation of neural tube and neural crest lineages of cultured ectoderm cells from Xenopus gastrula. *Development* **119**, 1067-1078.
Krupke, O. A. and Burke, R. D. (2014). Eph-Ephrin signaling and focal adhesion kinase regulate actomyosin-dependent apical constriction of ciliary band cells. *Development* **141**, 1075-1084.
Krupke, O., Yaguchi, S., Yaguchi, J. and Burke, R. D. (2014). Imaging neural development in embryonic and larval sea urchins. *Methods Mol. Biol.* **1128**, 147-160.
Lapraz, F., Röttinger, E., Duboc, V., Range, R., Duloquin, L., Walton, K., Wu, S.-Y., Bradham, C., Loza, M. A., Hibino, T. et al. (2006). RTK and TGF-beta signaling pathways genes in the sea urchin genome. *Dev. Biol.* **300**, 132-152.
Lapraz, F., Besnardeau, L. and Lepage, T. (2009). Patterning of the dorsal-ventral axis in echinoderms: insights into the evolution of the BMP-Chordin Signaling Network. *PLoS Biol.* **7**, e1000248.
Lemaire, P., Bertrand, V. and Hudson, C. (2002). Early steps in the formation of neural tissue in ascidian embryos. *Dev. Biol.* **252**, 151-169.
Li, E., Materna, S. C. and Davidson, E. H. (2012). Direct and indirect control of oral ectoderm regulatory gene expression by Nodal signaling in the sea urchin embryo. *Dev. Biol.* **369**, 377-385.
Mackie, G. O., Spencer, A. N. and Strathmann, R. (1969). Electrical activity associated with ciliary reversal in an echinoderm larva. *Nature* **223**, 1384.
Marlow, H., Tosches, M. A., Tomer, R., Steinmetz, P. R., Lauri, A., Larsson, T. and Arendt, D. (2014). Larval body patterning and apical organs are conserved in animal evolution. *BMC Biol.* **12**, 7.
Materna, S. C. and Davidson, E. H. (2012). A comprehensive analysis of Delta signaling in pre-gastrular sea urchin embryos. *Dev. Biol.* **364**, 77-87.
Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.
Nakajima, Y., Kaneko, H., Murray, G. and Burke, R. D. (2004). Divergent patterns of neural development in larval echinoids and asteroids. *Evol. Dev.* **6**, 95-104.
Ohtsuka, Y., Matsumoto, J., Katsuyama, Y. and Okamura, Y. (2014). Nodal signaling regulates specification of ascidian peripheral neurons through control of the BMP signal. *Development* **141**, 3889-3899.
Otim, O., Amore, G., Minokawa, T., McClay, D. R. and Davidson, E. H. (2004). SpHnf6, a transcription factor that executes multiple functions in sea urchin embryogenesis. *Dev. Biol.* **273**, 226-243.
Poustka, A. J., Kuhn, A., Radosavljevic, V., Wellenreuther, R., Lehrach, H. and Panopoulou, G. (2004). On the origin of the chordate central nervous system: expression of onecut in the sea urchin embryo. *Evol. Dev.* **6**, 227-236.
Poustka, A. J., Kühn, A., Groth, D., Weise, V., Yaguchi, S., Burke, R. D., Herwig, R., Lehrach, H. and Panopoulou, G. (2007). A global view of gene expression in lithium and zinc treated sea urchin embryos: new components of gene regulatory networks. *Genome Biol.* **8**, R85.
Rentzsch, F., Fritzenwanker, J. H., Scholz, C. B. and Technau, U. (2008). FGF signalling controls formation of the apical sensory organ in the cnidarian Nematostella vectensis. *Development* **135**, 1761-1769.
Richards, G. S. and Rentzsch, F. (2014). Transgenic analysis of a SoxB gene reveals neural progenitor cells in the cnidarian Nematostella vectensis. *Development* **141**, 4681-4689.
Robinow, S. and White, K. (1988). The locus Elav of Drosophila melanogaster is expressed in neurons at all developmental stages. *Dev. Biol.* **126**, 294-303.
Robinow, S. and White, K. (1991). Characterization and spatial distribution of the Elav protein during Drosophila melanogaster development. *J. Neurobiol.* **22**, 443-461.
Rottinger, E., Saudemont, A., Duboc, V., Besnardeau, L., McClay, D. and Lepage, T. (2008). FGF signals guide migration of mesenchymal cells, control skeletal morphogenesis of the skeleton and regulate gastrulation during sea urchin development. *Development* **135**, 353-365.

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Ryan, A. K. and Rosenfeld, M. G. (1997). POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev.* **11**, 1207-1225.

Satterlie, R. A. and Cameron, R. A. (1985). Electrical activity at metamorphosis in larvae of the sea urchin Lytechinus pictus (Echinoidea: Echinodermata). *J. Exp. Zool.* **235**, 197-204.

Saudemont, A., Haillot, E., Mekpoh, F., Bessodes, N., Quirin, M., Lapraz, F., Duboc, V., Röttinger, E., Range, R., Oisel, A. et al. (2010). Ancestral regulatory circuits governing ectoderm patterning downstream of Nodal and BMP2/4 revealed by gene regulatory network analysis in an echinoderm. *PLoS Genet.* **6**, e1001259.

Sinigaglia, C., Busengdal, H., Leclère, L., Technau, U. and Rentzsch, F. (2013). The bilaterian head patterning gene six3/6 controls aboral domain development in a Cnidarian. *PLoS Biol.* **11**, e1001488.

Strathmann, R. R. (2007). Time and extent of ciliary response to particles in a non-filtering feeding mechanism. *Biol. Bull.* **212**, 93-103.

Taguchi, S., Tagawa, K., Humphreys, T. and Satoh, N. (2002). Group B Sox genes that contribute to specification of the vertebrate brain are expressed in the apical organ and ciliary bands of hemichordate larvae. *Zool. Sci.* **19**, 57-66.

Wegner, M., Drolet, D. W. and Rosenfeld, M. G. (1993). POU-domain proteins: structure and function of developmental regulators. *Curr. Opin. Cell Biol.* **5**, 488-498.

Wei, Z., Yaguchi, J., Yaguchi, S., Angerer, R. C. and Angerer, L. M. (2009). The sea urchin animal pole domain is a Six3-dependent neurogenic patterning center. *Development* **136**, 1179-1189.

Wei, Z., Angerer, L. M. and Angerer, R. C. (2016). Neurogenic gene regulatory pathways in the sea urchin embryo. *Development* **143**, 298-305.

Yaguchi, S., Yaguchi, J. and Burke, R. D. (2006). Specification of ectoderm restricts the size of the animal plate and patterns neurogenesis in sea urchin embryos. *Development* **133**, 2337-2346.

Yaguchi, S., Yaguchi, J., Angerer, R. C., Angerer, L. M. and Burke, R. D. (2010). TGF beta signaling positions the ciliary band and patterns neurons in the sea urchin embryo. *Dev. Biol.* **347**, 71-81.

Yaguchi, S., Yaguchi, J., Wei, Z., Jin, Y., Angerer, L. M. and Inaba, K. (2011). Fez function is required to maintain the size of the animal plate in the sea urchin embryo. *Development* **138**, 4233-4243.

Yankura, K. A., Koechlein, C. S., Cryan, A. F., Cheatle, A. and Hinman, V. F. (2013). Gene regulatory network for neurogenesis in a sea star embryo connects broad neural specification and localized patterning. *Proc. Natl. Acad. Sci. USA* **110**, 8591-8596.

Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* **8**, 709-715.

Zhong, W. M. (2003). Diversifying neural cells through order of birth and asymmetry of division. *Neuron* **37**, 11-14.

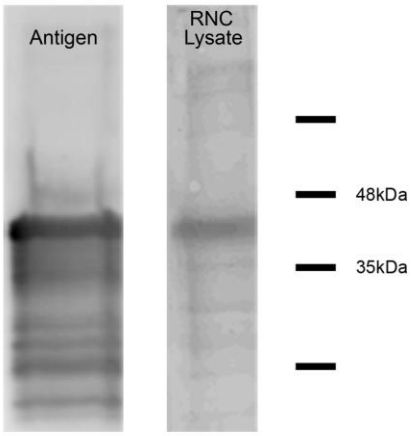
Supplemental Figure S1

SoxC

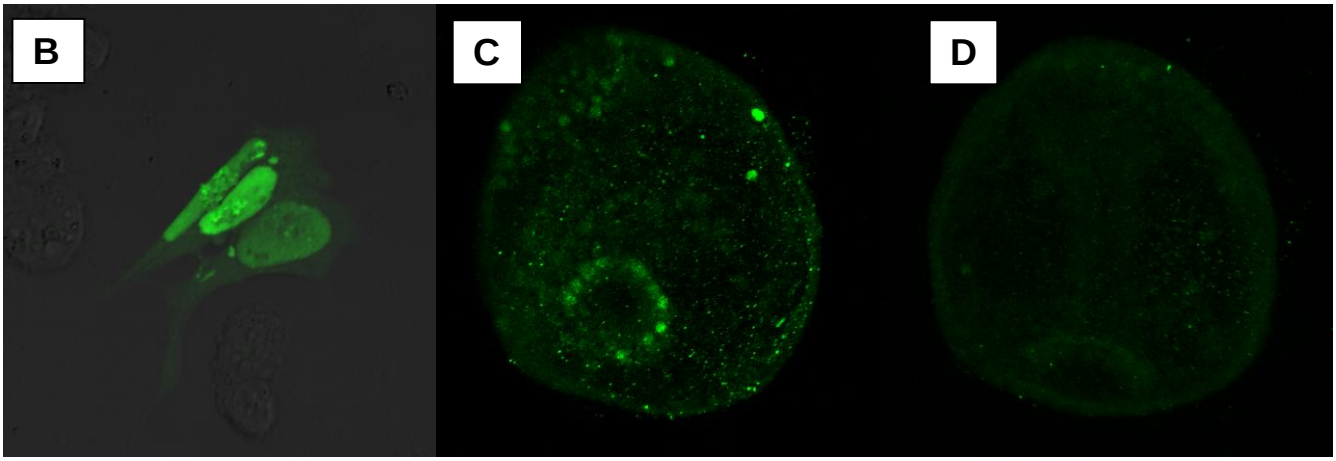
The predicted SoxC protein sequence is from the curated echinoderm genomic database, Echinobase (http://www.echinobase.org/Echinobase/). The bold sequence was amplified from cDNA, cloned, expressed and purified as described in Methods. A rat was immunized and a polyclonal serum prepared, as well, a mouse was immunized and a monoclonal antibody prepared. The two antibodies revealed identical immunolocalizations in doubly labelled preparations, and immunoreactivity was lost in embryos derived from eggs injected with a SoxC MASO. When HEK cells were transfected with a full length plasmid encoding urchin SoxC, they were immunoreactive to anti-SoxC.

>SPU_002603.3a Peptide Sequence
MVPQNLTSNGLITMQSQQIPNGHGSPGSTGSSEELRQSTLDIAEDICQTNWKGNNGHI
KRPMNAFMVWSQIERRRIMETTPDMHNAEISKRLGRRWKTLDEVAKSPYVEEAERLR
LLHMAQYPDYKY**RPRKKSKPTTKPEAAKTTSSKPKANKPKSSSKLTKMNGIVIDQMH**
PHQIVQSGRIEKIPKLKLTIDKKFRENMKASKIVELVPSQLTPPAKVPASPTGSNTDPC
NEQSLYEDYANIQHTYEMQRYEFGVPSGATSTTCSSPASSDVSQQSSMSTNSSVSS
MSTGSSYCAQQSIEDVVFPGGLTGSEFNNFNFGSVPDDLSPLDSVGSNGSHFEFPDY
TTPEVSELIDSDWLLSSMISAYN

Native protein predicted size: 41 kDa
Predicted expressed protein size: 30 kDa
A



Panel A. Lane 1 Immunoblot of antigen. The expressed SoxC protein used for immunization was probed with hybridoma supernatant that was derived from a mouse immunized with bacterially expressed SoxC protein. SoxC coding sequence was amplified from *S. pupuratus* cDNA and expressed in *E.coli* (see Methods).
Lane 2 Immunoblot of lysate of *S. pupuratus* radial nerve cord (RNC) probed with rat polyclonal antibodies directed against SoxC. Polyclonal antibodies were derived from serum of rats injected with bacterially expressed SoxC protein.



B. HEK cells transfected with pCS2+:SoxC, prepared with anti-SoxC antibody (rat serum) for immunofluorescence. Transfected cells were immunoreactive and indicate a nuclear localization of the SoxC protein.
C. Embryos (48h) from eggs injected with control MASO and prepared with anti-SoxC (mouse anti-SoxC hybridoma supernatant).
D. Embryos (48h) from eggs injected with SoxC MASO and prepared with anti-SoxC (mouse anti-SoxC hybridoma supernatant).

There are data from in situ hybridizations (Howard-Ashby et al. 2006; Poutska et al. 2007) with Sox C and the antibody localizations are consistent with the data and descriptions provided. In general there are more cells immunoreactive with the antisera than identified with in situ hybridization. However, this is typical of mRNA being less stable than protein and having a shorter half-life.

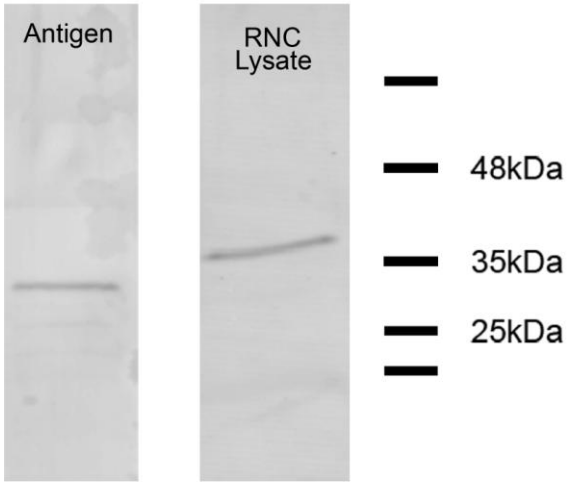
SoxB2

The predicted SoxB2 protein sequence is from the curated echinoderm genomic database, Echinobase (http://www.echinobase.org/Echinobase/). The bold sequence was amplified from cDNA, cloned, expressed and purified as described in Methods. A rat was immunized and a polyclonal serum prepared.

>SPU_025113.3a Peptide Sequence
MMMDSAMGKGTDHVKRPMNAFMVWSRGQRRKLAQENPKMHNSEISKRLGAEWKL LSEDDKRPFIDEAKRLRALHMKEHPDYKYRPRRKPKSLMKRDKYAFPIPCIPTSSPY QVATSQADIMNMASAEKARTYLSSHQHHAAAAAAAASQYSVLEHAQKLESPTSLIR DFPHHPALYPPPHMYPTSAGAVPGSAFGKLPGGSAAAAAAAAAAGYSAQPYMMPY PAWPGQDGVQRPVAYILVKPDMEPYGPTHPAIRPTMPLPTRPTAATAL

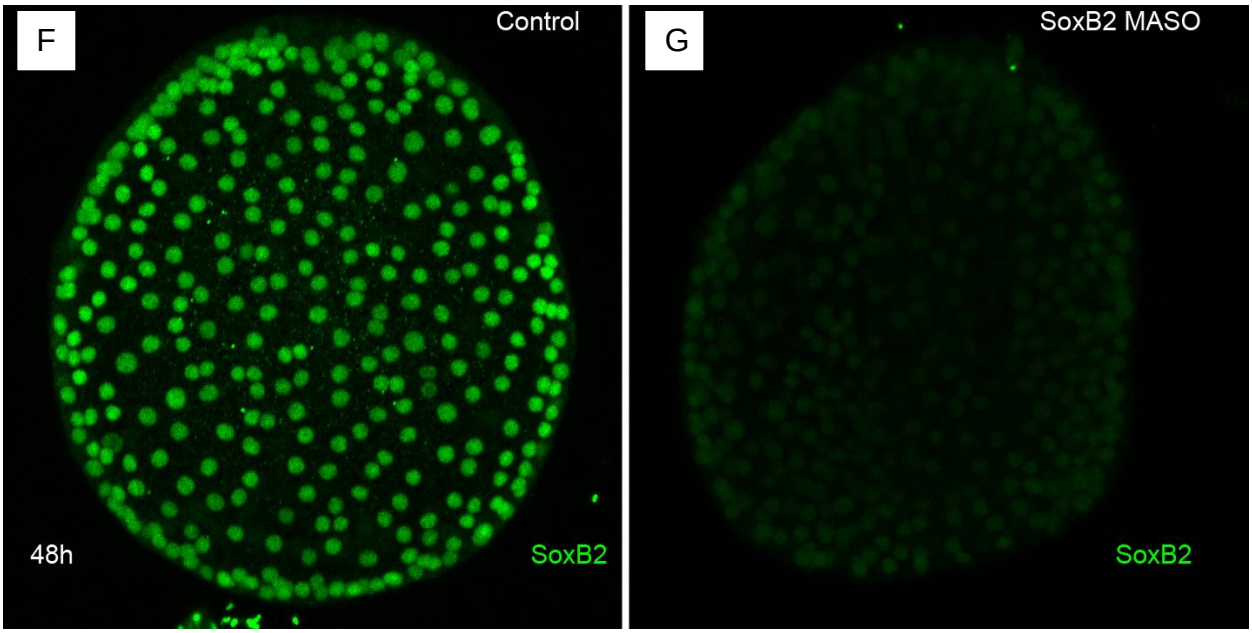
Native protein predicted size: 30 kDa
Predicted expressed protein size: 25 kDa

E



Panel E Lane 1 Immunoblot of SoxB2 antigen. The expressed SoxB2 protein used for immunization was separated by PAGE, transferred, and the membrane probed with serum of a rat immunized with bacterially expressed SoxB2 protein. SoxB2 coding sequence was amplified from *S. pupuratus* cDNA and expressed in *E.coli* (see Methods).

Lane 2 Immunoblot of lysate of *S. pupuratus* radial nerve cord (RNC) probed with rat serum immunized with SoxB2..



Maximum intensity projections of embryos prepared with anti-SoxB2 serum. F. Control, 48h embryo showing the normal distribution of SoxB2. G. Embryo derived from egg injected with 400 µM SoxB2 MASO, prepared as in panel F and imaged with identical settings. There is only a very weak signal indicating that there is reduced antibody binding and that the MASO has suppressed expression of the SoxB2 protein.

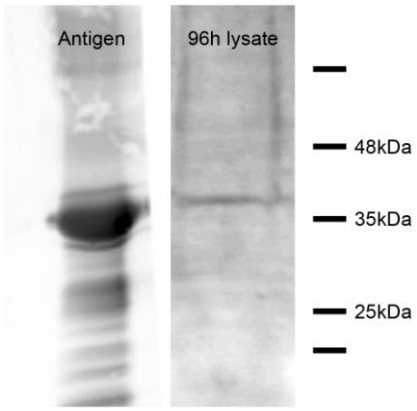
ELAV

The predicted ELAV protein sequence is from the curated echinoderm genomic database, Echinobase (http://www.echinobase.org/Echinobase/). The bold sequence was amplified from cDNA, cloned, expressed and purified as described in Methods. A rat was immunized and a polyclonal serum prepared, as well, a rabbit was immunized and a polyclonal serum prepared. The two antibodies revealed identical immunolocalizations in doubly labelled preparations.

>SPU_002324.3b Peptide Sequence
MINVIDNMEAQTVQPA**MQNGGLMKPNVVGVGGDEDSKTNLIVNYLPQNMAQDEMKS LFGKFGEIESCKLVRDKLTGQSLGYGFVNYLKPADALKAVKTLNGLRLQCKTIKVSFA RPSSQAIKDANLYISGIPKHYGQLDLDNLFNAFGRIICSRLLLDHECGRPRGVGFVRY DRRCEAEKAIEGLNGNIPHGGKDPLIVKFANNPGQHYQKCLQQMYQQMPIISPTLSP RRVGGPVSAGGSQNFIGPMRHMAHCFRWQKMGSKMQGLIGKLLPKNFMFNPMTSS DVISHMNLQAMTNNGQGWCIFVYNLPADCEDGLLWQLFGPYGAVTNVKVVRDQPN QRCKGYGFVNMLNYDEALSAINTLNGYQLNGKRTLQVSFKSSKQKS**
385 AA

Native protein predicted size:
SPU_002324.3b: 43 kDa
SPU_002324.3a: 41 kDa
SPU_002324.3b: 41 kDa
SPU_002324.3a: 40 kDa

H



Panel H. Lane 1 Immunoblot of ELAV antigen. The expressed ELAV protein used for immunization was separated by PAGE, transferred, and the membrane probed with serum of a rabbit immunized with bacterially expressed ELAV protein. ELAV coding sequence was amplified from *S. pupuratus* cDNA and expressed in *E.coli* (see Methods).

Lane 2 Immunoblot of lysate of *S. pupuratus* plutei (96h) probed with serum of a rabbit immunized with ELAV..

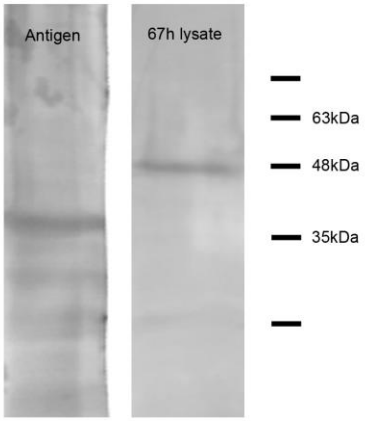
Brn1/2/4

The predicted Brn1/2/4 protein sequence is from the curated echinoderm genomic database, Echinobase (http://www.echinobase.org/Echinobase/). The bold sequence was amplified from cDNA, cloned, expressed and purified as described in Methods. A rat was immunized and a polyclonal serum prepared.

>SPU_016443.3a Peptide Sequence
METVISTPYSLSLSQADITPSSNSLILSSATDHIHHNITSLSDNSMQSGQVNVMYQKLGN
EFLQQQGGNGLPLAHHGAQWVTGLSAPPHADPTSHWAGVPAHLLGHGQDIKPNLGQ
TRDEINELHRSGHSHVQSAATWNTGNA**HMAMPMSMTMPMTTSSGGGPLGHTPTSA**
HPMYTYGAMNGMMSCAQQFGQNGPMRGVLGPGGGQLPSHNGSETVIEDDAPSSD
DLEQFAKTFKQRRIKLGYTQADVGLALGTLYGNVFSQTTICRFEALQLSFKNMCKLK
PLLAKWLEEADSTSGSPTSLDKIAAQGRKRKKRTSIEVTIKGALENAFLKQPKPSAQ
EISALADGLQLEKEVVRVWFCNRRQKEKRMTPPLNGIGPGGMQSADSPPPGQAATG
EHVLPHSTASGLHHHHHHHHPVITSLSSHAHHIGPGSSPIHGPPASVSPPAVHSPISSA
LTPHSQHQAQSVQ

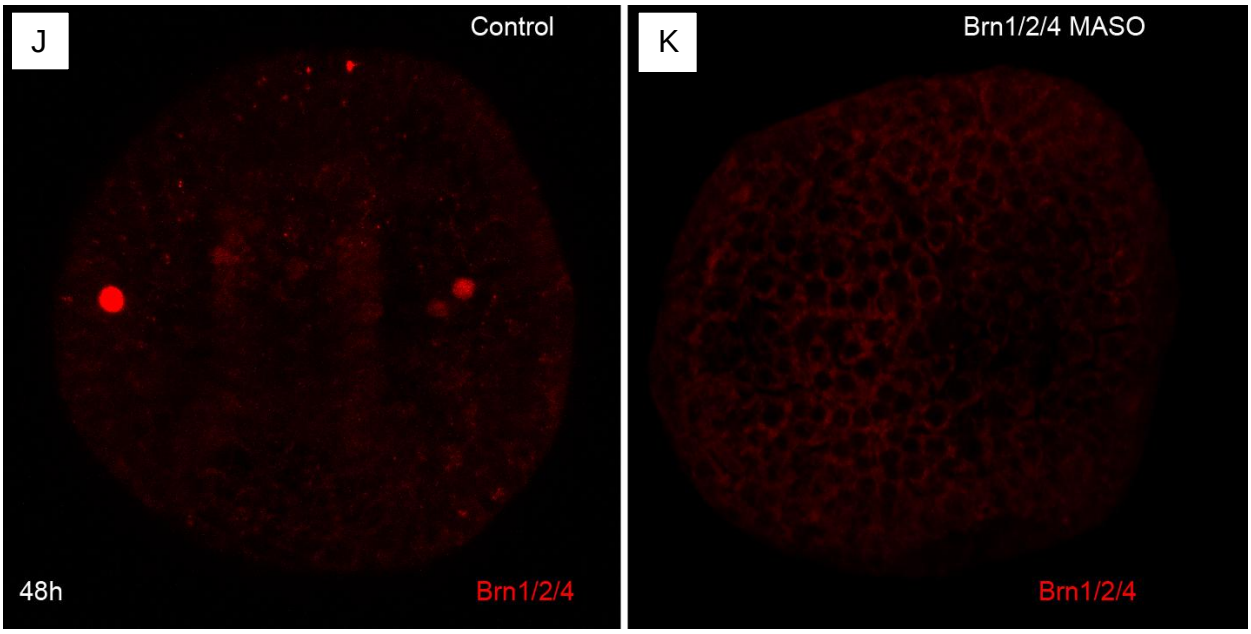
Native protein predicted size: 50 kDa
Predicted expressed protein size: 33 kDa

I



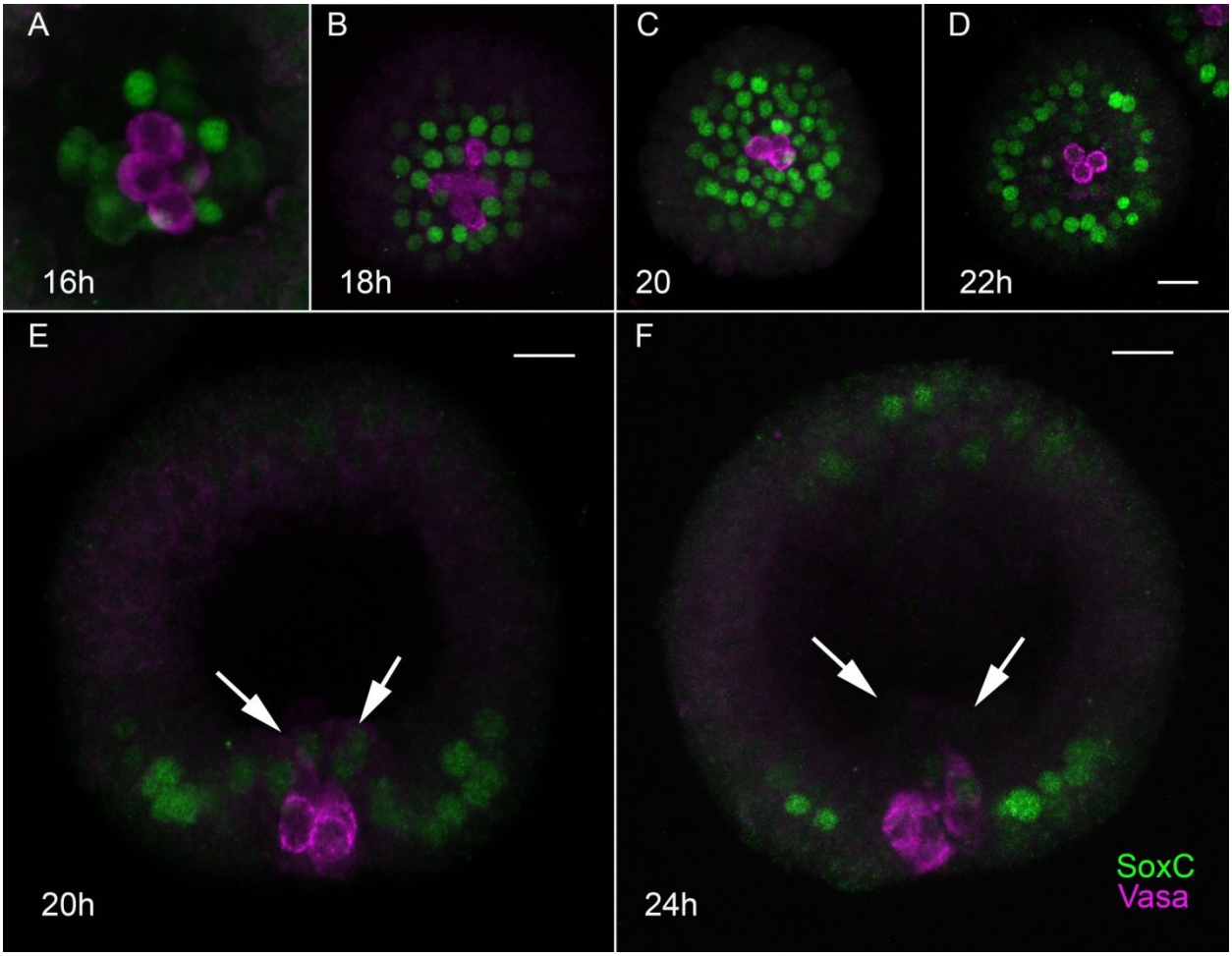
Panel I. Lane 1 Immunoblot of Brn1/2/4 antigen. The expressed Brn1/2/4 protein used for immunization was separated by PAGE, transferred, and the membrane probed with serum of a rat immunized with bacterially expressed Brn1/2/4 protein. Brn1/2/4 coding sequence was amplified from *S. pupuratus* cDNA and expressed in *E.coli* (see Methods).

Lane 2 Immunoblot of lysate of *S. pupuratus* prisms (67h) probed with serum of a rat immunized with Brn1/2/4..



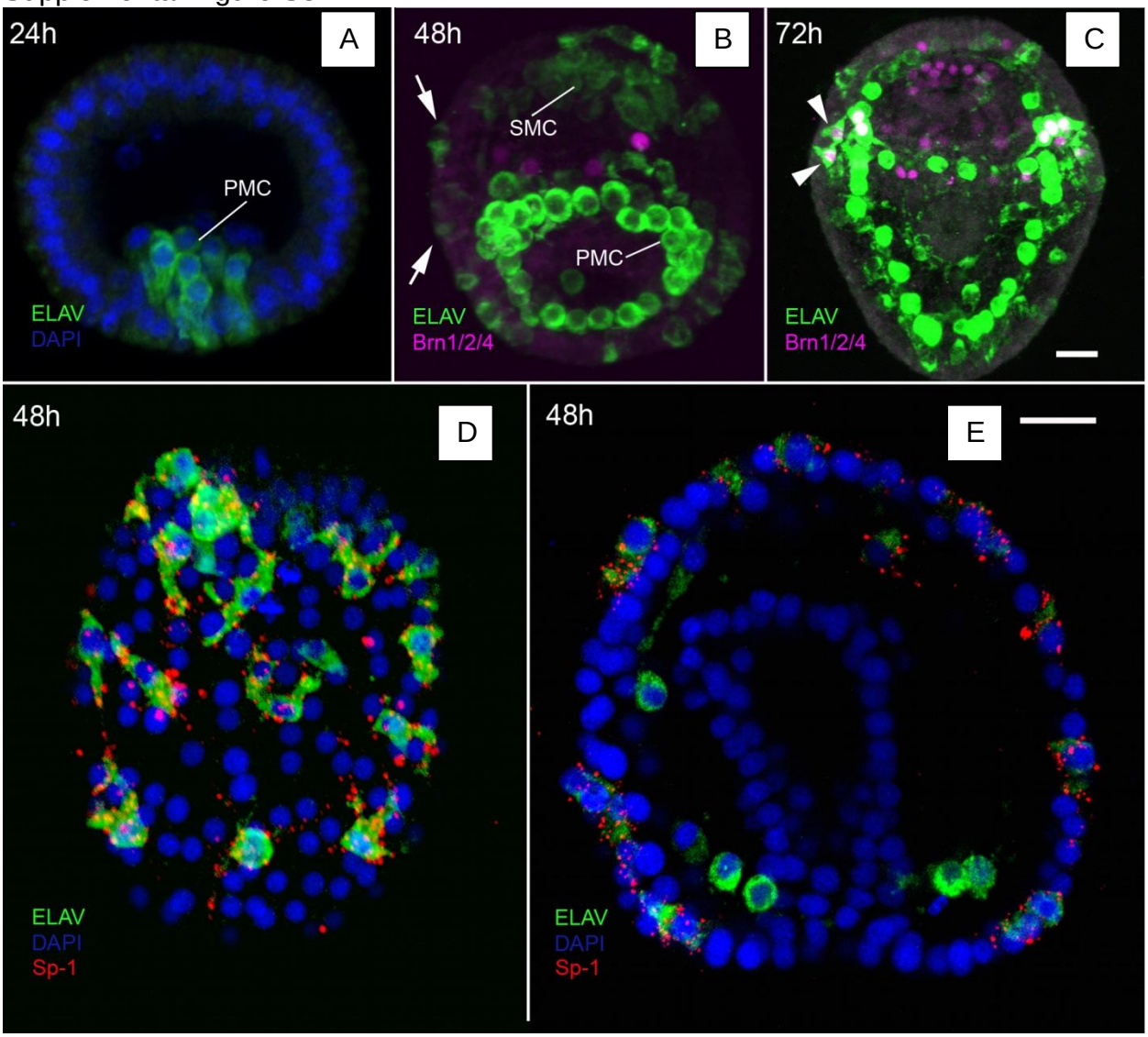
Maximum intensity projections of embryos prepared with anti-Brn1/2/4 serum. J Control, 48h embryo showing the normal distribution of Brn1/2/4. K Embryo derived from egg injected with 100 µM Brn1/2/4 MASO, prepared as in panel J and imaged with identical settings. There is only a very weak signal indicating that there is reduced antibody binding and that the MASO has suppressed expression of the Brn1/2/4 protein.

Supplemental Figure S2

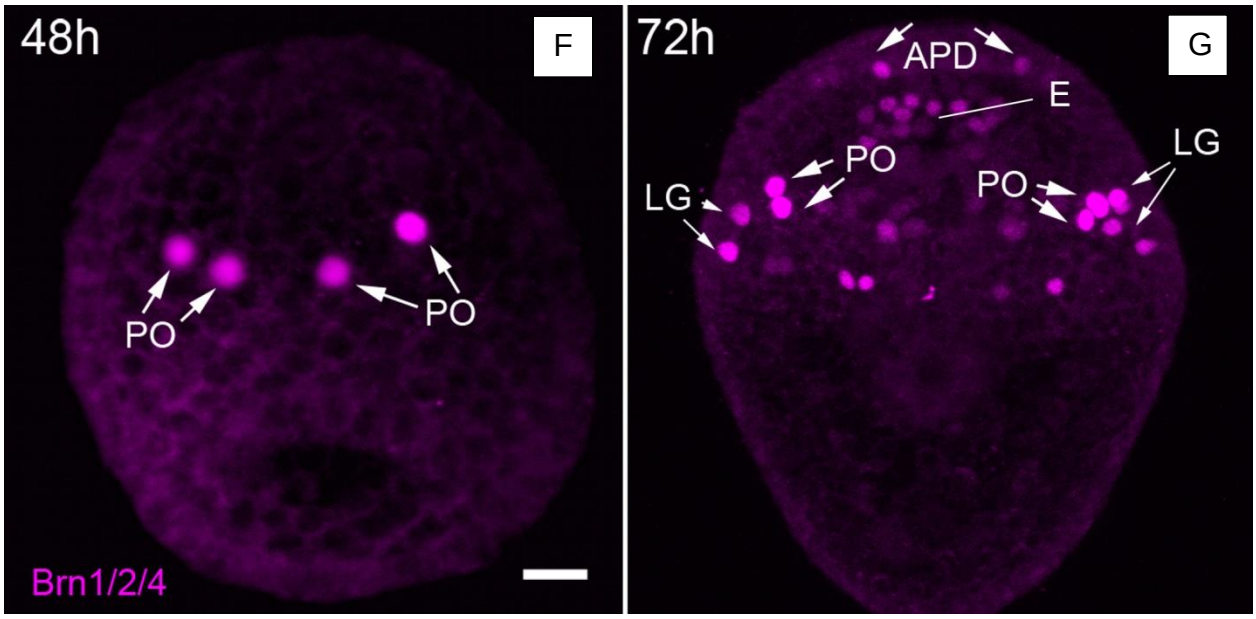


SoxC is initially expressed in late cleavage stages of *S. purpuratus* embryos in vegetal lineages. A-D Vegetal views. A. Expression in large micromeres, identified by their number and position relative to the small micromeres (Vasa) at 16 h. At subsequent stages (B –D) the number of cells expression SoxC increases as the expression domain expands outward. By 22 h, immediately prior to ingression of primary mesenchyme cells, SoxC is downregulated in micromere lineages. E. Lateral view of 20 h embryo in which expression of SoxC is throughout the cells of the endomesodermal lineages (arrows). F. Lateral view of 24 h embryo in which primary mesenchyme has begun to ingress (arrows) and expression of SoxC has returned to background levels. Note the initiation of expression in the animal pole domain at this stage. Bars = 10 µM.

Supplemental Figure S3



Ontogeny of expression of ELAV in mesodermal lineages. A. In mesenchyme blastulae, primary mesenchyme (PMC) express ELAV as a cytoplasmic protein as they ingress. B. In late gastrulae, the primary mesenchyme have formed a ring and continue to express ELAV, in addition, secondary mesenchyme (SMC) at the tip of the archenteron express ELAV. Note the cells within the plane of the ectoderm that express ELAV (arrows) C. In early plutei ELAV is expressed in skeletogenic mesenchyme and pigment cells. Presumptive neurons can be distinguished (arrowheads) by co-expression of Brn1/2/4. D, E. Pigment cells, identified by expression of the Sp1 antigen, co-express ELAV and have inserted in the aboral ectoderm of late gastrulae. Bars = 10 µM.



Embryonic expression of the transcription factor Brn1/2/4. F. Initial expression of Brn1/2/4 is at 48 h in late gastrulae. The protein localizes to the nucleus of 2-4 cells located in 2 bilateral clusters in the oral ectoderm; the presumptive PO neurons. G. Over the next 24 h expression in PO neural progenitors persists and cells in the aboral ectoderm adjacent to the ciliary band, which will form the lateral ganglia LG, begin expression. As well there is expression in cell along the dorsal periphery of the animal pole domain (APD). Expression in esophagus (E) and in neural progenitors was first reported by Cole and Arnone (2009).

Table S1: Antibodies and Dilutions

	Antibody	Species	Dilution	Reference
Primary	SoxC	Mouse (monoclonal)	1:10	This Study
		Rat (polyclonal)	1:600	This Study
	Hnf6	Rat	1:600	Yaguchi *et al.* 2010 Dev. Biol. 347: 71–81
	Nk2.1	Rabbit	1:500	Takacs *et al.* 2004 Dev. Biol., 269: 152–164
	Brn1/2/4	Rat	1:200	This Study
	ELAV	Rabbit	1:200	This Study
	SoxB2	Rat	1:500	This Study
	SynB (1E11)	Mouse (monoclonal)	1:5	Nakajima *et al.* 2004 Evol. Dev., 6: 95–104
Secondary	Alexa Fluor 488 IgG (H+L)	goat anti-mouse goat anti-rat goat anti-rabbit	1:12000	Invitrogen
	Alexa Fluor 568 IgG (H+L)	goat anti-mouse goat anti-rat goat anti-rabbit	1:12000	Invitrogen
	Alexa Fluor 633 IgG (H+L)	goat anti-rat	1:12000	Invitrogen
	Alexa Fluor 635 IgG (H+L)	goat anti-rabbit goat anti-mouse	1:12000	Invitrogen