

**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, 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 Δ cadherin 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 μ M; Yaguchi et al., 2010b), *Delta*-MO (2.0

mM), Nodal-MO (200 μ M; Yaguchi et al., 2010b), Lefty-MO (400 μ M; Yaguchi et al., 2010b), BMP2/4-MO (400 μ M; 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 μ g/ μ l; Logan et al., 1999), myc-mRNA (0.1 μ g/ μ l).

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 Δ cadherin (Δ cad) (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 Δ cadherin-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 Δ cad-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

Δ cad-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 Δ cad-injected embryos to eliminate indirect effects caused by Zfhx1/Z81 functions at earlier stages in other regions of the embryo. In Δ cad-injected Zfhx1/Z81 morphants *foxQ2* is expressed throughout the expanded animal plate as in control Δ cad 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 Δ cad-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 Δ cad alone-injected embryos (Fig. 5E). However, intriguingly in Δ cad-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 Δ cad-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 (Verschuere 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.

Acknowledgements

We thank Robert Angerer for fruitful comments on this manuscript and Robert Burke, Yoko Nakajima, and David McClay for essential reagents. We thank Masato Kiyomoto and Mamoru Yamaguchi for providing the adult sea urchins and Mrs. Y. Tsuchiya, T. Sato, H. Shinagawa, and Y. Yamada, Shimoda Marine Research Center, for collecting and keeping the adult sea urchins. This work was supported, in part, by Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (MEXT), by Grant-in Aid for Young Scientists (B No. 21770227 and No. 23770241), and Takeda Science Foundation to S.Y., in part by MEXT (No. 22370023) to K.I., and in part by the Intramural Program of the National Institutes of Health, National Institute for Dental and Craniofacial Research, to L.M.A. J. Y. was a Predoctoral Fellow of JSPS with research grant (23-3584).

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Figure legends

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 Δ cad-injected embryos. (N) *delta* expression pattern is unaltered in Δ cad-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.

Figure-1

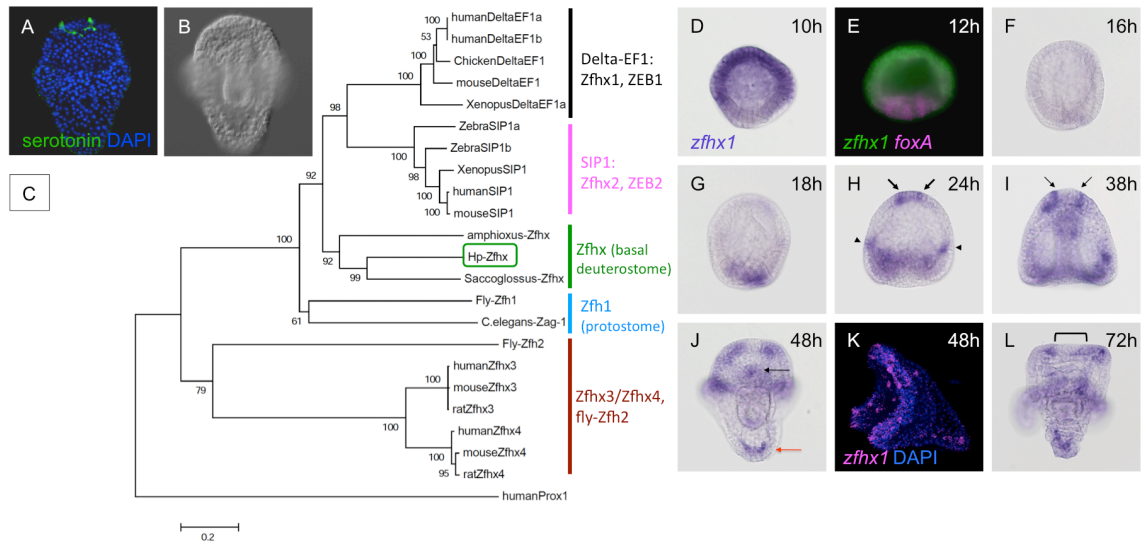


Figure-2

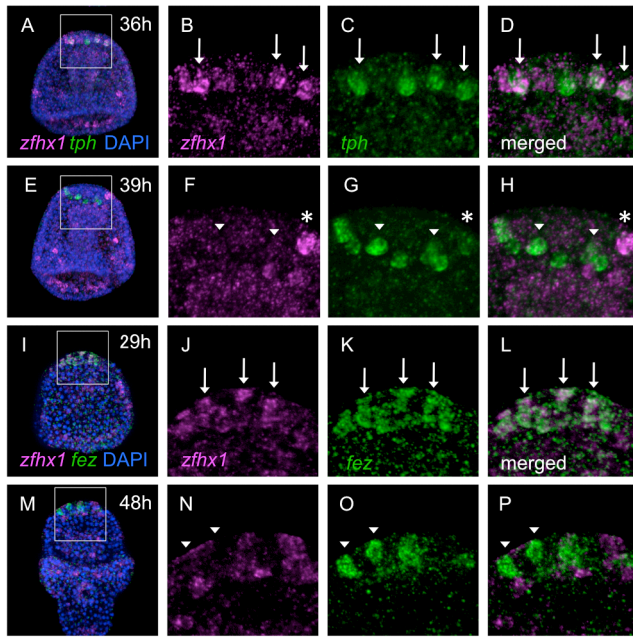


Figure-3

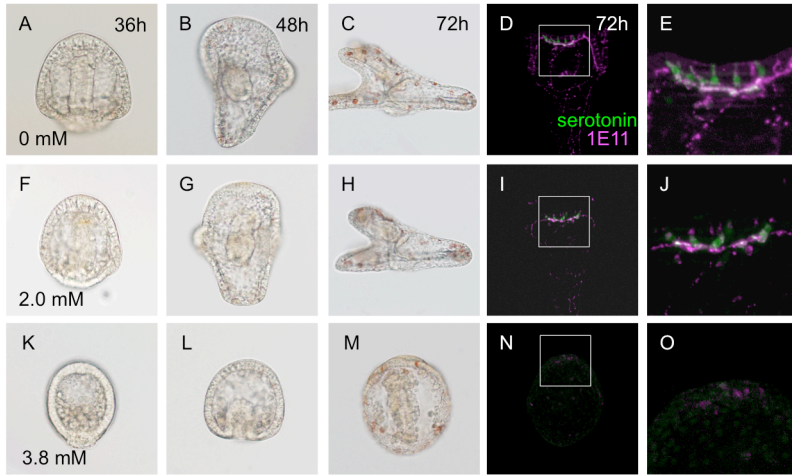


Figure-4

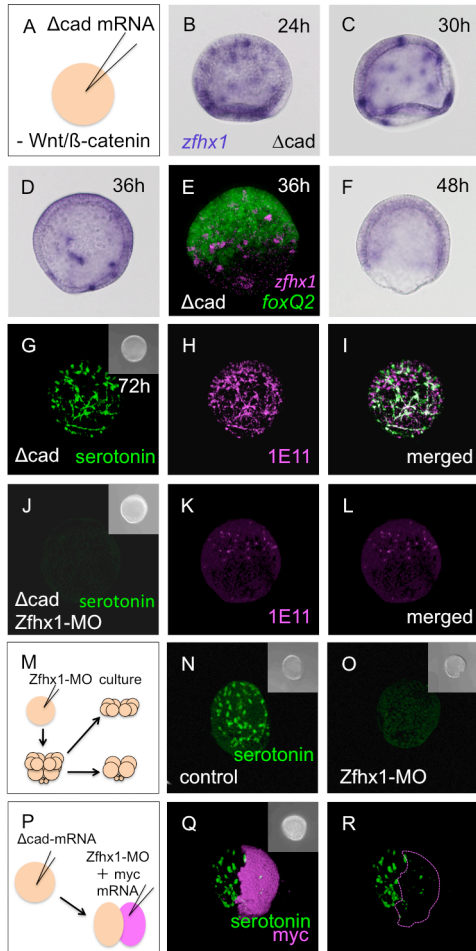


Figure-5

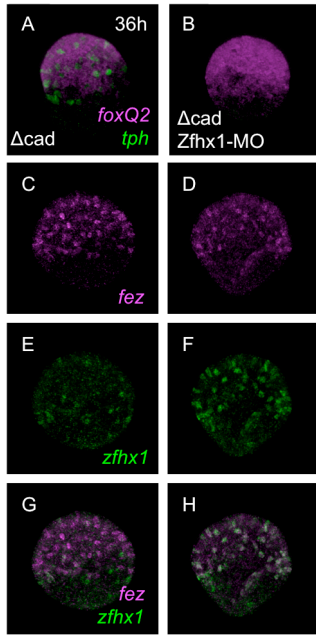


Figure-6

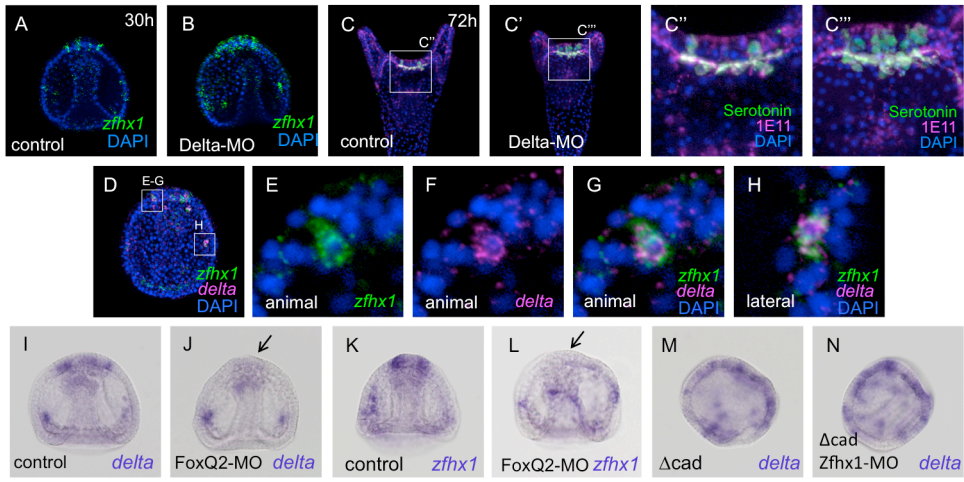


Figure-7

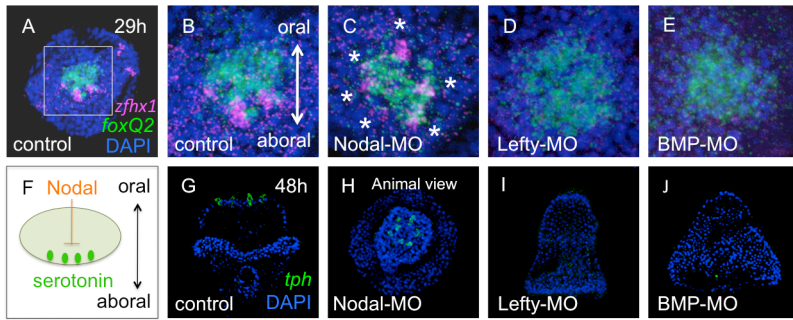
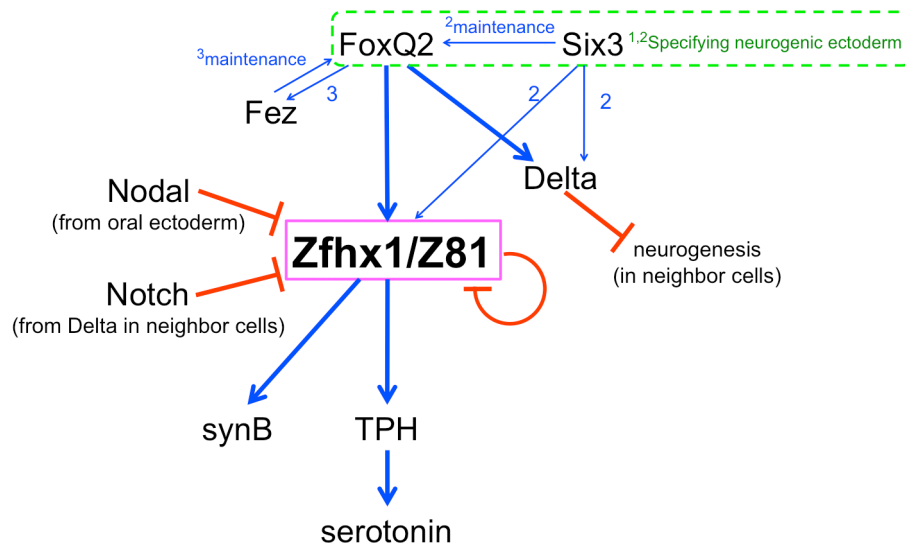


Figure-8



Supplemental Data

Supplemental Figure 1.

Sea urchin zinc-finger homeobox (*Zfhx1/Z81*) does not have a Smad binding domain (SDB). ClustalW alignment of amino acid sequences of zinc-finger E-box binding proteins, human-Delta-EF1a, human-Delta-EF1b, chicken-Delta-EF1, zebrafish-Kheper, human-SIP1, *Xenopus*-SIP1, zebrafish-SIP1a, zebrafish-SIP1b, *Hemicentrotus pulcherrimus* *Zfhx1/Z81* (Hp-*Zfhx*; bold), *Saccoglossus kowalevskii* *Zfhx*, amphioxus-*Zfhx*, and fly-*Zfh1*. The position of the SDB domain is highlighted in red. Green and Magenta squares indicate conserved zinc-finger domains and homeodomain, respectively, according to the domain search results by SMART. The numbers on the right ends show the amino acid positions within each protein sequence.

Supplemental Figure 1.

Hp-Zfhx	-----MGATMADSATIRCKRRKQANPQRKNVDIGNPKGMTQAIIA	41
Saccoglossus-Zfhx	-----MAEGP--RCTRRKQANPRRKKELLENE-----A	27
amphioxus-Zfhx	MVPLTMNQSRQLMSPRRPHANMHAGVTPVSESRICLNKNVEARNCGVTPVSESRICLN	60
humanDeltaEFlb	-----MADGP--RCKRRKQANPRRNN--VTNYNTIVVETNSDS	33
humanDeltaEFla	-----MK--VTNYNTIVVETNSDS	16
mouseDeltaEFl	-----MADGP--RCKRRKQANPRRNN--VTNYNTIVVETNSDS	33
chickenDeltaEFl	-----MADGP--RCKRRKQANPRRNN--VTNYNTIVVETNSDS	33
XenopusDeltaEFla	-----MADGP--RCKRRKQANPRRNN--VTNYNTIVVETNSDS	33
ZebraKheper	-----MADGP--RCKRRKQANPRRNN--VTNYNTIVVETNSDS	33
humanSIP1	-----MKQPIADGPF--RCKRRKQANPRRNN--VVDYDQVVDVTSGET	38
XenopusSIP1	-----MKQPIADGPF--RCKRRKQANPRRNN--VVDYDQVVDVTSGET	38
zebraSIP1b	-----MRELIMADGPF--RCKRRKQANPRRNNAAVLDFENVVDVTSGET	40
zebraSIP1a	-----MKQPIADGPF--RCKRRKQANPRRNN--VVDYDQVVDVTSGET	38
flyZfh1	-----	
Hp-Zfhx	DGNDFISPSPEN-----GYHSNSTIEDSDGDDKGSFMGDEEDEAEMDK--	86
Saccoglossus-Zfhx	EKCNKNNNSD-----GYHSN-----ENEINQDE--	52
amphioxus-Zfhx	-----EEDDEGAVLDDADASTAHMSDSGVADNNEPDQR	106
humanDeltaEFlb	DDEDKLHIVEEE-----SVTDAADCEG---VPEDDLPTDQIVLGRSSEREQNA	79
humanDeltaEFla	DDEDKLHIVEEE-----SVTDAADCEG---VPEDDLPTDQIVLGRSSEREQNA	62
mouseDeltaEFl	DDEDKLHIVEEE-----SITDAADCEGG---MPPDELPADQIVLPG--GSDRGGGA	79
chickenDeltaEFl	DDEDKLHIVEEE-----SITDAADCEGG---MPPDELPADQIVLPG--GSDRGGGA	79
XenopusDeltaEFla	DDEDKLHIVEEE-----SVTDAADCEG---VPEDDLPTDQIVLPG--GSDRGGGA	80
ZebraKheper	DDEDKLHIVEEE-----SLDGAADCEG---VPEDDLPTDQIVLPG--GSDRGGGA	80
humanSIP1	DEEDKLHIAEDDGIANPLDQETSPASVFNHE---SSPHVSQLPREEE--EDEIREGGV	93
XenopusSIP1	DEEDKLHIAEDDGIANPLDQETSPASVFNHE---SSPHVSQLPREEE--EDEIREGGV	93
zebraSIP1b	EEDRLLVSEED---ALLNGAGSPASLVNHESEAPPSTLSHTLLRKTVDDDDMKDST	97
zebraSIP1a	DDEDRLLVSEEG---GSPAGVPSLE---ASPRVAHALLSCRGEENESQDGAG	85
flyZfh1	-----	
Hp-Zfhx	-----STSDGDKDDGVDDLEPKDFHRKNGMHHLHPHSPFPHPHQFR	128
Saccoglossus-Zfhx	-----MSSNDGSHYGDGQYQSNKE LGED-----CAAPG----	81
amphioxus-Zfhx	LDDSL-----HMENGTPVQSPAGDDISTPTTPTGPHSNPPTGTPHRTDE	153
humanDeltaEFlb	--KNCWE-----DDR--KEG--QELGPEAQADEAGCTVKDECESDAEANEQNH	123
humanDeltaEFla	--KNCWE-----DDTGKEG--QELGPEAQADEAGCTVKDECESDAEANEQNH	107
mouseDeltaEFl	--KNCWQ-----DNN-----KDNCESDAEANEQNH	103
chickenDeltaEFl	--NSCWE-----DEG--KET--KEILGPEAQDEAVGCTVKDECESDAEANEQNH	123
XenopusDeltaEFla	--KSCWE-----DEEGADCGDELGPEAEADELGTCTVKDECESDAEANEQNH	126
ZebraKheper	-----WD-----DVK-----EECVSD--EDERSD	90
humanSIP1	--EHPWHNNEILQASVDGPEEMKEDYDTMGPEATIQTALNNGTVKNANCTSDFEYFAKR	151
XenopusSIP1	--DHNWHNNVILKASVDGSDMKEDYDTMGPEQVTHVTINNGTVKNANCTSDFEYFAKR	151
zebraSIP1b	--ENVWHNLLNASIDGTDLAKADYDTMGTDVSLPE--IGNGTVKSVCHTDFEDFFPKR	154
zebraSIP1a	--AHVWR-----HGLNGSEERKAEYNSMSPDISLHG--IGNGTVKGIDASSELESFFAKR	137
flyZfh1	-----	
Hp-Zfhx	NGEVGGGVINLEIYMNRSDTAIYPEPVEDMDGVNGDTPDPNTEPN--DNETEIEGEL	186
Saccoglossus-Zfhx	GGGGGGGRAGLNLQYLSRGTALIIYPEAPDEN-----SDPSGQNGE--SNDDTIL--	130
amphioxus-Zfhx	LADGTQAEHDIREYANRSDTAIYPEPVEDMDGVNGDTPDPNTEPN--DNETEIEGEL	205
humanDeltaEFlb	-----PNVEEFQQQDTAVIYPEAPEEDQR--QGTPEASGHDEN-----GTPDAF	166
humanDeltaEFla	-----PNVEEFQQQDTAVIYPEAPEEDQR--QGTPEASGHDEN-----GTPDAF	150
mouseDeltaEFl	-----PNVEEFQQQDTAVIYPEAPEEDQR--QGTPEASGHDEN-----GTPDAF	146
chickenDeltaEFl	-----PNVEEFQQQDTAVIYPEAPEEDQR--QGTPEASGHDEN-----GTPDAF	166
XenopusDeltaEFla	-----PNVEEFQQQDTAVIYPEAPEEDQR--QGTPEASGHDEN-----GTPDAF	169
ZebraKheper	-----ALVEEMLQQGDTAVIYPEAPEEDPR--QGTPEASGHDEN-----GTPDSF	133
humanSIP1	KLEERDGHAVSIEEYLQRSDTAIYPEAPEELSR--LGTPEANGQEN--DLPPGTPDAF	207
XenopusSIP1	KMDAGDSNGVSIAEYLQRSDTAIYPEAPEELSR--LGTPEANGQEN--DLPPGTPDAF	207
zebraSIP1b	KLVDTE SHVVSIAEYLQRSDTAIYPEAPEELSRSLATPEATGHEEN--DLPPGTPDAF	212
zebraSIP1a	KLDDGE GHAASIAEYLQ--DTVIIYEPDPEEGTR--LGTPEANGQENE NDLALRTPDAF	193
flyZfh1	-----	
Zinc-finger domains		
Hp-Zfhx	GKPEDCPYCDRSYKRLTSLKEHIKYRHEKTFNFSQCEPCNYCFAYKSQLERHMATHMPGR	246
Saccoglossus-Zfhx	---SCPYCDRVYKRLTSLKEHIKYRHEKNANNVACSECNYSFAYKSQLERHMATHMPGR	186
amphioxus-Zfhx	---CPYCDRGGYKRLTSLKEHIKYRHERITDSSYACNECNYSFAYKSQLERHMATHMPGR	260
humanDeltaEFlb	SQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	226
humanDeltaEFla	SQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	210
mouseDeltaEFl	SQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	206
chickenDeltaEFl	SQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	226
XenopusDeltaEFla	SQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	229
ZebraKheper	SQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	193
humanSIP1	AQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	267
XenopusSIP1	AQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	267
zebraSIP1b	AQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	272
zebraSIP1a	AQLTTCPCYCDRGGYKRLTSLKEHIKYRHEKNE DNFSCSLCSYTFAYRITQLERHMATHMPGR	253
flyZfh1	-----MSAAACLSSSTSSPEKTF-----CRICHKAFANVYRLORHMTSHDSESA	43
	. * . . * . . * * * * * ! * * * * ! * .	

Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFla
 humanDeltaEFla
 mouseDeltaEFla
 chickenDeltaEFla
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1
 zebraSIP1b
 zebraSIP1a
 flyZfh1

```

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DQVCERCNKA FVNIYRLQRHMLTHTSG--NRKFKCCECGKAFYKHHLKEHLRIHSGEKPY 319
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E-----QRHVTQSGGGNRKFKCTECGKAFYKHHLKEHLRIHSGEKPY 237
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D-----QPQLNEGAG--NRKFKCTECGKAFYKHHLKEHLRIHSGEKPY 296
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```

***** ** *****;*****;*****;

Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
 humanDeltaEFla
 mouseDeltaEFla
 chickenDeltaEFla
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1b
 zebraSIP1a
 flyZfh1

```

ECPTCKRFRSHSGSYSSHSI SSKKCI-----PVKEQPPALTRITGVP 346
ECTPCCKRFRSHSGSYSSHSI SSKKCI-----PSKYMP-SLLLSITKAA 285
ECPNCKRFRSHSGSYSSHSI SSKKCI GLISFRNMMAE-----MPNVVPTSMHLLMAQA 373
ECPNCKRFRSHSGSYSSHSI SSKKCI SLIPVNGRPT-----GLKTSQCS SPSLSASPGSP 323
ECPNCKRFRSHSGSYSSHSI SSKKCI SLIPVNGRPT-----GLKTSQCS SPSLSASPGSP 307
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ECSNCKRFRSHSGSYSSHSI SSKKCI GLISVNGRVRHG-----VNNKPGSSPNASAASPGSP 352
GCDNCKRFRSHSGSYSSHSI SSKKCI SMG-----LKLNNRA 112
  
```

* * * *****;*****;*****

Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
 humanDeltaEFla
 mouseDeltaEFla
 chickenDeltaEFla
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1
 zebraSIP1b
 zebraSIP1a
 flyZfh1

```

PVKVINLSPVLPQSP LSDIDTSES-----LPLEPKKSAPAYMTP LHIKI 392
PVKLISEDMSPVTG--PHNIHNYIPG-----SFYQSDKPSNDDDDALSINV 330
AANPTVSLPNGS ANPATNGLHASSTGLLQQAQLNLTLP SLLAATPPLGAASTLQNPVHVKT 433
TRPQIRQKIEN--KPLQEQ LSVNQIKTEP-----VDYEFKPVVAS--GINCSTPLQN 372
TRPQIRQKIEN--KPLQEQ LSVNQIKTEP-----VDYEFKPVVAS--GINCSTPLQN 356
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ARVQVRDLKDNS--KPLQEQ LPLTIQIKSEP-----LDYEFKPVVAS--SARGVN 342
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AITQLRHKLENGKPLMS EQPSGLLKIKTES-----LDFNDYKVLIMASHAFNGAHPFMN 418
AISQLRHKLENGKPLGLQDQSNHLNIRSEP-----LDFNDYKVLIMAS--HGATGSPFLN 422
ALAQLRHKLENGR SMLQDPSAHTDIKSEP-----MDFNEYRHLIASQQEYGASGAFLN 406
LLKRLKLESPGSSASARRSPSDHGKGLPE-----QPSPGLPHPMYSYFASDAQVQG 164
  
```

Smad-binding domain

Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
 humanDeltaEFla
 mouseDeltaEFla
 chickenDeltaEFla
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1
 zebraSIP1b
 zebraSIP1a
 flyZfh1

```

PNADGSVQGHPSNETLENGNTPLTSPAN-----DAVKKVLQIVAS--IVCRQOK 440
NTSDDQSQ-----DKSITITSPPN-----DAVKKVLQIVGA--IVSRQOK 368
EPMDTLSPHSSRS SSI SPS SSGVYV SSSGSS TE SPVKGDINQVKKVLQIVEN--IVTRQOK 492
GVFTGGGQLQATSSPQGMVQAVVLPVGLVS---PISINLSDIQNVLKVAVDGNVIRQVL 429
GVFTGGGQLQATSSPQGMVQAVVLPVGLVS---PISINLSDIQNVLKVAVDGNVIRQVL 413
GVFTSGGQLQATSSPQGMVQAVVLPVGLVS---PISINLSDIQNVLKVAVDGNVIRQVL 409
GVFTSGGQLQATSSPQGMVQAVVLPVGLVS---PISINLSDIQNVLKVAVDGNVIRQVL 429
GVFTSGSLQATSSPQGMVQAVVLPVGLVS---PISINLSDIQNVLKVAVDGNVIRQVL 432
GMFGGQ---AAAPLQAVQAVVLPVGLVS---PISINLSDIQNVLKVAVDGNVIRQVL 395
GGLGATSPVGVHP SAQSPHQHLGVGMEAPLLGFP TNN S NLSEVQKVLQIVDN--IVSRQOK 476
GGLGATSPVGLIHS SAPSPHQHLGVGMEAPLLGYP S LNNLSEVQKVLQIVDN--IVSRQOK 477
GGVRGSPVGLIHN--SQSP LQHLGMEIEGQMLGYP S LNNLSEVQKVLQIVDN--IVCRQOK 480
GGRRGSPVGMHSSQNPL QHLGIGSDSHPLGYTGFI NNMSEVQKVLQIVDN--IVCRQOK 465
GSAAPAPFPFPHPNYMAALLAFPHNFMAAAAGLDRVHPYSIQRLLQL SAAGQQOREE 224
  
```

! : ! * : ! : ! :

Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
 humanDeltaEFla
 mouseDeltaEFla
 chickenDeltaEFla
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1
 zebraSIP1b
 zebraSIP1a
 flyZfh1

```

DQOKTDISKLKKTKTSDTAPVVASPF EIRP F KLEKMAAVTLPKATPP PPSSENTPEKTP 500
EGQKNVAKLKKAKQPPMERE SKDPVNEVK-----TAEKIVLPEI PREETPAKSP 518
ETGAMSITWPRAVD NAAGNLSDEL SKIREN-----AKAMSLATEALS SDAARLPL 443
ENNQANLASKEQETINASP IQQGGH-----SVISAI S LPLVDQDGTITKI 473
ENNQANLASKEQETINASP IQQGGH-----SVISAI S LPLVDQDGTITKI 457
ETNQANLASKEQAVSASP IQQGGH-----SVISAI S LPLVDQDGTITKI 453
ENNHANLASKEQETINASP IQQGGH-----SLISAI S LPLVDQDGTITKI 473
ENNHANLASKEQETINASP IQQGGH-----SVISAI S LPLVDQDGTITKI 476
ESTQAKGQAGTGI V GAGGLAQATQ-----QVIQAL S LPLVDQDGNAKI 439
DCKAEEI S K L KGYHMKDPC SQPEEQ--GVT-----SPNTPVGLPVVSHNGATKS 524
ECKPEE I T K L KGYHMKDPC SQPEEQ--GVT-----SPNTPVGLPVVSHNGATKS 525
DCKPEE I S K L KAY--MKELGSHIEEQKQLN-----SSGGGQLPLINHGATKS 529
DGNPEE I S K L RAY--MKELGSHIEEQ--N-----RVLASQGSFLGVGHNSPTKT 510
REEQQKQQHDEEETPDEPKLVMDIE-----EPETKEMAPTEATEAATPI 270
  
```

Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
 humanDeltaEFla
 mouseDeltaEFl
 chickenDeltaEFl
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1
 zebraSIP1b
 zebraSIP1a
 flyZfh1

ESEKPKFTLNGNGVHQEKKSYNIVDYTLKVKVHEAQAINRCLETRFPSSQINLNPDRGTGCK 560
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 IINYSLEQPSQLQVVPQ-----NLKKE-NPVATNSCKSEKLPEDLTVKSEKDKS 505
 IINYSLEQPSQLQVVPQ-----NLKKE-IPATNSCKSEKLPEDLTVKSEKDKS 501
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 IIDYTLKVNKAKACQLSLTDSRRQISNLIKKEKRLTLDLVDKMIENHNIPTPFCQ 585
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Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
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 mouseDeltaEFl
 chickenDeltaEFl
 XenopusDeltaEFla
 ZebraKheper
 humanSIP1
 XenopusSIP1
 zebraSIP1b
 zebraSIP1a
 flyZfh1

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 FEGGVNDSTCLLDCDCPGDINALPELKHVDLQPTQPPPL----- 545
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 FEGETNDSTCLLDCDCPGDINALPELKHVDKMPPLQPS----- 561
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 -----DKTITLTVDEKSMHNDIL-LKHCQ----- 507
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 FCKESFPGPPIPLHQHERYLCMNEEIKAVLQPHENTVPMK----- 625
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 YCKETFPGPPIPLHQHERYLCMNEEIKAVLQPAQNALTNK----- 610
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Hp-Zfhx
 Saccoglossus-Zfhx
 amphioxus-Zfhx
 humanDeltaEFlb
 humanDeltaEFla
 mouseDeltaEFl
 chickenDeltaEFl
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 ZebraKheper
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 zebraSIP1a
 flyZfh1

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 -----SGTEAEKPESSAPSETGENNLSGQP--- 587
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Homeodomain

Hp-Zfhx
 Saccoglossus-Zfhx
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 zebraSIP1b
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 flyZfh1

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 -----PLKNLSSLKAYYALNAQPSAEELSKTADSVNLPDVLVKKWFEKMQ-- 617
 -----PLKNLSSLKAYYALNAQPSAEELSKTADSVNLPDVLVKKWFEKMQ-- 611
 -----PLKNLSSLKAYYALNAQPSAEELSKTADSVNLPDVLVKKWFEKMQ-- 633
 -----PLKNLSSLKAYYALNAQPSAEELSKTADSVNLPDVLVKKWFEKMQ-- 632
 -----PLKNLSSLKAYYALNNEPTKEELAKISESVLPAEVLVKKWFEKMQ-- 578
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 -----LYKDHMSVLKAYYAMNNEPNSDELLKISTAVGLPQEFVKEWFEQRK-- 697
 -----PYKDHMSVLKAYYAMNNEPNSDELLKISTAVGLPQEFVKEWFEQRK-- 701
 -----PYKDHMSLVNYSMNTEPNSDELLKISTAVGLPQEFVKAFAVQWKAQS 685
 -----TAINEEQQQQLKQHYSLNARFSDERFMTIARLQILDPRVVOVWQNNR-- 444

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 zebraSIP1b
 zebraSIP1a
 flyZfh1

EEEGSDPLKSLSLMRQSPVVTVPPTQORRIKEHTGAQAIDSKEACVKS PAENCDLETAS 799
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 AGQISVQSSEP-----SSPEPGKVNIPAKNNDPQSANANE PQDST 658
 AGQIPGQSPDP-----PSPGTGSVNIPTKIDEPQPADGNE PQEDS 652
 AGQISVQSSGP-----SSPEQVKSISPTDNDQAATTNESE PQNST 674
 SGEIPVDSQP-----SSPKPQNSVIENSVKSKTE SGLNDG--TS 671
 LGQISMDPSSP-----QHEE--EQITPVLDLGTGASPKPDLDEQM 617
 VYQYSNRSPSLERS--KPLAPNSNPTKDSL----LPRSPVKPMSDITSPSTAEIHNVS 751
 VYQYSNRSPSLERS--KPLAPNSNPTKDSL----LPRSPVKPMSDITSPSTAEIHNVS 752
 VFQYTSRTPPLDRSPVESIHPVSAHTPIKDSLGIRSPMSLVKGSDRITSPAIPELHN-- 759
 HHSFRKRSPPPERSG-----ETNHVRDFAFARSVSLGQYGDSTAEIQAITNGDSG 737
 SREKMQSFQN-----NQAGAAPPMPIDSQASLTREDQPLDLVSKR 486

Hp-Zfhx
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XenopusSIP1
zebraSIP1b
zebraSIP1a
flyZfh1

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VNLQSP-----KMTNSPVLVPGSTINGRSRSTP---SPSPLNLSR 698
TRGQSPV-----KIRSSPVLVPGSAMNGRSRSTP---SPSPLNLSR 692
NNSQNP-----NTSKSQTSGGSTQNGRSRSTP---SPSPLNLSR 714
VPLKSPK-----QEAEPL-----AVNGSESGHR---FSYITKLSLE 706
NSEKQEE-----RECCSPAEGIAASVNGIE SVPA---SPSPLNLS--- 654
TNCDPPL-----RLTKPSHFITNKP-VEKLDHSRSNTPSPPLNLSSTS 792
XenopusSIP1-----RLTKPSHFITNKP-VEKLDHSRSNTPSPPLNLSSTS 794
zebraSIP1b-----RLSKTPQYSNHKQLGKMDHSRSNTPSPPLNLSSTS 800
zebraSIP1a-----QITG-TRQITNEKP-LDSVDHLRGETSPPLNLSSTS 777
flyZfh1-----SESSPPYIAPSGEALNPEAINLSRKFSTSA 525

Hp-Zfhx
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humanDeltaEFla
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XenopusDeltaEFla
ZebraKheper
humanSIP1
XenopusSIP1
zebraSIP1b
zebraSIP1a
flyZfh1

MERDSRSPARSITPAKETRDSSPLDLSLPRKATPPPSLSRSPKIPKLVYGYNSALYSA 919
IVSEPSPPSQERTKNEQ---PLDLSMPKLLKVS--SKARR-----FPQTSQYHQ 805
LPPFGQGIAPFPFGYIDFLASPMTGAFNLGLSGP----- 870
NTQGYLYTAEG---AQEEPQVEPLDLSLPKQGG-----EL 746
humanDeltaEFla-----AQEEPQVEPLDLSLPKQGG-----EL 730
mouseDeltaEFla-----AQEEPQVEPLDLSLPKQGG-----EL 724
chickenDeltaEFla-----VQEEPMQVEPLDLSLPKQGG-----EL 746
XenopusDeltaEFla-----PDGEGQAEPLDLSLPKQGG-----NV 738
ZebraKheper-----EAGE---GPLDLSLPK--- 678
humanSIP1-----SKNSHSSSYTPNSFSSEELQAEPLDLSLPKQMKEP-----KSI IATKN 835
XenopusSIP1-----SKNSHSSSYTPNSFSSEELQAEPLDLSLPKLNES-----KTI IATKN 833
zebraSIP1b-----SKNSHTSYTPNSFTSEDLQAEPLDLSLPKMKEP-----KHII IATKS 843
zebraSIP1a-----SKNSHSSSYTPNSLTSSEDAHGEPLDLSLPKQVS-----KAER 814
flyZfh1-----SPASISPSAAALYFGAAPPSPNSQLDSTP----- 557

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zebraSIP1b
zebraSIP1a
flyZfh1

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-----YLTPGFNAATLSTKRPLDTPTRRRRRRRVY-----DNGSRVPHATQS- 914
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humanDeltaEFla-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 779
mouseDeltaEFla-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 773
chickenDeltaEFla-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 777
XenopusDeltaEFla-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 795
ZebraKheper-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 796
humanSIP1-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 795
XenopusSIP1-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 796
zebraSIP1b-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 796
zebraSIP1a-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 796
flyZfh1-----EPLNLSCA-KKEPKQKDCV-----TDSEPVVNVIPP 796

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humanSIP1
XenopusSIP1
zebraSIP1b
zebraSIP1a
flyZfh1

TSSASDAS ILLKRSYPELYAYLPTMGYAAKMRFAVDGYVPVAPPMMYGSGLQVPDVY 1033
NPSA--ARILTHAIELEPHLYAAHHHPG-----LHNSLAAAAPITQGPITKLLKMLD 909
-----VTPVQAPSPMHPMDDPTQSPAL 938
SAMP-----INI AIPVTVAQLPTTVAIADQNSVPC 825
humanDeltaEFla-----INI AIPVTVAQLPTTVAIADQNSVPC 809
mouseDeltaEFla-----INI AIPVTVAQLPTTVAIADQNSVPC 803
chickenDeltaEFla-----INI AIPVTVAQLPTTVAIADQNSVPC 825
XenopusDeltaEFla-----INI AIPVTVAQLPTTVAIADQNSVPC 816
ZebraKheper-----INI AIPVTVAQLPTTVAIADQNSVPC 750
humanSIP1-----LYTALPPQSAPPATFMPVQTSIPG 919
XenopusSIP1-----LYSALPPQSAPPATFMPVQTSIPG 919
zebraSIP1b-----MYTSLPPQSAPPATFMPVQTSIPG 930
zebraSIP1a-----MYTSLPPQSAPPATFMPVQTSIPG 903
flyZfh1-----RSGQAFPLGPPYMLPMSL PHEAL 580

Hp-Zfhx
Saccoglossus-Zfhx
amphioxus-Zfhx
humanDeltaEFla
humanDeltaEFla
mouseDeltaEFla
chickenDeltaEFla
XenopusDeltaEFla
ZebraKheper
humanSIP1
XenopusSIP1
zebraSIP1b
zebraSIP1a
flyZfh1

SALAKTGFMPSTMAHLGAAATGPNPFLPAMLNGGG-LHPNHAGADNLSDIASEDS LSDIG 1092
G-LMSTGSPDIMP--AGRHFMIEDEIRNSLGSNP-MSSYHPGAVHRDLSSEGLDES- 964
AQETAEGSPSPSPSGTYPGYIGDSASSPGSPA-LSTGTP----- 979
humanDeltaEFla-----LRLAANKQTILLIPQVAYTYSTIVS-PAVQEPPLK-VIQPNQNDERQDTSSEGVSNVED 883
humanDeltaEFla-----LRLAANKQTILLIPQVAYTYSTIVS-PAVQEPPLK-VIQPNQNDERQDTSSEGVSNVED 867
mouseDeltaEFla-----LRLAANKQTILLIPQVAYTYSTIVS-PAVQEPPLK-VIQPNQNDERQDTSSEGVSNVED 861
chickenDeltaEFla-----LRLAANKQTILLIPQVAYTYSTIVS-PAVQEPPLK-VIQPNQNDERQDTSSEGVSNVED 883
XenopusDeltaEFla-----LRLAANKQTILLIPQVAYTYSTIVS-PAVQEPPLK-VIQPNQNDERQDTSSEGVSNVED 874
ZebraKheper-----LRLAANKQTILLIPQVAYTYSTIVS-PAVQEPPLK-VIQPNQNDERQDTSSEGVSNVED 810
humanSIP1-----LRYPGLDQMSFLPHMAYTYPGAATFADMQRK-YQRKQGFQGLDGAQDYMSGLDD 978
XenopusSIP1-----LRYPGLDQMSFLPHMAYTYPGAATFADMQRK-YQRKQGFQGLDGAQDYMSGLDD 978
zebraSIP1b-----LRYPGLDQMSFLPHMAYTYPGAATFADMQRK-YQRKQGFQGLDGAQDYMSGLDD 989
zebraSIP1a-----LRYPGLDQMSFLPHMAYTYPGAATFADMQRK-YQRKQGFQGLDGAQDYMSGLDD 962
flyZfh1-----FKMRPGDFASNAHLMNSIKLPDYRGTSLSPGGSE-KRSWRDDDSRI SHEDEFGAGVLM 639

Zinc-finger domains

Table listing amino acid sequences for Zinc-finger domains across various species (Hp-ZfHX, Hp-ZfHX, Hp-ZfHX) and their corresponding protein IDs (1151, 1024, 1033, 943, 927, 921, 943, 934, 870, 1038, 1038, 1049, 1022, 699, 1201, 1069, 1080, 1000, 984, 978, 1000, 991, 930, 1095, 1095, 1106, 1079, 747, 1241, 1087, 1044, 1028, 1026, 1045, 1033, 981, 1144, 1144, 1157, 1134, 1256, 1099, 1083, 1085, 1089, 1068, 1041, 1188, 1188, 1197, 1180, 1124, 1108, 1117, 1114, 1091, 1078, 1214, 1214, 1219, 1201).

Supplemental Figure 2.

Forebrain embryonic zinc finger, *Fez*, is not required for the expression of *zfhx1/z81*. *Zfhx1/z81* is expressed in the animal plate region of (A) control (Gly; glycerol-injected) embryo (arrows) and (B) *Fez* morphants (arrows) at 36-hours post-fertilization. Note that the animal plate in which serotonergic neurons develop is smaller in *Fez* morphants (Yaguchi et al., submitted).

Supplemental Figure 2.

