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Zinc finger homeobox is required for the differentiation of serotonergic neurons in the sea urchin embryo

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Abstract

Serotonergic neurons differentiate in the neurogenic animal plate ectoderm of the sea urchin embryo. The regulatory mechanisms that control the specification or differentiation of these neurons in the sea urchin embryo are not yet understood, although, after the genome was sequenced, many genes encoding transcription factors expressed in this region were identified. Here, we report that *zinc finger homeobox* (*zfhx1/z81*) is expressed in serotonergic neural precursor cells, using double *in situ* hybridization screening with a serotonergic neural marker, *tryptophan 5-hydroxylase* (*tph*) encoding a serotonin synthase that is required for the differentiation of serotonergic neurons. *zfhx1/z81* begins to be expressed at gastrula stage in individual cells in the anterior neuroectoderm, some of which also express *delta*. *zfhx1/z81* expression gradually disappears as neural differentiation begins with *tph* expression. When the translation of Zfhx1/Z81 is blocked by morpholino injection, embryos express neither *tph* nor the neural marker *synaptotagminB* in cells of the animal plate, and serotonergic neurons do not differentiate. In contrast, Zfhx1/Z81 morphants do express *fez*, another neural precursor marker, which appears to function in the initial phase of specification/differentiation of serotonergic neurons. In addition, *zfhx1/z81* is one of the targets suppressed in the animal plate by anti-neural signals such as Nodal as well as Delta-Notch. We conclude that Zfhx1/Z81 functions during the specification of individual anterior neural precursors and promotes the expression of *tph* and *synaptotagminB*, required for the differentiation of serotonergic neurons.

Introduction

The presence of serotonergic neurons in anterior neuroectoderm, as in a brain or an apical organ, is conserved in all metazoans except for sponges and ctenophores (Hay-Schmidt, 2000). Although a number of previous studies have revealed some of the regulatory mechanisms involved in serotonergic neuron development (reviewed in Cordes, 2005), the whole pathway from specification to terminal differentiation still needs to be elucidated,

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especially in invertebrates. Because the regulatory state of ectoderm in absence of signals supports neural differentiation in vertebrates and sea urchin embryos (Levine and Brivanlou, 2007: Tropepe et al., 2001; Vallier et al., 2004; Watanabe et al., 2005), researchers have focused more on the mechanisms of how this state is protected from anti-neural signals like BMP (De Robertis and Kuroda, 2004; Bradham et al., 2009; Lapraz et al., 2009; Yaguchi et al., 2010a). However, in order to understand how specific neurons differentiate within the neuroectoderm, it is important to decipher the underlying regulatory mechanisms that promote it.

In sea urchin embryos, the two early neurogenic ectoderm territories are the anterior neuroectoderm, which includes animal plate and adjacent cells, and the ciliary band ectoderm (reviewed in Angerer et al., 2011). Each of these is specified separately and patterned by combined functions of maternal factors and different zygotic signaling molecules. Under the control of those factors, a number of neurons differentiate at specific locations in each region. The first neurogenic territory to be specified is the anterior neuroectoderm. Within this region, serotonin-positive neurons appear at the aboral edge of animal plate of late gastrula (Bisgrove and Burke, 1986; 1987). They progressively increase in number and at pluteus stage their axons extend to form a plexus (Yaguchi et al., 2000). In embryos, in which all signals are shut down by injecting Δcadherin or discarding the vegetal half (Logan et al., 1999; Wikramanayake and Klein, 1997; Duboc et al., 2004), most of the prospective ectoderm becomes the animal plate and consequently many serotonergic neurons differentiate throughout it but, unlike in the normal embryo, they are scattered without any orderly pattern (Yaguchi et al., 2006). These findings suggest that the state of sea urchin embryo blastomeres in the absence of Wnt/β-catenin or Nodal/BMP2/4 signaling supports differentiation of anterior neuroectoderm, which contains the animal plate. Subsequently Wnt/β-catenin signals convert blastomere fates to endoderm, mesoderm and, within the ectoderm, eliminates anterior neuroectoderm fates except at the animal pole.

After the animal plate is restricted to the animal pole at early blastula stage, the differentiation of serotonergic neurons is prevented on the oral side by Nodal signals. In contrast to the process of ciliary band formation (Yaguchi et al., 2010a), Nodal is not involved in the specification of the animal plate (Yaguchi et al., 2006) but in patterning the region along oral-aboral axis (Yaguchi et al., 2007). In the absence of Nodal signaling, serotonergic neurons develop radially around the animal plate, while in its presence they are restricted to the aboral edge (Yaguchi et al., 2006, 2007). However, it is yet unclear how this patterning leads to serotonergic neurons differentiating only at the aboral edge of the animal plate. Here we show that Zinc finger homeobox (Zfhx1/Z81) is the earliest known transcription factor to be expressed specifically in individual serotonergic neural precursor cells in the animal plate, to be required for their differentiation and to be repressed on the oral side by Nodal signaling. Furthermore, it is co-expressed with Delta and repressed by Delta/Notch-mediated lateral inhibition. We show that Zfhx1/Z81 is required for synthesis of serotonin and that it depends on FoxQ2, which is essential for animal plate formation. This work establishes an important layer of regulatory control for the development and precise patterning of serotonergic neurons in the anterior neurogenic ectoderm of sea urchin embryos.

Materials and Methods

Animals and embryo culture

Embryos of *Hemicentrotus pulcherrimus* collected around Shimoda Marine Research Center, University of Tsukuba, and around Marine and Coastal Research Center, Ochanomizu University were used. The gametes were collected by intrablastocoelar

injection of 0.5 M KCl and the embryos were cultured by standard methods with filtered natural seawater (FSW) at 15 °C.

Whole-mount *in situ* hybridization and immunohistochemistry

Whole-mount *in situ* hybridization was performed as described previously (Minokawa et al., 2004; Yaguchi et al., 2010b). Immunohistochemistry for detecting serotonin, synaptotagminB (synB), and c-myc was performed as described previously (Yaguchi et al., 2006). The primary antibodies were detected with secondary antibodies conjugated with Alexa-568 and Alexa-488 (Life Technologies, Carlsbad, CA, USA). The specimens were observed with a Zeiss Axio Imager.Z1 equipped with Apotome system, and optical sections were stacked and analyzed with ImageJ and Adobe Photoshop. Panels and drawings for figures were made with Microsoft PowerPoint.

Microinjection of morpholino antisense oligonucleotides (MO)

Microinjection into fertilized eggs and one blastomere of two-cell stage were performed as described previously (Yaguchi et al., 2006; Yaguchi et al., 2010b). We used the following morpholinos (Gene Tools, Philomath, OR, USA) at the indicated concentrations in 24% glycerol in injection needles: Two different morpholinos blocking expression of Zfhx1/Z81 [Zfhx1/Z81-MO1 (2.0 mM), Zfhx1/Z81-MO2 (1.9–3.8 mM)] were used to confirm the specificity of Zfhx1/Z81 function. The phenotypes obtained with FoxQ2-MO (200 μM; Yaguchi et al., 2010b), Delta-MO (2.0 mM), Nodal-MO (200 μM; Yaguchi et al., 2010b), Lefty-MO (400 μM; Yaguchi et al., 2010b), BMP2/4-MO (400 μM; Yaguchi et al., 2010b) were the same as published previously in *H. pulcherrimus* or other species (Duboc et al., 2004; Duboc et al., 2008; Yaguchi et al., 2008; Lapraz et al., 2009). The morpholino sequences were the following: Zfhx1/Z81-MO1: 5'-ACGTAGGTATGTTCCAAAACACAAG -3', and Zfhx1/Z81-MO2: 5'-CAGAAGGCAGAGTCCCACAGTCCCA -3'. mRNAs were synthesized from linearized plasmids using the mMessage mMachine kit (Life Technologies, Carlsbad, CA, USA), and injected at the indicated concentrations in 24% glycerol: Δ-cadherin (0.3–0.6 μg/μl; Logan et al., 1999), myc-mRNA (0.1 μg/μl).

Results

Expression of *zfhx1/z81* during development

During the annotation of the sea urchin genome sequence (Sodergren et al., 2006), the spatial patterns of expression of a number of predicted genes encoding putative transcription factors were determined. Among those that were expressed in the anterior neuroectoderm (ANE) was one encoding a zinc finger-containing protein, called Z81 (Materna et al., 2006). Further studies showed that its expression in the ANE depended on Six3, a factor required for neural development (Wei et al., 2009). This gene (Z81; SPU_022242) was initially annotated as zfh-1 (Sodergren et al., 2006) and has subsequently been called Smad Interacting protein, Sip1 or SmadIP (Saudemont et al., 2010) or SpSip1 (Su et al., 2009). As shown below, we confirmed previously reported expression patterns in other species (Howard-Ashby et al., 2006; Materna et al., 2006; Saudemont et al., 2010) in *Hemicentrotus pulcherrimus* and observed that this gene is expressed in individual cells of the ANE arranged in a pattern suggesting they could be serotonergic precursors (Fig. 1A, B). Because revealing the transcription factor activities required for specification or differentiation of serotonergic neurons in sea urchin embryos is the primary goal, we selected this gene for further study. We cloned and sequenced it using a Japanese sea urchin, *H. pulcherrimus,* employed 5'RACE to determine the 5' end of the ORF (accession number: AB630322), and found that it lacks the first two exons included in the predicted sequence, SPU_022242. We analyzed its phylogenetic position in detail and found that the gene belongs to the E-box

binding zinc finger protein family including delta-EF and smad-interacting protein1 (SIP1). Based on the phylogenetic tree, it belongs to neither of these but is very closely related to non-vertebrate zinc finger homeobox proteins (Saccoglossus-Zfhx and Amphioxus-Zfhx: Fig. 1C, supplemental Fig. 1). Among the 4 classes of vertebrate Zfhx proteins, this non-vertebrate, deuterostome group type is more closely related to Zfhx1 (Delta-EF; ZEB1) and Zfhx2 (SIP1; ZEB2) than to Zfhx3 and Zfhx4. Among other invertebrate proteins, Fly-Zfh-1 and C. elegans Zag-1 are the closest. Therefore, we named it Hp-Zfhx1/Z81 (Zfhx1/Z81 hereafter in this paper).

zfhx1/z81 is not expressed maternally (Wei et al., 2006), but just before embryo hatching, the mRNA appears in a broad region except at the vegetal plate, which expresses *foxA* (Fig. 1D, E). The function of Zfhx1/Z81 at this early time is discussed in elsewhere (Su et al., 2009). Expression in this domain disappears when the embryo hatches (Fig. 1F), and appears in a new set of cells in the endomesoderm region at mesenchyme blastula stage (Fig. 1G). Adding to the vegetal expression, when the gut begins to invaginate, *zfhx1/z81* is expressed in a few cells in the animal plate region as well as a few cells in the lateral ectoderm, where the lateral ganglion will form (Fig. 1H, arrows and arrowheads, respectively; Howard-Ashby et al., 2006). At later stages, *zfhx1/z81* is expressed in a pattern like that of the future ciliary band neurons (Fig. 1J–L; most clearly revealed in the fluorescent *in situ* hybridization in panel K)(Bisgrove and Burke, 1986; Nakajima et al., 2004). Here we focus only on *zfhx1/z81* expression in the animal plate because the pattern of its expression is similar to that of serotonergic neurons (Fig. 1A). At the prism stage, *zfhx1/z81* continues to be expressed in similar regions as those in gastrulae, but disappears from the central part of the animal plate (Fig. 1I, between arrows). In pluteus larvae, the gene expression patterns of the ciliary band are the same as those in prism stage, and lower lip cells and mesenchymal cells at the vertex begin to express *zfhx1/z81* (Fig. 1J–L; black and red arrow, respectively). In contrast, the expression in animal plate region begins to disappear at this stage (Fig. 1L, bracket).

zfhx1/z81 expression is transient in neural precursor cells, disappearing after *tryptophan 5-hydroxylase* expression begins

To investigate when and where *zfhx1/z81* is expressed in the animal plate region in detail, we performed double fluorescent *in situ* hybridization detecting *zfhx1/z81* and *tryptophan 5-hydroxylase* (*tph*), which encodes the rate-limiting enzyme in serotonin synthesis and therefore is a differentiation marker specific for serotonergic neurons in the sea urchin embryo (Yaguchi and Katow, 2003). *zfhx1/z81*-expressing cells in the animal plate (as described in Figure 1) begin to express *tph* at late gastrula stage (36 hours post fertilization (hpf); Fig. 2A–D, arrows). This indicates that *zfhx1/z81* is expressed in serotonergic neural precursor cells. However, although these neural precursors express both genes at 36-hpf (Fig. 2A–D), at 39-hpf most of them lack *zfhx1/z81* transcripts (Fig. 2E–H, arrowheads), suggesting that *zfhx1/z81* expression precedes *tph*. At this stage, a cell appears which expresses *zfhx1/z81* strongly but *tph* weakly and is likely to be a new serotonergic precursor cell (Fig. 2E–H, asterisk). Next, we compared distributions of *zfhx1/z81* and *fez*, *forebrain embryonic zinc finger*, which we recently reported as being expressed in the entire animal plate during blastula stages and subsequently in serotonergic neurons and their precursors (Yaguchi et al., 2011). When the blastula-stage expression of *fez* begins to fade and is progressively replaced by stronger signals in a few individual cells in the animal plate region at mid-gastrula stage (Fig. 2K), *zfhx1/z81* mRNA is present in the same cells (Fig. 2I–L, arrows). Afterward, *zfhx1/z81* transcripts disappear by the prism stage, whereas *fez* mRNA remains in the serotonergic neurons (Fig. 2M–P, arrowheads). Taken together, *zfhx1/z81* is expressed in neural precursors at beginning of gastrulation and disappears soon after these cells begin to differentiate, as indicated by *tph* expression at late gastrula stage.

Zfhx1/Z81 is required for the differentiation of serotonergic neurons

The spatial and temporal expression pattern of *zfhx1/z81* suggests that it might be involved in the specification and/or differentiation of serotonergic neurons in the sea urchin embryo. To examine this, we blocked the translation of *zfhx1/z81* by injecting morpholino anti-sense oligonucleotide (MO; Zfhx1/Z81-MO represents Zfhx1/Z81-MO2 throughout this study otherwise indicated). In embryos injected with Zfhx1/Z81-MO at 2 mM, gastrulation is delayed (Fig. 3F) and their body size becomes smaller than normal (Fig. 3A–C, F–H). The number of serotonergic neurons decreases in morphants, but those that do form still extend axons to form a complex in the animal plate region as they do normal embryos (Fig. 3D, E, I, J). Although serotonergic neurons do not appear in 3.8 mM Zfhx1/Z81-MO-injected embryo as well as in the 2.0 mM Zfhx1/Z81-MO1-injected embryo (data not shown), it is unclear whether this effect results directly from blocking Zfhx1/Z81 function in neural precursor cells or because of indirect effects that drastically delay gastrulation and lead to ectoderm patterning defects, including loss of oral-aboral polarity (Fig. 3K–O). Indirect effects are possible because *zfhx1/z81* is expressed broadly in ectoderm early (Saudemont et al., 2010) and then in animal and vegetal cells (Howard-Ashby et al., 2006) and is thought to play a role in oral-aboral polarity (Su et al., 2009) (also see Fig. 1),

To eliminate possible indirect effects, we examined Zfhx1/Z81 function in two types of embryos that lack vegetal signals that are necessary for endomesoderm development and for Nodal expression that regulates oral-aboral polarity. These are embryos either injected with Δcadherin (Δcad) (Logan et al., 1999; Wikramanayake et al., 1998; Yaguchi et al., 2008) or lacking the vegetal half starting from 8-cell or 16-cell stages (Wikramanayake et al., 1995; Yaguchi et al., 2006; Yaguchi et al., 2008). These two types of embryos are thus far not detectably different as monitored by gene expression and responses to experimental perturbations (Logan et al., 1999; Yaguchi et al., 2006; Yaguchi et al., 2007; Yaguchi et al., 2008; Sasaki and Kominami, 2008). In Δcadherin-injected embryos, the expanded animal plate contains a greatly increased number of serotonergic neurons as reported previously (Yaguchi et al., 2006). As expected, *zfhx1/z81*-expressing cells are scattered throughout the expanded animal plate of these embryos at 24-hpf (Fig. 4B, C). As development proceeds, the number of *zfhx1/z81*-positive cells gradually decreases, as observed in normal embryos (Fig. 4A–D), especially, in the central part of the expanded animal plate where *foxQ2* is strongly expressed (Fig. 4E). At 2 days after fertilization, the Δcad-injected embryo lacks *zfhx1/z81* expression in individual cells completely (Fig. 4F). Therefore, the expression patterns of *zfhx1/z81* in the expanded animal plate reflect the behavior of *zfhx1/z81* in normal embryos. If Zfhx1/Z81 is knocked down in these embryos, development of serotonergic neurons is strongly inhibited (3.8 mM Zfhx1/Z81-MO2 injection; Fig. 4J–L). This morpholino effect is confirmed by injecting 2.0 mM Zfhx1/Z81-MO1 (data not shown). This is also true in animal-half embryoids (Fig. 4M, O), because loss of Zfhx1/Z81 completely eliminates the large number of serotonergic neurons normally present in them (Yaguchi et al., 2006) (Fig. 4N; *cf*. with G). To confirm that the requirement for Zfhx1/Z81 for serotonergic neuron differentiation is cell-autonomous, Zfhx1/Z81-MO and mRNA encoding 5 myc epitopes as a lineage tracer were injected into one blastomere of 2-cell embryos already containing Δcad-mRNA (Fig. 4P). In these embryos, the serotonergic neurons differentiate normally in the myc-negative, Zfhx1/Z81-positive side but not in the myc-positive, Zfhx1/Z81-negative region (Fig. 4Q, R). The lack of serotonergic neurons at the border of first cleavage plane next to Zfhx1/Z81-positive cells strongly supports the idea that Zfhx1/Z81 is not required for even short-range signals promoting serotonergic neuron differentiation, but rather acts cell-autonomously. Together, these results indicate that Zfhx1/Z81 is required for the differentiation of serotonergic neurons in the anterior neuroectoderm.

Zfhx1/Z81 is required for the expression of *tph* but not early neuronal genes

To examine at which step Zfhx1/Z81 is involved during the specification and differentiation of serotonergic neurons, we examined Zfhx1/Z81 morphants for expression of *foxQ2*, normally in all cells of the animal plate, *tph*, and *fez*, an early serotonergic neural marker (Yaguchi et al., 2011). We again used Δcad-injected embryos to eliminate indirect effects caused by Zfhx1/Z81 functions at earlier stages in other regions of the embryo. In Δcad-injected Zfhx1/Z81 morphants *foxQ2* is expressed throughout the expanded animal plate as in control Δcad alone-injected embryos (*cf.* Fig. 5A with B) but *tph* is not expressed at all (Fig. 5B), indicating that Zfhx1/Z81 is required for *tph* expression but not for *foxQ2*. As well, *fez*, another serotonergic neural marker, is expressed in Δcad-injected Zfhx1/Z81 morphants as in control embryos, indicating that Zfhx1/Z81 is not required for neuron-specific expression of *fez* (Fig. 5C, D). Conversely, *zfhx1/z81* expression does not require Fez (Supplemental Figure 2), indicating that these two genes, while co-expressed in individual cells at the animal plate of early gastrulae, function in parallel pathways. As shown in Figure 2, *zfhx1/z81* transcripts gradually start to disappear from the animal plate in control Δcad alone-injected embryos (Fig. 5E). However, intriguingly in Δcad-injected Zfhx1/Z81 morphants, *zfhx1/z81* transcripts remain (Fig. 5F), indicating that *zfhx1/z81* is regulated by auto-repression mechanism in these embryos (Fig. 5G, H). These results support the temporal expression data (Fig. 1, 2), which suggests that *zfhx1/z81* and *fez* transcripts appear after *foxQ2* is expressed, but before the serotonin synthase tryptophan 5-hydroxylase gene, *tph*. Although both *zfhx1/z81* and *fez* depend on FoxQ2 and are co-expressed in cells in the *foxQ2*-positive animal plate (see below, Fig. 7), they have independent roles in these serotonergic precursors, since Zfhx1/Z81 is required for differentiation of these neurons while Fez is not (Yaguchi et al., 2011).

It has been supposed that Delta functions in neurogenesis in the sea urchin embryo based on its expression pattern in ectoderm (Röttinger et al., 2006; Lapraz et al., 2009; Saudemont et al., 2010) and the fact that DAPT, which inhibits Notch signaling and lateral inhibition, results in significant increases in neuron number (Wei et al., 2011; Yaguchi et al., 2011). Further support that it is Delta that mediates lateral inhibition in the anterior neuroectoderm through Notch signaling is that a cluster of contiguous serotonergic neurons develops on the aboral side of the animal plate (Fig. 6C–C’’’), exactly as observed previously in DAPT-treated embryos (Yaguchi et al., 2011). These facts suggest that *delta* is specifically expressed in neural precursors in sea urchin embryos and could be co-expressed with *zfhx1/z81*. This is in fact the case since fluorescent double *in situ* hybridizations showed that it is co-expressed with *zfhx1/z81* in serotonergic neuron precursors in the animal plate (Fig. 6D–H; stacks of a few optical sections). In contrast, *delta* is not expressed in differentiating *tph*-positive neurons (data not shown). Taken together, these results show that, in animal plate neurons, transient expression of *delta* and *zfhx1/z81* is followed by *tph*.

To establish regulatory relationships between FoxQ2, Delta and Zfhx1/Z81, we carried out a series of morpholino-mediated knock-downs. In FoxQ2 morphants, in which serotonergic neurons fail to differentiate, neither *delta* nor *zfhx1/z81* is expressed in the animal plate region (Fig. 6I–L, arrows). In contrast, both genes are expressed in lateral regions, as expected, since FoxQ2 is not expressed at these sites. Thus, animal plate expression of *delta* and *zfhx1/z81* requires FoxQ2 function. When the translation of *delta* is blocked by injecting Delta-MO, *zfhx1/z81*-positive cells increase in number and are immediately adjacent to each other, making a cluster in the animal plate region (*cf.* Fig. 6A with B; stacks of a few optical sections), as do serotonergic neurons (Fig. 6C–C’’’). These data suggest that Delta functions to inhibit neighboring cells, but not its own expressing cells, from differentiating as Zfhx1/Z81-expressing serotonergic neuronal precursors. Delta expression in animal plate cells does not require Zfhx1/Z81 because it is expressed in the same scattered pattern as serotonergic neurons in Δcad-injected embryos that either contain or lack Zfhx1/Z81 (Fig. 6M, N). Taken

together, Zfhx1/Z81 appears in animal plate cells during gastrulation where it is required for *tph* expression and subsequent serotonin synthesis, but not for the early regulatory genes like *foxQ2*, *fez* and *delta*.

Nodal signaling suppresses *zfhx1/z81* expression

Previous studies showed that serotonergic neurons differentiate only at the aboral/lateral edge of the animal plate, and this asymmetry is caused by Nodal signaling from cells on the oral side of the plate (Fig. 7F; Yaguchi et al., 2007). As expected, in normal embryos, *zfhx1/z81* is also expressed in cells at the aboral/lateral edge of the *foxQ2*-positive animal plate region at gastrula stage (Fig. 7A, B), and at prism and pluteus stages the serotonergic neurons expressing *tph* gene are aligned similarly (Fig. 7G). When the translation of Nodal is blocked by injecting Nodal-MO, *zfhx1/z81*- and *tph*-positive cells surround the animal plate (Fig. 7C, asterisks; 7H, respectively). In contrast, when Nodal signaling is enhanced and extends to the aboral side of the animal plate (Duboc et al., 2004; Duboc et al., 2008) by blocking the translation of Lefty, an endogenous antagonist of Nodal signaling, neither *zfhx1/z81* nor *tph* is expressed in the animal plate (Fig. 7D, I). When translation of BMP2/4, another TGF-β member involved in cell fate specification along the aboral side of the embryo, is blocked, the morphants also do not express *zfhx1/z81* and *tph* (Fig. 7E, J). In these morphants Nodal signaling extends further to the aboral side (Yaguchi et al., 2010a), where it suppresses expression of *zfhx1/z81* and differentiation of serotonergic neurons. Taken together, Nodal signals in the oral ectoderm suppress the expression of *zfhx1/z81* and subsequently *tph*, leading to development of serotonergic neurons only on the aboral edge of the animal plate.

Discussion

The data presented here show that Zfhx1/Z81 is required cell-autonomously for the differentiation of serotonergic neurons in sea urchin embryos. Most of the transcription factors expressed early throughout the animal plate are required for the specification and differentiation of this territory (Yaguchi et al., 2008; Wei et al., 2009). When the function of those genes is blocked, the animal plate is lost as are the neurons that develop within it as well as the apical tuft (Yaguchi et al., 2010b). Therefore, it was not clear how these early regulatory activities were connected to the specification of individual neurons expressing the terminal differentiation genes, *tph* and *synptotagminB*, at late gastrula stage (Yaguchi and Katow, 2003; Burke et al., 2006). Here we show that Zfhx1/Z81 is one of the intermediate factors downstream of genes specifying the early animal plate and upstream of those sponsoring terminal differentiation of serotonergic neurogenesis. Knock-down of either FoxQ2 or Zfhx1/Z81 significantly decreases the number of serotonergic neurons (Yaguchi et al., 2008; this study) and FoxQ2 morphants do not express *zfhx1/z81*. Furthermore, *zfhx1/z81* is co-expressed with *delta* at early gastrula stage, the first direct demonstration that *delta* is expressed in neural cells in the animal plate of sea urchin embryos. As in other embryos, we show here that Delta functions in neuronal precursors to limit the number of cells in the animal plate that differentiate as neurons through lateral inhibition. Thus, Delta and Zfhx1/Z81 mark neuronal precursors. As well, the expression pattern and timing of *zfhx1/z81* relative to terminal differentiation genes is appropriate for its requirement for the differentiation of serotonergic neurons. Zfhx1/Z81 could be a direct activator of *tph* since it is co-expressed with *tph* as serotonergic neurons begin to differentiate. In contrast, *delta* and *tph* are rarely co-expressed in normal embryos, consistent with the sequential waves of expression of *delta*, *zfhx1/z81* and *tph*. Together, the expression patterns and loss-of-function data indicate that FoxQ2 is required for *delta* and *zfhx1/z81* expression in neuronal precursors. Delta/Notch signaling limits the number of these precursors and Zfhx1/Z81 then

is required of expression of genes necessary for the terminal differentiation of serotonergic neurons.

The results reported here indicate that Nodal signaling-mediated suppression of serotonergic neural differentiation on the oral side of the animal plate (Yaguchi et al., 2007) must occur downstream of FoxQ2 and at or upstream of *zfhx1/z81* expression because here we show that Nodal suppresses *zfhx1/z81* expression, but has no detectable effect on *foxQ2* expression. Thus, this work fills an important gap in our understanding of the regulatory path that links specification of the neurogenic field to the differentiation of individual neurons in sea urchin embryos.

Zfh/ZEB family members have a characteristic molecular structure; N- and C-terminal zinc finger domains and a central homeodomain (Fortini et al., 1991; Genetta et al., 1994). It has been reported that these transcription factors bind to E-boxes and have been shown to play a role in regulating myogenesis in vertebrates and invertebrates (Postigo et al., 1999). In addition, the vertebrate-type family of ZEB factors includes branches to delta-EF1 and SIP1. They attenuate BMP signaling with Smad-interacting activity (Postigo, 2003), and the Smad-binding domain (SBD) in SIP1 has been already identified (Verschueren et al., 1999). In contrast, the amino acid sequence alignment shows the sea urchin Zfhx1/Z81 as well as fly Zfh-1 have no conserved SDB sequence (supplemental Fig. 1). Although it was annotated as SIP1 after the sea urchin genome was sequenced (Su et al., 2009; Saudemont et al., 2010), there is no evidence that it interacts with the Smad family; instead our phylogenetic analysis suggests that this gene, SPU_022242, does not belong to the SIP1 branches but is most closely related to the invertebrate-type ZEB member, Zfhx (Fig. 1).

In flies and worms, Zfh-1 and Zfh-2 were reported to possess both zinc fingers and homeodomains, and both are expressed in the nervous system. Zfh-2 contains 17 zinc-finger domains and 3 homeodomains, and in *Drosophila* it binds to a regulatory region of the *DOPA decarboxylase* gene, which is essential for the second step of biosynthesis of dopamine and serotonin (Lundell and Hirsh, 1992). The homolog of vertebrate *zfh-2* in sea urchins is *atbf1* (SPU_017348), suggesting that Zfhx-1, the gene studied here, and Zfh-2 also have different functions in the sea urchin. The function of Zfh-1 in flies is not well understood but it is expressed in the serotonergic lineage in their central nervous system where its expression is regulated by Notch signaling and Eagle transcription factor (Lai et al., 1991; Lee and Lundell, 2007). In *C. elegans*, a homolog of Zfh-1, Zag-1, is expressed several neuronal lineages including those leading to head and tail ganglia, dorsal and ventral cords, and some of them express *tph* and synthesize serotonin (Sze et al., 2002; Wacker, et al., 2003). Among those serotonergic neurons, the HSN serotonergic motor neurons require Zag-1 for expression of *tph* (Clark and Chiu, 2003). However, because *tph* expression in the head region is not affected in *zag-1* mutants, the function of Zfh-1/Zag-1 in the serotonergic neuron-lineage in the anterior neuroectoderm of an ecdysozoan invertebrate differs from the role of Zfhx-1 in this region of sea urchin embryos. Whether Zfhx proteins are involved in development of serotonergic neurons in other deuterostomes is not yet known, although predictions from genome sequences of hemichordate and amphioxus reveal that they have the same invertebrate-type Zfhx (XM_002740578.1; XM_002592121.1, Putnam et al., 2008),

A diagram summarizing the mechanism and timing of Zfhx1/Z81 function is presented in Figure 8. At the beginning of neurogenesis in the animal plate of the sea urchin embryos, FoxQ2 and Six3 are required for formation of the animal plate and expression of downstream genes like *fez* and *nk2.1*, which are expressed uniformly in this territory (Yaguchi et al., 2011; Yaguchi et al., 2008; Wei et al., 2009). Whereas Nk2.1 is involved in formation of the long immotile cilia of the apical tuft, (Dunn et al., 2007; Yaguchi et al.,

2010b), Fez functions in controlling animal plate size and ultimately the number of serotonergic neurons, but is not required for nerve cell differentiation itself (Yaguchi et al., 2011). *delta* is expressed in neural precursors in the animal plate starting at late mesenchyme blastula stage and Delta signals through Notch to neighboring cells preventing their differentiation to serotonergic neurons. Shortly thereafter, *zfhx1/z81* and *fez* are expressed in these neural precursors. However, the expression of these three genes, *delta*, *zfhx1/z81* and *fez*, is regulated by independent mechanisms because knock-downs of each does not affect the expression of other two (Fig. 5, 6; supplemental Fig. 2; Yaguchi et al., 2011).

At least, three independent signaling cascades regulate the differentiation of serotonergic neurons: Wnt/β-catenin positions the animal plate at the anterior end of the embryo where serotonergic neurons develop and Delta/Notch and Nodal determine, respectively, the number and position of these neurons. *zfhx1/z81* expression exclusively in serotonergic neuron precursors in the animal plate depends on at least one or two positive inputs (FoxQ2 and Six3), and three negative inputs (Nodal, Notch and Zfhx1/Z81 itself). *zfhx1/z81* expression depends on Six3 (Wei et al., 2009) and FoxQ2 (this work). The fact that Six3 is important for maintaining *foxQ2* (Wei et al., 2009), may explain these observations (Fig. 6). Although FoxQ2 could provide direct inputs into regulating *zfhx1/z81* transcription, this would occur well after initial formation of the animal plate. Furthermore, it is clearly not sufficient to control its spatial pattern since *zfhx1/z81* is expressed in only a subset of animal plate cells. The mechanism that activates expression of *zfhx1/z81* and *delta* in this subset is not yet understood. Negative regulation of serotonergic neural development by Nodal from the oral side or by Delta/Notch-mediated lateral inhibition in the animal plate acts at or upstream of *zfhx1/z81*. Finally, Zfhx1/Z81-mediated negative auto-regulation of *zfhx1/z81* transcription implies tight regulation of Zfhx1/Z81 levels is required in these neural cells. All of these mechanisms help to ensure *zfhx1/z81* expression in a few neural precursors on the aboral side of the animal plate, where it activates expression of genes required for serotonergic differentiation. The regulatory relationships established here provide an important framework for the eventual construction of the serotonergic neural gene regulatory network in the sea urchin embryo.

Highlights

> Serotonergic neurons differentiate at aboral edge of the anterior neuroectoderm in sea urchin embryos. > *zinc-finger homeobox* (*zfhx1/z81*) is co-expressed with serotonin synthase gene, *tryptophan 5-hydroxylase* (*tph*). > Zfhx1/Z81 is required for *tph* expression. > *zfhx1/z81* depends on FoxQ2, which is required for formation of the anterior neuroectoderm. > We conclude that Zfhx1/Z81 is an important intermediate regulator in serotonergic neurogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Angerer LM, Yaguchi S, Angerer RC, Burke RD. The evolution of nervous system patterning: insights from sea urchin development. Development. 2011; 138:3613–3623. [PubMed: 21828090]

Bisgrove BW, Burke RD. Development of serotonergic neurons in embryos of the sea urchin, *Strongylocentrotus purpuratus*. Development Growth & Differentiation. 1986; 28:569–574.

Bisgrove BW, Burke RD. Development of the nervous system of the pluteus larva of *Strongylocentrotus droebachiensis*. Cell and Tissue Research. 1987; 248:335–343.

Burke RD, Angerer LM, Elphick MR, Humphrey GW, Yaguchi S, Kiyama T, Liang S, Mu X, Agca C, Klein WH, Brandhorst BP, Rowe M, Wilson K, Churcher AM, Taylor JS, Chen N, Murray G, Wang D, Mellott D, Olinski R, Hallbook F, Thorndyke MC. A genomic view of the sea urchin nervous system. Dev Biol. 2006; 300:434–460. [PubMed: 16965768]

Clark SG, Chiu C. C. elegans ZAG-1, a Zn-finger-homeodomain protein, regulates axonal development and neuronal differentiation. Development. 2003; 130:3781–3794. [PubMed: 12835394]

Cordes SP. Molecular genetics of the early development of hindbrain serotonergic neurons. Clin Genet. 2005; 68:487–494. [PubMed: 16283875]

De Robertis EM, Kuroda H. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annu Rev Cell Dev Biol. 2004; 20:285–308. [PubMed: 15473842]

Dunn EF, Moy VN, Angerer LM, Angerer RC, Morris RL, Peterson KJ. Molecular paleoecology: using gene regulatory analysis to address the origins of complex life cycles in the late Precambrian. Evol Dev. 2007; 9:10–24. [PubMed: 17227363]

Fortini ME, Lai ZC, Rubin GM. The *Drosophila* zfh-1 and zfh-2 genes encode novel proteins containing both zinc-finger and homeodomain motifs. Mech Dev. 1991; 34:113–122. [PubMed: 1680376]

Genetta T, Ruezinsky D, Kadesch T. Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. Mol Cell Biol. 1994; 14:6153–6163. [PubMed: 8065348]

Hay-Schmidt A. The evolution of the serotonergic nervous system. Proc Biol Sci. 2000; 267:1071–1079. [PubMed: 10885511]

Howard-Ashby M, Materna SC, Brown CT, Chen L, Cameron RA, Davidson EH. Identification and characterization of homeobox transcription factor genes in *Strongylocentrotus purpuratus*, and their expression in embryonic development. Dev Biol. 2006; 300:74–89. [PubMed: 17055477]

Lai ZC, Fortini ME, Rubin GM. The embryonic expression patterns of zfh-1 and zfh-2, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. Mech Dev. 1991; 34:123–134. [PubMed: 1680377]

Lee HK, Lundell MJ. Differentiation of the *Drosophila* serotonergic lineage depends on the regulation of Zfh-1 by Notch and Eagle. Mol Cell Neurosci. 2007; 36:47–58. [PubMed: 17702602]

Levine AJ, Brivanlou AH. Proposal of a model of mammalian neural induction. Dev Biol. 2007; 308:247–256. [PubMed: 17585896]

Logan CY, Miller JR, Ferkowicz MJ, McClay DR. Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. Development. 1999; 126:345–357. [PubMed: 9847248]

Lundell MJ, Hirsh J. The zfh-2 gene product is a potential regulator of neuron-specific *dopa decarboxylase* gene expression in *Drosophila*. Dev Biol. 1992; 154:84–94. [PubMed: 1426635]

Materna SC, Howard-Ashby M, Gray RF, Davidson EH. The C2H2 zinc finger genes of *Strongylocentrotus purpuratus* and their expression in embryonic development. Dev Biol. 2006; 300:108–120. [PubMed: 16997293]

Nakajima Y, Kaneko H, Murray G, Burke RD. Divergent patterns of neural development in larval echinoids and asteroids. Evol Dev. 2004; 6:95–104. [PubMed: 15009122]

Postigo AA. Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. EMBO J. 2003; 22:2443–2452. [PubMed: 12743038]

Postigo AA, Ward E, Skeath JB, Dean DC. zfh-1, the *Drosophila* homologue of ZEB, is a transcriptional repressor that regulates somatic myogenesis. Mol Cell Biol. 1999; 19:7255–7263. [PubMed: 10490660]

Poustka AJ, Kuhn A, Groth D, Weise V, Yaguchi S, Burke RD, Herwig R, Lehrach H, Panopoulou G. A global view of gene expression in lithium and zinc treated sea urchin embryos: new components of gene regulatory networks. Genome Biol. 2007; 8:R85. [PubMed: 17506889]

Putnam NH, Butts T, Ferrier DE, Furlong RF, Hellsten U, Kawashima T, Robinson-Rechavi M, Shoguchi E, Terry A, Yu JK, Benito-Gutierrez EL, Dubchak I, Garcia-Fernandez J, Gibson-Brown JJ, Grigoriev IV, Horton AC, de Jong PJ, Jurka J, Kapitonov VV, Kohara Y, Kuroki Y, Lindquist E, Lucas S, Osoegawa K, Pennacchio LA, Salamov AA, Satou Y, Sauka-Spengler T, Schmutz J, Shin IT, Toyoda A, Bronner-Fraser M, Fujiyama A, Holland LZ, Holland PW, Satoh N, Rokhsar DS. The amphioxus genome and the evolution of the chordate karyotype. Nature. 2008; 453:1064–1071. [PubMed: 18563158]

Rottinger E, Croce J, Lhomond G, Besnardeau L, Gache C, Lepage T. Nemo-like kinase (NLK) acts downstream of Notch/Delta signalling to downregulate TCF during mesoderm induction in the sea urchin embryo. Development. 2006; 133:4341–4353. [PubMed: 17038519]

Sasaki H, Kominami T. Specification process of animal plate in the sea urchin embryo. Development Growth & Differentiation. 2008; 50:595–606.

Saudemont A, Haillot E, Mekpoh F, Bessodes N, Quirin M, Lapraz F, Duboc V, Rottinger E, Range R, Oisel A, Besnardeau L, Wincker P, Lepage T. Ancestral regulatory circuits governing ectoderm patterning downstream of Nodal and BMP2/4 revealed by gene regulatory network analysis in an echinoderm. PLoS Genet. 2010; 6 e1001259.

Sodergren E, Weinstock GM, Davidson EH, Cameron RA, Gibbs RA, Angerer RC, Angerer LM, Arnone MI, Burgess DR, Burke RD, Coffman JA, Dean M, Elphick MR, Ettensohn CA, Foltz KR, Hamdoun A, Hynes RO, Klein WH, Marzluff W, McClay DR, Morris RL, Mushegian A, Rast JP, Smith LC, Thorndyke MC, Vacquier VD, Wessel GM, Wray G, Zhang L, Elsik CG, Ermolaeva O, Hlavina W, Hofmann G, Kitts P, Landrum MJ, Mackey AJ, Maglott D, Panopoulou G, Poustka AJ, Pruitt K, Sapojnikov V, Song X, Souvorov A, Solovyev V, Wei Z, Whittaker CA, Worley K, Durbin KJ, Shen Y, Fedrigo O, Garfield D, Haygood R, Primus A, Satija R, Severson T, Gonzalez-Garay ML, Jackson AR, Milosavljevic A, Tong M, Killian CE, Livingston BT, Wilt FH, Adams N, Belle R, Carbonneau S, Cheung R, Cormier P, Cosson B, Croce J, Fernandez-Guerra A, Geneviere AM, Goel M, Kelkar H, Morales J, Mulner-Lorillon O, Robertson AJ, Goldstone JV, Cole B, Epel D, Gold B, Hahn ME, Howard-Ashby M, Scally M, Stegeman JJ, Allgood EL, Cool J, Judkins KM, McCafferty SS, Musante AM, Obar RA, Rawson AP, Rossetti BJ, Gibbons IR, Hoffman MP, Leone A, Istrail S, Materna SC, Samanta MP, Stolc V, Tongprasit W, Tu Q, Bergeron KF, Brandhorst BP, Whittle J, Berney K, Bottjer DJ, Calestani C, Peterson K, Chow E, Yuan QA, Elhaik E, Graur D, Reese JT, Bosdet I, Heesun S, Marra MA, Schein J, Anderson MK, Brockton V, Buckley KM, Cohen AH, Fugmann SD, Hibino T, Loza-Coll M, Majeske AJ, Messier C, Nair SV, Pancer Z, Terwilliger DP, Agca C, Arboleda E, Chen N, Churcher AM, Hallbook F, Humphrey GW, Idris MM, Kiyama T, Liang S, Mellott D, Mu X, Murray G, Olinski RP, Raible F, Rowe M, Taylor JS, Tessmar-Raible K, Wang D, Wilson KH, Yaguchi S, Gaasterland T, Galindo BE, Gunaratne HJ, Juliano C, Kinukawa M, Moy GW, Neill AT, Nomura M, Raisch M, Reade A, Roux MM, Song JL, Su YH, Townley IK, Voronina E, Wong JL, Amore G, Branno M, Brown ER, Cavalieri V, Duboc V, Duloquin L, Flytzanis C, Gache C, Lapraz F, Lepage T, Locascio A, Martinez P, Matassi G, Matranga V, Range R, Rizzo F, Rottinger E, Beane W, Bradham C, Byrum C, Glenn T, Hussain S, Manning G, Miranda E, Thomason R, Walton K, Wikramanayke A, Wu SY, Xu R, Brown CT, Chen L, Gray RF, Lee PY, Nam J, Oliveri P, Smith J, Muzny D, Bell S, Chacko J, Cree A, Curry S, Davis C, Dinh H, Dugan-Rocha S, Fowler J, Gill R, Hamilton C, Hernandez J, Hines S, Hume J, Jackson L, Jolivet A, Kovar C, Lee S, Lewis L, Miner G, Morgan M, Nazareth LV, Okwuonu G, Parker D, Pu LL, Thorn R, Wright R. The genome of the sea urchin *Strongylocentrotus purpuratus*. Science. 2006; 314:941–952. [PubMed: 17095691]

Southwick SM, Paige S, Morgan CA 3rd, Bremner JD, Krystal JH, Charney DS. Neurotransmitter alterations in PTSD: catecholamines and serotonin. Semin Clin Neuropsychiatry. 1999; 4:242–248. [PubMed: 10553029]

Su YH, Li E, Geiss GK, Longabaugh WJ, Kramer A, Davidson EH. A perturbation model of the gene regulatory network for oral and aboral ectoderm specification in the sea urchin embryo. Dev Biol. 2009; 329:410–421. [PubMed: 19268450]

Sze JY, Zhang S, Li J, Ruvkun G. The C. elegans POU-domain transcription factor UNC-86 regulates the tph-1 tryptophan hydroxylase gene and neurite outgrowth in specific serotonergic neurons. Development. 2002; 129:3901–3911. [PubMed: 12135927]

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony, methods. Mol Biol Evol. 2011; 28:2731–2739. [PubMed: 21546353]

Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron. 2001; 30:65–78. [PubMed: 11343645]

Tu Q, Brown CT, Davidson EH, Oliveri P. Sea urchin Forkhead gene family: phylogeny and embryonic expression. Dev Biol. 2006; 300:49–62. [PubMed: 17081512]

Vallier L, Reynolds D, Pedersen RA. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev Biol. 2004; 275:403–421. [PubMed: 15501227]

Verschueren K, Remacle JE, Collart C, Kraft H, Baker BS, Tylzanowski P, Nelles L, Wuytens G, Su MT, Bodmer R, Smith JC, Huylebroeck D. SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. J Biol Chem. 1999; 274:20489–20498. [PubMed: 10400677]

Wacker I, Schwarz V, Hedgecock EM, Hutter H. zag-1, a Zn-finger homeodomain transcription factor controlling neuronal differentiation and axon outgrowth in *C. elegans*. Development. 2003; 130:3795–3805. [PubMed: 12835395]

Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y. Directed differentiation of telencephalic precursors from embryonic stem cells. Nat Neurosci. 2005; 8:288–296. [PubMed: 15696161]

Wei Z, Angerer RC, Angerer LM. A database of mRNA expression patterns for the sea urchin embryo. Dev Biol. 2006; 300:476–484. [PubMed: 17007833]

Wei Z, Angerer RC, Angerer LM. Direct development of neurons within foregut endoderm of sea urchin embryos. Proc Natl Acad Sci U S A. 2011

Wei Z, Yaguchi J, Yaguchi S, Angerer RC, Angerer LM. The sea urchin animal pole domain is a Six3-dependent neurogenic patterning center. Development. 2009; 136:1179–1189. [PubMed: 19270175]

Wikramanayake AH, Klein WH. Multiple signaling events specify ectoderm and pattern the oral-aboral axis in the sea urchin embryo. Development. 1997; 124:13–20. [PubMed: 9006063]

Yaguchi S, Kanoh K, Amemiya S, Katow H. Initial analysis of immunochemical cell surface properties, location and formation of the serotonergic apical ganglion in sea urchin embryos. Development Growth & Differentiation. 2000; 42:479–488.

Yaguchi S, Katow H. Expression of tryptophan 5-hydroxylase gene during sea urchin neurogenesis and role of serotonergic nervous system in larval behavior. J Comp Neurol. 2003; 466:219–229. [PubMed: 14528449]

Yaguchi S, Yaguchi J, Angerer RC, Angerer LM. A Wnt-FoxQ2-nodal pathway links primary and secondary axis specification in sea urchin embryos. Dev Cell. 2008; 14:97–107. [PubMed: 18194656]

Yaguchi S, Yaguchi J, Angerer RC, Angerer LM, Burke RD. TGF-beta signaling positions the ciliary band and patterns neurons in the sea urchin embryo. Dev Biol. 2010a; 347:71–81. [PubMed: 20709054]

Yaguchi S, Yaguchi J, Burke RD. Specification of ectoderm restricts the size of the animal plate and patterns neurogenesis in sea urchin embryos. Development. 2006; 133:2337–2346. [PubMed: 16687447]

Yaguchi S, Yaguchi J, Burke RD. Sp-Smad2/3 mediates patterning of neurogenic ectoderm by nodal in the sea urchin embryo. Dev Biol. 2007; 302:494–503. [PubMed: 17101124]

Yaguchi S, Yaguchi J, Wei Z, Jin Y, Angerer LM, Inaba K. Fez function is required to maintain the size of the animal plate in the sea urchin embryo. Development. 2011; 138:4233–4243. [PubMed: 21852402]

Yaguchi S, Yaguchi J, Wei Z, Shiba K, Angerer LM, Inaba K. ankAT-1 is a novel gene mediating the apical tuft formation in the sea urchin embryo. Dev Biol. 2010b; 348:67–75. [PubMed: 20875818]

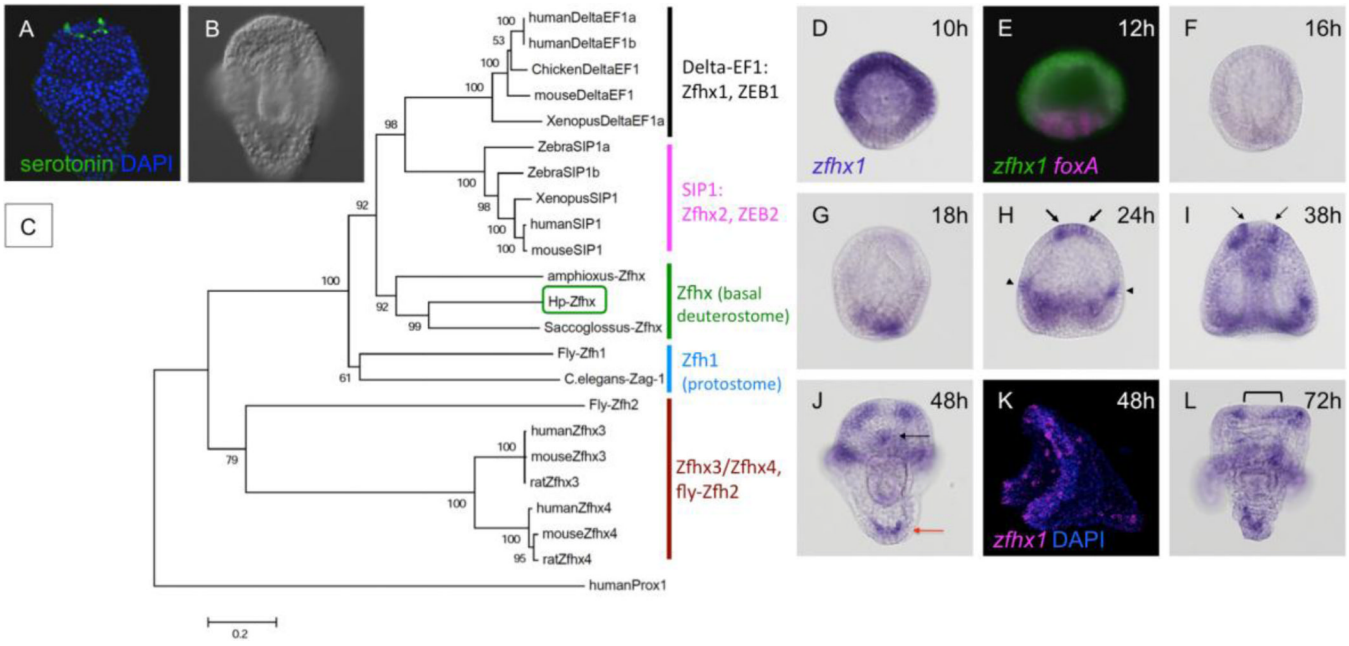

Figure 1.

zfhx1/z81 is expressed in serotonergic neurons in the animal plate. The animal pole of embryos in each microscopic image is at the top unless otherwise indicated. (A) Serotonergic neurons in a prism larva of the sea urchin, *Hemicentrotus pulcherrimus* (green). (B) DIC image of (A). (C) Phylogenetic tree drawn using MEGA 5 (Tamura et al., 2011) shows that Hp-Zfhx1/Z81 belongs to basal deuterostome-type Zfhx/Zfh branch. ZEB1 and ZEB2, zinc finger E-box binding protein 1 and 2, respectively. SIP1, smad-interacting protein 1. humanProx, prospero-related homeobox of human. Numbers on the branches show the bootstrap value (%; 1,000 replicates). The scale bar indicates 0.2 amino acid substitutions per position in sequence. (D–L) Expression of *zfhx1/z81* at the following stages. (D) unhatched blastula, 10-hpf (10h). (E) double fluorescent *in situ* hybridization with *zfhx1/z81* (green) and *foxA* (magenta) in unhatched blastula, 12-hpf (12h). (F) hatched blastula, 16-hpf (16h). (G) mesenchyme blastula, 18-hpf (18h). (H) early gastrula, 24-hpf (24h). Arrows and arrowheads show *zfhx1/z81* expression in the animal plate and future ciliary band region, respectively. (I) prism larva, 38-hpf (38h). The arrows indicate the outer edge of the central part of animal plate, where *zfhx1/z81* is missing. (J) pluteus larva, 48-hpf (48h). Black and red arrow shows *zfhx1/z81* gene expression in lower lip region and posterior mesenchyme cells, respectively. (K) lateral view of pluteus larva, fluorescent *in situ* hybridization. (L) 72-hpf pluteus stage (72h).

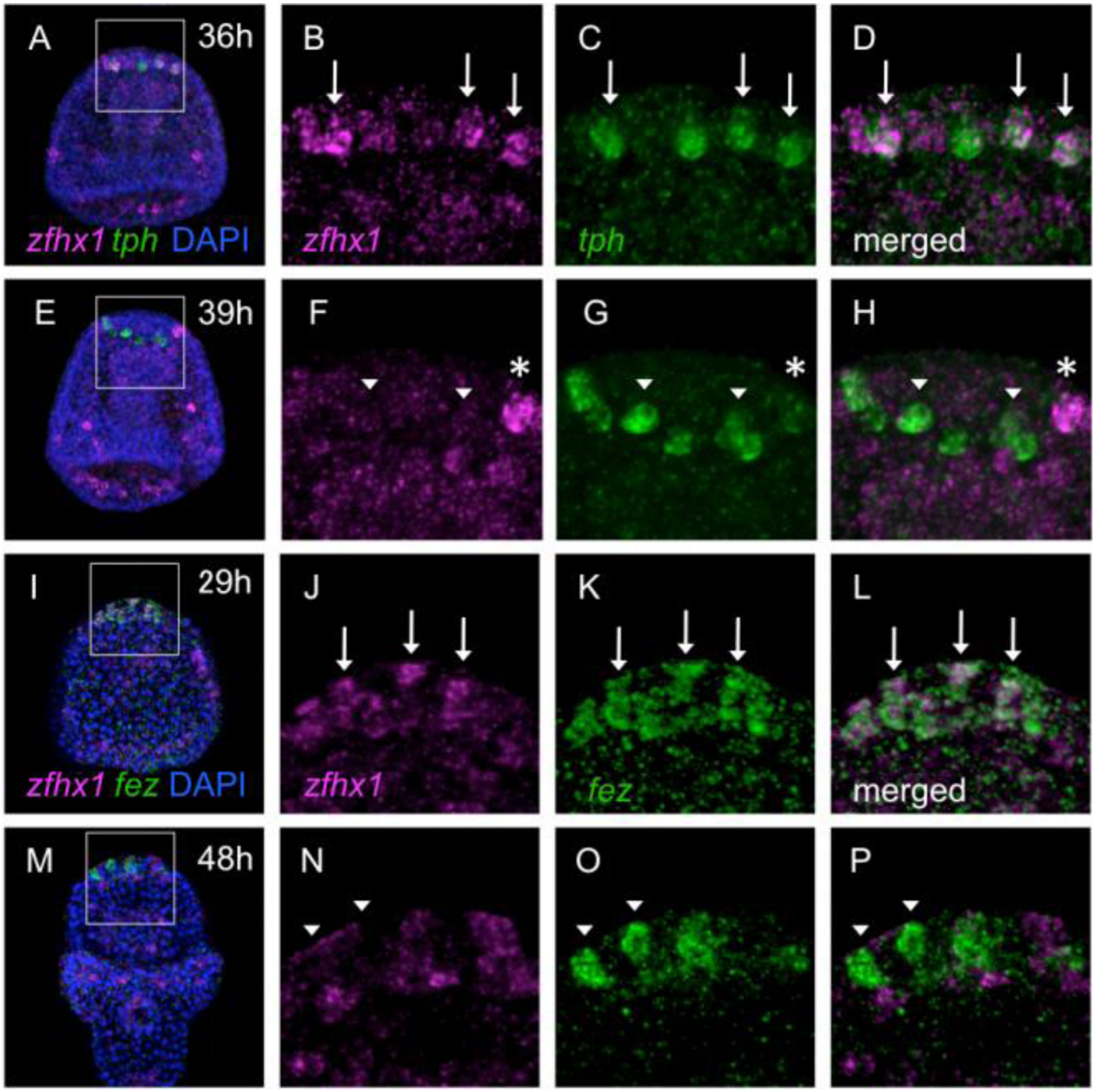

Figure 2.

zfhx1/z81 is transiently expressed in serotonergic neural precursor cells. (A–H) Double fluorescent *in situ* hybridization detecting *zfhx1/z81* and *tph* in 36-hpf (A–D) and 39-hpf (E–H) embryos. (A) *zfhx1/z81* is expressed in the animal plate region. A square region is magnified in (B–D). (B) *zfhx1/z81* is expressed in a few cells (arrows). (C) *tph* at the same region (arrows). (D) Merged image of (B) and (C). Arrows show the cells expressing both *zfhx1/z81* and *tph*. (E) Most of *zfhx1/z81* disappears from the animal plate in 39-hpf embryo. A square shows the region that is magnified in (F–H). (F) *zfhx1/z81* is not expressed in *tph*-positive cells (arrowheads). Asterisk shows *zfhx1/z81*-positive cell. (G) *tph* expression in the same region. Arrowheads indicate the cells expressing *tph* strongly. Asterisk shows a cell

expressing *tph* weakly. (H) Merged image of (F) and (G). (I–P) Double fluorescent *in situ* hybridization detecting *zfhx1/z81* and *fez* in 29-hpf (I–L) and 48-hpf (M–P) embryos. (I) *zfhx1/z81* is expressed in the animal plate region in 29-hpf. The square shows the region that is magnified in (J–L). (J) *zfhx1/z81*-expressing cells in the animal plate (arrows). (K) *fez*-expressing cells in the same region. (L) Merged image of (J) and (K). (M) *zfhx1/z81* is down regulated in a 48-hpf embryo. (N) *zfhx1/z81* is not detected in the cells in which *fez* is expressed (arrowheads). (O) *fez* expression in the same region. (P) Merged image of (N) and (O). *zfhx1/z81*-positive cells (magenta) in (M–P) are non-serotonergic neurons in the animal plate.

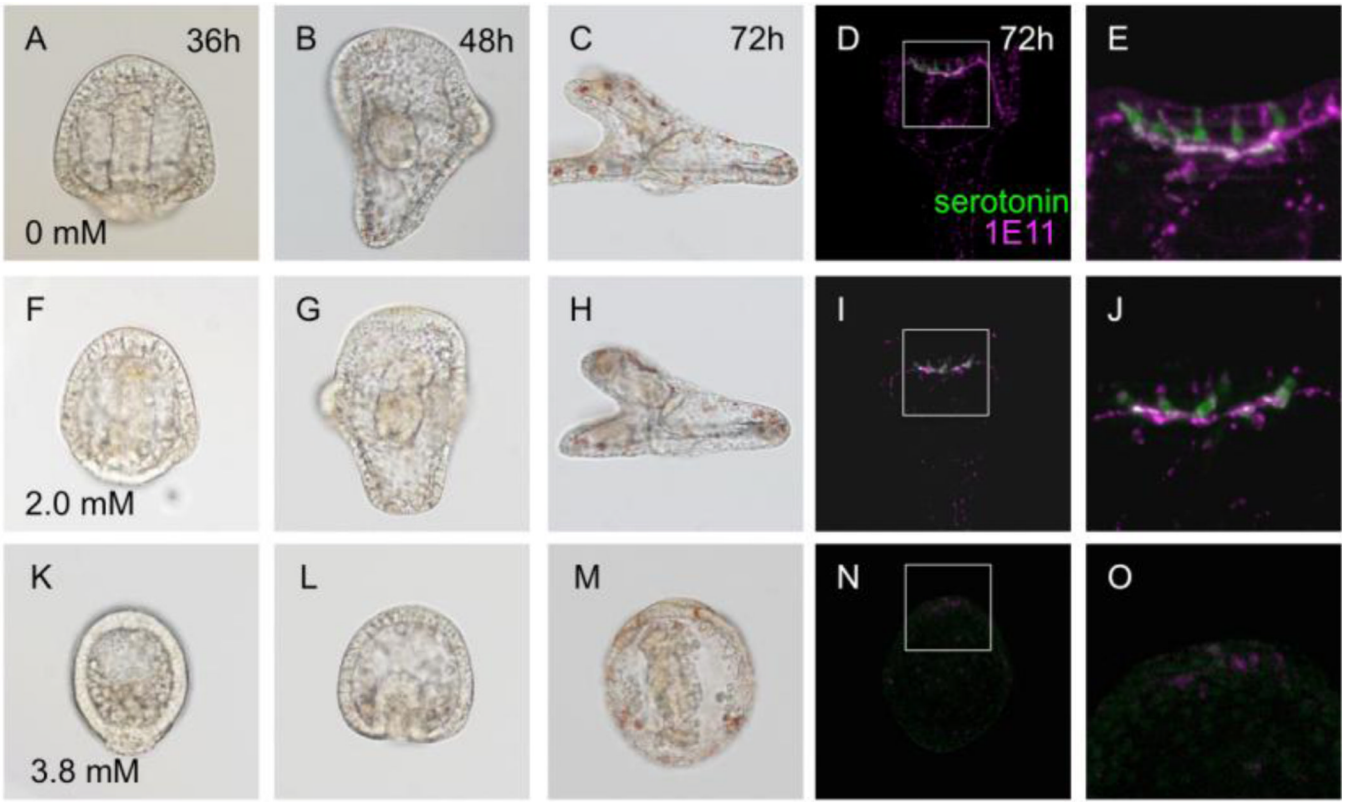

Figure 3.

Knockdown of Zfhx1/Z81 not only decreases the number of serotonergic neurons but also inhibits normal vegetal tissue development and oral/aboral polarity. (A–E) Control embryos (glycerol-injected). (A) 36-hpf prism stage. (B) 48-hpf pluteus stage. (C) 72-hpf early 4-arm pluteus stage, lateral view. (D) Immuno-fluorescent image of a 72-hpf embryo stained for serotonin and synaptotagminB (1E11); the rectangle shows the region magnified in (E). (E) Seven serotonergic neurons are present in this embryo. (F–J) 2.0 mM Zfhx1/Z81-MO-injected embryos. (F) 36-hpf. (G) 48-hpf. (H) 72-hpf. The length of the body along the anterior-posterior axis is shorter than that of normal embryos (C). (I) The development of the nervous system is incomplete in the morphant. The square shows the region magnified in (J). (J) The number of serotonergic neurons is less than that of control. (K–O) 3.8 mM Zfhx1/Z81-MO-injected embryos. (K) 36-hpf. (L) 48-hpf. (M) 72-hpf. (N) This morphant has no detectable neurons in the animal plate. Square shows the region magnified in (O). (O) Neural development is strongly suppressed in the morphants.

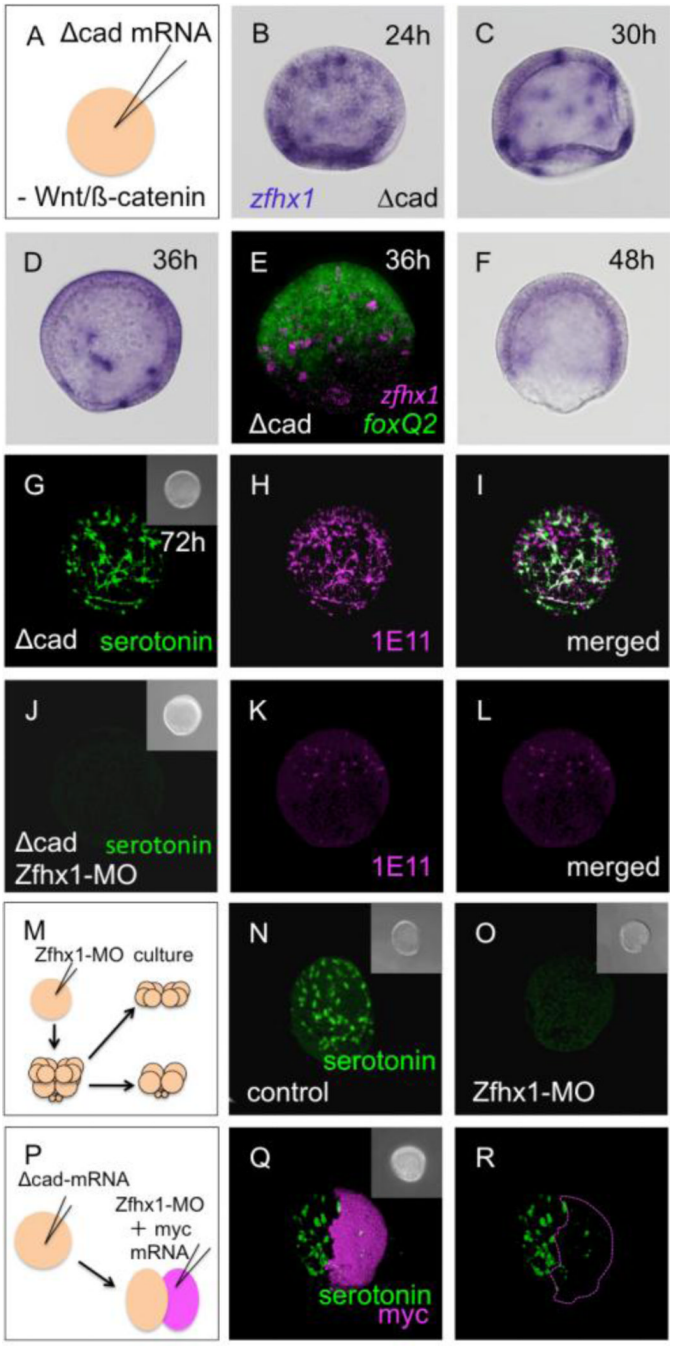

Figure 4.

Zfhx1/Z81 is required for the differentiation of serotonergic neurons. (A) Microinjection to inhibit canonical Wnt signaling. (B–F) The expression patterns of *zfhx1/z81* in Δcad-injected embryos. (B) *zfhx1/z81*-positive neural precursors are scattered in the expanded 24-hpf embryo. (C) 30-hpf embryo. (D) 36-hpf embryo; the number of *zfhx1/z81* cells decreased. (E) Double fluorescent *in situ* hybridization shows that *zfhx1/z81* disappears from the central part of the animal plate. (F) *zfhx1/z81* is down regulated in 48-hpf Δcad-injected embryos. The apparent staining in this embryo is background diffuse staining that is higher in the thickened ectoderm of these embryos. (G) Many serotonergic neurons differentiate in the expanded animal plate in Δcad-injected embryo. (H) All of serotonergic and non-

serotonergic neurons in the animal plate are synaptotagminB (1E11 antigen)-positive. (I) Merged image of (G) and (H). (J) Δcad-injected Zfhx1/Z81 morphants have no serotonergic neurons at 72-hpf. (K) Serotonin-negative 1E11 neurons begin to differentiate in morphants. (L) Merged image of (J) and (K). (M) Method for creating animal caps from Zfhx1/Z81 morphants. (N) Serotonergic neurons differentiate in the glycerol-injected control animal cap. (O) No serotonergic neurons differentiate in the animal cap of Zfhx1/Z81 morphants. (P) Method to inject Zfhx1/Z81-MO and myc mRNA into one of two blastomeres derived from a Δcad-injected egg. (Q) Nearly all of the serotonergic neurons differentiate in the myc (i.e. Zfhx1/Z81-MO)-negative half of the embryo. (R) Only the outline of myc-positive, Zfhx1/Z81-deficient region of (Q) is shown. Insets are DIC images for each panel.

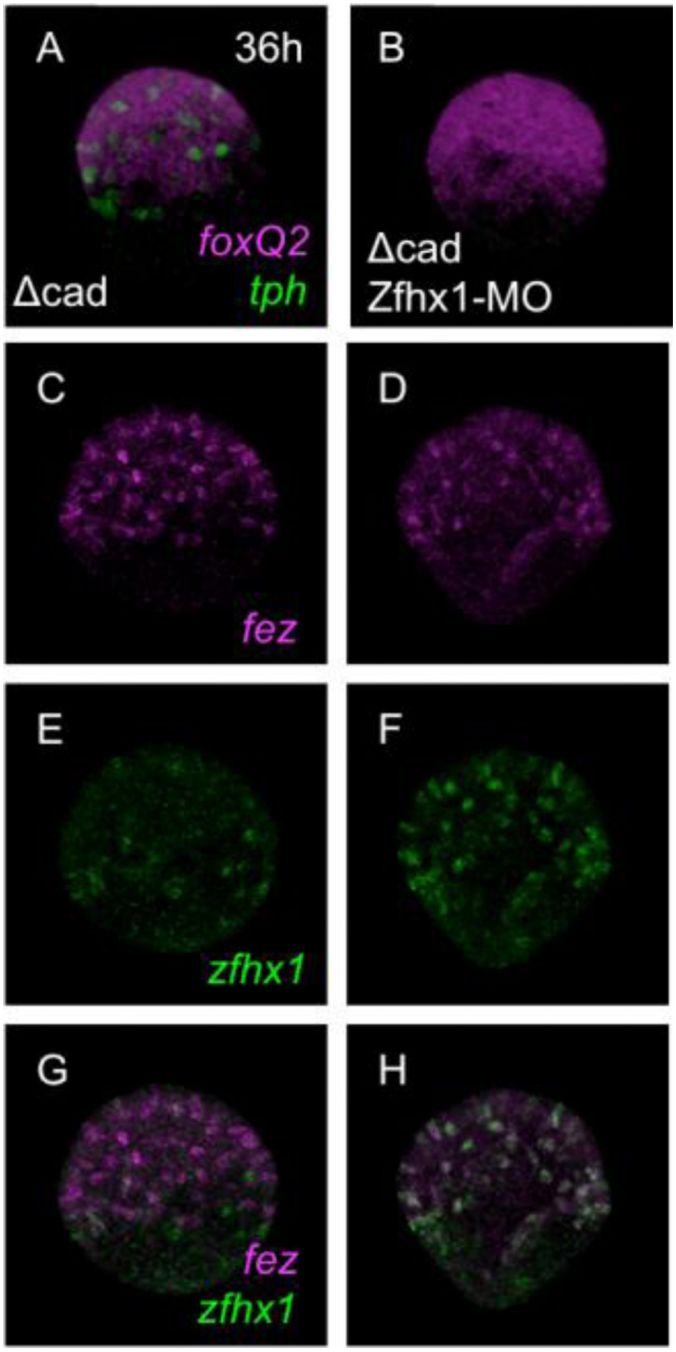

Figure 5.
Zfhx1/Z81 is not required for expression of genes involved in early specification of the animal plate. (A) *foxQ2* and *tph* in a Δcad-injected embryo at 36-hpf. (B) The expression pattern of *foxQ2* is not altered in Δcad-injected Zfhx1/Z81 morphants, whereas no *tph* expression is detected. (C, E, G) Δcad-alone-injected control embryo. (D, F, H) Δcad-injected Zfhx1/Z81 morphant. (C, D) The expression patterns of *fez* at 36-hpf. (E, F) The expression patterns of *zfhx1/z81*. (G, H) Merged images of (C) and (E), and (D) and (F), respectively.

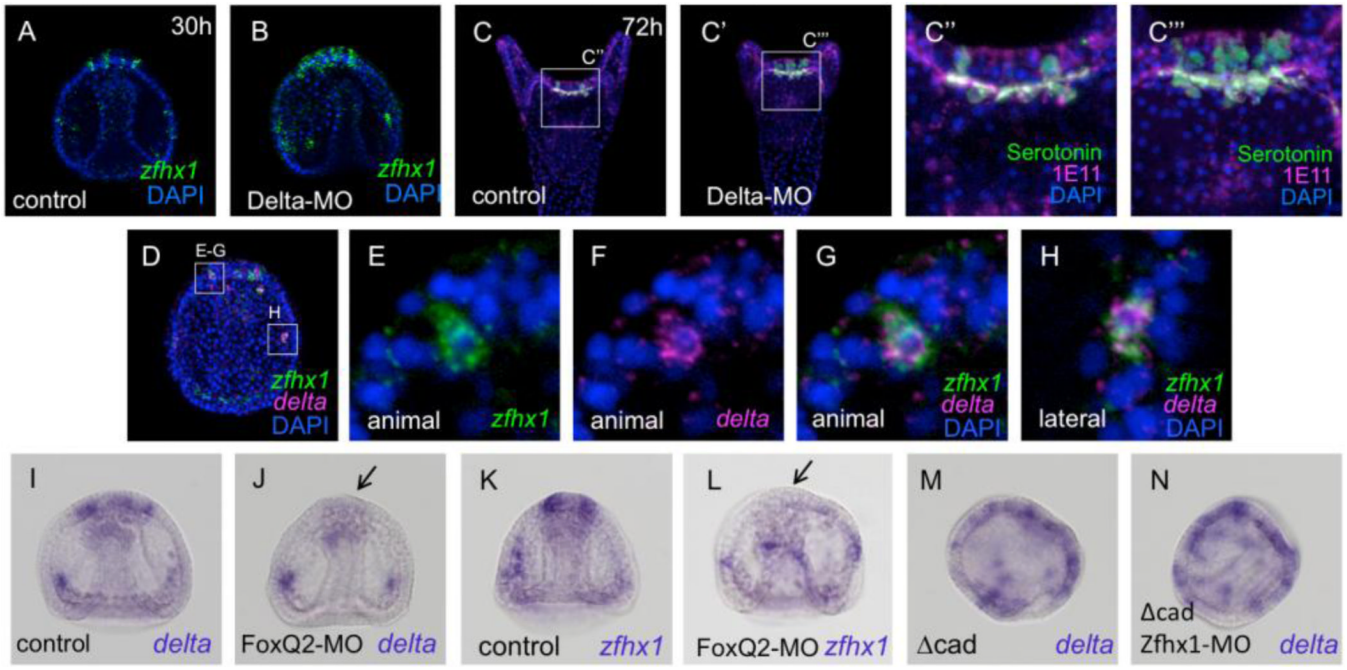

Figure 6.

delta is a specific neural marker in the animal plate. (A) *zfhx1/z81* expression in the animal plate of 30-hpf (30h) embryo detected with fluorescent in situ hybridization. (B) More cells express *zfhx1/z81* and make a cluster in the animal plate of Delta morphants. (C) The normal patterning of serotonergic neurons in 72-hpf embryo. A square shows the region magnified in (C''). 1E11, a pan-neural marker (magenta); serotonin (green). (C') A cluster of serotonergic neurons is formed in the animal plate of Delta morphants. A square shows the region magnified in (C'''). (C'') Magnified image of the square region in (C). (C''') Magnified image of the square region in (C'). (D) Double fluorescent *in situ* hybridization detects *zfhx1/z81* and *delta* co-expression at gastrula stage. The magnified images are shown in (E–G) for animal plate and (H) for lateral regions. (E) A cell expressing *zfhx1/z81* in the animal plate. (F) *delta* expression. (G) Merged image of (E) and (F). (H) A cell expressing *zfhx1/z81* (green) and *delta* (magenta) in the lateral region. (I) *delta* expression in the control (glycerol-injected) late gastrula. (J) *delta* expression in the animal plate is suppressed in FoxQ2 morphants (arrow). (K) *zfhx1/z81* in the control late gastrula. (L) *zfhx1/z81* expression in the animal plate requires FoxQ2 (arrow). (M) Many *delta*-expressing cells are present in the expanded animal plate of Δcad-injected embryos. (N) *delta* expression pattern is unaltered in Δcad-injected Zfhx1/Z81 morphants.

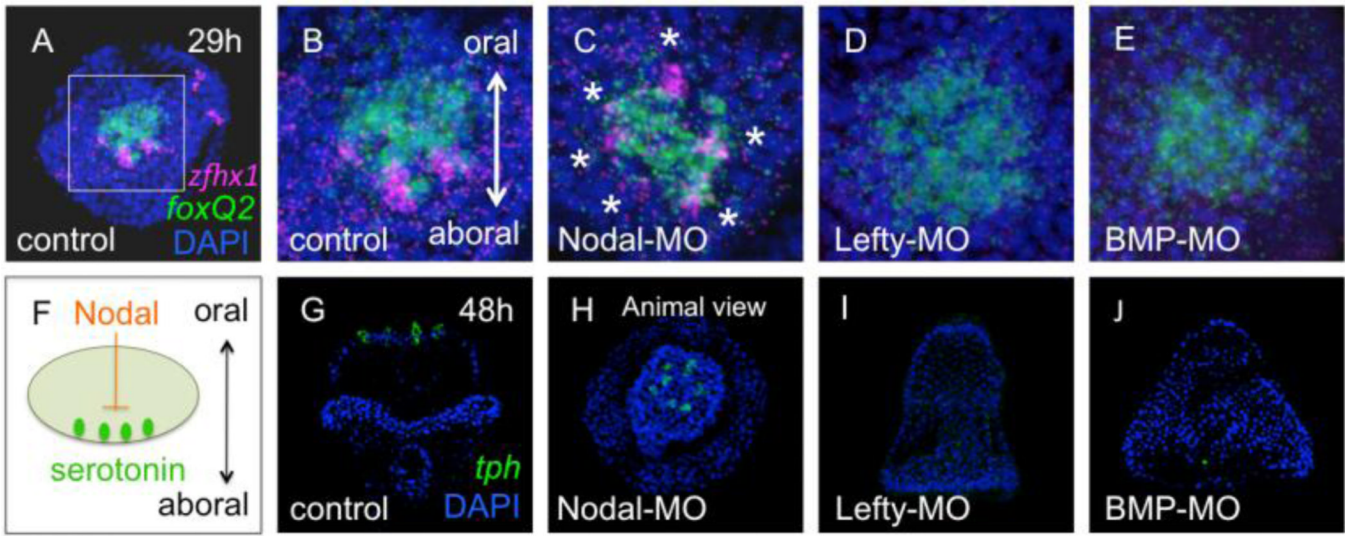

Figure 7.

Nodal suppresses the expression of *zfhx1/z81* on the oral side of the animal plate. (A) The expression pattern of *zfhx1/z81* (magenta) in the animal plate of control (glycerol-injected) embryos is marked by *foxQ2* (green) expression. A square shows the region magnified in (B). Animal pole view. (B) *zfhx1/z81* is expressed in cells along the aboral edge of the animal plate. (C) *zfhx1/z81* is expressed all around the circumference of the animal plate in Nodal morphants (asterisks). (D) *zfhx1/z81* is not expressed in Lefty or BMP morphants, in which Nodal expression extends around the animal plate (E). (F) Schematic illustrating that Nodal suppresses the differentiation of serotonergic neurons on the oral side of the animal plate. (G) The expression pattern of *tph* in the control (glycerol-injected) embryo (green). Oral view. (H) *tph* is radially expressed in the animal plate in Nodal morphants. Animal pole view. (I, J) *tph* is not expressed in either Lefty or BMP morphants.

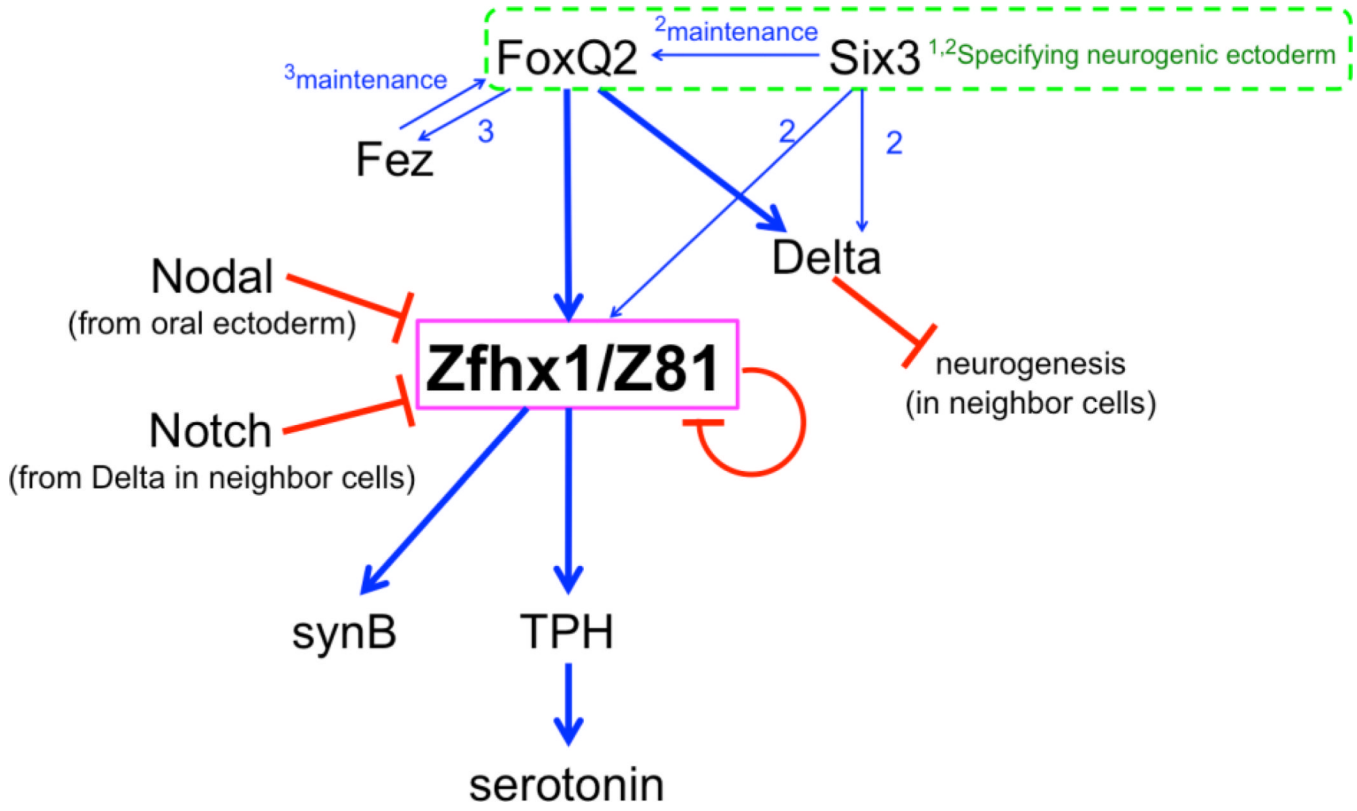

Figure 8.

Model of the regulatory mechanisms controlling differentiation of serotonergic neurons in the sea urchin embryo. FoxQ2 and Six3 are involved in the specification of the animal plate during early development (1; Yaguchi et al., 2008, 2; Wei et al., 2009). FoxQ2 is required for *fez* expression and then Fez maintains *foxQ2* expression on the aboral side of the animal plate (3; Yaguchi et al., 2011). Both FoxQ2 and Six3 regulate *zfhx1/z81* and *delta* expression and Six3 supports FoxQ2 expression (2). Zfhx1/z81 is required for the expression of *tph*, which is required for serotonin synthesis, and for *synaptotagminB* (*synB*). Delta-Notch signaling limits the number of differentiating neurons by lateral inhibition and Nodal inhibits their development on the oral side of the animal plate. Zfhx1/Z81 suppresses its own expression.