Gene regulatory network for neurogenesis in a sea star embryo connects broad neural specification and localized patterning

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A great challenge in development biology is to understand how interacting networks of regulatory genes can direct the often highly complex patterning of cells in a 3D embryo. Here, we detail the gene regulatory network that describes the distribution of ciliary band-associated neurons in the bipinnaria larva of the sea star. This larva, typically for the ancestral deuterostome dipleurula larval type that it represents, forms two loops of ciliary bands that extend across much of the anterior-posterior and dorsal-ventral ectoderm. We show that the sea star first likely uses maternally inherited factors and the Wnt and Delta pathways to distinguish neurogenic ectoderm from endomesoderm. The broad neurogenic potential of the ectoderm persists throughout much of gastrulation. Nodal, bone morphogenetic protein 2/4 (Bmp2/4), and Six3-dependent pathways then sculpt a complex ciliary band territory that is defined by the expression of the forkhead transcription factor, *foxg*. Foxg is needed to define two molecularly distinct ectodermal domains, and for the formation of differentiated neurons along the edge of these two territories. Thus, significantly, Bmp2/4 signaling in sea stars does not distinguish differentiated neurons from nonneuronal ectoderm as it does in many other animals, but instead contributes to the patterning of an ectodermal territory, which then, in turn, provides cues to permit the final steps of neuronal differentiation. The modularity between specification and patterning likely reflects the evolutionary history of this gene regulatory network, in which an ancient module for specification of a broad neurogenic potential ectoderm was subsequently overlaid with a module for patterning.

echinoderm | neural patterning | evolution

The fascinating challenge of developmental biology is to determine how gene regulatory networks (GRNs), which are encoded within the genome and initiated by anisotropies in the zygote, can orchestrate the complex final pattern of appropriately differentiated cells in time and 3D space. The specification and patterning of the nervous system has received particular attention from developmental biologists, as it is such a defining feature of the body plan. Many animals have diffuse nervous systems that form a neural net (1). Other animals have highly patterned centralized nervous systems (CNSs), of which the best characterized are the ventral nerve cord of *Drosophila* and the dorsal hollow neural tube of vertebrates. In both of these very disparate taxa, the position of the neuroectoderm is established by gradients of bone morphogenetic protein (BMP) and its antagonist, Chordin/short gastrulation (Sog), along the dorsal-ventral (DV) axis (2, 3). High concentrations of BMP promote the formation of nonneurogenic ectoderm, whereas neurogenic ectoderm forms where BMP concentration is low (4). Although there are other types of nervous system localizations (1), much less is known of the GRNs that lead to their final pattern. We were especially intrigued by the particularly distinctive localized pattern of neurons associated with the ciliary bands of the bipinnaria larva of the sea star, *Patiria miniata*. The sea star (Phylum Echinodermata) bipinnaria has two loops of ciliary bands: one that loops above the mouth and one below it, which extends from the ventral surface to the anterior, dorsal margins

of the ectoderm (Fig. 1*A*). As there are two loops of ciliary bands, a particular anterior-posterior (AP) coordinate may have neurons along two locations within the DV axis and vice versa. These neurons coordinate the action of the cilia to enable the larvae to swim and feed in response to environmental cues within the water column (5). This design is very different from the DV-restricted patterning of the CNS in vertebrates and *Drosophila*, and even the echinoderm pluteus larva, which develops neurons associated with a single ciliary band that is restricted along the DV axis (Fig. 1*A*). Indeed, in sea stars there seems to be no simple AP and DV positioning mechanism that could establish the pattern of these neurons. However, unlike other well-characterized deuterostome embryos, this distinctive bipinnaria pattern of neurons is a common feature of the larvae of members of several classes of echinoderms [i.e., the auricularia of sea cucumbers (6) and the larvae of some basal crinoids (7)] and also some groups of hemichordates (i.e., tornaria larvae). This larval type has therefore arguably been defined as the ancestral deuterostome form and, as such, has featured strongly in evolutionary hypotheses of vertebrate CNS origins (8–10). However, very little is known of the mechanisms of development of this larva, particularly of the ectoderm. Thus, the motivation of this study was to understand the GRN that explains a distinctive nervous system pattern in what might be the basal representative of a large clade of animals.

The initial placeholders of the GRN for this developmental process are the signaling events that first establish the AP and DV axes and distinguish ectoderm from endoderm and mesoderm. Sea star embryos undergo equal cleavage and hatch as a blastula at around 24 h after fertilization. The endoderm and mesoderm form at the vegetal pole and invaginate during gastrulation, leaving the ectoderm as a ciliated outer domain. Recent studies indicate that canonical Wnt (cWnt) pathways may have an ancestral role in establishing early animal (anterior)-vegetal (posterior) axes and distinguishing endomesoderm from ectoderm (11, 12). We therefore start our examination of the AP axis formation and the establishment of the ectoderm by considering the role of cWnt signaling in the sea star. The first morphological evidence of DV patterning in sea stars is the formation of an invagination (where
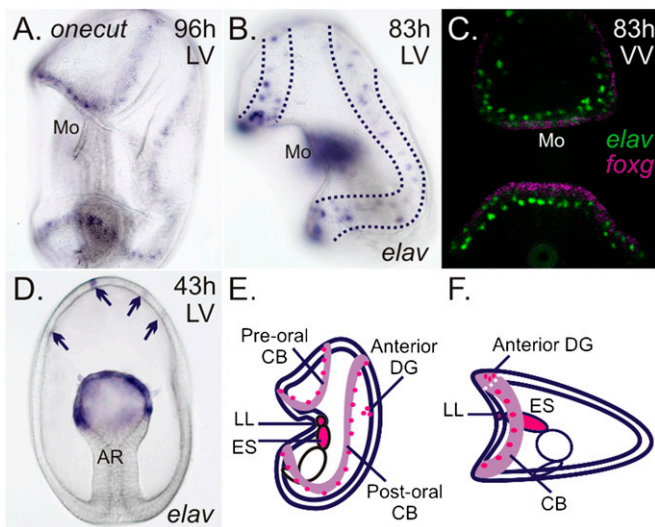
DEVELOPMENTAL BIOLOGY

Fig. 1. Expression of *elav* in *P. miniata*. In all figure plates, unless noted, sea star embryos are oriented with the anterior pole up and with ventral to the left; h, hours postfertilization; LV, lateral view; VV, ventral view. (*A*) Expression of *onecut* within the ectoderm marks the ciliary bands that loop above and below the mouth (Mo) of 96-h-old larvae (WMISH). (*B*) Dotted lines highlight *elav*-expressing ectodermal cells in two rows above and below the mouth. *elav*-expressing cells are also detected within the mesoderm and mesenchyme (not in plane of focus in *B*) (WMISH). (*C*) Two rows of *elav*-expressing cells (green) form near the ciliary bands, marked by *foxg* (purple) (FISH). (*D*) Transcripts of *elav* are first observed within cells of the anterior ectoderm (arrows) and in the mesodermal bulb of the archenteron (AR) of gastrulae (WMISH). Schematic of sea star (*E*) and sea urchin (*F*) larvae, viewed laterally, depicts expression of *elav* (magenta) in all known neural territories; the larval ciliary bands (CB) are shown in light purple. DG, dorsal ganglion; ES, esophagus; LL, lower lip. (Magnification: 200×.)

the mouth will later from) on the ventral surface at around 72 h. This formation is preceded by the expression of the transcription factor *foxa* in this oral territory before gastrulation (13). In sea urchins, Nodal is needed to establish the mouth ectoderm and BMP gradients are used to establish territories along the DV ectodermal axis (14–17). These pathways therefore also serve as starting points for our investigations of DV patterning in the sea star.

Results and Discussion

Localized Patterns of Neurons in the Sea Star Larva. We first characterized the pattern of neurons by examining the expression of the single *P. miniata* ortholog of *elav* (Fig. S1). Elav is an RNA binding protein that plays a role in the transition from neural progenitor to committed and differentiated states (18) and is a conserved molecular marker of postmitotic neurons (19, 20). Expression is detected in two rows of ectodermal cells along the pre- and postoral ciliary bands that surround the mouth (Fig. 1 *B* and *C* and Fig. S2*A*); additionally, *elav* expression is present in the lower lip of the mouth and esophagus (Fig. S2*B*) and within the bilateral anterior dorsal ganglia (Fig. S2*C*). Earlier in development, *elav* is first observed within several cells in the anterior pole ectoderm of 2-d-old (gastrula stage) embryos (Fig. 1*D*). We think that these *elav*-positive cells will later coalesce to form the bilateral, anterior dorsal ganglia of larvae, rather than neurons of the ciliary bands. Previous studies have characterized the distribution of neurons in the sea star bipinnaria based on immunoreactivity to the serotonin precursor, 5-hydroxytryptophan, and synaptotagmin-B (21). These studies identify neurons associated with the anterior ectoderm, larval ciliary bands, lower lip of the mouth, and esophagus (22, 23). Thus, we are confident that *elav* expression within the ectoderm serves as a marker of most, if not all, differentiated neurons. Expression of *elav* is observed

additionally within the mesodermal bulb at the top of the archenteron of gastrulae (Fig. 1*D*), and later in these cells as they ingress into the blastocoel. It is not yet known if any of these *elav*-expressing mesenchymal cells are neurons.

This finding is very different from the pattern of neurons found in the two closest relatives to the sea star for which any mechanisms of neural specification are known (i.e., the sea urchin and the directly developing hemichordate *Saccoglossus*, which contrasts to the indirectly developing hemichordates that develop tornaria larvae). In sea urchins, a single ciliary band and its associated neurons (22) form at the boundary between the oral (ventral) and aboral (dorsal) ectoderm (14–17, 24) (Fig. 1*F*). In *Saccoglossus*, *elav* is expressed throughout the ectoderm with no apparent DV patterning (20).

Neurogenesis Occurs Broadly Throughout the Ectoderm During Gastrulation. We previously identified many transcription factors expressed within the sea star ectoderm (25–27) that, based on their orthology to other taxa, likely have roles in neurogenesis. *P. miniata* orthologs of *otx* (in particular the ectodermally localized *otxβb* isoform) (26), *onecut* (see ref. 27 and Fig. 2*A*), and *soxb1* (Fig. 2*B*) are expressed throughout the ectoderm through gastrulation. Quantitative reverse-transcription PCR (qPCR) reveals that transcripts of sea star orthologs of these genes, as well
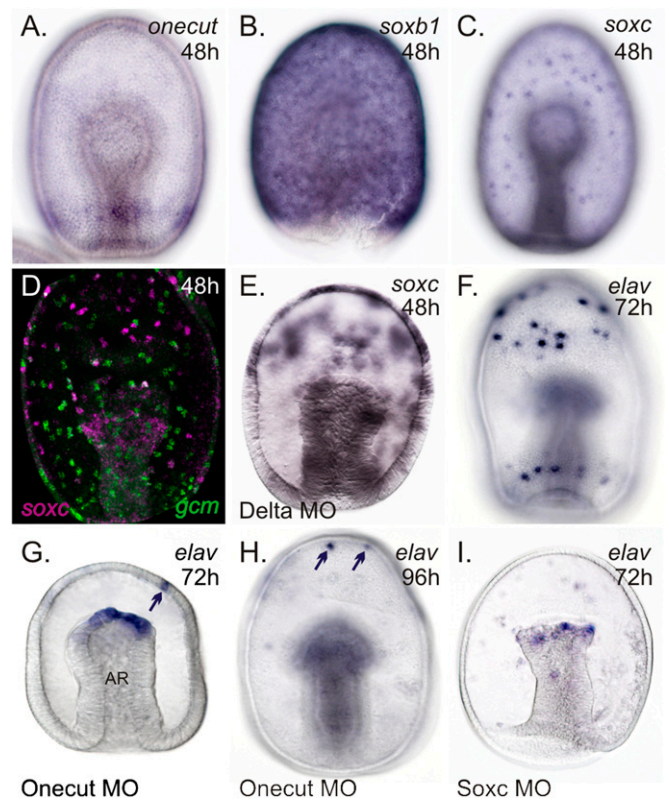

Fig. 2. Sea star gastrulae develop a broad neurogenic potential ectoderm. Transcripts of *onecut* (*A*), *soxb1* (*B*), and *soxc* (*C*) are found throughout the ectoderm of gastrulae. (*D*) *soxc*-expressing cells (purple) do not colocalize with those expressing *glial cells missing* (*gcm*; green) (32). (*E*) Ectopic expression of *soxc* within the ectoderm of Delta morphants (MO). (*F*) Transcripts of *elav* in the ciliary band neurons of an ~72-h-old larva. In Onecut morphants at ~72 h (*G*) or 96 h (*H*) postfertilization, *elav*-positive ciliary band neurons are not observed. (*I*) Both anterior and ciliary band *elav*-expressing neurons are lost in Soxc morphants around 72 h postfertilization. *elav* remains expressed in the mesoderm (*G–I*) at the tip of the archenteron (AR), later in mesenchyme (not in plane of focus in *H* and *I*) and in some anterior ectodermal cells (arrows in *G* and *H*). (Magnification: 200×.)

as other pan-ectodermal–expressed regulatory genes (25), are abundant within the fertilized eggs (Figs. S3 and S4), and therefore may operate near the top of a developmental GRN hierarchy for specification of the ectoderm. When cWnt signaling is blocked by injecting one-cell–stage embryos with a construct that blocks the nuclearization of β-catenin (Δ-cadherin) (28), the expression of these genes is found throughout the later embryo (Fig. S5 *A* and *B*). *elav*-expressing cells are also found scattered throughout this nβ-catenin–deficient ectoderm (Fig. S5C) rather than being restricted the anterior pole neural territory (e.g., compare with Fig. 1D). Thus, we suggest that similar to sea urchins and many other taxa, cWnt signaling is used to initially distinguish ectoderm from endomesoderm. In particular, cWnt signaling in sea stars segregates—as we show further below—the broad neurogenic potential ectoderm from the endomesoderm.

In vertebrates, and possibly protostomes (29), the expression of *sox* gene-family members marks a progression along a pathway toward neural commitment; *soxb* orthologs function during the early specification of neurons, and the *soxc* orthologs play a role in the commitment of neurons (30). We find here that in sea stars, *soxb1* is expressed throughout the ectoderm of gastrulae (Fig. 2B), but transcripts of *soxc* are also found throughout the ectoderm, but only within distinct cells, giving the appearance of a spotted pattern of localization (Fig. 2 *C* and *D*). Delta Notch lateral inhibition is a conserved mechanism for distinguishing one cell type from another, particularly neuronal from nonneuronal, within an epithelium (31). In sea stars, *delta* is expressed in a spotted pattern throughout the ectoderm at the gastrulae stage (32) and, when disrupted, results in an expansion of *soxc* expression to adjacent cells within the ectoderm (compare Fig. *C* and *E*). This finding suggests that a lateral inhibition mechanism may also be used to segregate *soxc*-expressing cells within the ectoderm.

We next sought to determine whether the expression of *elav* depends on these pan-ectodermal–expressed genes. Perturbation, by injecting zygotes with a sequence-specific translation blocking morpholinos (hereafter referred to as a morphant) of *onecut* results in the loss of *elav*-expressing cells associated with the developing ciliary bands (compare Fig. 2 *F* and *G*). Some *elav*-expressing cells remain within the anterior pole territory in what we think are cells that will later become the dorsal ganglia (Fig. 2G). To confirm that the loss of ciliary band neurons in Onecut morphants is not because of a developmental delay resulting from the morpholino injection, we show that these neurons remain absent from the ectoderm of Onecut morphants even at 4 d postfertilization (early larval stage) (Fig. 2H). We additionally show that perturbing the function of Onecut does not broadly respecify the ectoderm, as another cell type that is marked by the expression of *gcm* is expressed normally in these morphants (Fig. S6). We also demonstrate that the sea star ortholog of Soxc is required for *elav* expression. In Soxc morphants at 3 d following fertilization, a loss of *elav*-positive cells was observed from both the anterior pole territory and ectoderm near the developing ciliary bands (Fig. 2I).

Taken together, these data suggest that a suite of genes, which we term the pan-neurogenic suite, are first expressed within the sea star fertilized egg, are restricted by cWnt signaling, and initiate a regulatory program for the specification of a broad neurogenic potential ectodermal territory. We do not know yet if these factors are directly involved in the neural differentiation. However, the pan-neurogenic suite, which includes at least *onecut*-, *soxb1*-, and *soxc*-expressing cells, forms throughout the ectoderm and are therefore not regulated by AP or DV patterning mechanisms within the ectoderm. In this respect, the sea star develops much like *Saccoglossus*, which maintains a pan-neurogenic ectoderm throughout gastrulation (20). It has been argued that the ectoderm of sea urchins will become broadly neurogenic in the absence of signaling (33); but, in normal development, this neurogenic potential is rapidly restricted to the anterior pole territory (34, 35)

and ciliary band domain (16, 17). The presence of a broad neurogenic potential ectoderm, therefore, may be a basal feature of all echinoderms and hemichordates maintained in common for almost 800 million y (36), although the particular details of the GRN associated with this may differ. The striking difference is that during development, *elav*-expressing differentiated neurons become localized to ciliary bands and the anterior ganglia in echinoderms, but in *Saccoglossus* differentiated neurons remain throughout the ectoderm.

Patterning a *Foxg*-Defined Ciliary Band Domain. To understand how the broad neurogenic potential ectoderm nonetheless leads to the localized expression of *elav*, we next considered the role of AP and DV patterning mechanisms in sea stars. We have shown previously
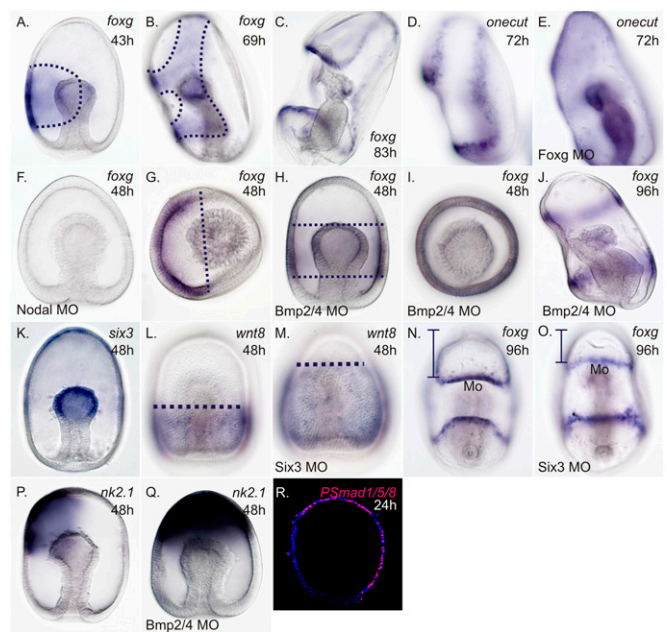

Fig. 3. Patterning of a *foxg*-expressing CBD. (*A*) Transcripts of *foxg* are initially localized within one ectodermal domain on the ventral side of 43-h-old gastrulae. (*B*) By 69 h, *foxg* transcripts are cleared within the stomodeal (mouth) ectoderm and remain within in a single domain that forms around the mouth and loops over the anterior pole. (*C*) In 83-h-old larvae, transcripts of *foxg* are clearly localized within the ectoderm of both ciliated bands. (*D*) Transcripts of *onecut* localize to the ectoderm of the ciliary bands in ∼72-h-old larvae. (*E*) Around 72 h, transcripts of *onecut* are found throughout the ectoderm of Foxg morphants (MO). (*F*) Expression of *foxg* is lost from the ectoderm in Nodal morphants around 48 h. (*G*) Anterior pole view (APV) shows *foxg* transcripts within only the ventral ectoderm. (*H* and *I*) In Bmp2/4 morphants, *foxg* transcripts are detected within a concentric domain around the middle of gastrulae. (*I*) APV of *foxg* expression in Bmp2/4 morphants. (*J*) In Bmp2/4 morphants at around 96-h postfertilization, *foxg* is expressed in two concentric loops above and below the mouth that correspond to the pre- and postoral ciliary bands, respectively; *foxg* expression does not extend into the anterior pole ectoderm. (*K*) Transcripts of *six3* localize within the anterior ectoderm at 48 h. (*L*) Transcripts of *wnt8* localize within the posterior ectoderm at 48 h, and (*M*) expand anteriorly in Six3 morphants. (*N*) Ventral view of *foxg* expression within the ectoderm of the pre- and postoral ciliary bands, above and below the mouth (Mo), respectively. (*O*) Ventral view of *foxg* expression shows that preoral ciliary band above the mouth shifts anteriorly in Six3 morphants. Vertical bracketed bars in (*N* and *O*) highlight the reduction in the anterior oral hood ectoderm of Six3 morphants compared with controls. (*P*) Transcripts of *nk2.1* within the ventral anterior pole ectoderm at the gastrula-stage. (*Q*) Expression of *nk2.1* expands dorsally in Bmp2/4 morphants. (*R*) The transcriptional effector of BMP2/4 signaling, phosphorylated Smad1/5/8 (pSmad1/5/8) is localized throughout the dorsal ectoderm (pink); DAPI staining shows nuclei (blue). (Magnification: 200×.)

that there are at least four molecularly distinct AP restricted domains within the sea star ectoderm that are similar to orthologous domains observed within the hemichordate ectoderm and the developing vertebrate nervous system (25). For example, we find concentric domains of expression of *zic*, *foxq2/retinal homeobox* (*rx*), *six3*, and *nk1* along the AP axis (25). Although these domains are less apparent in the sea urchin, we have suggested previously that at least some of these AP domains may exist and that they are likely compressed into the anterior pole ectoderm much earlier in development (25).

In addition to AP domains, sea star gastrulae also have distinct domains of gene expression within the ectoderm along the DV axis. For example, we have previously shown that *foxg* and *foxa* are expressed on the ventral surface of gastrulae (13, 25). We also cloned *nodal* and *bmp2/4* orthologs from *P. miniata* and examined their expression. Similarly to sea urchin, and as expected, the expression of *nodal* (Fig. S7A) and *bmp2/4* (Fig. S7B) is restricted along the DV axis early in development, although by late gastrulation *bmp2/4* expression is no longer detected in the ectoderm. We additionally examined the localization of activated Smad1/5/8, the transcriptional effector for BMP2/4 signaling. Just as has been shown in sea urchins (15), we find that phosphorylated Smad1/5/8 is found throughout the dorsal ectoderm of blastula (see, for example, Fig. 4). Again, by around 60 h of development (late gastrula) we no longer detect pSmad1/5/8 by immunohistochemistry. Thus, it is immediately evident that neurogenesis can occur broadly throughout the ectoderm without regard to the presence of active BMP signaling.

Of the DV restricted genes, the ectodermal expression of *foxg* is quite different from that of other sea star regulatory genes that are later expressed within the ectoderm of the ciliary bands. It is first observed relatively much later in development and only on

Fig. 4. Linking the Foxg-CBD to localized neural patterning. (A) In Foxg morphants at about 96 h, *elav*-expressing ciliary band associated neurons are absent from the lateral surface; however, *elav*-expressing ciliary band neurons remain on the ventral surface (arrows in A) above and below the mouth (Mo). Expression of *elav* remains in the mesoderm (not in plane of focus). (B) A surface ectodermal view of A shows the presence of anterior dorsal *elav*-expressing neurons in Foxg morphants. (C) In Bmp2/4 morphants at ~96 h, loops of *elav* expression within the ectoderm appear similar to *foxg* (see Fig. 3J). (D) *elav* expression within the ectoderm near the pre- and postoral ciliary bands, above and below the mouth. (E) Expression of *elav* within the preoral ciliary band above mouth shifts anteriorly in Six3 morphants similar to *foxg* (see Fig. 3O). Expression of *elav* remains unchanged in the mesoderm (not in plane of focus in D and E). Vertical bracketed bars in (D and E) highlight the reduction in the anterior oral hood ectoderm of Six3 morphants compared with controls. (F) Transcripts of *eph* are found throughout the ectoderm of the CBD around 72 h. (G) Transcripts of *efn* are found throughout the non-CBD ectoderm in normal embryos, but (H) throughout the ectoderm in Foxg morphants. (I) Preoral (dotted lines) and postoral (arrows) *elav*-expressing ciliary band neurons above and below the mouth, respectively, around 72 h. The asterisk marks *elav*-expressing anterior dorsal neurons. (J) In ~72-h-old Eph morphants, *elav*-expressing ciliary band neurons are largely absent from the lateral surface; however, *elav*-expressing ciliary band neurons remain on the ventral surface (arrows) and within the anterior, dorsal ectoderm (dotted line). (Magnification: 200×.)

the ventral side of early gastrulae (Fig. 3A) (25). As development proceeds, *foxg* expression clears from the stomodeal (mouth) ectoderm and remains expressed within a mostly concentric domain that appears not unlike the expression of its ortholog in the sea urchin (37) (Fig. S8). A critical difference, however, is that *foxg* expression in sea stars is detected shortly thereafter in a domain, which we term the ciliary band domain (CBD), that starts to extend dorsally and anteriorly over the anterior pole (Fig. 3B). By larval stage, *foxg* expression is restricted to both ciliary bands (Fig. 3C) in what we think is the edge of its former domain. We observe a loss of localized *onecut* expression within the ectoderm of the ciliary bands of Foxg morphants (compare Fig. 3 D and E). This finding suggests that one of the roles of Foxg is to organize gene expression within the ectoderm of the pre- and postoral ciliary bands. Therefore, it is possible that the observed role of Onecut in neurogenesis (Fig. 2 G and H) may occur only after its expression is restricted to the ciliary bands. The spatial relationships of *foxg*, *onecut*, and *elav* within the ectoderm are summarized in Fig. S8.

We next sought to understand how this *foxg*-CBD is shaped. Expression of *foxg* within the ectoderm is lost in Nodal morphants (compare Fig. 3 A and F, and Fig. S7), as is *foxa* expression within the stomodeal ectoderm (Fig. S7). Thus, Nodal signaling establishes two nested ectodermal domains on the ventral side of sea star gastrulae, the *foxg*-expressing domain that includes and surrounds the more restricted *foxa*-expressing stomodeal domain.

In addition, *foxg* is no longer polarized to the ventral surface of gastrulae when Bmp2/4 signaling is disrupted using a targeted morpholino. Expression of *foxg* is observed in a ring around the middle of Bmp2/4 morphants (compare Fig. 3 A and G to H and I, respectively); however, the AP boundary of *foxg* expression above and below the mouth does not appear to change (compare Fig. 3 A and H). This finding suggests that AP positioning of the *foxg*-CBD domain is established independently of DV patterning mechanisms. In 4-d-old Bmp2/4 morphants, expression of *foxg* is similarly radialized, although *foxg* expression has properly resolved into distinct pre- and postoral ciliary bands (Fig. 3J).

We next examined how AP patterning mechanisms may affect *foxg* expression. In vertebrates, Six3 gene products repress Wnt-mediated posteriorization of the neuroectoderm (38, 39). The sea urchin ortholog of Six3 also has the potential to repress Wnt signaling, because overexpression of Six3 represses the expression of several *wnts*, although their expression remains unchanged in Six3 morphants (35). In 2-d-old sea star gastrulae, *six3* is expressed in the anterior ectoderm (Fig. 3K), in a domain that seemingly does not overlap with *wnt8* expression within the posterior ectoderm (Fig. 3L). To determine if Six3 promotes anterior ectodermal identity by repression of *wnt8*, we blocked the translation of *six3* using a specific translation blocking morpholino. When the function of Six3 is disrupted, the expression of *wnt8* expands anteriorly, suggesting that Six3 antagonism of Wnt signaling establishes a boundary between the anterior and posterior ectoderm (compare Fig. 3 L and M) and that this mechanism is conserved between these echinoderms and vertebrates. Consistent with this finding, in Six3 morphants at 4 d postfertilization (early larval stage) there is a morphologically obvious reduction in the anterior region of larvae (i.e., the oral hood ectoderm above the mouth is reduced). In addition, the archenteron, a structure that derives from vegetal (posterior)-most epithelium and forms the endoderm and mesoderm, appears elongated. Importantly, expression of *foxg* indicates that the position of the preoral ciliary band above the mouth shifts anteriorly in Six3 morphants; the position postoral ciliary band below the mouth, as expected, remains relatively unchanged (compare Fig. 3 N and O). Thus, shifting the Wnt8-Six3 boundary that respectively demarcates the posterior versus anterior ectoderm, results in an anterior shift in *foxg* expression.

Although the finer details of how the *foxg*-CBD extends up through the anterior pole ectoderm are not yet understood, we
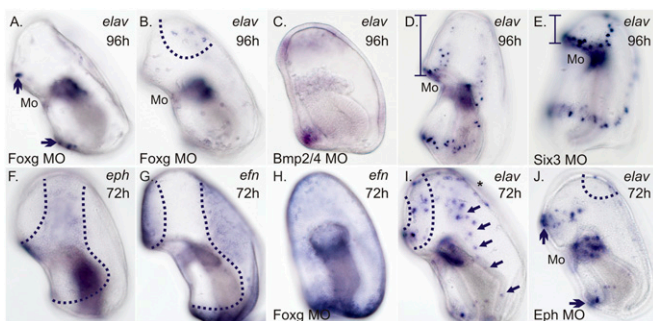
suggest that a change in Bmp2/4-mediated gene expression within the anterior pole ectoderm might also be involved in the shaping of the CBD in this territory. We find that *nk2.1* normally is expressed within the anterior pole ventrally to the domain of *foxg* expression in this territory. In Bmp2/4 morphants, *nk2.1* is now expressed throughout the anterior pole (compare Fig. 3 *P* and *Q*) and *foxg* expression concomitantly fails to move into the anterior (Fig. 3). It is possible that DV restriction of the gene expression within the anterior pole ectoderm permits the extension of *foxg* expression that is observed in late gastrulae. Thus, the normal processes of ventralization and dorsalization of the anterior pole ectoderm may allow for the extension of the CBD into this territory.

In summary, we have shown that the sea star first produces a broad neurogenic potential ectoderm that is not initially patterned along the DV or AP ectoderm axis. This broad neurogenic potential ectoderm is then overlaid with a highly patterned CDB. The AP and DV patterning mechanisms that shape the expression of this CBD and rely on interactions between Nodal, BMP2/4, Wnt and Six3, at least, to establish this *foxg*-expression territory.

Linking Patterning to the Final Steps of Neurogenesis. What remains to be shown is how the final differentiation to *elav*-expressing neurons is achieved. Crucially, we show that *elav*-expressing ciliary band neurons, but not anterior neurons that will likely form the dorsal ganglia, are largely absent from the ectoderm of *foxg* morphants (Fig. 4 *A* and *B*). We note that this aspect is unlike Soxc morphants, in which both ciliary band and anterior dorsal neurons are absent (Fig. 2*I*). We find that the localization of *elav*-expressing ciliary band neurons within the ectoderm also shifts when the shape of the *foxg*-CBD is altered. The expression of *elav* is radialized in two distinct rings above and below the mouth in these Bmp2/4 morphants (Fig. 4*C*) and the positioning of the *elav* moves anteriorly in Six3 morphants (compare Fig. 4 *D* and *E*). Importantly, the presence of *elav*-expressing cells does not appear to change in response to these perturbations, rather only the location of *elav*-positive cells changes within the ectoderm.

Therefore, *elav*-expressing cells can form in many locations within the ectoderm as long as they are associated with *foxg* and only after *foxg* becomes localized into ciliary bands. Additionally these *elav*-expressing cells only appear after phosphorylated Smad1/5/8 is no longer detected within the ectoderm. However, *elav*-positive cells do not overlap with *foxg* expression and only form along the outer edge of its domain of expression and not, for instance, within the former *foxg*-CBD territory (Fig. 1*C*). An additional mechanism is required to allow final neural differentiation and this is likely controlled by the action of some transcription

factor or signaling molecule that forms a boundary demarcated by *foxg* expression. We observed that the *ephrin receptor* (*eph*) is expressed throughout the *foxg*-CBD of late gastrulae (Fig. 4*F*), but the *ephrin ligand* (*efn*) is expressed exactly opposite to this, throughout the non-CBD ectoderm (Fig. 4*G*). The expression of these genes therefore forms a border at the edge of the *foxg* domain. In Foxg morphants, we observed a nearly ubiquitous expression of *efn* throughout the ectoderm (Fig. 4*H*). Therefore, an additional function of Foxg is to segregate out two molecularly distinct ectodermal domains, such that there is a boundary of gene-expression profiles; this is represented by, at least, the apposed expression of *eph* and *efn* at the edge of its territory. In vertebrates, interactions of Eph and Efn can mediate directed cell migrations (40). Therefore, we suspect that one of the roles of the Eph/Efn boundary in the sea star is to guide cells to a closer association with the ciliary bands. In sea stars, Eph morphants have a reduction of differentiated neurons along the lateral parts of the ciliary bands (compare Fig. 4 *I* and *J*), which could suggest that cell migration is needed to bring predifferentiated cells from the pan-neurogenic ectoderm to a closer association with the *foxg*-CBD before they can express *elav*. It is also possible that the Eph and Efn are simply involved in establishing two distinct territories that are needed to permit final differentiation and have no direct role in neural migrations. At present, we do not know how many times *soxc*-expressing neural precursor cells divide and thus, for now, cannot compare numbers of *soxc* versus *elav*-expressing cells to help distinguish between these alternatives. This is a direction of further study.

Conclusions

This study was driven by the important need to understand the developmental mechanisms that lead to a complex distribution of neurons across the ectoderm of a dipleurula-like larva, which is considered the basal larval type of deuterostomes. The data presented here clearly explain how *elav*-expressing cells are patterned across the AP and DV axes in association with the two ciliary bands in sea stars (summarized in Fig. 5 and Fig. S9). The sea star first likely uses maternally inherited anisotropies and the cWnt pathway to distinguish endomesoderm from neurogenic ectoderm that persists through gastrulation. This endomesoderm is then overlaid with a *foxg*-patterning module, which is shaped by Nodal signaling from the mouth and is patterned by Bmp2/4 and Six3. Foxg, in turn, is needed to form two distinct ciliary bands at the edge of this domain and to distinguish a molecular boundary between the CBD and other ectoderm. Thus, significantly, Bmp2/4 signaling in sea stars does not distinguish differentiated neurons
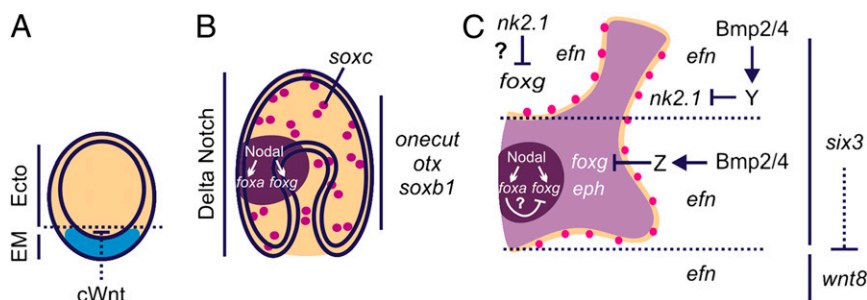

Fig. 5. Model for localized patterning of neurons within a broad neurogenic potential ectoderm. (*A*) Canonical Wnt (cWnt) signaling initially establishes endomesoderm (EM, blue) from ectoderm (Ecto, tan). A suite of neurogenic genes including *onecut-*, *otxßb-*, and *soxb1* are expressed throughout the ectoderm of (*A*) blastulae and (*B*) gastrulae. (*B*) Delta Notch signaling patterns distinct *soxc*-expressing cells within the broad neurogenic potential ectoderm. Nodal signaling establishes *foxg* and *foxa* expression within the ventral ectoderm (dark purple). (*C*) During late gastrulation, *foxg* expression clears from the stomodeal (mouth) ectoderm and a *foxg*-CBD (light purple) is shaped by Bmp2/4 signaling and Six3. The creation of this *foxg*-CBD, distinguishes between *eph*- and *efn*-expressing ectodermal territories. Differentiated neurons (magenta) form along this boundary. Dotted lines in *C* denote the position of the *foxg*-CBD within the ectoderm along the AP axis; *six3*-expressing anterior ectoderm extends upward from the bottom dotted line and includes the anterior pole ectoderm, above the top dotted line.

from nonneuronal ectoderm but instead contributes to the patterning of the CBD, which then in turn provides cues to permit the final steps of neuronal differentiation. The final fate commitment to neuronal cell type in sea star may instead be governed by a temporal loss of BMP signaling rather than a spatial gradient as observed in many other taxa. This functional modulation between specification and patterning likely reflects the evolutionary history of this GRN, in which an ancestral module for specification of a broad neurogenic potential ectoderm was subsequently overlaid with a GRN for patterning. Such patterning could allow a swimming larval form to more efficiently coordinate ciliary beating.

Experimental Procedures

Embryo Culture and Characterization of *P. miniata* Gene Expression. Embryos were cultured in seawater at 15 °C and fixed for in situ hybridization, as previously described (26). Partial sequences of *P. miniata* orthologs were obtained via screening a *P. miniata* arrayed cDNA library (26) or were obtained from the publically available transcriptome. Whole-mount in situ hybridization (WMISH) and FISH was performed as previously described (25, 26).

qPCR and NanoString nCounter Assay. RNA from developmentally staged *P. miniata* embryos was extracted using GenElute Mammalian Total RNA Kit

(Sigma-Aldrich). cDNA was synthesized using iScript Select cDNA Synthesis Kit (Bio-Rad). Abundance of gene transcripts was determined using the nCounter Gene Expression Assay (NanoString Technologies) as described by ref. 41 and using custom *P. miniata* Reporter CodeSet and Capture ProbeSet.

Microinjection. Microinjection was performed as described previously (26). Morpholino antisense oligonucleotides were designed by GeneTools. Approximately 20–40 injected embryos were used in spatial analyses of gene expression. Experiments were repeated at least twice. Similar phenotypes were observed when a second morpholino targeted to the same transcript was used. The embryos presented are representative of changes observed in greater than 90% of injected embryos.

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