Neurogenic gene regulatory pathways in the sea urchin embryo
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ABSTRACT
During embryogenesis the sea urchin early pluteus larva differentiates 40-50 neurons marked by expression of the pan-neural marker synaptotagmin B (SynB) that are distributed along the ciliary band, in the apical plate and pharyngeal endoderm, and 4-6 serotonergic neurons that are confined to the apical plate. Development of all neurons has been shown to depend on the function of Six3. Using a combination of molecular screens and tests of gene function by morpholino-mediated knockdown, we identified SoxC and Brn1/2/4, which function sequentially in the neurogenic regulatory pathway and are also required for the differentiation of all neurons. Misexpression of Brn1/2/4 at low dose caused an increase in the number of serotonin-expressing cells and at higher doses converted most of the embryo to a neurogenic epithelial sphere expressing the Hnf6 ciliary band marker. A third factor, Z167, was shown to work downstream of the Six3 and SoxC core factors and to define a branch specific for the differentiation of serotonergic neurons. These results provide a framework for building a gene regulatory network for neurogenesis in the sea urchin embryo. KEY WORDS: Sea urchin, Strongylocentrotus purpuratus, Neurogenesis, Embryogenesis, SoxC, Brn1/2/4, Six3

INTRODUCTION
At the end of embryogenesis the nervous system of the sea urchin early pluteus larva includes an apical ganglion in the apical plate at the anterior (animal) pole, a set of 40-50 peripheral neurons, most of which differentiate in, or adjacent to, the ciliary band, and a few neurons that differentiate in the pharyngeal endoderm (Yaguchi et al., 2006; Wei et al., 2009; Range et al., 2013; Burke et al., 2014) (Fig. S1). The apical organ develops within a distinct regulatory domain termed the animal pole domain (APD) (Angerer et al., 2011), which encompasses 10-15% of the blastula surrounding the anterior pole. The patterns of gene expression in the APD become increasingly complex during later larval development and, in addition to the apical ganglion, it generates the apical tuft of cilia and several different groups of cells identified by expression of specific genes with neurogenic functions in other organisms (Burke et al., 2014); the first of these to appear are 2-6 neurons at the posterior margin within the APD, which express serotonin and tryptophan hydroxylase (Tph), an enzyme in the serotonin biosynthesis pathway. The peripheral neurons send projections along the ciliary band to the apical organ and out into the dorsal ectoderm (Burke et al., 2014); the apical ganglion is thought to have sensory and integrating functions, while the ciliary band neurons are thought to have sensory function, and together they are proposed to coordinate the ciliary beat. All of these differentiated neurons can be identified by expression of the pan-neuronal marker synaptotagmin B (SynB) (Burke et al., 2006).

The initial positioning of the neurons in the sea urchin embryo ectoderm is achieved in part through downregulation of neurogenic potential by separate signaling mechanisms operating along the maternally established animal-vegetal (future anterior-posterior) axis and the embryonic dorsal-ventral axis (Angerer et al., 2011). When all known early cell-cell signaling in the embryo is blocked by inhibiting the inductive cascade normally initiated at the posterior (vegetal) pole, a strong underlying maternally driven neurogenic bias is revealed: virtually the whole embryo activates a developmental program characteristic of the APD that generates the apical organ and ciliary tuft, and serotonergic and non-serotonergic neurons differentiate throughout the hyperciliated embryo (Yaguchi et al., 2006; Wei et al., 2009; Range et al., 2013). This is the severely ‘animalized’ embryo of classical experimental embryology (Hörstadius, 1973). In normal embryos a cascade of multiple Wnt signaling events, initiated at the posterior pole at fertilization and operating, at least in part, through the FoxQ2 transcription factor, progressively restricts the posterior margin of the APD toward the anterior pole (Range et al., 2013; Yaguchi et al., 2008). By the blastula stage, activation of Wnt antagonists within the APD opposes this posterior signaling and defines the final APD domain. Comparison of functional and expression data from ambulacarians, invertebrate chordates and vertebrates strongly suggests that this Wnt network may be a mechanism that is shared by deuterostomes for positioning the anterior neuroectoderm (Range, 2014). Along the ventral-dorsal axis, differentiation of non-serotonergic neurons in the lateral ectoderm is restricted to a region close to, or within, the ciliary band by a combination of Nodal signaling on the ventral side and the functions of BMP2/4 and the atypical BMP ligand ADMP on the future dorsal side of the embryo (Yaguchi et al., 2010; Lapraz et al., 2009, 2015). Surprisingly, relatively late in embryogenesis, two additional neurons that are thought to function in feeding differentiate within the pharyngeal endoderm (Wei et al., 2011).

Although we have some understanding of the mechanisms that position neurogenic territories in the sea urchin embryo, little information is available in this or other lower deuterostome embryos on the gene regulatory networks (GRNs) that direct the differentiation of different types of neurons. To begin such an analysis, in a previous study we used the leverage provided by the difference in the phenotype and gene expression of ‘animalized’ versus normal embryos to search for potential regulatory genes that might function in neurogenesis. This effort identified a large number of orthologs of vertebrate genes that are expressed in the APD and function in neurogenesis in other systems, especially in the vertebrate forebrain (Wei et al., 2009). One of these, Six3, was found to be required for the differentiation of all neurons in the embryo (Wei et al., 2009), and global misexpression of Six3 was shown to drive embryos to the expanded APD phenotype. Six3 expression begins during cleavage stages, broadly and
encompassing the neurogenic regions of the embryo. The fact that its expression is not restricted to individual neural precursors suggests that Six3 is involved in establishing neuroectoderm territories but is not directly involved in initiating the terminal differentiation of neurons. In this work we have identified three factors that function downstream of Six3 in the pathways to serotonergic and SynB-positive neurons using RNA-Seq-based screening and analysis by multicolor in situ hybridization: SoxC and Brn1/2/4 function in the specification of all neurons, whereas Z167 defines a branch point leading to the differentiation of serotonergic neurons.

**RESULTS**

**SoxC function is required downstream of Six3 for development of all neurons**

SoxC is an early, zygotically activated gene that is expressed in scattered individual cells in the lateral ectoderm [Fig. 1A,B (red) and G-I (green)] and in the APD (future apical plate), as well as in a group of contiguous cells at the posterior end (vegetal plate; Fig. 1A, arrowheads) at the blastula stage and foregut at the gastrula stage (Fig. 1B, arrowheads) (Pourtsa et al., 2007). In embryos developed from eggs injected with a Six3 morpholino (morphants), SoxC mRNA levels were greatly reduced, as shown here by in situ hybridization (Fig. 1C) and consistent with previous microarray and qPCR experiments (Wei et al., 2009). Simultaneous hybridization with probes for Hnf6 (also known as onecut), which is an APD/ciliary band marker, and SoxC transcripts showed that SoxC-expressing cells lie adjacent to, or within, the ciliary band and APD (Fig. 1G-I, arrowheads), as expected for neural precursors (Burke et al., 2014). Further supporting this identity, cells in both the apical plate and lateral ectoderm that express SoxC also express Delta (Fig. 1D,E), which is characteristic of neural precursors in embryos (Vässin et al., 1987; Yang et al., 2009). Furthermore, when Delta-Notch signaling is blocked by treatment with DAPT, which is a gamma secretase inhibitor of Notch signaling, SoxC-expressing cells increase in number and form small clusters (Fig. 1F), reflecting the loss of lateral inhibition that is also characteristic of neural precursors (Formosa-Jordan et al., 2013). The number and positions of SoxC-expressing cells varied among embryos at the same time of development (Fig. 1G,H,I), suggesting expression is asynchronous and/or that SoxC is expressed transiently.

To determine whether it is required for the differentiation of some or all neurons, we knocked down SoxC by morpholino injection and stained with antibodies specific for serotonergic neurons in the APD and for all neurons as recognized by the pan-neural marker SynB. As shown in Fig. 2, signals for both neural markers were greatly reduced and, in many cases, completely eliminated, with minimal effect on other structures of the embryo (Fig. 2A versus B and C). The same phenotype was observed with two different morpholinos, one of which was tested and shown to prevent accumulation of SoxC protein (Fig. S2A,B). This suggested that SoxC could be an immediate regulator of neural terminal differentiation. However, in double in situ hybridization experiments for SoxC and SynB transcripts, the two were rarely found colocalized (Fig. 2D-F). Similarly, in embryos treated with DAPT, both SoxC-positive and SynB-positive cells formed clusters, but doubly labeled clusters were very rare (Fig. 2G-I). This suggests that SoxC has an early transient function in neurogenesis and is not an immediate regulator of terminal differentiation genes, such as SynB.

In vertebrates, the SoxC family member Sox11 functions in neural progenitors or neuroblasts (Hong and Saint-Jeannet, 2005; Reiprich and Wegner, 2015), suggesting that SoxC might play a similar role in sea urchin embryos. To test this possibility, we determined whether some SoxC-expressing cells are still dividing. We labeled embryos with anti-phospho-Histone H3 (H3p) antibody, which specifically labels chromatin between prophase and anaphase (Hendzel et al., 1997). In embryos analyzed at late gastrula and early prism stages, the metaphase nuclei of a few SoxC-expressing cells were labeled by the antibody (Fig. 3A-D, arrowheads). The frequency of doubly labeled cells was low, consistent with the low rate of cell division at this stage, the small number of SoxC-expressing cells in any individual embryo and the short duration of metaphase of dividing cells. We conclude that at least some SoxC-expressing cells are still cycling, consistent with SoxC

![Fig. 1. SoxC expression in control sea urchin embryos and embryos treated with DAPT or Six3 morpholino.](image-url)
operating early in the neurogenesis pathway. In a few cases, nuclei of two adjacent cells were labeled by H3p (Fig. 3E–N, arrowheads), only one of which was SoxC positive, raising the possibility that SoxC might continue to be expressed in one daughter of an asymmetric division. Although it is not possible from these data to define the fates of these two cells, we suggest it is likely that the SoxC-positive cell has retained its precursor identity.

Identifying genes that function downstream of SoxC

The above observations suggested that regulators of terminal differentiation genes function downstream of SoxC. To search for such factors we carried out an RNA-Seq screening experiment to identify genes that depend on SoxC for expression. We tested RNA from 30 h SoxC morpholino-injected embryos because, at this stage, many neural genes are likely to be active, but mRNAs characteristic of differentiated SynB-positive neurons have not yet appeared (Burke et al., 2006). Among mRNAs affected we identified a few that encode transcription factors, the abundance of which decreased significantly in the absence of SoxC. We pursued Z167 and Brn1/2/4, which were suppressed ∼8-fold and ∼4-fold, respectively, as candidates for intermediate functions in the neural development pathway.

Z167 is encoded by one of 377 zinc finger transcription factor genes that have been annotated in the sea urchin genome (Materna et al., 2006); a specific vertebrate homolog cannot be determined because of the high degree of sequence conservation among zinc finger proteins. Brn1/2/4 is related to three Class III POU domain transcription factors expressed in vertebrate embryos that have demonstrated roles in neural specification (Ryan and Rosenfeld, 1997; Wegner et al., 1993). During mouse embryogenesis, Brn1 (Pou3f3), Brn2 (Pou3f2) and Brn4 (Pou3f4) are expressed in all levels of the CNS and their expression is more tissue-restricted in adults (Wegner et al., 1993). Deletion of Brn2 is lethal in mouse but not until as late as embryonic day 10. No general defects in neurogenesis were identified, but an essential role for Brn2 has been demonstrated relatively late in the differentiation of specific neural lineages of the endocrine hypothalamus and anterior pituitary (Nakai et al., 1995; Schonemann et al., 1995). Brn2 is highly overexpressed in melanoma, in which it behaves as a pro-proliferative transcription factor (Vance and Goding, 2004). It is required for melanocyte development and lack of Brn2 expression in migrating melanoblasts has led to the suggestion that it is required early and transiently (Goding, 2008).

Z167 links SoxC function to the differentiation of serotonergic neurons in the APD

Consistent with the large reduction in Z167 expression observed in the screen, in control embryos Z167 is expressed only in individual cells in the APD (Fig. 4A, red), and transcripts are essentially undetectable in SoxC knockdowns (Fig. 4B). As is observed for cells expressing SoxC or SynB, Z167-expressing cells increase in number and form small clusters when embryos are treated with DAPT (Fig. 4C, green). These observations suggested that the
Z167-positive cells might be developing serotonergic neurons. As an initial test of whether Z167 links SoxC function to the expression of neural terminal differentiation genes, we performed a triply labeled in situ hybridization for SoxC, Z167 and Tph [a marker specific for serotonergic neurons (Yaguchi and Katow, 2003)]. Analysis of a representative embryo is shown in Fig. 4D-I. Shown in these optical sections are five SoxC-positive cells (Fig. 4H, blue), three Tph-positive cells (Fig. 4E, green) and three Z167-expressing cells (Fig. 4G, red). All three Tph-positive and all three Z167-positive cells are in the APD, whereas only two of the five SoxC-positive cells are located there. When the image is separated into more ventral (Fig. 4F) and dorsal (Fig. 4I) planes, the ventral image shows two Tph-positive cells and one Z167-expressing cell (arrowhead). In the dorsal image, two of the three Z167-positive cells also express SoxC, while the third also expresses Tph (arrowhead; the signal from this cell is also shown in the ventral section). Therefore, some cells express both SoxC and Z167.

Fig. 4. Z167 is required for differentiation of serotonergic neurons in the APD. (A,B) Doubly labeled in situ hybridization for Z167 (red) and SoxC (green), showing that Z167 is expressed in individual cells at the anterior pole of a control embryo (A), whereas its expression is greatly reduced in SoxC morphants (B). (C) Z167-expressing cells (green) form clusters in embryos treated with DAPT. (D-I) Triple in situ hybridization for SoxC (blue), Z167 (red) and Tph (green), showing that some Z167-expressing cells also express SoxC, whereas others also express Tph, a marker for serotonergic neurons. (D) The stack of multiple optical sections further analyzed in E-I. (E) Tph, (G) Z167, (H) SoxC. The combined signals are shown in more ventral (F) and more dorsal (I) optical sections. (J-L) Immunostaining for serotonin (green) and SynB (red), showing that serotonin expression is reduced in Z167 morphants. (J) Control; (K,L) two morphants. Nuclei in A-C and J-L are labeled with DAPI (blue). Embryos in A-I are at 46 h and in J-L at 70 h. All embryos are shown with anterior poles at the top. Scale bar: 20 µm.

Fig. 5. Brn1/2/4 is required downstream of SoxC for differentiation of all neurons. (A,A′) Brn1/2/4 (red) is expressed in individual cells in ectoderm and in a group of contiguous cells in the foregut (arrowhead) of a control embryo (A); the number of individual labeled cells is greatly reduced in SoxC morphants (A′). (B,B′) Brn1/2/4 and SoxC doubly labeled in situ hybridization of an embryo, showing a few Brn1/2/4-expressing cells (red) that also express SoxC (green, arrowheads). (B′) Red channel only. (C,C′) Doubly labeled in situ hybridization for Brn1/2/4 and SynB in two different embryos, showing that some Brn1/2/4-expressing cells (red) also express SynB (green, arrowheads). (D-F) Brn1/2/4 morpholino greatly reduced the number of neurons (green, serotonin; red, SynB). (D) Control; (E,F) two morphants. (D′-F′) Corresponding DIC images. (G-I) Immunostaining for serotonin (green) and SynB (red) in a 3-day control embryo (G) and a Brn1/2/4 mRNA-injected (H) embryo (higher dose). The latter embryo is an epithelial sphere that lacks serotonin staining but has expanded SynB signal. (I) DIC image of embryo in H. (J-L) Three different injected embryos showing that, at the lower Brn1/2/4 mRNA dose, the serotonergic cell number is increased. At ~1.0 µg/µl Brn1/2/4 mRNA, most embryos resembled those in J-L. Embryos in A-C are at 46 h and in D-L at 72 h. All embryos are shown with the anterior poles at the top, except H,I,L; L is an anterior view of the APD. Nuclei are labeled with DAPI (blue). Scale bar: 20 µm.
whereas others express both Z167 and Tph. These observations are consistent with a dynamic transition in the regulatory state of serotonergic neurons: SoxC→Z167→Tph/serotonin.

To determine whether Z167 activity links SoxC function to the differentiation of serotonergic neurons, we characterized a morpholino knockdown. As shown in Fig. 4, Z167 morphants have greatly reduced immunostaining for serotonin in the anterior pole but, as expected, staining throughout embryos for the pan-neural marker SynB was little affected (Fig. 4J versus K and L). Interestingly, knockdown of Z167 also resulted in loss of Z167 transcripts (Fig. S2C versus D), suggesting that, once activated, it is locked in a positive autoregulatory loop. Although Z167 expression was reduced in Z167 morphants, SoxC expression was not reduced (Fig. S2D), indicating that Z167 does not reciprocally activate SoxC.

**Brn1/2/4 function is required downstream of SoxC for all neurons**

Since SoxC is required for the development of all neurons, whereas Z167 is required only for serotonergic neurons, other factors must relay SoxC function in other neural precursors. Of the genes affected by SoxC knockdown, Brn1/2/4 was selected for further study because, like SoxC, it is expressed in scattered individual cells in the ectoderm, including the APD, and throughout the foregut region (Cole and Arnone, 2009) (Fig. 5A, red). Brn1/2/4 is also expressed maternally, and transcripts gradually decrease in abundance until they are undetectable at the blastula stage (Fig. S3). It is likely that these early transcripts are uniformly distributed in the embryo since they have not been detected by in situ hybridization (Cole and Arnone, 2009; Z.W., unpublished observations).

The dependence of Brn1/2/4 on SoxC function was confirmed by morpholino-mediated knockdown and in situ hybridization analysis: in SoxC morphants, labeling of individual ectoderm cells for Brn1/2/4 transcripts was dramatically reduced, whereas expression in the foregut was not detectably affected (Fig. 5A,A′, arrowhead). To examine the possibility that Brn1/2/4 links SoxC function to the expression of terminal differentiation genes in neurons, we carried out double in situ hybridizations of Brn1/2/4 and SoxC or SynB. In embryos probed for SoxC and Brn1/2/4, some Brn1/2/4-expressing cells also expressed SoxC (Fig. 5B,B′, arrowheads); as was the case for Z167, only a few cells were doubly labeled. Similarly, some Brn1/2/4-positive cells also expressed SynB (Fig. 5C,C′, arrowheads). These results are consistent with Brn1/2/4 linking SoxC function to terminal differentiation of neurons throughout the ectoderm. To determine whether Brn1/2/4 is required for neurogenesis, we carried out morpholino-mediated knockdown. At a morpholino dose that minimized other developmental effects, the number of neurons was markedly reduced (Fig. 5D,D′ versus E,E′ and F,F′) for both serotonergic and other SynB-positive neurons. At this dose, many embryos gastrulated and appeared relatively normal with respect to major structures; some were noticeably smaller, with fewer nuclei (Fig. S2). In Brn1/2/4 morphants, SoxC expression was not detectably affected (Fig. S2E,F), indicating that Brn1/2/4 functions downstream of SoxC, rather than in a parallel, cross-regulatory mode.

To determine whether Brn1/2/4 is sufficient to drive neural fate in the embryo, we also tested the effects of gain-of-function by misexpressing synthetic Brn1/2/4 mRNA. At a low dose, the number of serotonergic neurons approximately doubled (Fig. 5G versus J-L, green), whereas endomesoderm development was variably reduced. At a higher dose, the resulting embryos formed epithelial spheres with SynB signal expanded to encompass the whole embryo and no detectable staining for serotonin (Fig. 5G versus H and I, red). Further analysis of the high-dose phenotype with probes for territory-specific proteins showed that most nuclei contained Hnf6 protein (Fig. S4A-A′ versus A″, red), which in the normal embryo is expressed only in ciliary band and the APD. The embryos appeared to be radialized around the anterior-posterior (animal-vegetal) axis, since oral ectoderm-specific GSC was not detectable (Fig. S4C-C″ versus D-D″), and Spec1, which is confined to the aboral ectoderm of control embryos, accumulated in all cells (Fig. S4A versus A′). Differentiation of the endomesoderm, as indicated by the Endo1 marker for mid- and hindgut, was greatly suppressed (Fig. S4C′ versus D′); only a few cells continued to express Endo1, marking the location of the vegetal pole. The APD territory was at least partially retained, as evidenced by a contiguous patch of expression of Nk2.1, which is a driver of a separate regulatory pathway that constructs the apical tuft of cilia within the APD (Dunn et al., 2007) (Fig. S4E versus F and G). We conclude that Brn1/2/4 is required for the development of serotonergic and all SynB-expressing neurons and can directly or indirectly drive the production of excess neurons at low dose, although high-level global misexpression produces additional gain-of-function effects.

If Brn1/2/4 functions in a ‘single-file’ regulatory pathway leading to neural differentiation, then it might be sufficient to rescue neural differentiation in the absence of SoxC. We tested this possibility by co-injecting SoxC morpholino and synthetic Brn1/2/4 mRNA. SoxC morpholino (Fig. 6B) and Brn1/2/4 RNA (Fig. 6C) individually showed their expected phenotypes. When the two were co-injected, the embryoid phenotype resembled that produced by Brn1/2/4 RNA misexpression alone, but without any detectable SynB or serotonin (Fig. 6D). This result suggests that other factors working in parallel with Brn1/2/4 are required downstream of SoxC.

**DISCUSSION**

In this work we have used molecular screens and gene knockdown approaches to define a temporal and functional sequence of gene
activities required for the development of all neurons and a branch in that pathway that is specific for serotonergic neurons in the APD. Our entry point to this pathway was the observation that the transcription factor Six3 is required for the expression of many candidate neurogenic genes. From this set, SoxC was selected as a likely positive regulator of neurogenesis because it is downregulated in the absence of Six3 function and is expressed in all neurogenic territories, including isolated individual cells in the lateral ectoderm, which is characteristic of presumptive neurons. Morpholino-mediated knockdown of SoxC function confirmed its requirement for the differentiation of all neurons in the embryo (Fig. 2). In vertebrates, expression of SoxC proteins (Sox 4, 11 and 12) marks the transition from neuroepithelial cells to dividing neuroblasts (Reiprich and Wegner, 2015). A similar function is suggested for the single Sox C representative in sea urchin embryos by the observation that the number of SoxC-expressing cells increases when Notch signaling is inhibited by DAPT treatment (Fig. 2) and our finding that at least some SoxC-positive cells are still dividing (Fig. 3). Although overexpression of Sox4, 11 or 12 in chicken and mouse has been shown to have an inductive effect on pan-neuronal gene expression and differentiation (Reiprich and Wegner, 2015), in our hands overexpression of SoxC mRNA in sea urchin embryos did not produce a detectable phenotype, suggesting that SoxC is necessary, but not sufficient, to specify neurons (Garner et al., 2016; Z.W., unpublished observations). In addition, the fact that, like Six3, SoxC is clearly expressed in cells that never adopt neural fate (e.g. the high-level expression in early endomesoderm; Fig. 1), suggests that it has additional functions in sea urchin embryos and is not sufficient to specify neuronal fate.

The fact that SoxC expression appears to be transient and that individual cells did not co-express differentiated neuronal markers implied that another regulatory layer connects SoxC function to the expression of genes involved in terminal differentiation, such as SynB. In addition, in the absence of lineage tracing, it prevented a firm conclusion as to whether SoxC function in neurogenesis is cell-autonomous and whether SoxC-expressing cells include actual neural precursors. Therefore, we carried out an additional molecular screen to identify genes that might link SoxC function to differentiating neurons. This effort identified Brn1/2/4 as a potential downstream effector of SoxC action, based on its similar pattern of expression and its association with neural development in other embryos (Ryan and Rosenfeld, 1997; Wegner et al., 1993). Brn1/2/4 expression was shown to depend on SoxC and, in turn, was shown to be required for the differentiation of serotonergic neurons in the APD and non-serotonergic neurons in the ectoderm and foregut (Fig. 5). The distribution of Brn1/2/4-expressing cells in the ectoderm varied among embryos at the same time of development, suggesting that it also is expressed asynchronously and in individual neural precursors. Brn1/2/4 thus provided a link between SoxC and SynB, since some Brn1/2/4-expressing cells were shown to express SoxC, whereas others expressed SynB (Fig. 5).

In vertebrates, Brn2 expression, in combination with that of two other transcription factors, can convert fibroblasts into neural cells (Pang et al., 2011; Pfisterer et al., 2011). Interestingly, we observed that misexpression of Brn1/2/4 in sea urchin embryos at low dose increased the number of serotonergic neurons, whereas at higher dose it converted the whole embryo to a novel phenotype (Fig. 5 and Fig. S4). Almost all cells in these embryos accumulated the APD/ciliary band transcription factor Hnf6, with SynB-positive fibers distributed throughout the embryo. Anterior-posterior polarity was retained but differentiation of endomesoderm derivatives at the vegetal pole was dramatically reduced. By contrast, the APD at least partially differentiated, as shown by expression of the Nk2.1 transcription factor, which functions in production of the apical tuft of cilia through a different regulatory pathway (Dunn et al., 2007). This phenotype suggests the interesting possibility that the two phases of Brn1/2/4 expression (Wei et al., 2006; Materna et al., 2010) (Fig. S3) might have different but related functions: the early, uniform expression, which is amplified and prolonged by Brn1/2/4 uniform misexpression, might promote or insulate a common precursor to ciliary band cells and neurons, whereas later Brn1/2/4 might function in a much more limited set of neural precursors.

The SoxC knockdown screen also identified Z167 as a transcription factor that is specifically expressed in, and required for, development of serotonergic neurons in the APD. Although another zinc finger protein, Zlhx/Z81 (Yaguchi et al., 2012), has been shown to be required for the development of serotonergic neurons, and several other transcription factors have been shown to be expressed within them (Yaguchi et al., 2011), Z167 is the first factor to be shown to functionally link to the shared core regulatory pathway of Six3→SoxC→Brn1/2/4 that is required for all neurons.

Among the outstanding questions for the future is the identity of mechanisms by which the expression of genes in the neurogenic regulatory pathways becomes restricted to individual neural precursors. Neurons in all three major regions – APD, ciliary band and foregut – have no known origins in cell lineage. Although the APD arises from the an1 octet of blastomeres of the 32-cell embryo (Hörstadius, 1973), the arrangement of cell types within it is only determined after dorsal-ventral polarity is established. For example, the serotonergic neurons discussed in this work normally differentiate...
at the dorsal edge of the APD, but can develop throughout the APD under a variety of experimental treatments that radialize the embryo. The distribution of other molecular markers further indicates a complex and evolving fine-scale patterning of different cell types. The ciliary band is defined by persistence of the neurogenic bias, driven at least in part by maternal and early zygotic SoxB1 (Barsi et al., 2015). Recent work by these authors shows that the ciliary band comprises four separate gene regulatory territories, the dorsal and ventral (oral and aboral) borders of which are defined by the combined functions of a set of at least ten transcriptional repressors. Neurons appear to be specified within the ciliary band progressively and asynchronously, and except for the involvement of Delta-Notch signaling, nothing is known about the local mechanisms that position them. Finally, the specification of the pair of individual pharyngeal neurons poses an intriguing regulatory phenomenon: they arise within endoderm after it is specified via an extensively characterized GRN (http://sugp.caltech.edu/endomes/) through a neurogenic pathway that includes Six3 and Nkx3.2 (Wei et al., 2011), which we now show also includes SoxC and Brn1/2/4. Interestingly, all of these factors are initially more broadly expressed in endomesoderm, implying that additional regulatory interactions among cells are required for specifying the neurons.

The data presented here allow us to outline a model for gene regulatory pathways leading to the differentiation of SynB-positive and serotonergic neurons in the sea urchin embryo (Fig. 7). The most upstream gene, Six3, is activated in early cleavage, probably by maternal factors among those that are sufficient to initiate the APD developmental program throughout the embryo (Yaguchi et al., 2006; Wei et al., 2009; Range et al., 2013). Although Six3 is necessary for differentiation of all neurons in the embryo, its function is neither specific nor sufficient for their production. Instead, it is crucial for development of the APD per se: embryos lacking Six3 fail to produce neurons, the apical ciliary tuft and the cuboidal epithelium characteristic of the APD, whereas global misexpression converts the embryo to an expanded APD (Wei et al., 2009). Downstream of Six3, SoxC and Delta are activated by unknown mechanisms in isolated individual cells that have properties of neural precursors. These are capable of proliferation and probably undergo asymmetric division, with one daughter cell activating Brn1/2/4 and/or Z167 expression. SoxC expression is transient, and it is required for all neurogenesis, but its overexpression is not sufficient to overproduce neurons, indicating that it must operate in parallel with other factors at this point. As SoxC expression declines, Brn1/2/4 and Z167 mRNAs begin to accumulate; their expression also is transient and leads to the activation of differentiated gene products, including SynB, Tph and serotonin.

The SoxC→Brn1/2/4→SynB pathway is clearly incomplete. Homologs of a number of genes implicated in neurogenesis in other systems have been identified that also depend on Six3 for expression (Wei et al., 2009) and the requirement for several has been demonstrated by functional assays, including Zihx for serotonergic neurons (Yaguchi et al., 2012) and Nkx3.2 for pharyngeal neurons (Wei et al., 2009). Here, we have provided a scaffold pathway for integrating other genes into a GRN for neurogenesis in sea urchin embryos.

**MATERIALS AND METHODS**

**Embryo culture**

Adult sea urchins (Strongylocentrotus purpuratus, Stimpson 1857) were obtained from Monterey Abalone (Goleta, CA, USA) and Patrick Leahy (Point Loma, CA, USA) and maintained in seawater at 10°C. Embryos were cultured in artificial seawater (ASW) at 15°C. In some experiments, embryos were cultured in 5 μM N-[3,5-difluorophenacyl]-L-alanyl-2-phenylglycine-1,1-dimethyl ester (DAPT) beginning at 4 h after fertilization.

**Microinjection of morpholino antisense oligonucleotides (MOs) and synthetic mRNAs**

Eggs were prepared as described previously (Wei et al., 2009). Approximately 2 pl of solution containing 25% glyceral and morpholin (Gene Tools) were injected. The knockdown phenotypes were confirmed in each case by two different morpholinos with non-overlapping or partially overlapping sequences. Morpholino sequences and concentrations were as follows (5′-3′): SoxCMO1, TACTCCCTGCCCCTGATCTGATG (0.3 μM); Brn1/2/4MO2, GATCTGAGTAGCAAAACACCCAG (0.6 μM); SoxCMO2, CTTGAAGTGACATTCATTTTGACG (1.2 μM); Brn1/2/4MO1, TACTCCCTGCCCCTGATCTGATG (0.3 μM); Z167MO1, GAAAGCGGCTCTGCTTACTGCGTG (0.3 μM); Z167MO2, CTGTCCATCTACCTCAGAT (1.2 μM). All data shown were derived using SoxCMO1, Z167MO1 and Brn1/2/4MO2. Embryos showed a steep dose-response to both Brn1/2/4 morpholinos, and careful calibration of the dosage was required to separate loss of neurons from effects on other tissues (see main text). Both morpholinos gave the same phenotype, although the dose response sometimes differed. For SoxC, an antibody was available and the morpholinos were shown to efficiently eliminate the protein signal (Fig. S2). For all microinjection experiments, at least three repeats were performed and for each experiment 50-100 embryos were analyzed. Brn1/2/4 mRNA was synthesized using the mMMESSAGE mMACHINE Kit (Ambion), according to the manufacturer’s protocol. The injection solution was 0.5 μg/μl or 1.0 μg/μl Brn1/2/4 mRNA.

**Whole-mount in situ hybridization**

Embryos were fixed, hybridized and stained as described previously (Minokawa et al., 2004), except that each RNA in situ hybridization probe was purified with a Qiagen QuiaQuik PCR column after adding EDTA to 10 mM. SoxC, Delta, Z167, Brn1/2/4, Tph and SynB (Syt1) probes were labeled with digoxigenin, FITC or DNP and detected with Cy3-TSA, FITC-TSA or Cy5-TSA (PerkinElmer). Two-color or three-color fluorescent in situ hybridization was carried out as described previously (Yaguchi et al., 2008; Sethi et al., 2014). The concentration of probes was 0.1 ng/μl.

**Immunohistochemistry**

Embryos were fixed in 2% formaldehyde for 10 min at room temperature. They were incubated overnight at 4°C with primary antibodies using the following dilutions: serotonin, 1:1000 (S5545, Sigma); synaptotagmin B, 1:2000 (Garner et al., 2016); phospho-Histone H3 (H3p), 1:4000 (06-570, Upstate Cell Signaling Solutions); SoxC, 1:500 (gift of Robert Burke). Bound primary antibodies were detected by incubation with Alexa Fluor-coupled secondary antibodies (A11034, Invitrogen) for 1 h and nuclei were stained with DAPI. Embryos were observed using a Zeiss Axiosvert 200 M microscope. Optical sections were obtained with an Apotome unit (Zeiss) and stacked images were prepared using Adobe Photoshop.

**RNA-Seq screening**

RNA from 800-1000 glycerol-injected control or SoxC morpholino-injected embryos was purified using Nucleospin columns (Macherey-Nagel). Further RNA-Seq processing and sequencing were carried out by Beckman Coulter Genomics service. PERL programming was used for data analysis and exploration. The RNA-Seq data are available at GEO under accession number GSE76067.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Z.W., L.M.A. and R.C.A. planned experiments and analyzed the data. Z.W. performed experiments. Z.W. and R.C.A. prepared the manuscript.
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