TGFβ signaling positions the ciliary band and acts indirectly to pattern neurons in the sea urchin embryo

Shunsuke Yaguchi[1,2,3], Junko Yaguchi[1,2,3], Robert C. Angerer[3], Lynne M. Angerer[3], Robert D. Burke[2]*
[1]Shimoda Marine Research Center, University of Tsukuba, Japan,
[2]Dept of Biochemistry and Microbiology, University of Victoria, Canada,
[3]National Institute of Dental and Craniofacial Research, National Institutes of Health, USA

Corresponding Author: Robert D. Burke,
Biochemistry/Microbiology, University of Victoria, POB 3020, STN CSC, 3800 Finnerty Rd, Victoria, BC Canada, V8W 3N5
Phone 250 721 7105
Fax 250 721 7120
Email rburke@uvic.ca

ABSTRACT

The ciliary band is a distinct region of embryonic ectoderm that is specified between oral and aboral ectoderm. Flask-shaped ciliary cells and neurons differentiate in this region and they are patterned to form an integrated tissue that functions as the principal swimming and feeding organ of the larva. TGFβ signaling, which is known to mediate oral and aboral patterning of the ectoderm, has been implicated in ciliary band formation. We have used morpholino knockdown and ectopic expression of RNA to alter TGFβ signaling at the level of ligands, receptors, and signal transduction components and assessed the differentiation and patterning of the ciliary band cells and associated neurons. These results suggest that the effect of TGFβ signaling on the development of most neurons is indirect. We propose that the primary effects of these signals are to position the ciliary cells, which in turn support neural differentiation. Nodal signaling determines the position of the oral margin of the ciliary band, while BMP signals regulate the width of the band. Since both Nodal and BMP2/4 signaling produce ectoderm that does not support neurogenesis, we propose that formation of a ciliary band requires protection from these signals. In addition, our studies reveal that interfering with the expression of the only sea urchin BMP receptor, Alk3/6, produces a ciliary band phenotype that is different from that resulting from the loss of BMP2/4. This suggests that at least one additional component that signals through Alk3/6 is part of normal ectoderm signaling. We propose a model that incorporates spatially regulated control of Nodal and BMP signaling to determine the position and differentiation of the ciliary band, and subsequent neural patterning.

INTRODUCTION

Members of the transforming growth factor-β (TGF-β) superfamily play central roles in cell fate specification in development. TGF-β ligands are secreted proteins that diffuse from their source and activate complex signaling networks that regulate differentiation. TGF-β signaling patterns are complicated because a range of factors modify ligand availability and receptor and signal transduction functions, creating complex developmental patterns from seemingly simple arrangements of localized signaling sources and widespread receptors. Well-known examples are Nodal and BMP4. In vertebrates, *nodal* is expressed on the left side of the embryo and its localized effects are controlled by Lefty-1 and Lefty-2 (Meno et al., 1996). Leftys bind to the EGF-CFC proteins that are required for Nodal to bind to the activin-like kinase (Alk) receptor at the midline of the body, thereby blocking Nodal binding, and preventing Nodal signals from spreading to right side (Chen and Shen, 2004). BMP4, which is expressed on the future ventral side of vertebrate ectoderm, diffuses throughout the embryo, but is antagonized by the direct binding of Chordin, which is expressed in the dorsal organizer. The consequence is that dorsal tissues form where BMP4's ventralizing effects are blocked (De Robertis and Kuroda 2004). It is a hallmark of TGFβ signaling that molecular antagonists pattern the effects of the secreting ligands with surprising precision.

In sea urchin embryos four regions of ectoderm – the animal plate, oral ectoderm, aboral ectoderm and ciliary band – are produced by animal hemisphere blastomeres (Davidson et al., 1998; Yaguchi et al., 2006). Incompletely characterized events,

dependent on vegetal canonical Wnt, restrict the animal plate to the animal pole (Yaguchi et al., 2006) and eliminate a repressor of *nodal* expression (Yaguchi et al., 2008). As a consequence, the TGF-β signals, Nodal and subsequently BMP2/4, begin to pattern the remaining ectoderm in the animal hemisphere, producing oral, aboral and ciliary band ectoderm (Duboc et al., 2004, Yaguchi et al., 2006). Models of ectodermal specification suggest that Nodal signaling is limited to the oral ectoderm by Lefty, which depends on Nodal and has long-range inhibitory functions (Duboc et al., 2008). A reaction-diffusion model in which Lefty acts as a feedback inhibitor has been proposed to explain how it restricts Nodal signaling to oral ectoderm (Duboc et al., 2008; Bolouri and Davidson, 2009). BMP2/4, which also acts downstream of Nodal, is transcribed in the oral ectoderm (Angerer et al., 2000, Duboc et al., 2004), yet acts outside of oral ectoderm to induce aboral ectoderm Lapraz et al., 2009). Bradham et al. (2009) and Lapraz et al. (2009) showed that Chordin, expressed in the oral ectoderm under the control of Nodal, blocks BMP2/4 activity. In its absence, or in the absence of Nodal, differentiation of ciliary band neurons is altered as well as the normal expression pattern of a ciliary band marker. Although TGFβ signaling accounts for many aspects of oral and aboral ectoderm specification, we understand very little of the mechanisms involved in ciliary band formation, and the differentiation of ciliary band neurons.

The ciliary band is the principal swimming and feeding organ of the larva. It is a tightly packed strip of flask-shaped, ciliary cells that beat away from the mouth, producing a force that moves the larva forward and captures food particles deflected by ciliary reversals (Strathmann, 2007). In addition to the ciliary cells, there is a series of neurons, mostly on the oral side of the ciliary band, that have short, microvillar dendritic

processes on their surface (Burke, 1978). A tract of axons that lies at the base of the ciliary cells interconnects the nerve cells. The nervous system is thought to regulate the direction of ciliary beat, as depolarization of the ciliary cells accompanies reversals of ciliary beat (Mackie et al., 1969; Satterlie and Cameron, 1985). Thus, the ciliary band is an integrated tissue innervated by neurons arranged in a precise pattern.

Our objective was to determine how components of the oral-aboral signaling network specify and pattern the ciliary cells and neurons of the ciliary band. We manipulated the signaling network by knocking down ligands and receptors with morpholinos and expressing RNAs encoding antagonists and dominant negative, or constitutively active signal transduction components. We anticipated that by assessing the distribution of different types of ectoderm and neurons, we would be able to deduce how oral/aboral ectoderm patterning mechanisms regulate formation of the ciliary band. Our results indicate that the ciliary band is positioned by TGFβ signaling, yet it is a region in which TGFβ signaling is suppressed. As well, the ciliary band cells are required for the differentiation and patterning of ciliary band neurons. We identify several novel roles for known components of the oral-aboral signaling network and for other components that appear to be missing.

MATERIALS AND METHODS

Animals and embryo culture

Strongylocentrotus purpuratus were collected near Victoria, BC or purchased from The Cultured Abalone, Goleta, CA. Gametes were obtained by intra-coelomic injection of 0.5M KCl and embryos were cultured by standard methods with filtered

seawater (FSW) or artificial seawater at 15˚C.

Microinjection of morpholino anti-sense oligonucleotides (MO) and mRNAs

Eggs were prepared as described previously (Yaguchi et al., 2006). Morpholinos (Gene Tools, Eugene, OR) were microinjected in 22.5% glycerol with the following concentration in the injection needles: nodal-MO (600μM), lefty-MO (200μM), BMP2/4-MO (150μM), and Alk3/6-MO (200μM). The morpholino sequences are:

nodal-MO: 5'-GATGTCTCAGCTCTCTGAAATGTAG-3'
lefty-MO: 5'-AGCACCGAGTGATAATTCCATATTG-3'
BMP2/4-MO: 5'-GTGGTAACCATGATGGGTCTGAAAG-3'
Alk3/6-MO: 5'-TAGTGTTACATCTGTCGC[CAT]ATTC-3'

The preparation and concentration for *nodal*, *lefty*, modified *smad2/3* and *BMP2/4* mRNAs are described previously (Yaguchi et al., 2006; Yaguchi et al., 2007). To misexpress modified *smad1/5*, the C-terminal of Sp-Smad1/5 was substituted or deleted in a manner similar to that described for Smad2/3 modification (Yaguchi et al., 2007). The concentration of act-*smad*1/5 and dn-*smad1/5* were 3.0μg/μl in injection needles.

Immunohistochemistry

Immunohistochemistry was done as described previously (Yaguchi et al., 2006). Primary antibodies were incubated overnight at 4˚C using the following dilutions:

Synaptotagmin (1E11, 1:800; Nakajima et al., 2004), Goosecoid (Gsc, 1:600; Kenny et al., 2003), Hnf6 (1:500), serotonin (1:1000, Sigma), and Nk2.1 (1:800; Takacs et al., 2004). The specimens were observed using Leica (DM6000) and Zeiss (Axiovert 200M and LSM410) microscopes. The images were analyzed with ImageJ (NIH) and Adobe Photoshop and the figures were prepared with Canvas8.

RESULTS

Ciliary Band Neurons

The ciliary band in a pluteus larva is composed of 3-4 rows of columnar cells that surround the oral ectoderm (Fig. 1A, green line). To identify differentiating ciliary band cells, we prepared an antibody to Hnf6, a transcription factor of the ONECUT family, which is expressed in the ciliary band (Otim et al., 2004; Poustka et al., 2004). In prism and pluteus stages, Hnf6 protein is detected in the nuclei of the tightly packed columnar cells of the ciliary band. Double staining for Goosecoid (Gsc), a transcription factor expressed in squamous oral ectoderm (Angerer et al., 2001) and Hnf6 shows that these do not co-localize and ciliary band is a distinct region of ectoderm (Fig 1C-E).

In the early pluteus larva some neurons are in the thickened animal plate and around the mouth, but most reside in the ciliary band (Fig. 1B, H-K). The cell bodies of ciliary band neurons are predominantly on the oral side of the ciliary band and bundled axon tracts connect the neurons and encircle the oral ectoderm (Fig. 1F-G). Synaptotagmin B-containing projections from the ciliary band neurons underlie the aboral ectoderm, are oriented toward the posterior end of the larva, and are not bundled. On the left and right sides of the pluteus, lateral ganglia each include a cluster of 2-4

neural cell bodies beneath the aboral ectoderm that extend projections posteriorly and into the axonal tracts of the ciliary band. The only neural projections under the oral ectoderm are two bundles of axons that cross the oral ectoderm at the base of the postoral arms (Fig 1B, arrow). The serotonergic neurons are restricted to the animal plate at this stage of development (Fig 1L-N). Thus, the types and organization of neurons and neural projections are distinctive in the oral and aboral ectoderm as well as in different parts of the ciliary band. The key features of the ciliary band neurons are: 1. Neuronal cell bodies are restricted to the oral side of the ciliary band; 2. Axons in the ciliary band form bundles; 3. Unbundled axons project posteriorly under the aboral ectoderm; 4. Only two axonal tracks at the base of the postoral arms project under the oral ectoderm.

Suppressing Nodal signaling

Injecting eggs with an Sp-nodal morpholino oligonucleotide (nodal-MO) results in embryos that are radialized with a gut that elongates toward the animal plate, yet no mouth forms (Duboc et al., 2004; Fig. 2A inset). A more severe phenotype with an everted archenteron, which has not been reported in *Paracentrotus lividus* (Duboc et al., 2004) or *Hemicentrotus pulcherrimus* (data not shown), is common in *S. purpuratus*. At the end of gastrulation, there is a large disk of tightly packed, columnar cells, 10-14 cells wide, that contain nuclear Hnf6. This disk includes the animal plate, as it expresses Nk2.1 (Fig. 2A-F; Yaguchi et al., 2006; Takacs et al., 2004) and serotonergic neurons (Fig. 2D-F), and the remaining ectoderm derived from animal blastomeres. There is another region lacking nuclear Hnf6, but marked by small, non-nuclear spots of non-

specific staining in the basal bodies. Cells in this region are less densely packed, squamous, and express the aboral ectoderm, Spec1 (data not shown). Thus in nodal-MO embryos, the ectoderm is specified as animal plate, surrounded by an expanded region of ciliary band cells, and a squamous epithelium that has features of aboral ectoderm.

Synaptotagmin-expressing neurons differentiate throughout much of the ectoderm in nodal-MO embryos, although most of the cell bodies are associated with the ciliary band. Much of the Synaptotagmin signal is in growth cones on neurites projecting toward the blastopore beneath the aboral ectoderm (Fig. 2A-F). The neurons in the ciliary band region do not interconnect and form bundled axon tracts (Fig 2A, D). An alternative, although not equivalent, means of suppressing Nodal signaling is over-expression of *lefty*, an endogenous antagonist of Nodal (Duboc et al., 2008). As expected, the Nodal-dependent gene, *gsc*, is not expressed in these embryos (Fig. 2G, I; Gsc protein), which are similar in form to nodal-MO embryos, with a radialized ectoderm, a straight archenteron and no mouth. The Hnf6 expression pattern, the distribution of neurons and axon projections are also the same in *lefty* RNA-injected embryos as described for nodal-MO containing embryos (Fig. 2G-L). Similarly, embryos injected with a dominant negative version of *smad2/3* (Yaguchi et al., 2007), a downstream effector of Nodal signals, have the same phenotype as nodal-MO- or *lefty* RNA-injected embryos: Synaptotagmin neurons differentiate along the margin of the thickened ciliary band ectoderm and extend neurites posteriorly (Fig. 2M-O**).** Taken together, these results show that embryos lacking Nodal function have 3 types of ectoderm: animal plate, a region with some ciliary band features, and a more vegetal

region with aboral ectoderm features. Most of nerve cell bodies are near the ciliary band, but they lack the neural patterning characteristic of ciliary band.

Enhancing Nodal signaling

Injecting eggs with *nodal* RNA also produces a radialized embryo, but in this case, four regions of ectoderm are present and arranged along the animal-vegetal axis: the animal plate, and successive rings of oral ectoderm, ciliary band, and aboral-like ectoderm (Duboc et al., 2004; Yaguchi et al., 2006). Hnf6 protein is detected in cells of the animal plate and in a thin, interrupted strip of ciliary band, 1-2 cells wide (Fig. 3B), confirming previously reported *in situ* hybridization data (Yaguchi et al., 2006). In *nodal* RNA-injected embryos, the cell bodies of Synaptotagmin-expressing neurons are predominantly in the ciliary band, axons form a single tract that joins the neurons and short neurites project posteriorly. (Fig. 3A, C, D, F). In 4-day plutei, neurons expressing Synaptotagmin appear in the animal plate, but there are no cells expressing serotonin (Fig. 3D-F).

Lefty-MO embryos are similar to *nodal* mRNA-injected embryos (Duboc et al., 2008). The expression of the oral ectoderm marker, Gsc, is radialized in both cases (Fig. 3G-I; Duboc et al., 2008) and serotonergic neurons do not differentiate in the animal plate (Fig. 3J-L; Yaguchi et al., 2007). However, there is no ciliary band, as Hnf6 protein (Fig. 3H) and Synaptotagmin neurons are found only in the animal plate (Fig. 3J-L) in lefty-MO-injected embryos. Embryos injected with act-*smad2/3* mRNA are similar in form to lefty-MO-injected embryos, being radialized, lacking ciliary bands and serotonergic neurons (Fig. 3N and O) (Yaguchi et al., 2007). However, unlike lefty-MO

embryos, there are no Synaptotagmin expressing animal plate neurons (Fig. 3M-O).

Injection of *nodal* RNA results in ectopic Nodal signaling and also ectopic expression of Nodal-dependent genes like *lefty*, *BMP2/4* and *chordin*. The neural patterning is normal in the ciliary band that forms in these embryos as cells are interconnected with bundled axons and extend aboral projections. The fact that misexpressed *nodal* can still direct the formation of a set of fully integrated ectodermal tissues supporting the differentiation and patterning of neural components reinforces the idea that it functions near the top of the oral/aboral ectoderm specification pathway. The loss of a ciliary band in lefty-MO embryos or those misexpressing act-*smad2/3* suggests that if Nodal signaling is not suppressed by Lefty, or if its downstream effector is present throughout the embryo, then development of this tissue is blocked.

Suppressing BMP2/4 signaling

BMP2/4-MO-injected embryos developed as previously described (Duboc et al., 2004). They are not radialized, as the gut bends to the oral side and fuses to form a mouth (Fig. 4B, inset). The overall form of the embryo is distorted, but they contain four regions of ectoderm: animal plate, oral ectoderm, ciliary band, and aboral ectoderm. However, the oral ectoderm marker, Gsc, is not restricted to the oral side but surrounds the animal plate (Fig. 4D-F). A band 5-6 cells wide of Hnf6-expressing ciliary cells, slightly wider than ciliary bands in control embryos, is present but is displaced to the aboral side and does not intersect the ciliated cells in the animal plate, as in normal embryos (Fig. 4A-F). The squamous ectoderm opposite to the oral ectoderm has a low cell density and expresses the aboral ectoderm marker, Spec1 (data not shown). Synaptotagmin-expressing neurons differentiate in embryos injected with BMP2/4-MO;

however, they are not restricted to the ciliary band but the cell bodies are predominantly in the oral half of the embryo (Fig. 4A, C). The cell bodies are multipolar and neurites project randomly without forming bundled tracts. Few neurites associate with the oral ectoderm and no serotonergic neurons develop in the animal plate.

Similarly, knockdown of Alk3/6, the only BMP receptor in the sea urchin genome, produced embryos with some features in common with embryos in which BMP2/4 expression is blocked (Fig. 4G-I). Again, the ciliary band shifts toward the aboral side around the animal plate (Fig. 4H and I), oral ectoderm, marked by Gsc expression, surrounds the animal plate (Fig. 4J and L), and the animal plate marker Nk2.1 is expressed in animal pole cells, although this domain is larger than normal (Fig. 4L). Alk3/6-MO injected embryos are more radial than BMP2/4-MO injected embryos as the ciliary band is in a plane almost orthagonal to the animal-vegetal axis. The major difference between the BMP2/4-MO and the Alk3/6-MO embryos is that the band of Hnf6-expressing cells is much wider, 10-12 cells, than it is in BMP2/4-MO embryos. Synaptotagmin-expressing neurons differentiate throughout this broad band of cells expressing Hnf6 as well as ectopically throughout the aboral region. The neurons project neurites randomly throughout the non-oral half of the embryo and the neurons interconnect, but axon tracts fail to form. Thus, embryos in which expression of the Alk3/6 receptor is blocked are not identical to embryos in which one of the ligands, BMP2/4, is suppressed.

When RNA encoding a dominant-negative form of *smad1/5* (dn-*smad1/5*) is injected into eggs, the embryos are similar in form to embryos injected with Alk3/6-MO. The oral territory is expanded to surround the animal plate and the Hnf6-expressing

ciliary band is shifted aborally and is as wide as that in Alk3/6-MO-injected embryos (Fig. 4M-O). Similarly, embryos expressing dn-*smad1/5* have Synaptotagmin neurons throughout the aboral ectoderm, only short randomly oriented projections form, the cells are not interconnected nor do axons bundle into tracts (Fig. 4P-R). Thus, suppressing signaling that specifies aboral ectoderm with either Alk3/6-MO or dn-*smad1/5* results in ectoderm that supports the differentiation but not the patterning of neurons. These neurons are not associated with the band of Hnf-6 cells, indicating BMP ligands are involved in the process that patterns neurons within the ciliary band.

Enhancing BMP2/4 signaling

Embryos injected with *BMP2/4* mRNA develop as previously described (Angerer et al., 2000; Yaguchi et al., 2006). Most of the ectoderm expresses Spec1 but not Gsc (Fig. 5G-I). As well, neither ciliary band cells nor Synaptotagmin expressing, ciliary band neurons differentiate (Fig. 5A-F). The animal plate is pronounced, expressing Hnf6 and Nk2.1 and contains serotonergic neurons that express Synaptotagmin (Fig. 5C, F, and I). This phenotype also results in embryos expressing act-*smad1/5* (Fig. 5J-O), as neither ciliary band ectoderm nor ciliary band neurons differentiate (Fig. 5J and N). However, these embryos lack serotonin-containing neurons in the animal plate. Taken together, these experiments indicate that BMP2/4 can inhibit formation of ciliary band and suppress differentiation of ciliary band neurons.

DISCUSSION

TGFβ signaling acts indirectly on differentiation of neurons

Treatments that enhance BMP2/4 or Nodal signaling appear to inhibit neural differentiation. Misexpressed ligands could be acting on neural progenitors directly, or they could be acting indirectly on the non-neural ectoderm, which in turn either supports or suppresses neural differentiation. If signaling acts directly on neural progenitors to prevent their differentiation, then blocking of that signaling with either a receptor morpholino or mRNA encoding dn-*smads* should result in a cell-autonomous increase in the number of neurons. However, if this happens it must affect only a small fraction of the ciliary band neurons, indicating that most, if not all of them respond indirectly to TGFβ signals. Our model proposes that the indirect effect of TGFβ signaling is to provide the appropriate environment for neural development and the Hnf6-expressing ciliary cells provide this environment.

Much of the behavior of neurons reported in untreated embryos and in embryos resulting from the perturbations described here support a model in which the ciliary band is required for the differentiation of neurons and the outgrowth and bundling of axons. Neurons do not differentiate in treatments that result in the loss of the ciliary band and when the ciliary band is displaced, neurons differentiate at the new site. Our data show that oral ectoderm does not support any of these events and aboral ectoderm only promotes growth of unbundled neurites. There are numerous situations in neural development of other metazoans in which neural progenitors must receive appropriate neurotrophic support to differentiate and neurite outgrowth is directed by axon guidance cues that determine the direction of neurite growth and regulate axon

bundling by regulating adhesion (Chilton, 2006). We propose that the ciliary band provides an environment conducive to neural development and organization.

A model for patterning the ciliary band

When Nodal is expressed in the oral ectoderm, it initiates a sequence of signaling and differentiation events that includes the expression of factors that antagonize or modify Nodal and BMP2/4 signals (Fig. 6). So far, Lefty and Chordin have this role (Duboc et al., 2004, 2008; Bradham et al., 2009), and we propose that they exclude TGFβ signals from the ciliary band. Here we show that Nodal signaling localized by Lefty, positions the oral margin of the ciliary band. Signaling from BMP2/4 and at least one additional BMP narrows the potential width of the band on the aboral side from about 12 cells to 4. Although Nodal and BMP2/4 signaling block development of ciliary band neurons, the correct patterning of these cells within the ciliary band depends on both of these signals being present. In the normal embryo, the ectoderm that is subject to these TGFβ signals includes all of the ectoderm except the animal plate and the ectoderm surrounding the blastopore. Little is known about specification of the vegetal ectoderm, but it likely involves canonical Wnt signaling, which is active in precursors during cleavage stages. However, the specification mechanisms of the ectoderm at the animal pole (Wei et al., 2009) and that of oral and aboral ectoderm, which require TGFβ signals, are beginning to emerge (Duboc et al., 2004, Range et al., 2007, Nam et al., 2007, Bradham et al., 2009, Su et al., 2009, Lapraz et al., 2009). Here we have examined how these signals position the cells expressing Hnf6 that will become the ciliary cells of the ciliary band.

Nodal is sufficient for differentiation and neural patterning of the ciliary band

All the data presented here suggest that Nodal initiates a series of events, including expression of another TGFβ, BMP2/4, that are required for the differentiation and patterning of the neural components of the ciliary band in the TGFβ-responsive ectoderm. We found that neither a ciliary band nor differentiated synaptotagmin expressing neurons form in embryos mis-expressing act-*smad2/3*, which transduces the effects of Nodal signaling throughout the embryo, and bypasses negative feedback regulation by Lefty, a Nodal antagonist. In contrast, an innervated ciliary band can form in embryos mis-expressing *nodal*, and its downstream target *lefty*, even though *nodal* is initially expressed uniformly throughout the embryo.

Our data support the proposition that Lefty is a critical regulator of ciliary band formation. When Lefty expression is blocked, the domain of endogenous Nodal signaling expands and no ciliary band is detectable by Hnf6 staining. In addition, the ectoderm that results does not support differentiation of Synaptotagmin expressing neurons. The loss of ciliary band and neurons in embryos with suppressed expression of Lefty argues that in the normal embryo prevention of ectopic Nodal signaling by Lefty is an essential feature of patterning of the TGFβ-responsive ectoderm as proposed by Duboc and Lepage (2008).

When Nodal signaling is blocked, all but the most vegetal ectoderm continues to express Hnf6, a transcription factor that is a component of not only the basal regulatory state of ectoderm operating before Nodal or BMP2/4 signaling begins, but also of the mature ciliary band. When BMP signaling is blocked, ciliary band cells, expressing Hnf6,

are present, whereas in embryos mis-expressing BMP2/4, they are absent (this paper; Angerer et al., 2000; Duboc et al., 2004; Bradham et al., 2009; Lapraz et al., 2009). Control of BMP signaling is likely to be mediated, at least in part, by Chordin, which has been shown to antagonize BMP2/4 in sea urchin embryos and is necessary for correct formation of the ciliary band and development of ciliary band neurons (Bradham et al., 2009; Lapraz et al., 2009). Taken together, these observations suggest that TGFβ signaling transforms most of the early ectoderm into an epidermal regulatory state except in cells where these signals are excluded; the site of ciliary band formation. It follows that the ciliary band, and subsequently the development and patterning of neurons within it, require protection from Nodal and BMP.

Restriction of the ciliary band to a narrow strip of cells expressing Hnf6 follows shortly after the activation of the oral signaling network. The levels of *nodal* and *lefty* mRNAs increase significantly during early blastula stages and *chordin* and *BMP2/4* transcription is up-regulated a few hours later during mesenchyme blastula stage (Angerer et al., 2000; Bradham et al., 2009). This precedes by only a few hours the emergence of the ciliary band at late mesenchyme blastula stage (Otim et al., 2004; Poustka et al., 2004). Exactly how spatially regulated TGFβ signaling restricts the expression of *hnf6* is not yet clear, however, it is likely that mechanisms that control the levels and distribution of Lefty, Chordin and other TGFβ antagonists are involved.

Nodal positions the oral boundary of the ciliary band, and BMP regulates its width

The position and size of the band of cells expressing Hnf6 is dramatically altered when the domain of Nodal expression is altered. When it is blocked, the band is 10-14 cells wide and shifts toward the animal pole of the embryo, whereas, when it is over

expressed, the band is reduced to a width of only 1 cell and shifts toward the vegetal end of the embryo. In normal embryos, Gsc, a target of Nodal signaling, is expressed in a domain that directly abuts the ciliary band. These observations suggest that Nodal signaling regulates the position of the oral margin of Hnf-6 expressing ciliary band cells.

BMP2/4 appears to determine the position of the posterior margin of the band of cells expressing Hnf6. When embryos are injected with *BMP2/4* RNA or act-*smad1/5* no band of Hnf6 cells forms. As well when BMP2/4 signaling is suppressed (BMP2/4-MO, dn*smad1/5* RNA, Alk3/6-MO), the band of Hnf-6 cells that forms is shifted away from the animal plate toward the vegetal pole and is wider. Its posterior margin is restricted by at least two factors that signal through ALK3/6, yet BMP2/4 appears to play a relatively small role in this process. Loss of BMP2/4 increases the band from 4 to 5 or 6 cells in width while loss of Alk3/6 further increases it to 10-12 cells. This is the first demonstration that at least one additional BMP must be involved in patterning of this region of the aboral ectoderm. However, Lapraz et al. (2009) do not report a similar difference between the effects of BMP2/4-MO and Alk3/6-MO on embryos of *Paracentrotus lividus.* Leaving open the possibility that there is an incomplete suppression of BMP2/4 in *S. purpuratus* or species differences in regulation of aboral ectoderm specification by BMP pathways.

Vegetal ectoderm is resistant to TGFβ signals

The specification and differentiation of the most vegetal region of ectoderm is poorly understood. Perturbations of Nodal or BMP signaling make it clear that this ectoderm responds differently than more animal ectoderm. Although loss of BMP

signaling results in expansion of the ciliary band, it does not extend into the vegetal ectoderm. Loss of Nodal signaling, and consequently BMP2/4, reveals that the vegetal ectoderm continues to express aboral markers (Duboc et al., 2004) rather than Hnf6 as most of the rest of the ectoderm does (this work). Although misexpression of *nodal* generates a ciliary band near the vegetal pole, there remains a vegetal strip of aboral ectoderm (this work, Duboc et al., 2004). At least some of this ectoderm is probably derived from veg1 blastomeres, which surround to blastopore (Davidson et al., 1998), and as such its regulatory state is likely to be different from animal blastomere-derived ectoderm as a result of vegetal Wnt signaling (Davidson et al., 2002).

Patterning of the ciliary band nervous system

Nodal and BMP2/4 specify oral and aboral ectoderm, and suppression of these signals in a narrow region of ectoderm between them produces the ciliary band. These tissues appear to control how the neurons develop within this band. Oral ectoderm inhibits differentiation of neurons and outgrowth of neurites, but aboral ectoderm supports outgrowth of unbundled neurites. The ciliary band cells are under the influence of a gene regulatory network that includes *hnf6*, but the presence of Hnf6 is not sufficient to ensure correct patterning of ciliary band neurons. The Hnf6-expressing cells are capable of forming a thickened, ciliated epithelium but, in the absence of TGFβ signals, they do not support correct formation of bundled axonal tracts that interconnect. The mechanisms by which TGFβ signaling affects the direction of neural projections and the interactions among them are not understood. Rigorous testing will be required to understand the intricate mechanisms by which TGFβ signaling patterns the elegant,

yet relatively simple, tissues that serve the critical functions of swimming and feeding in the larva.

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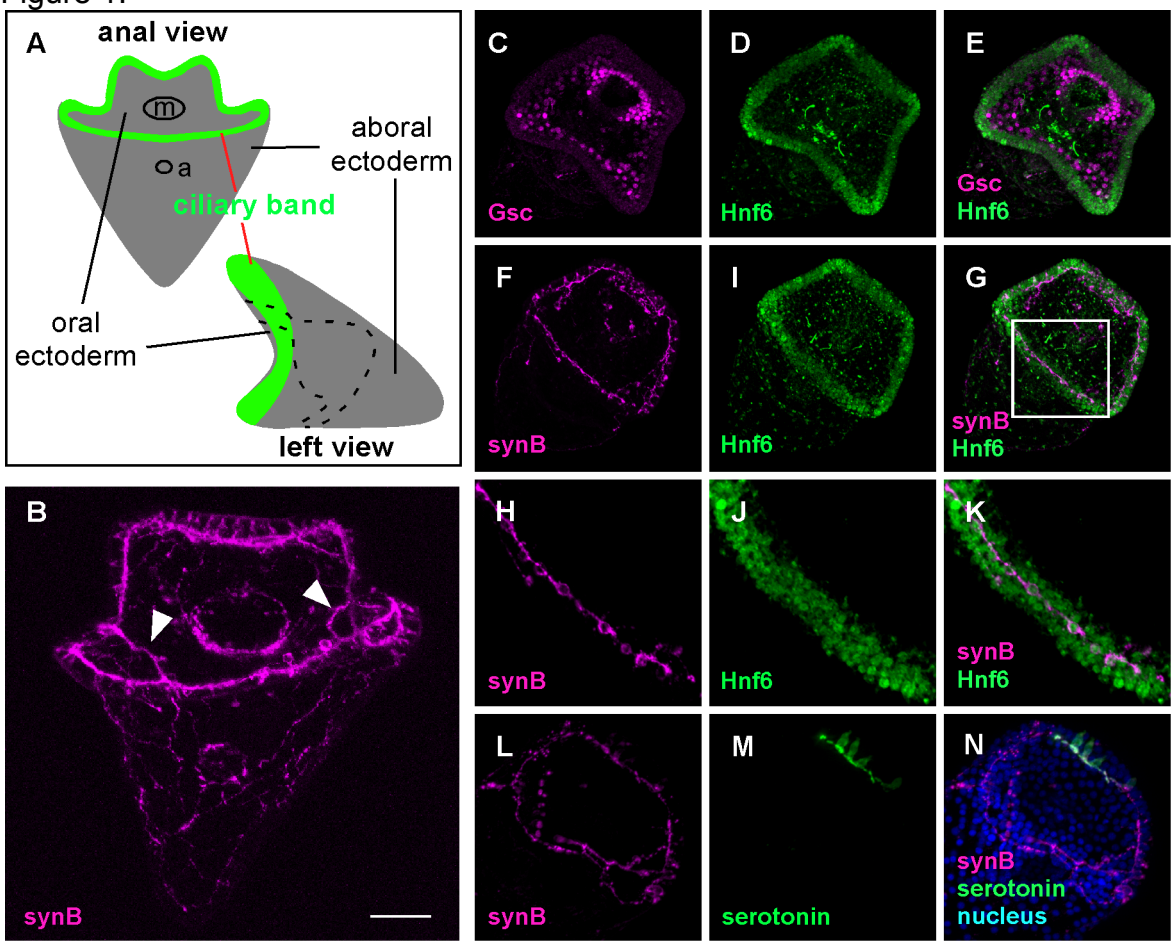
Figure 1.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anal view
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aboral ectoderm
ciliary band
oral ectoderm
left view
B
synB
C
Gsc
D
Hnf6
E
Gsc
Hnf6
F
synB
I
Hnf6
G
synB
Hnf6
H
synB
J
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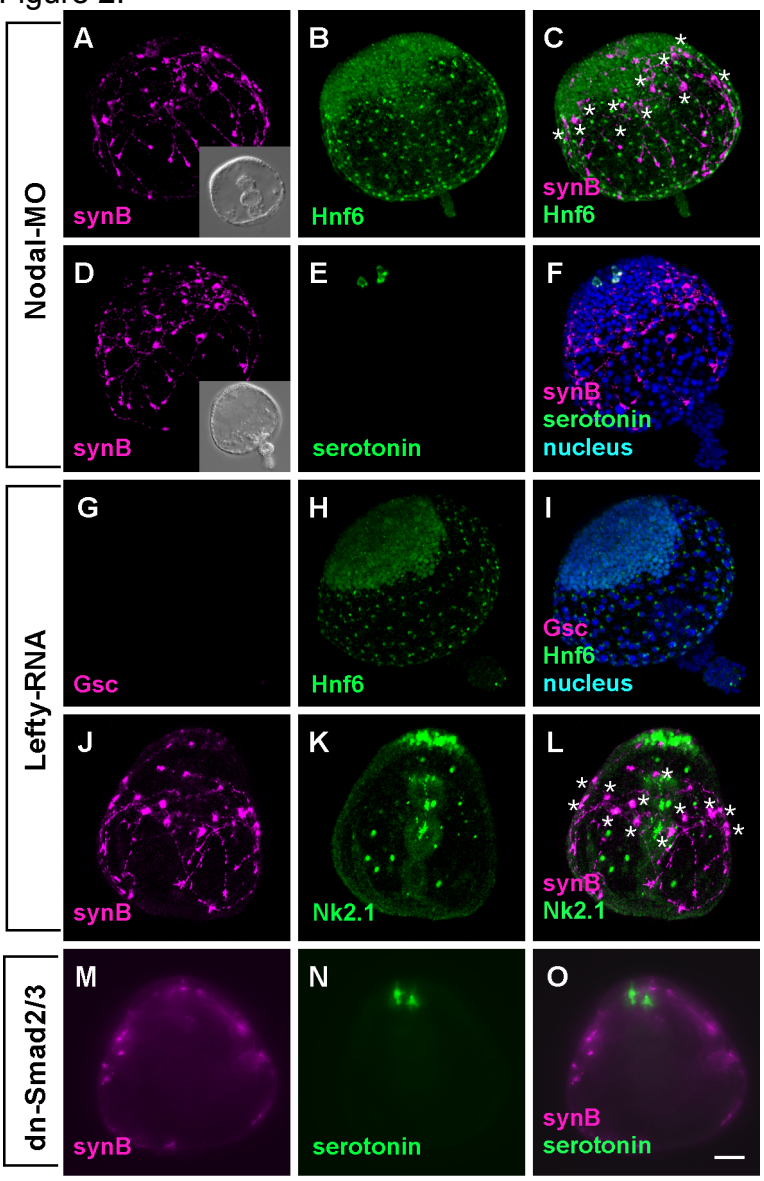
K
synB
Hnf6
L
synB
M
serotonin
N
synB
serotonin
nucleus

Figure 2.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synB
B
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C
synB
Hnf6
D
synB
E
serotonin
F
synB
serotonin
nucleus
G
Gsc
H
Hnf6
I
Gsc
Hnf6
nucleus
J
synB
K
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L
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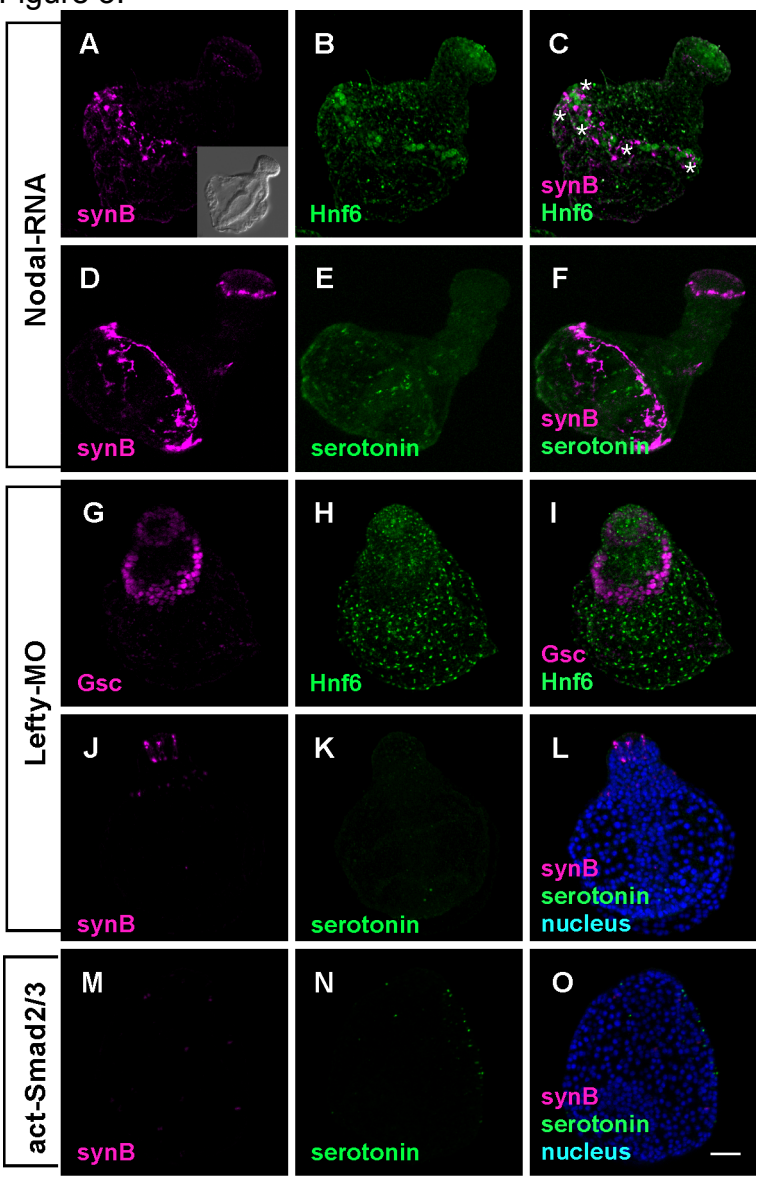
M
synB
N
serotonin
O
synB
serotonin

Figure 3.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Lefty-MO
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synB
B
Hnf6
C
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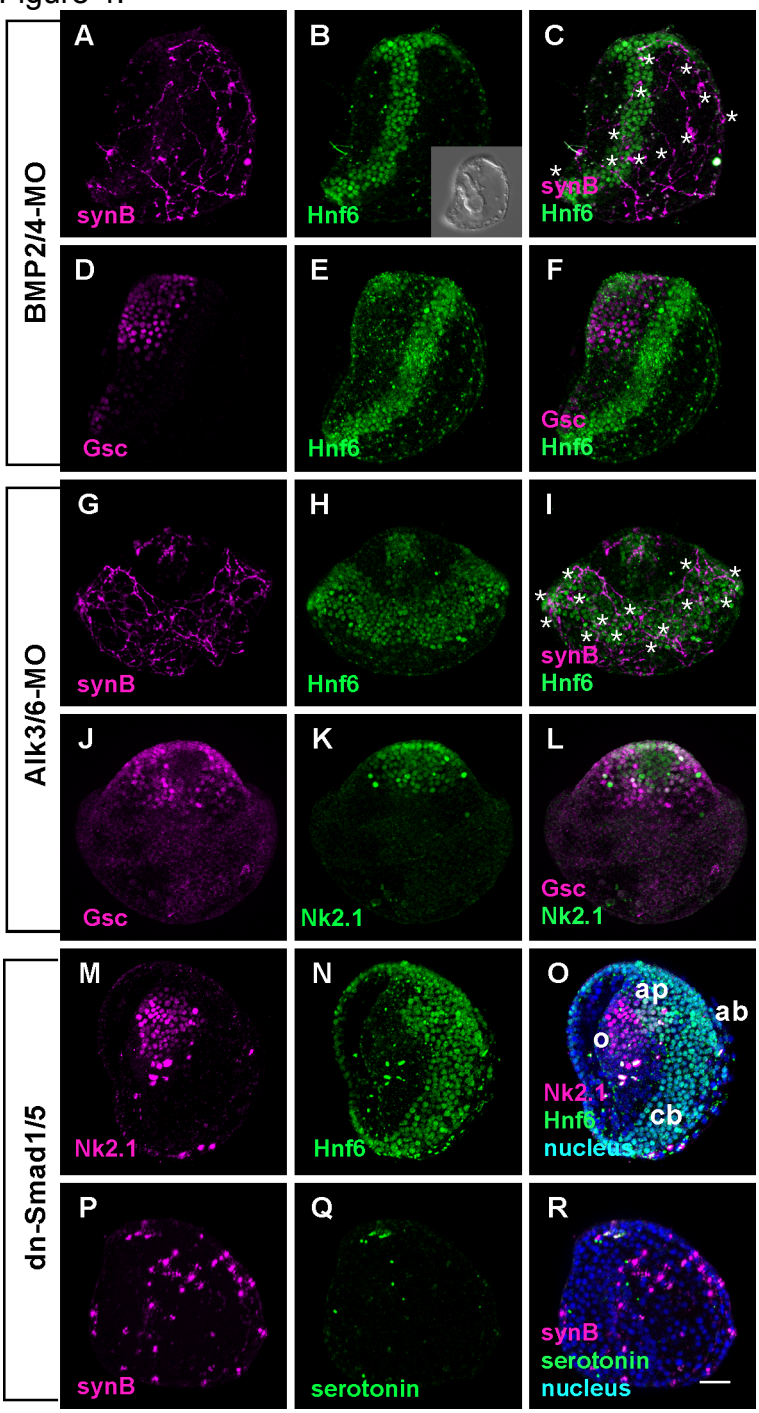
Hnf6
D
synB
E
serotonin
F
synB
serotonin
G
Gsc
H
Hnf6
I
Gsc
Hnf6
J
synB
K
serotonin
L
synB
serotonin
nucleus
M
synB
N
serotonin
O
synB
serotonin
nucleus

Figure 4.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synB
B
Hnf6
C
synB
Hnf6
D
Gsc
E
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F
Gsc
Hnf6
Alk3/6-MO
G
synB
H
Hnf6
I
synB
Hnf6
J
Gsc
K
Nk2.1
L
Gsc
Nk2.1
dn-Smad1/5
M
Nk2.1
N
Hnf6
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ap
ab
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cb
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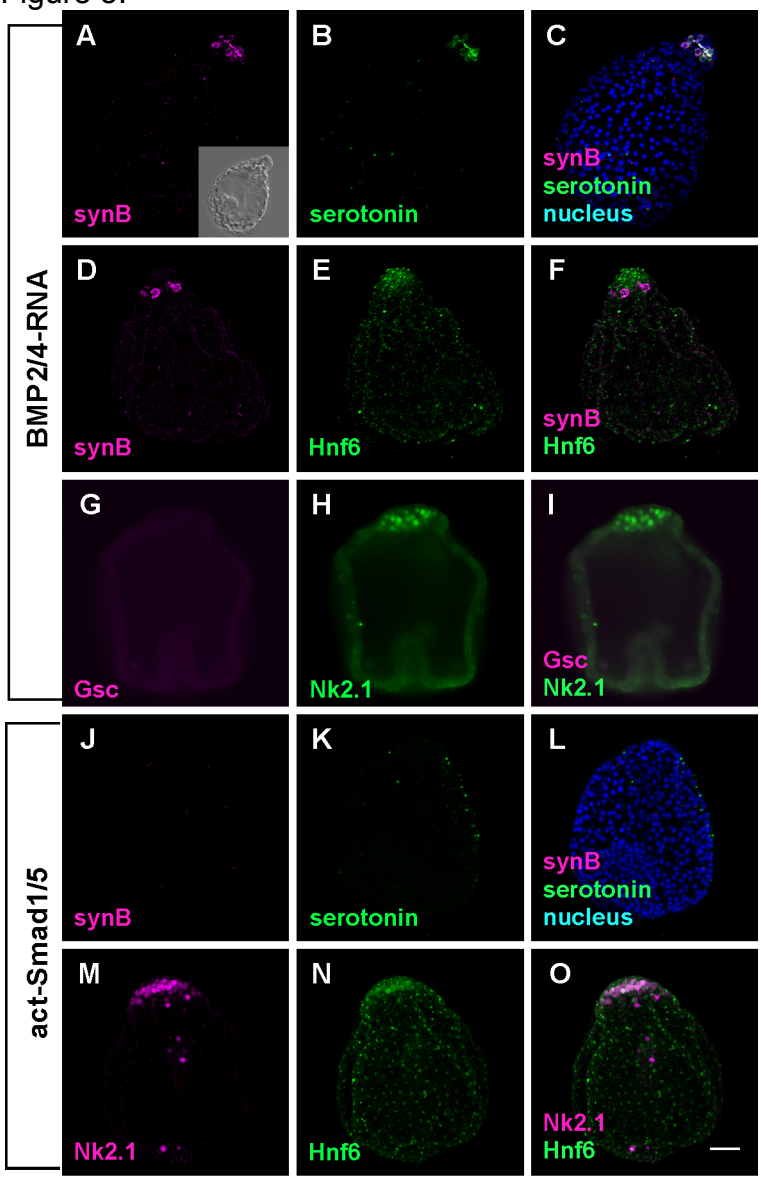
serotonin
R
synB
serotonin
nucleus

Figure 5.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G
Gsc
H
Nk2.1
I
Gsc
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J
synB
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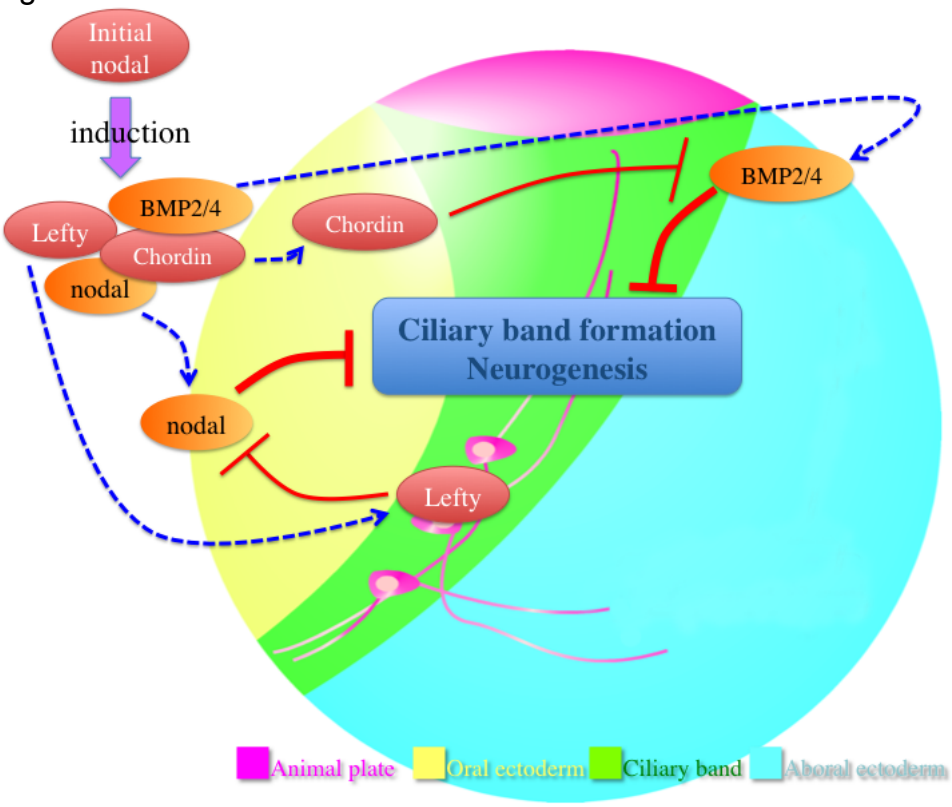
Hnf6

Figure 6.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BMP2/4
Lefty
Chordin
nodal
Chordin
BMP2/4
Ciliary band formation
Neurogenesis
nodal
Lefty
Animal plate
Oral ectoderm
Ciliary band
Aboral ectoderm