Molecular Mechanisms of Development of Serotonergic Neurons in the Sea Urchin Embryo

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Abstract

In neurogenic ectoderm at animal pole (animal plate) in sea urchin embryo, various gene expressions are localized along the dorsoventral axis, and serotonergic neurons are differentiated only at dorsal/lateral side of the plate. It is already reported that the localization of serotonergic neurons is influenced by TGF-ß signals from outside of nueroectoderm, but the detailed mechanism and the gene regulatory network of the differentiation of serotonergic neurons are not understood yet. It is shown that some genes expressing at neurogenic animal plate of sea urchin embryo are orthologous to those in vertebrate forebrain, suggesting that the study of neural differentiation in echinoderms lead us to uncover the universal developmental mechanism of neurogenic ectoderm and the evolution of concentrated nervous system in deuterostomes. Here, I revealed the molecular mechanisms of differentiation of serotonergic neurons through the analysis of two transcription factors, Zinc finger homeobox 1 (Zfhx1) and Homeobrain (Hbn). With loss-of-function analysis, I showed that both of them are required for differentiation of the serotonergic neurons. As well, the expression of mRNA encoding those proteins is strongly regulated by TGF-ß signals. In this study, I provide important observations and insights toward entire elucidation of the gene regulatory network for the development of anterior neurogenic ectoderm including the serotonergic neurons.

General Introduction

A fraction of the large group of Six3-dependent regulatory proteins in the sea urchin embryos are orthologous to those expressed in vertebrate forebrains, suggesting that they controlled formation of the early neurogenic domain in the common deuterostome ancestor of echinoderms and vertebrates (Wei et al., 2009). This consequence shows that the study of neurogenesis in sea urchins not only provide elucidation of universal mechanisms of neural development but also offer valuable insight for thinking about emergence of concentrated nervous system, central nervous system, and brain at the evolutionary process. In variable animals, it is being advanced to reveal of mechanisms of genes that necessary for process of differentiation of neurons and nervous system, also in sea urchin, large number of genes that indicate the participation in neurogenesis is reported from the genomic information and the knowledge is being accumulated (Burke et al., 2006, Angerer et al., 2011). However the molecular pathway that controls the differentiation of neurons and the detailed gene functions involved in the pathway are less information in variable animal yet.

Serotonergic neurons in the sea urchin embryos are formed at the dorsal edge of the neurogenic animal plate by 3 days post-fertilization (Yaguchi et al., 2000). This deflection of localization of the neurons to the dorsal edge is intriguing, because the entire animal plate has a potential to produce serotonergic neurons everywhere if any known transforming growth factor-ß (TGF-ß) signals are absent in this region (Yaguchi et al., 2010a). This suggests that TGF-ß signaling system decides the precise position of

the serotonegic neurons in the animal plate. In this respect, the sea urchin embryo is a great model to understand the relationship between the cell fate specification/patterning of neurogenic ectoderm as well as serotonergic neurons and the secreted signaling molecules like members of TGF-ß family, because the simultaneously functional analysis of multiple genes including complicated microinjection methods based on embryology is applicable. In addition, the echinoderms including the sea urchin phylogenetically share the common ancestors with chordates, suggesting that the study of the sea urchin nervous system will let us know the universal mechanisms regulating the formation of nervous system, at least among the deuterostomes, during animal evolution. However, although the knowledge on development of nervous system of the sea urchin is being accumulated, still only a countable number of papers that challenged to understand the molecular pathway for neural differentiation are published so far. Two papers, in which I was one of the authors, revealed the involvement of FoxQ2 and Six3 in animal plate formation. These two factors are one of the earliest genes zygotically expressed in the animal pole region and are required for specification of the entire neurogenic ectoderm; i.e. FoxQ2 or Six morphants lose the neurogenic ectoderm (Yaguchi et al., 2008; Wei et al., 2009). So, because the morphants lack the entire animal plate, we could not understand the molecular pathway to form serotonergic neurons in these studies. In this thesis, I show how the serotonergic neurons are formed at the precise position, and draw the molecular pathway from the initial input like FoxQ2 activity to the terminal differentiation of serotonergic neurons through analyzing the molecular function of two transcription factors, Zinc finger homeobox 1 (Zfhx1)

and Homeobrain (Hbn), in the sea urchin embryos. Simultaneously, I show their gene expressions and the consequent functions are strongly regulated by TGF-ß signals that play an essential role on the secondary axis specification/formation in the sea urchin embryo.

Part I.

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

Summary

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, I reveal that *zinc finger homeobox* (*zfhx1*) 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* begins to be expressed at gastrula stage in individual cells in the animal plate, some of which also express *delta*. *zfhx1* expression gradually disappears as neural differentiation begins with *tph* expression. When the translation of Zfhx1 is blocked by morpholino injection, embryos express neither *tph* nor the neural marker *synaptotagmin B* in cells of the animal plate, and serotonergic neurons do not differentiate. In contrast, Zfhx1 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* is one of the targets suppressed in the animal plate by anti-neural signals such as Nodal as well as Delta-Notch. I conclude that Zfhx1 functions during the specification of individual anterior neural precursors and promotes the expression of *tph* and *synaptotagmin B*, required for the differentiation of serotonergic neurons.

Introduction

The presence of serotonergic neurons in animal plate, 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 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/ß-catenin or Nodal/BMP2/4 signaling supports differentiation of anterior neuroectoderm, which contains the animal plate. Subsequently Wnt/ß-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 I show that Zinc finger homeobox (Zfhx1) 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. I show that Zfhx1 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 neurogenic animal plate 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, synaptotagmin B (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). I 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 [Zfhx1-MO1 (2.0 mM), Zfhx1-MO2 (1.9-3.8 mM)] were used to confirm the specificity of Zfhx1 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-MO1: 5'- ACGTAGGTATGTTCCAAAACACAAG -3', and

Zfhx1-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* 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 neurogenic animal plate ectoderm was one encoding a zinc finger-containing protein, called Z81 (Materna et al., 2006). Further studies showed that its expression in the animal plate 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, I 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 animal plate arranged in a pattern suggesting they could be serotonergic precursors (Fig. 1-1A, B). Because revealing the transcription factor activities required for specification or differentiation of serotonergic neurons in sea urchin embryos is the primary goal, I selected this gene for further study. I 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. I 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. 1-1C, Fig. 1-2). Among the 4 classes of vertebrate Zfhx proteins, this non-vertebrate, deuterostome group type is more closely related to Zfhx1a (Delta-EF; ZEB1) and Zfhx1b (SIP1; ZEB2) than to Zfhx3 and Zfhx4. Among other invertebrate proteins, Fly-Zfh-1 and C. elegans Zag-1 are the closest. Therefore, I named it Hp-Zfhx1 (Zfhx1 hereafter in this paper).

zfhx1 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. 1-1D, E). The function of Zfhx1 at this early time is discussed in elsewhere (Su et al., 2009). Expression in this domain disappears when the embryo hatches (Fig. 1-1F), and appears in a new set of cells in the endomesoderm region at mesenchyme blastula stage (Fig. 1-1G). Adding to the vegetal expression, when the gut begins to invaginate, *zfhx1* 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. 1-1H, arrows and arrowheads, respectively; Howard-Ashby et al., 2006). At later stages, *zfhx1* is expressed in a pattern like that of the future ciliary band neurons (Fig. 1-1J-L; most clearly revealed in the fluorescent *in situ* hybridization in panel K) (Bisgrove and Burke, 1986; Nakajima et al., 2004). Here I focus only on *zfhx1* expression in the animal plate because the pattern of its expression is similar to that of serotonergic neurons (Fig.

1-1A). At the prism stage, *zfhx1* continues to be expressed in similar regions as those in gastrulae, but disappears from the central part of the animal plate (Fig. 1-1I, 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* (Fig. 1-1J-L; black and red arrow, respectively). In contrast, the expression in animal plate region begins to disappear at this stage (Fig. 1-1L, bracket).

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

To investigate when and where *zfhx1* is expressed in the animal plate region in detail, I performed double fluorescent *in situ* hybridization detecting *zfhx1* 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*-expressing cells in the animal plate (as described in Figure 1-1) begin to express *tph* at late gastrula stage (36 hours post fertilization (h); Fig. 1-3A-D, arrows). This indicates that *zfhx1* is expressed in serotonergic neural precursor cells. However, although these neural precursors express both genes at 36 h (Fig. 1-3A-D), at 39 h most of them lack *zfhx1* transcripts (Fig. 1-3E-H, arrowheads), suggesting that *zfhx1* expression precedes *tph*. At this stage, a cell appears which expresses *zfhx1* strongly but *tph* weakly and is likely to be a new serotonergic precursor cell (Fig. 1-3E-H, asterisk). Next, I compared distributions of *zfhx1* and *fez*, *forebrain embryonic zinc finger*, 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. 1-3K), *zfhx1* mRNA is present in the same cells (Fig. 1-3I-L, arrows). Afterward, *zfhx1* transcripts disappear by the prism stage, whereas *fez* mRNA remains in the serotonergic neurons (Fig. 1-3M-P, arrowheads). Taken together, *zfhx1* 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 is required for the differentiation of serotonergic neurons

The spatial and temporal expression pattern of *zfhx1* suggests that it might be involved in the specification and/or differentiation of serotonergic neurons in the sea urchin embryo. To examine this, I blocked the translation of *zfhx1* by injecting morpholino anti-sense oligonucleotide (MO; Zfhx1-MO represents Zfhx1-MO2 throughout this study otherwise indicated). In embryos injected with Zfhx1-MO at 2 mM, gastrulation is delayed (Fig. 1-4F) and their body size becomes smaller than normal (Fig. 1-4A-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. 1-4D, E, I, J). Although serotonergic neurons do not appear in 3.8 mM Zfhx1-MO-injected embryo as well as in the 2.3 mM Zfhx1-MO1-injected embryo (Fig. 1-4P), it is unclear whether this effect results directly

from blocking Zfhx1 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. 1-4K-O). Indirect effects are possible because *zfhx1* is expressed broadly in ectoderm at early blastula stage (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. 1-1).

To eliminate possible indirect effects, I examined Zfhx1 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*-expressing cells are scattered throughout the expanded animal plate of these embryos at 24 h (Fig. 1-5A, B). As development proceeds, the number of *zfhx1*-positive cells gradually decreases, as observed in normal embryos (Fig. 1-5B-D), especially, in the central part of the expanded animal plate where *foxQ2* is strongly expressed (Fig. 1-5E). At 2 days after fertilization, the Δcad-injected embryo lacks *zfhx1*

expression in individual cells completely (Fig. 1-5F). Therefore, the expression patterns of *zfhx1* in the expanded animal plate reflect the behavior of *zfhx1* in normal embryos. If Zfhx1 is knocked down in these embryos, development of serotonergic neurons is strongly inhibited (3.8 mM Zfhx1-MO2 injection; Fig. 1-5J-L). This morpholino effect is confirmed by injecting 2.0 mM Zfhx1-MO1 (data not shown). This is also true in animal-half embryoids (Fig. 1-5M, O), because loss of Zfhx1 completely eliminates the large number of serotonergic neurons normally present in them (Yaguchi et al., 2006) (Fig. 1-5N; *cf.* with G). To confirm that the requirement for Zfhx1 for serotonergic neuron differentiation is cell-autonomous, Zfhx1-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. 1-5P). In these embryos, the serotonergic neurons differentiate normally in the myc-negative, Zfhx1-positive side but not in the myc-positive, Zfhx1-negative region (Fig. 1-5Q, R). The lack of serotonergic neurons at the border of first cleavage plane next to Zfhx1-positive cells strongly supports the idea that Zfhx1 is not required for even short-range signals promoting serotonergic neuron differentiation, but rather acts cell-autonomously. Together, these results indicate that Zfhx1 is required for the differentiation of serotonergic neurons in the animal plate.

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

To examine at which step Zfhx1 is involved during the specification and differentiation of serotonergic neurons, I examined Zfhx1 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). I again used Δcad-injected embryos to eliminate indirect effects caused by Zfhx1 functions at earlier stages in other regions of the embryo. In Δcad-injected Zfhx1 morphants *foxQ2* is expressed throughout the expanded animal plate as in control Δcad alone-injected embryos (*cf*. Fig. 1-6A with B) but *tph* is not expressed at all (Fig. 1-6B), indicating that Zfhx1 is required for *tph* expression but not for *foxQ2*. As well, *fez*, another serotonergic neural marker, is expressed in Δcad-injected Zfhx1 morphants as in control embryos, indicating that Zfhx1 is not required for neuron-specific expression of *fez* (Fig. 1-6C, D). Conversely, *zfhx1* expression does not require Fez (Figure 1-7), 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 1-3, *zfhx1* transcripts gradually start to disappear from the animal plate in control Δcad alone-injected embryos (Fig. 1-6E). However, intriguingly in Δcad-injected Zfhx1 morphants, *zfhx1* transcripts remain (Fig. 1-6F), indicating that *zfhx1* is regulated by auto-repression mechanism in these embryos (Fig. 1-6G, H). These results support the temporal expression data (Fig. 1-1, 1-3), which suggests that *zfhx1* and *fez* transcripts appear after *foxQ2* is expressed, but before the serotonin synthase tryptophan 5-hydroxylase gene, *tph*. Although both *zfhx1* and *fez* depend on FoxQ2 and are co-expressed in cells in the *foxQ2*-positive animal plate (see below, Fig. 1-8), they have independent roles in these serotonergic precursors, since Zfhx1 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 animal plate through Notch signaling is that a cluster of contiguous serotonergic neurons develops on the aboral side of the animal plate (Fig. 1-8C-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*. This is in fact the case since fluorescent double *in situ* hybridizations showed that it is co-expressed with *zfhx1* in serotonergic neuron precursors in the animal plate (Fig. 1-8D-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* is followed by *tph*.

To establish regulatory relationships between FoxQ2, Delta and Zfhx1, I carried out a series of morpholino-mediated knock-downs. In FoxQ2 morphants, in which serotonergic neurons fail to differentiate, neither *delta* nor *zfhx1* is expressed in the animal plate region (Fig. 1-8I-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* requires FoxQ2 function. When the translation of *delta* is blocked by injecting Delta-MO, *zfhx1*-positive cells increase in number and are immediately adjacent to each other, making a cluster in the animal plate region (*cf*. Fig.

1-8A with B; stacks of a few optical sections), as do serotonergic neurons (Fig. 1-8C-C'''). These data suggest that Delta functions to inhibit neighboring cells, but not its own expressing cells, from differentiating as Zfhx1-expressing serotonergic neuronal precursors. Delta expression in animal plate cells does not require Zfhx1 because it is expressed in the same scattered pattern as serotonergic neurons in Δcad-injected embryos that either contain or lack Zfhx1 (Fig. 1-8M, N). Taken together, Zfhx1 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* 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. 1-9F; Yaguchi et al., 2007). As expected, in normal embryos, *zfhx1* is also expressed in cells at the aboral/lateral edge of the *foxQ2*-positive animal plate region at gastrula stage (Fig. 1-9A, B), and at prism and pluteus stages the serotonergic neurons expressing *tph* gene are aligned similarly (Fig. 1-9G). When the translation of Nodal is blocked by injecting Nodal-MO, *zfhx1*- and *tph*-positive cells surround the animal plate (Fig. 1-9C, asterisks; 1-9H, 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* nor *tph* is expressed in the

animal plate (Fig. 1-9D, I). When translation of BMP2/4, another TGF-ß member involved in cell fate specification along the aboral side of the embryo, is blocked, the morphants also do not express *zfhx1* and *tph* (Fig. 1-9E, J). In these morphants Nodal signaling extends further to the aboral side (Yaguchi et al., 2010a), where it suppresses expression of *zfhx1* and differentiation of serotonergic neurons. Taken together, Nodal signals in the oral ectoderm suppress the expression of *zfhx1* 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 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 *synaptotagmin B*, at late gastrula stage (Yaguchi and Katow, 2003; Burke et al., 2006). Here I show that Zfhx1 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 significantly decreases the number of serotonergic neurons (Yaguchi et al., 2008; this study) and FoxQ2 morphants do not express *zfhx1*. Furthermore, *zfhx1* 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, I 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 mark neuronal precursors. As well, the expression pattern and timing of *zfhx1* relative to terminal differentiation genes is appropriate for its requirement for the differentiation of serotonergic neurons. Zfhx1

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* and *tph*. Together, the expression patterns and loss-of-function data indicate that FoxQ2 is required for *delta* and *zfhx1* expression in neuronal precursors. Delta/Notch signaling limits the number of these precursors and Zfhx1 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* expression because here I show that Nodal suppresses *zfhx1* 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 as well as fly Zfh-1 have no conserved SBD sequence (Fig. 1-2). 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 my 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. 1-1).

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 animal plate 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 function is presented in Figure 1-10. 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* and *fez* are expressed in these neural precursors. However, the expression of these three genes, *delta*, *zfhx1* and *fez*, is regulated by independent mechanisms because knock-downs of each does not affect the expression of other two (Fig. 1-6, 1-7, 1-8; Yaguchi et al., 2011).

At least, three independent signaling cascades regulate the differentiation of

serotonergic neurons: Wnt/ß-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* 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 itself). *zfhx1* expression depends on Six3 (Wei et al., 2009) and FoxQ2 . The fact that Six3 is important for maintaining *foxQ2* (Wei et al., 2009), may explain these observations (Fig. 1-8). Although FoxQ2 could provide direct inputs into regulating *zfhx1* 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* is expressed in only a subset of animal plate cells. The mechanism that activates expression of *zfhx1* 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*. Finally, Zfhx1-mediated negative auto-regulation of *zfhx1* transcription implies tight regulation of Zfhx1 levels is required in these neural cells (Fig1-6E-H). All of these mechanisms help to ensure *zfhx1* 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.

Figures

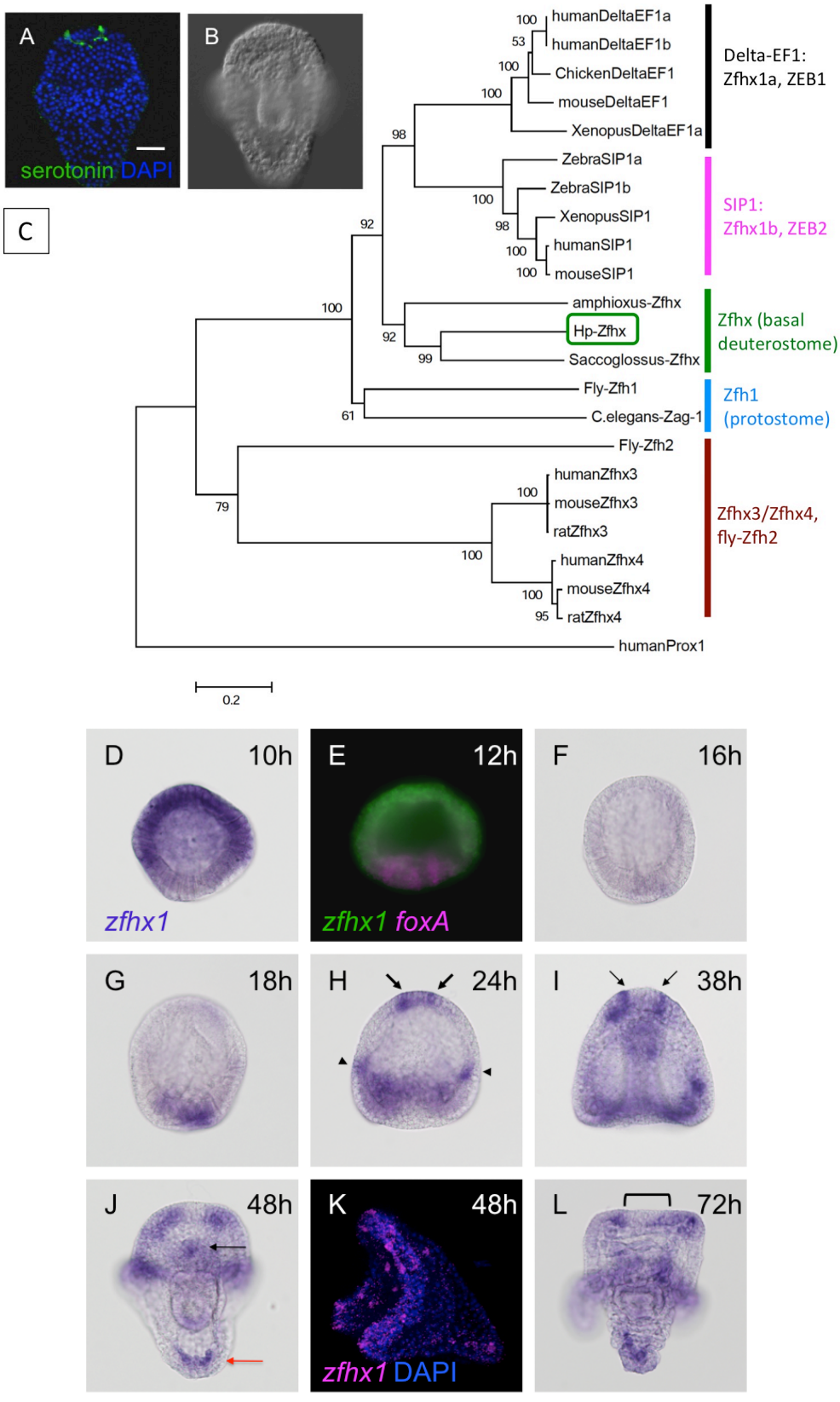
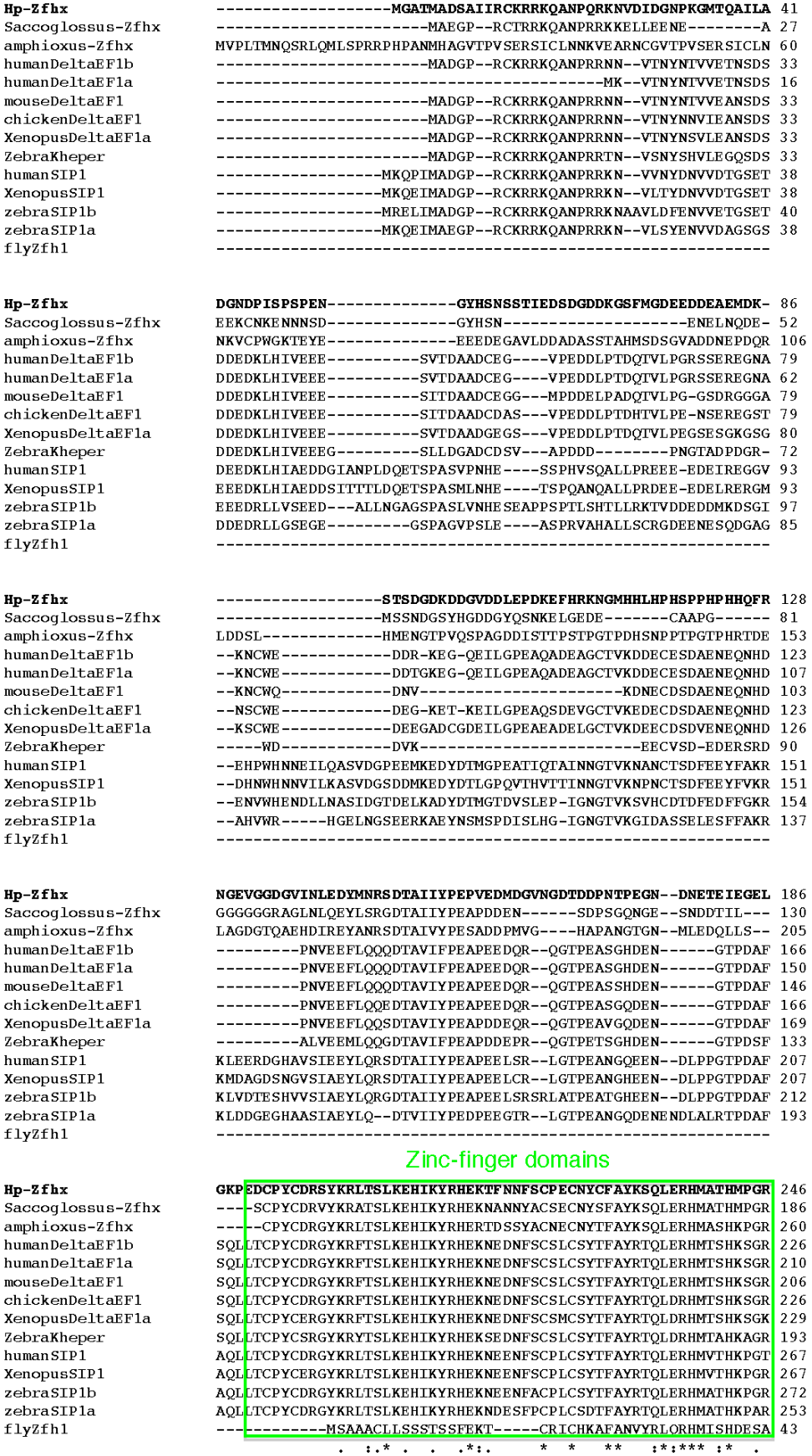

A
serotonin DAPI
B
C
humanDeltaEF1a
humanDeltaEF1b
ChickenDeltaEF1
mouseDeltaEF1
XenopusDeltaEF1a
Delta-EF1:
Zfhx1a, ZEB1
ZebraSIP1a
ZebraSIP1b
XenopusSIP1
humanSIP1
mouseSIP1
SIP1:
Zfhx1b, ZEB2
amphioxus-Zfhx
Hp-Zfhx
Saccoglossus-Zfhx
Zfhx (basal
deuterostome)
Fly-Zfh1
C.elegans-Zag-1
Zfh1
(protostome)
Fly-Zfh2
humanZfhx3
mouseZfhx3
ratZfhx3
humanZfhx4
mouseZfhx4
ratZfhx4
Zfhx3/Zfhx4,
fly-Zfh2
humanProx1
0.2
D 10h
zfhx1
E 12h
zfhx1 foxA
F 16h
G 18h
H 24h
I 38h
J 48h
K 48h
zfhx1 DAPI
L 72h

Figure 1-1. *zfhx1* 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 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* at the following stages. (D) unhatched blastula, 10 h. (E) double fluorescent *in situ* hybridization with *zfhx1* (green) and *foxA* (magenta) in unhatched blastula, 12 h. (F) hatched blastula, 16 h. (G) mesenchyme blastula, 18 h. (H) early gastrula, 24 h. Arrows and arrowheads show *zfhx1* expression in the animal plate and future ciliary band region, respectively. (I) prism larva, 38 h. The arrows indicate the outer edge of the central part of animal plate, where *zfhx1* is missing. (J) pluteus larva, 48 h. Black and red arrow shows *zfhx1* gene expression in lower lip region and posterior mesenchyme cells, respectively. (K) lateral view of pluteus larva, fluorescent *in situ* hybridization. (L) pluteus stage, 72 h. Scale bar in (A): 20 μm.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Saccoglossus-Zfhx ----------------------MAEGP--RCTRRKQANPRRKKELLEENE--------A 27
amphioxus-Zfhx MVPLTMNQSRLQMLSPRRPHPANMHAGVTPVSERSICLNNKVEARNCGVTPVSERSICLN 60
humanDeltaEF1b ----------------------MADGP--RCKRRKQANPRRNN--VTNYNTVVETNSDS 33
humanDeltaEF1a --MK--VTNYNTVVETNSDS 16
mouseDeltaEF1 ----------------------MADGP--RCKRRKQANPRRNN--VTNYNTVVEANSDS 33
chickenDeltaEF1 ----------------------MADGP--RCKRRKQANPRRNN--VTNYNNVIEANSDS 33
XenopusDeltaEF1a ----------------------MADGP--RCKRRKQANPRRNN--VTNYNSVLEANSDS 33
ZebraKheper ----------------------MADGP--RCKRRKQANPRRTN--VSNYSHVLEGQSDS 33
humanSIP1 -------------------MKQPIMADGP--RCKRRKQANPRRKN--VVNYDNVVDTGSET 38
XenopusSIP1 -------------------MKQEIMADGP--RCKRRKQANPRRKN--VLTYDNVVDTGSET 38
zebraSIP1b -------------------MRELIMADGP--RCKRRKQANPRRKNAAVLDFENVVETGSET 40
zebraSIP1a -------------------MKQEIMAEGP--RCKRRKQANPRRKN--VLSYENVVDAGSGS 38
flyZfh1 --
Hp-Zfhx DGNDPISPSPEN--------------GYHSNSSTIEDSDGDDKGSFMGDEEDDEAEMDK- 86
Saccoglossus-Zfhx EEKCNKENNNSD--------------GYHSN--------------------ENELNQDE- 52
amphioxus-Zfhx NKVCPWGKTEYE--------------EEEDEGAVLDDADASSTAHMSDSGVADDNEPDQR 106
humanDeltaEF1b DDEDKLHIVEEE----------SVTDAADCEG----VPEDDLPTDQTVLPGRSSEREGNA 79
humanDeltaEF1a DDEDKLHIVEEE----------SVTDAADCEG----VPEDDLPTDQTVLPGRSSEREGNA 62
mouseDeltaEF1 DDEDKLHIVEEE----------SITDAADCEGG---MPDDELPADQTVLPG-GSDRGGGA 79
chickenDeltaEF1 DDEDKLHIVEEE----------SITDAADCDAS---VPEDDLPTDHTVLPE-NSEREGST 79
XenopusDeltaEF1a DDEDKLHIVEEE----------SVTDAADGEGS---VPEDDLPTDQTVLPEGSESGKGSG 80
ZebraKheper DDEDKLHIVEEEG---------SLLDGADCDSV---APDDD--------PNGTADPDGR- 72
humanSIP1 DEEDKLHIAEDDGIANPLDQETSPASVPNHE----SSPHVSQALLPREEE-EDEIREGGV 93
XenopusSIP1 EEEDKLHIAEDDSITTTLDQETSPASMLNHE----TSPQANQALLPRDEE-EDELRERGM 93
zebraSIP1b EEEDRLLVSEED---ALLNGAGSPASLVNHESEAPPSPTLSHTLLRKTVDDEDDMKDSGI 97
zebraSIP1a DDEDRLLGSEGE---------GSPAGVPSLE----ASPRVAHALLSCRGDEENESQDGAG 85
flyZfh1 --
Hp-Zfhx -------------------STSDGDKDDGVDDLEPDKEFHRKNGMHHLHPHSPPHPHHQFR 128
Saccoglossus-Zfhx -------------------MSSNDGSYHGDDGYQSNKELGEDE-------CAAPG------ 81
amphioxus-Zfhx LDDSL-------------HMENGTPVQSPAGDDISTTPSTPGTPDHSNPPTPGTPHRTDE 153
humanDeltaEF1b --KNCWE------------DDR-KEG-QEILGPEAQADEAGCTVKDDECESDAENEQNHD 123
humanDeltaEF1a --KNCWE------------DDTGKEG-QEILGPEAQADEAGCTVKDDECESDAENEQNHD 107
mouseDeltaEF1 --KNCWQ------------DNV----------------------KDNECDSDAENEQNHD 103
chickenDeltaEF1 --NSCWE------------DEG-KET-KEILGPEAQSDEVGCTVKEDECDSDAENEQNHD 123
XenopusDeltaEF1a --KSCWE------------DEEGADCGDEILGPEAEADELGCTVKDEECDSDVENEQNHD 126
ZebraKheper -----WD------------DVK------------------------EECVSD-EDERSRD 90
humanSIP1 --EHPWHNNEILQASVDGPEEMKEDYDTMGPEATIQTAINNGTVKNANCTSDFEEYFAKR 151
XenopusSIP1 --DHNWHNNVILKASVDGSDDMKEDYDTLGPQVTHVTTINNGTVKNPNCTSDFEEYFVKR 151
zebraSIP1b --ENVWHENDLLNASIDGTDELKADYDTMGTDVSLEP-IGNGTVKSVHCDTDFEDFFGKR 154
zebraSIP1a --AHVWR-----HGELNGSEERKAEYNSMSPDISLHG-IGNGTVKGIDASSELESFFAKR 137
flyZfh1 --
Hp-Zfhx NGEVGGDGVINLEDYMNRSDTAIIYPEPVEDMDGVNGDTDDPNTPEGN--DNETEIEGEL 186
Saccoglossus-Zfhx GGGGGGRAGLNLQEYLSRGDTAIIYPEAPDDEN------SDPSGQNGE--SNDDTIL--- 130
amphioxus-Zfhx LAGDGTQAEHDIREYANRSDTAIVYPESADDPMVG----HAPANGTGN--MLEDQLLS-- 205
humanDeltaEF1b ---------PNVEEFLQQQDTAVIFPEAPEEDQR--QGTPEASGHDEN------GTPDAF 166
humanDeltaEF1a ---------PNVEEFLQQQDTAVIFPEAPEEDQR--QGTPEASGHDEN------GTPDAF 150
mouseDeltaEF1 ---------PNVEEFLQQQDTAVIYPEAPEEDQR--QGTPEASSHDEN------GTPDAF 146
chickenDeltaEF1 ---------PNVEEFLQQEDTAVIYPEAPEEDQR--QGTPEASGQDEN------GTPDAF 166
XenopusDeltaEF1a ---------PNVEEFLQQSDTAVIYPEAPDDEQR--QGTPEAVGQDEN------GTPDAF 169
ZebraKheper ---------ALVEEMLQQGDTAVIFPEAPDDEPR--QGTPETSGHDEN------GTPDSF 133
humanSIP1 KLEERDGHAVSIEEYLQRSDTAIIYPEAPEELSR--LGTPEANGQEEN--DLPPGTPDAF 207
XenopusSIP1 KMDAGDSNGVSIAEYLQRSDTAIIYPEAPEELCR--LGTPEANGHEEN--DLPPGTPDAF 207
zebraSIP1b KLVDTESHVVSIAEYLQRGDTAIIYPEAPEELSRSRLATPEATGHEEN--DLPPGTPDAF 212
zebraSIP1a KLDDGEGHAASIAEYLQ--DTVIIYPEDPEEGTR--LGTPEANGQDENENDLALRTPDAF 193
flyZfh1 --
Zinc-finger domains
Hp-Zfhx GKPEDCPYCDRSYKRLTSLKEHIKYRHEKTFNNFSCPECNYCFAYKSQLERHMATHMPGR 246
Saccoglossus-Zfhx ----SCPYCDRVYKRATSLKEHIKYRHEKNANNYACSECNYSFAYKSQLERHMATHMPGR 186
amphioxus-Zfhx -----CPYCDRGYKRLTSLKEHIKYRHERTDSSYACNECNYTFAYKSQLERHMASHKPGR 260
humanDeltaEF1b SQLLTCPYCDRGYKRFTSLKEHIKYRHEKNEDNFSCSLCSYTFAYRTQLERHMTSHKSGR 226
humanDeltaEF1a SQLLTCPYCDRGYKRFTSLKEHIKYRHEKNEDNFSCSLCSYTFAYRTQLERHMTSHKSGR 210
mouseDeltaEF1 SQLLTCPYCDRGYKRFTSLKEHIKYRHEKNEDNFSCSLCSYTFAYRTQLERHMTSHKSGR 206
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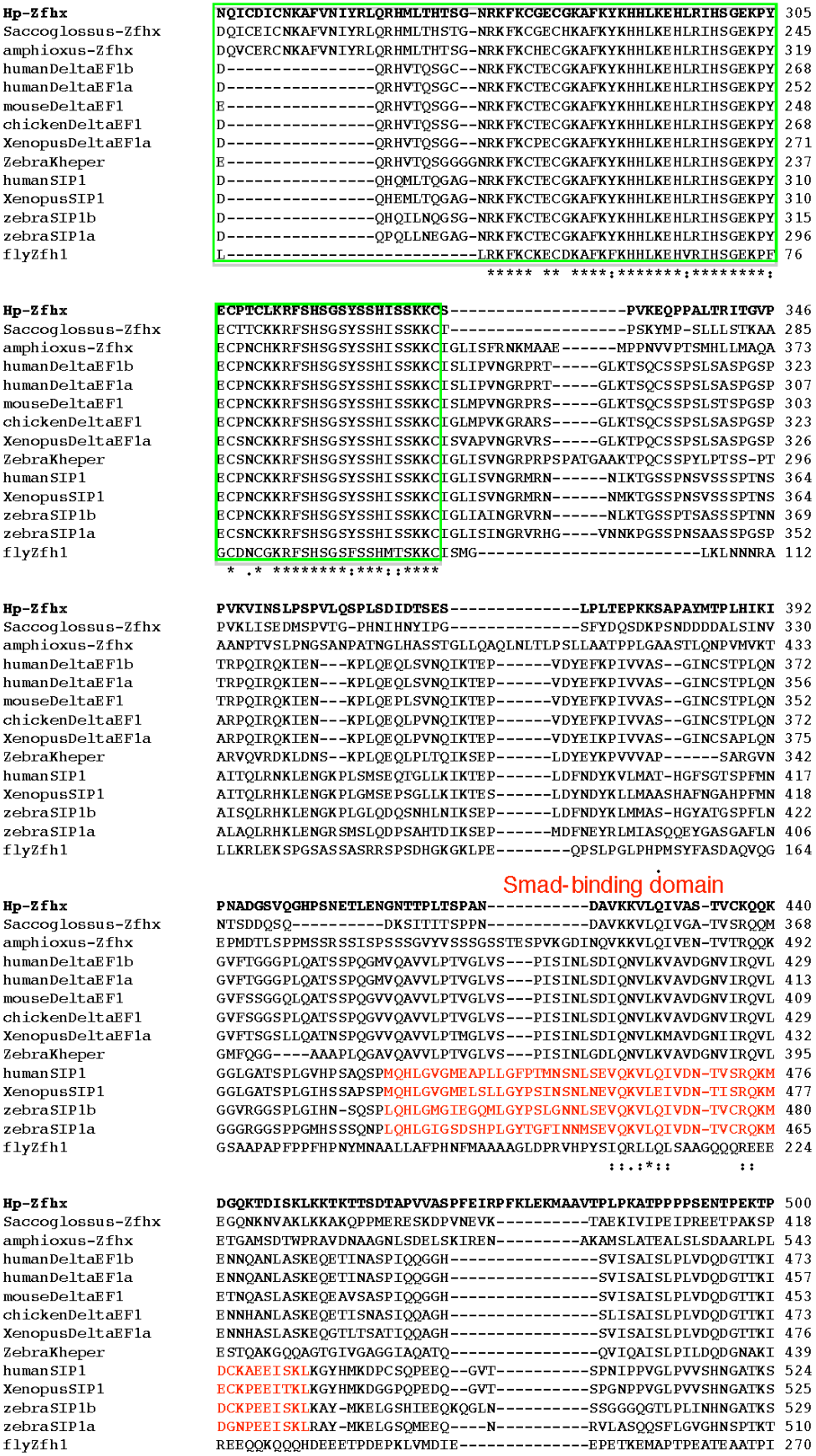
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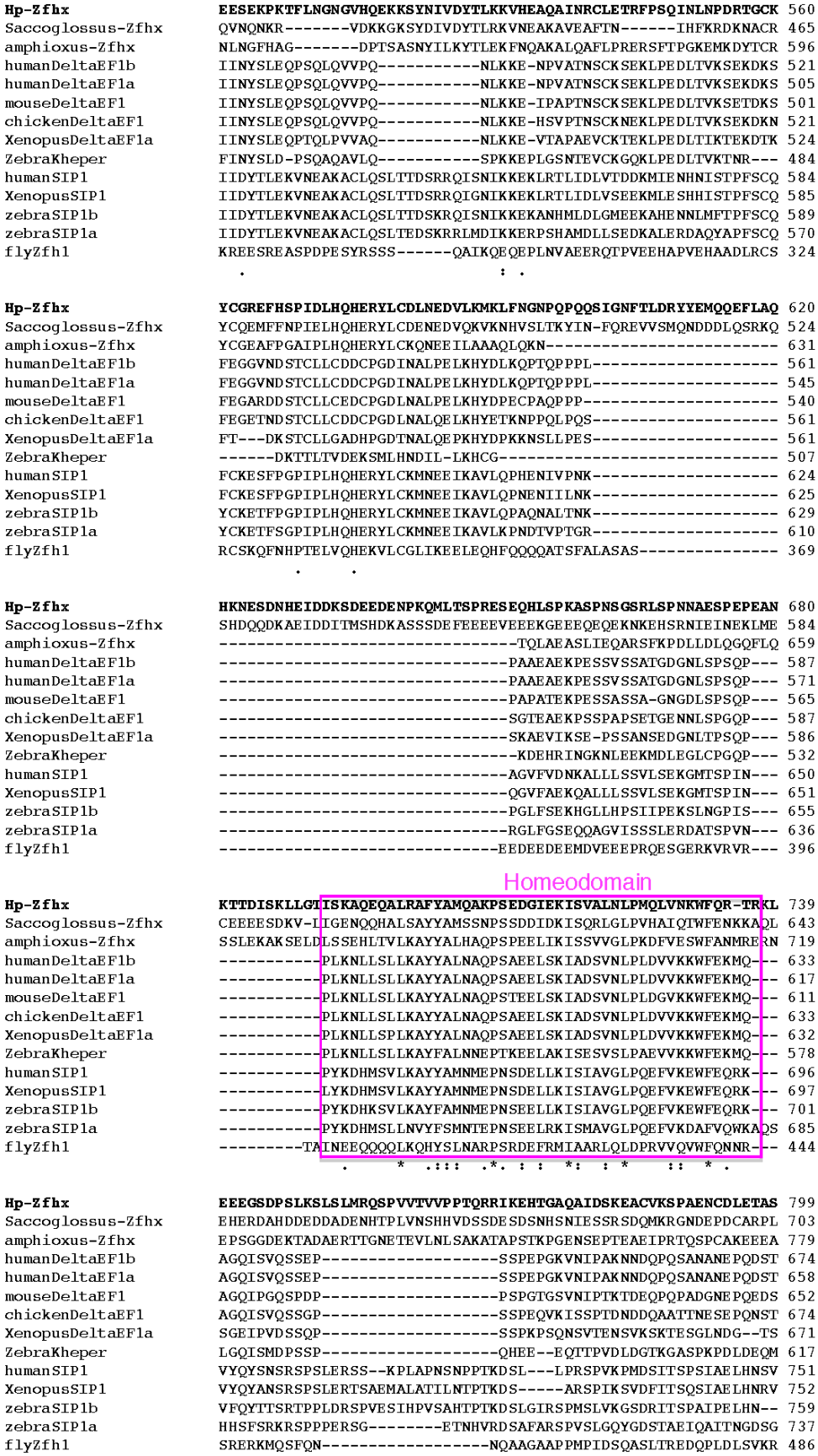
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amphioxus-Zfhx NLNGFHAG-------DPTSASNYILKYTLEKFNQAKALQAFLPRERSFTPGKEMKDYTCR 596
humanDeltaEF1b IINYSLEQPSQLQVVPQ----------NLKKE-NPVATNSCKSEKLPEDLTVKSEKDKS 521
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mouseDeltaEF1 IINYSLEQPSQLQVVPQ----------NLKKE-IPAPTNSCKSEKLPEDLTVKSETDKS 501
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chickenDeltaEF1 FEGETNDSTCLLCDDCPGDLNALQELKHYETKNPPQLPQS---------- 561
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XenopusDeltaEF1a ----------PLKNLLSPLKAYYALNAQPSAEELSKIADSVNLPLDVVKKWFEKMQ--- 632
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XenopusSIP1 ----------LYKDHMSVLKAYYAMNMEPNSDELLKISIAVGLPQEFVKEWFEQRK--- 697
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Saccoglossus-Zfhx EHERDAHDDEDDADENHTPLVNSHHVDSSDESDSNHSNIESSRSDQMKRGNDEPDCARPL 703
amphioxus-Zfhx EPSGGDEKTADAERTTGNETEVLNLSAKATAPSTKPGENSEPTEAEIPRTQSPCAKEEEA 779
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humanDeltaEF1a AGQISVQSSEP----------SSPEPGKVNIPAKNNDQPQSANANEPQDST 658
mouseDeltaEF1 AGQIPGQSPDP----------PSPGTGSVNIPTKTDEQPQPADGNEPQEDS 652
chickenDeltaEF1 AGQISVQSSGP----------SSPEQVKISSPTDNDDQAATTNESEPQNST 674
XenopusDeltaEF1a SGEIPVDSSQP----------SSPKPSQNSVTENSVKSKTESGLNDG--TS 671
ZebraKheper LGQISMDPSSP----------QHEE--EQTTPVDLDGTKGASPKPDLDEQM 617
humanSIP1 VYQYSNSRSPSLERSS--KPLAPNSNPPTKDSL---LPRSPVKPMDSITSPSIAELHNSV 751
XenopusSIP1 VYQYANSRSPSLERTSAEMALATILNTPTKDS-----ARSPIKSVDFITSQSIAELHNRV 752
zebraSIP1b VFQYTTSRTPPLDRSPVESIHPVSAHTPTKDSLGIRSPMSLVKGSDRITSPAIPELHN-- 759
zebraSIP1a HHSFSRKRSPPPERSG--------ETNHVRDSAFARSPVSLGQYGDSTAEIQAITNGDSG 737
flyZfh1 SRERKMQSFQN----------NQAAGAAPPMPIDSQASLTREDQPLDLSVKR 486

```
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amphioxus-Zfhx       QPQSDSD----APMDLTVPKTEASKSKPNSVVTIYNKRKSAMFAPIPDPLLTNPAAYYAA 835
humanDeltaEF1b       VNLQSPL------------------KMTNSPVLPVGSTTNGSRSSTP--SPSPLNLSSSR 714
humanDeltaEF1a       VNLQSPL------------------KMTNSPVLPVGSTTNGSRSSTP--SPSPLNLSSSR 698
mouseDeltaEF1        TRGQSPV------------------KIRSSPVLPVGSAMNGSRSCTS--SPSPLNLCSAR 692
chickenDeltaEF1      NNSQNPA------------------NTSKSQTSSGGSTQNGSRSSTP--SPSPLNLSSSR 714
XenopusDeltaEF1a     VPLKSPK------------------QEAEEPL-----AVNGSESGHR--FSYTTKLSSLE 706
ZebraKheper          NSEKQEE------------------RECCSPAEGIAASVNGIESVPA--SPSPLNLS--- 654
humanSIP1            TNCDPPL------------------RLTKPSHFTNIKP-VEKLDHSRSNTPSPLNLSSTS 792
XenopusSIP1          SNCDTPL------------------RLTKSNHFASMKPVLDKLDHSRSNTPSPLNLSSTS 794
zebraSIP1b           -NCDTPL------------------RLSKTPQYSNHKQLGDKMDHSRSNTPSPLNLSSAS 800
zebraSIP1a           HKLSRPH------------------QITG-TRQTNEKP-LDSVDHLRGETPSPLNLSSSS 777
flyZfh1              DPLTPK---------------------SESSPPYIAPPSGEALNPEAINLSRKFSTSASM 525

Hp-Zfhx              MERDSRSPARSLTPAKETRDSSPLDLSLPKRKATPPPSLSRSSIPPKLVYGYNYSALYSA 919
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amphioxus-Zfhx       LPFFGQGGIPAPFPGYIDFLASPMTGAFLNGLGSP-------------------------- 870
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mouseDeltaEF1        NPQGYSCVAEG---AQEEPQVEPLDLSLPKQQG-------------------------EL 724
chickenDeltaEF1      NSQGYTYTAEG---VQEEPQMEPLDLSLPKQHG-------------------------EL 746
XenopusDeltaEF1a     NSDGAACTDGT---PDGEQQAEPLDLSSPKPQG-------------------------NV 738
ZebraKheper          -ADGPVPARTL---EEGE---GPLDLSLPKS---------------------------- 678
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XenopusSIP1          SKNSHSSSYTPNSFSSEELQAEPLDLTVPKLLNES-----------------KTIIATKN 837
zebraSIP1b           SKNSHTSSYTPNSFTSEDLQAEPLDLSLPKLMKEP-----------------KHILTVKS 843
zebraSIP1a           SKHSHSSSYTPNSLTSEDAHGEPLDLSLPKQVS-----------------------KAER 814
flyZfh1              SPASISPSSAAALYFGAAPPPSPPNSQLDSTP---------------------------- 557
                                        *       .

Hp-Zfhx              AAAAAANGYLLPPAYAPSEPLPSSLYENSIKAHKSRTSPSP------GKTTPPSSTATTN 973
Saccoglossus-Zfhx    QQFDAARSVFHMKGYHSTPNTTPDFYFSSFKVDPRLAQPYF------ENNIKAHCNGQAH 859
amphioxus-Zfhx       ---------YLTPGFNAATLSTKRPRLDTPTRGRRGRRRVY------DNGSRVPMATQS- 914
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humanDeltaEF1a       LERSTITSVYQNSVYSVQE---EPLNLSCA-KKEPQKDSCV-------TDSEPVVNVIPP 779
mouseDeltaEF1        LERSTVSSVYQNSVYSVQE---EPLNLSCA-KKEPQKDSCV-------TDSEPVVNVVPP 773
chickenDeltaEF1      LERSTITSVYQNSVYSVQE---EPLNLTCA-KKEPQKDNSI-------TDSDPIVNVIPP 795
XenopusDeltaEF1a     PERPSITSVYQSSSYSVQE---EPLNLSCV-KKEPMQVDNA-------AKAETTL-IINP 786
ZebraKheper          ATTASVASGHANTVYSAQE---EPLNLTCT-KKELLSN----------ASTNAAIYASQP 724
humanSIP1            KTKASSISLDHNSVSSSSENSDEPLNLTFI-KKEF--SNSNNL---DNKSTNPVFSMNPF 889
XenopusSIP1          KSKPNNITVDHNSVSLSSETVDEPLNLTYI-KKEF--CNAN-----MDKSTSPLFGLNPF 889
zebraSIP1b           RLKLNSGPMDHNHVATPREHADEPLNLAYLSKKEFGSPNANSN---LDKSSSPMFGLNPF 900
zebraSIP1a           RAKPNGFSIEHTSNSTAREPGTEPLNLAHI-KKEFNGPNSLGNENQMDKSSSPIFSINPF 873
flyZfh1              ------------------------------------------------------------

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Saccoglossus-Zfhx    NPSA--ARILTHAIELPHLYAAHHHPG--------LHNSLLAAAAPTQGPTKKLKLMDSL 909
amphioxus-Zfhx       ----------------------------------VTPVQAPSPMHPSMDPDTQSPLAL 938
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XenopusDeltaEF1a     SANP-----------------------------INIAIPTVTAQLPTIVAIADQNGVPC 816
ZebraKheper          SANP-----------------------------INIMT----TQLPTLVAITDQGQAQC 750
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flyZfh1              ----------------------------------RSGQAFPGLPPYMLPMSLPMEAL 580

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amphioxus-Zfhx       AQETAEGSPSIPSPSPSGTYPGYIGDSASSPGSPA-LSTGTP------------------ 979
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XenopusDeltaEF1a     LRALAANKQTILIPQVTYTYSTTAS-PAVPEPQVK-NIQPNGNQDERQDTSSEGVSNVED 874
ZebraKheper          LRALTTTKQTILIPQLTYSYTTTSSSPAGTDTPQKNILHVNGIKEEKQEMGSEATSILEE 810
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zebraSIP1b           LRPYPSLDQISFLPHMAYTYAAGAASFAEMQQRRK-YQRKPGFQSELLDGPADYLSSLDD 989
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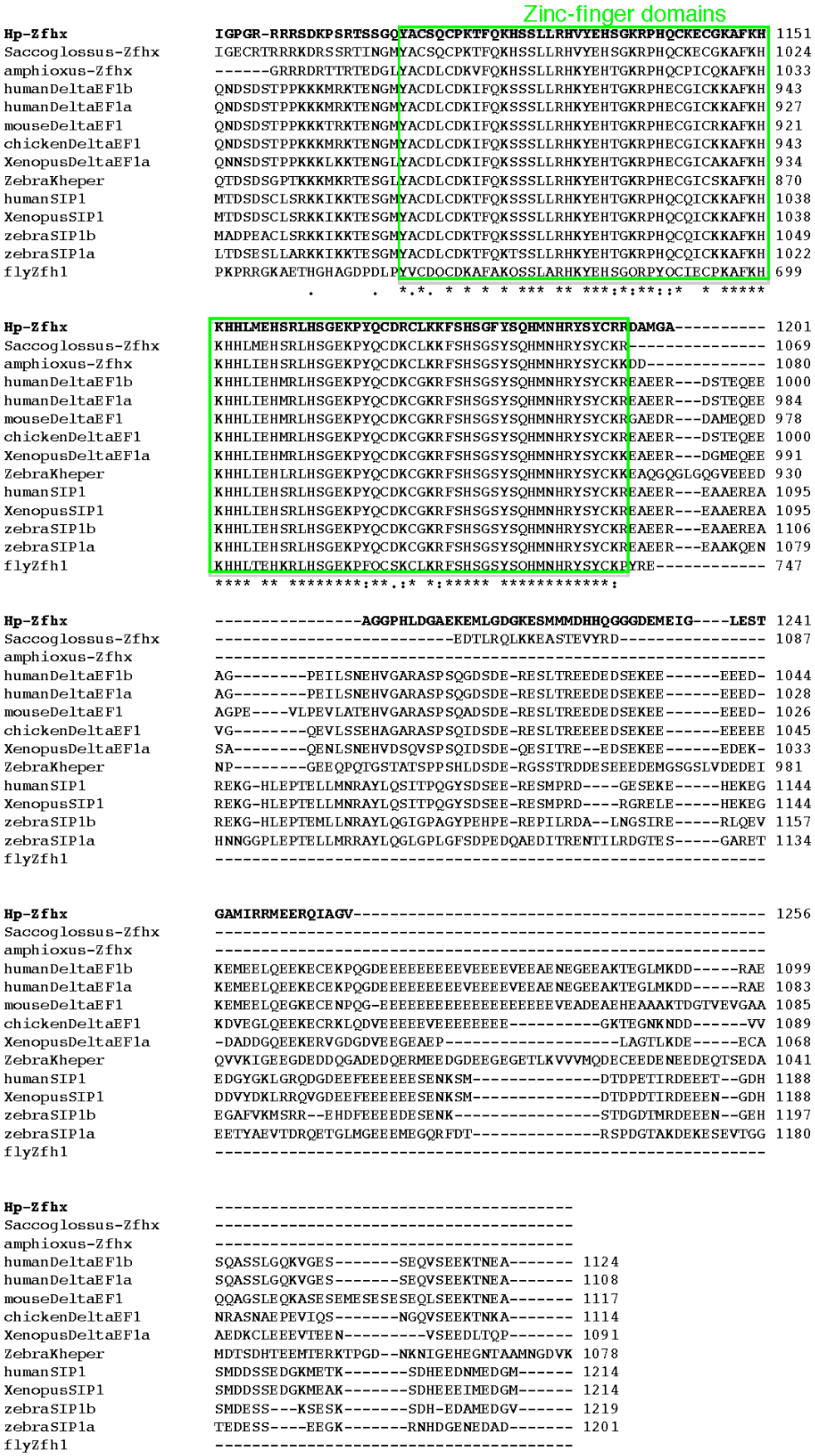
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amphioxus-Zfhx ------GRRRDRTTRTEDGLYACDLCDKVFQKHSSLLRHKYEHTGKRPHQCPICQKAFKH 1033
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chickenDeltaEF1 QNDSDSTPPKKKMRKTENGMYACDLCDKIFQKSSSLLRHKYEHTGKRPHECGICKKAFKH 943
XenopusDeltaEF1a QNNSDSTPPKKKLKKTENGLYACDLCDKIFQKSSSLLRHKYEHTGKRPHECGICAKAFKH 934
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zebraSIP1a LTDSESLLARKKIKKTESGMYACDLCDKTFQKTSSLLRHKYEHTGKRPHQCQICKKAFKH 1022
flyZfh1 PKPRRGKAETHGHAGDPDLPYVCDQCDKAFAKQSSLARHKYEHSGQRPYQCIECPKAFKH 699
. . *.*. * * * * *** ** ***:*:**::* * *****
Hp-Zfhx KHHLMEHSRLHSGEKPYQCDRCLKKFSHSGFYSQHMNHRYSYCRRDAMGA---------- 1201
Saccoglossus-Zfhx KHHLMEHSRLHSGEKPYQCDKCLKKFSHSGSYSQHMNHRYSYCKR---------- 1069
amphioxus-Zfhx KHHLIEHSRLHSGEKPYQCDKCLKRFSHSGSYSQHMNHRYSYCKKDD---------- 1080
humanDeltaEF1b KHHLIEHMRLHSGEKPYQCDKCGKRFSHSGSYSQHMNHRYSYCKREAEER---DSTEQEE 1000
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chickenDeltaEF1 KHHLIEHMRLHSGEKPYQCDKCGKRFSHSGSYSQHMNHRYSYCKREAEER---DSTEQEE 1000
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flyZfh1 KHHLTEHKRLHSGEKPFQCSKCLKRFSHSGSYSQHMNHRYSYCKPYRE---------- 747
**** ** ********:**.:* *:***** ************:
Hp-Zfhx ----------AGGPHLDGAEKEMLGDGKESMMMDHHQGGGDEMEIG----LEST 1241
Saccoglossus-Zfhx ----------EDTLRQLKKEASTEVYRD---------- 1087
amphioxus-Zfhx ----------
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XenopusDeltaEF1a SA--------QENLSNEHVDSQVSPSQIDSDE-QESITRE--EDSEKEE------EDEK- 1033
ZebraKheper NP--------GEEQPQTGSTATSPPSHLDSDE-RGSSTRDDESEEEDEMGSGSLVDEDEI 981
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XenopusSIP1 REKG-HLEPTELLMNRAYLQSITPQGYSDSEE-RESMPRD----RGRELE-----HEKEG 1144
zebraSIP1b REKG-HLEPTEMLLNRAYLQGIGPAGYPEHPE-REPILRDA--LNGSIRE-----RLQEV 1157
zebraSIP1a HNNGGPLEPTELLMRRAYLQGLGPLGFSDPEDQAEDITRENTILRDGTES-----GARET 1134
flyZfh1 ----------
Hp-Zfhx GAMIRRMEERQIAGV---------- 1256
Saccoglossus-Zfhx ----------
amphioxus-Zfhx ----------
humanDeltaEF1b KEMEELQEEKECEKPQGDEEEEEEEEEVEEEEVEEAENEGEEAKTEGLMKDD-----RAE 1099
humanDeltaEF1a KEMEELQEEKECEKPQGDEEEEEEEEEVEEEEVEEAENEGEEAKTEGLMKDD-----RAE 1083
mouseDeltaEF1 KEMEELQEGKECENPQG-EEEEEEEEEEEEEEEEEEEVEADEAEHEAAAKTDGTVEVGAA 1085
chickenDeltaEF1 KDVEGLQEEKECRKLQDVEEEEEVEEEEEEEE----------GKTEGNKNDD------VV 1089
XenopusDeltaEF1a -DADDGQEEKERVGDGDVEEGEAEP----------LAGTLKDE-----ECA 1068
ZebraKheper QVVKIGEEGDEDDQGADEDQERMEEDGDEEGEGETLKVVVMQDECEEDENEEDEQTSEDA 1041
humanSIP1 EDGYGKLGRQDGDEEFEEEEEESENKSM----------DTDPETIRDEEET--GDH 1188
XenopusSIP1 DDVYDKLRRQVGDEEFEEEEEESENKSM----------DTDPDTIRDEEEN--GDH 1188
zebraSIP1b EGAFVKMSRR--EHDFEEEEDESENK----------STDGDTMRDEEEN--GEH 1197
zebraSIP1a EETYAEVTDRQETGLMGEEEMEGQRFDT----------RSPDGTAKDEKESEVTGG 1180
flyZfh1 ----------
Hp-Zfhx ----------
Saccoglossus-Zfhx ----------
amphioxus-Zfhx ----------
humanDeltaEF1b SQASSLGQKVGES-------SEQVSEEKTNEA------- 1124
humanDeltaEF1a SQASSLGQKVGES-------SEQVSEEKTNEA------- 1108
mouseDeltaEF1 QQAGSLEQKASESEMESESESEQLSEEKTNEA------- 1117
chickenDeltaEF1 NRASNAEPEVIQS-------NGQVSEEKTNKA------- 1114
XenopusDeltaEF1a AEDKCLEEEVTEEN---------VSEEDLTQP------- 1091
ZebraKheper MDTSDHTEEMTERKTPGD--NKNIGEHEGNTAAMNGDVK 1078
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XenopusSIP1 SMDDSSEDGKMEAK-------SDHEEEIMEDGM------ 1214
zebraSIP1b SMDESS---KSESK-------SDH-EDAMEDGV------ 1219
zebraSIP1a TEDESS----EEGK-------RNHDGENEDAD------- 1201
flyZfh1 ----------

Figure 1-2. Sea urchin zinc-finger homeobox (Zfhx1) does not have a Smad binding domain (SBD).

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 (Hp-Zfhx; bold), *Saccoglossus kowalevskii* Zfhx, amphioxus-Zfhx, and fly-Zfh1. The position of the SBD 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.

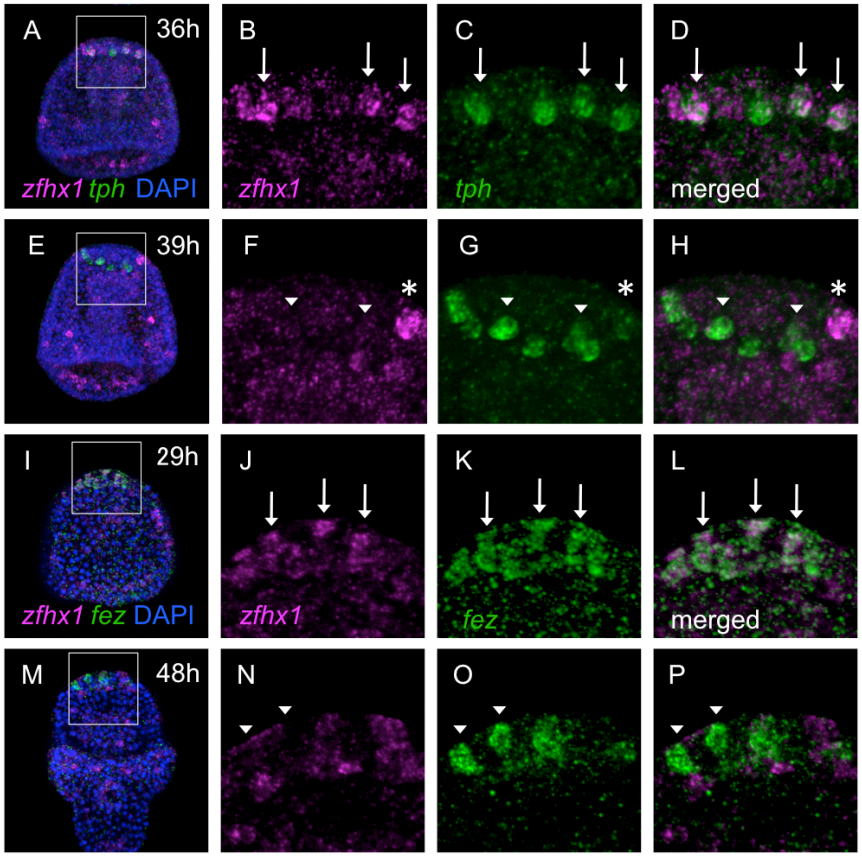

Figure 1-3. *zfhx1* is transiently expressed in serotonergic neural precursor cells.

(A-H) Double fluorescent *in situ* hybridization detecting *zfhx1* and *tph* in 36-h (A-D) and 39-h (E-H) embryos. (A) *zfhx1* is expressed in the animal plate region. A square region is magnified in (B-D). (B) *zfhx1* 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* and *tph*. (E) Most of *zfhx1* disappears from the animal plate in 39-h embryo. A square shows the region that is magnified in (F-H). (F) *zfhx1* is not expressed in *tph*-positive cells (arrowheads). Asterisk shows *zfhx1*-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* and *fez* in 29-h (I-L) and 48-h (M-P) embryos. (I) *zfhx1* is expressed in the animal plate region in 29 h. The square shows the region that is magnified in (J-L). (J) *zfhx1*-expressing cells in the animal plate (arrows). (K) *fez*-expressing cells in the same region. (L) Merged image of (J) and (K). (M) *zfhx1* is down regulated in a 48 h embryo. (N) *zfhx1* 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*-positive cells (magenta) in (M-P) are non-serotonergic neurons in the animal plate.

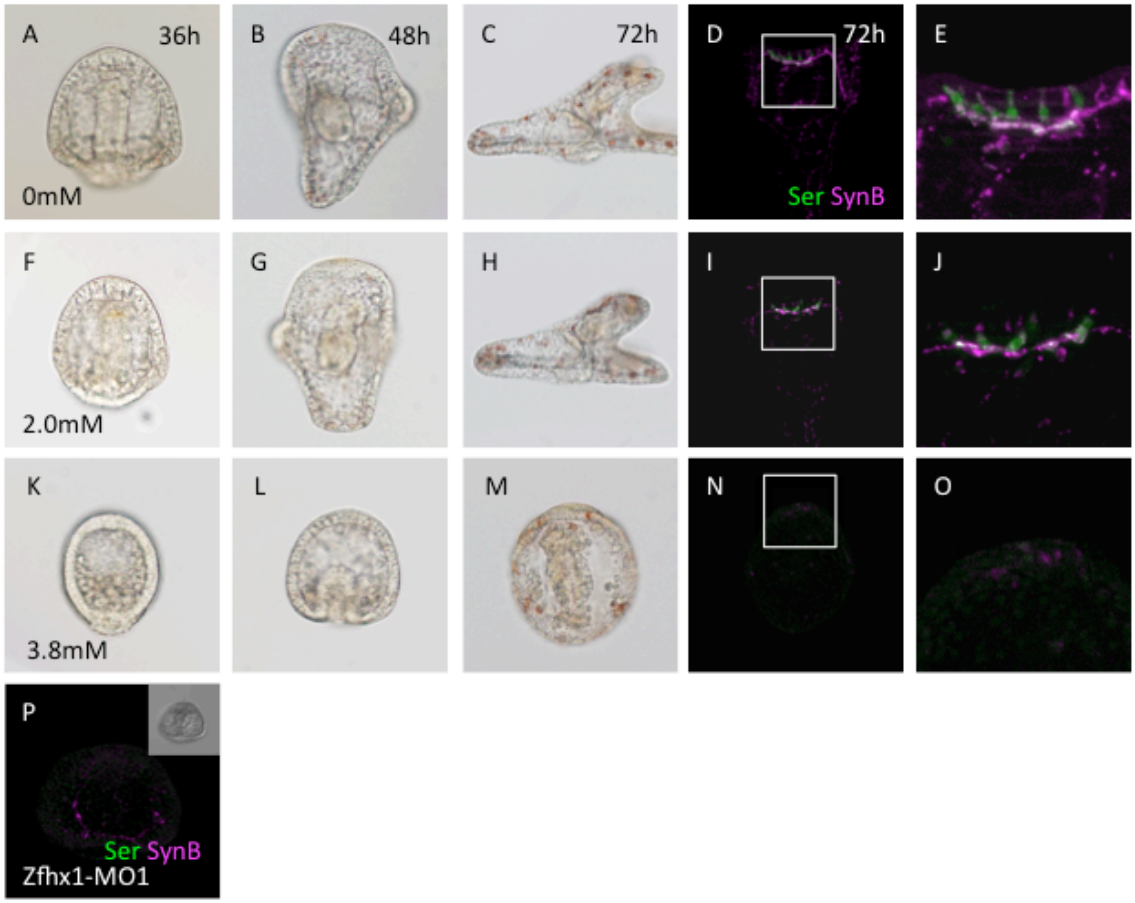

Figure 1-4. Knockdown of Zfhx1 not only decreases the number of serotonergic neurons but also inhibits normal vegetal tissue development and oral/aboral polarity.

(A-E) Control embryos (glycerol-injected). (A) 36-h prism stage. (B) 48-h pluteus stage. (C) 72-h early 4-arm pluteus stage, lateral view. (D) Immuno-fluorescent image of a 72-h embryo stained for serotonin and synaptotagmin B; the rectangle shows the region magnified in (E). (E) Seven serotonergic neurons are present in this embryo. (F-J) 2.0 mM Zfhx1-MO-injected embryos. (F) 36 h. (G) 48 h. (H) 72 h. 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-MO-injected embryos. (K) 36 h. (L) 48 h. (M) 72 h. (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. (P) 2.3mM Zfhx1-MO1-injected embryo.

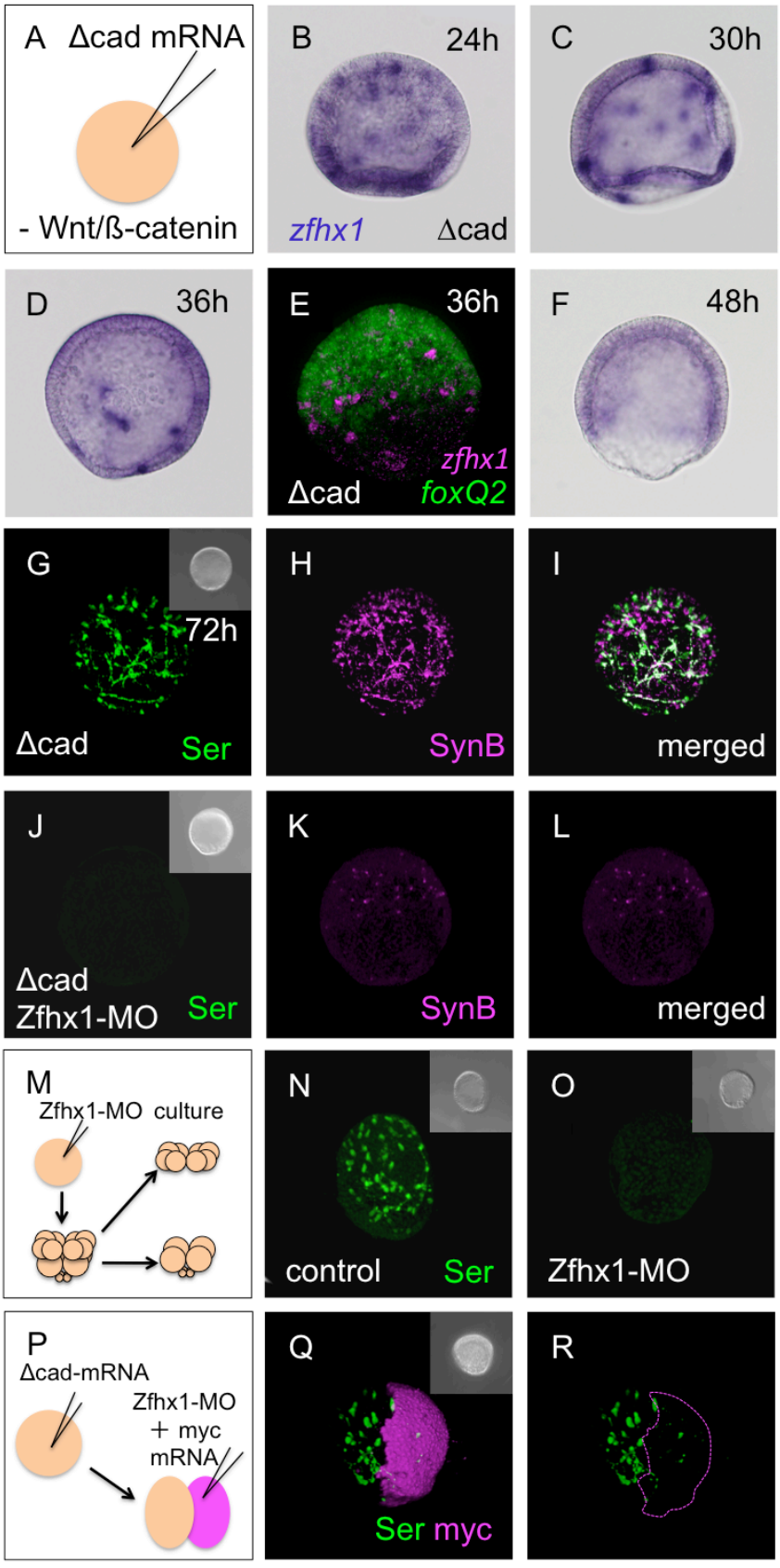
A Δcad mRNA
- Wnt/ß-catenin
B 24h
zfhx1
Δcad
C 30h
D 36h
E 36h
zfhx1
foxQ2
Δcad
F 48h
G
72h
Δcad
Ser
H
SynB
I
merged
J
Δcad
Zfhx1-MO
Ser
K
SynB
L
merged
M
Zfhx1-MO culture
N
control
Ser
O
Zfhx1-MO
P
Δcad-mRNA
Zfhx1-MO
+ myc
mRNA
Q
Ser myc
R

Figure 1-5. Zfhx1 is required for the differentiation of serotonergic neurons.

(A) Microinjection to inhibit canonical Wnt signaling. (B-F) The expression patterns of *zfhx1* in Δcad-injected embryos. (B) *zfhx1*-positive neural precursors are scattered in the expanded 24-h embryo. (C) 30-h embryo. (D) 36-h embryo; the number of *zfhx1* cells decreased. (E) Double fluorescent *in situ* hybridization shows that *zfhx1* disappears from the central part of the animal plate. (F) *zfhx1* is down regulated in 48-h 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 synaptotagmin B-positive. (I) Merged image of (G) and (H). (J) Δcad-injected Zfhx1 morphants have no serotonergic neurons at 72 h. (K) Serotonin-negative synaptotagmin B-positive neurons begin to differentiate in morphants. (L) Merged image of (J) and (K). (M) Method for creating animal caps from Zfhx1 morphants. (N) Serotonergic neurons differentiate in the glycerol-injected control animal cap. (O) No serotonergic neurons differentiate in the animal cap of Zfhx1 morphants. (P) Method to inject Zfhx1-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-MO)-negative half of the embryo. (R) Only the outline of myc-positive, Zfhx1-deficient region of (Q) is shown. Insets are DIC images for each panel.

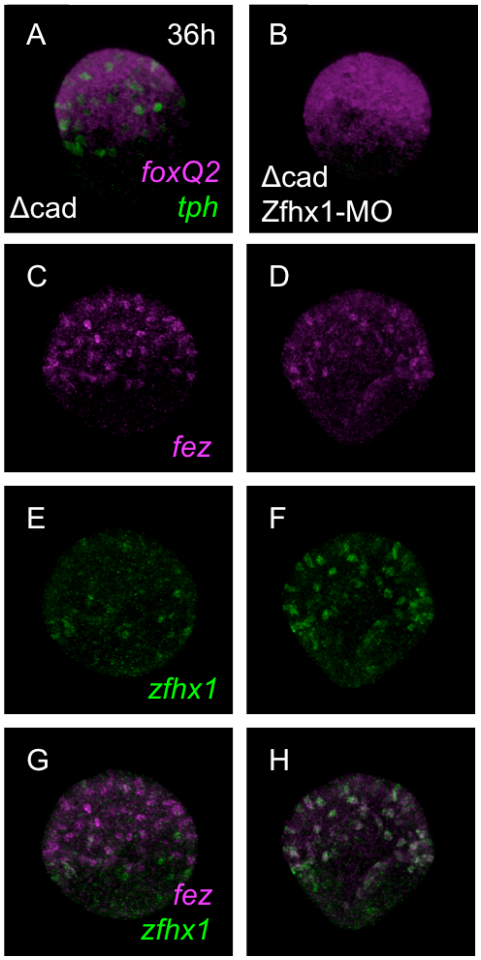

Figure 1-6. Zfhx1 is not required for expression of genes involved in early specification of the animal plate.

(A) *foxQ2* and *tph* in a Δcad-injected embryo at 36 h. (B) The expression pattern of *foxQ2* is not altered in Δcad-injected Zfhx1 morphants, whereas no *tph* expression is detected. (C, E, G) Δcad-alone-injected control embryo. (D, F, H) Δcad-injected Zfhx1 morphant. (C, D) The expression patterns of *fez* at 36 h. (E, F) The expression patterns of *zfhx1*. (G, H) Merged images of (C) and (E), and (D) and (F), respectively.

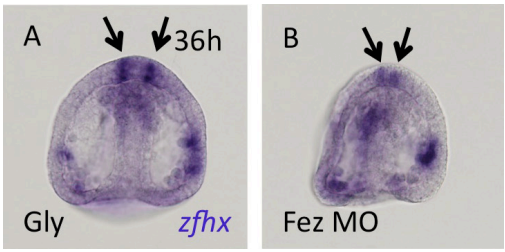

Figure 1-7. Forebrain embryonic zinc finger, Fez, is not required for the expression of *zfhx1*.

Zfhx1 is expressed in the animal plate region of (A) control (Gly; glycerol-injected) embryo (arrows) and (B) Fez morphants (arrows) at 36 h. Note that the animal plate in which serotonergic neurons develop is smaller in Fez morphants (Yaguchi et al., 2011).

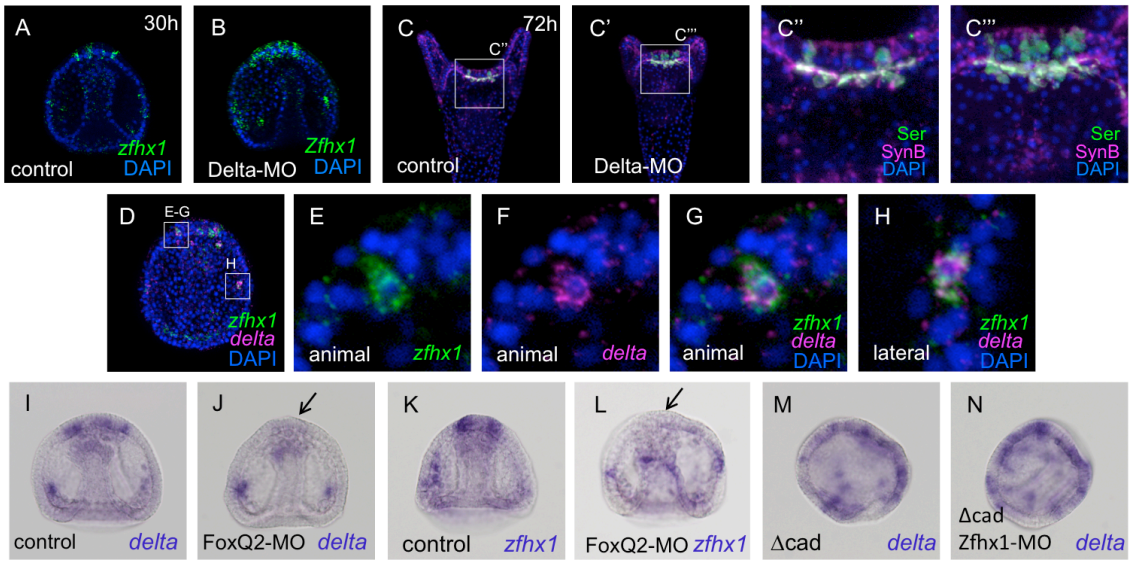

Figure 1-8. ***delta*** **is a specific neural marker in the animal plate.**

(A) *zfhx1* expression in the animal plate of 30 h embryo detected with fluorescent in situ hybridization. (B) More cells express *zfhx1* and make a cluster in the animal plate of Delta morphants. (C) The normal patterning of serotonergic neurons in 72-h embryo. A square shows the region magnified in (C'). Synaptotagmin B, 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* 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* in the animal plate. (F) *delta* expression. (G) Merged image of (E) and (F). (H) A cell expressing *zfhx1*

(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* in the control late gastrula. (L) *zfhx1* 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 morphants.

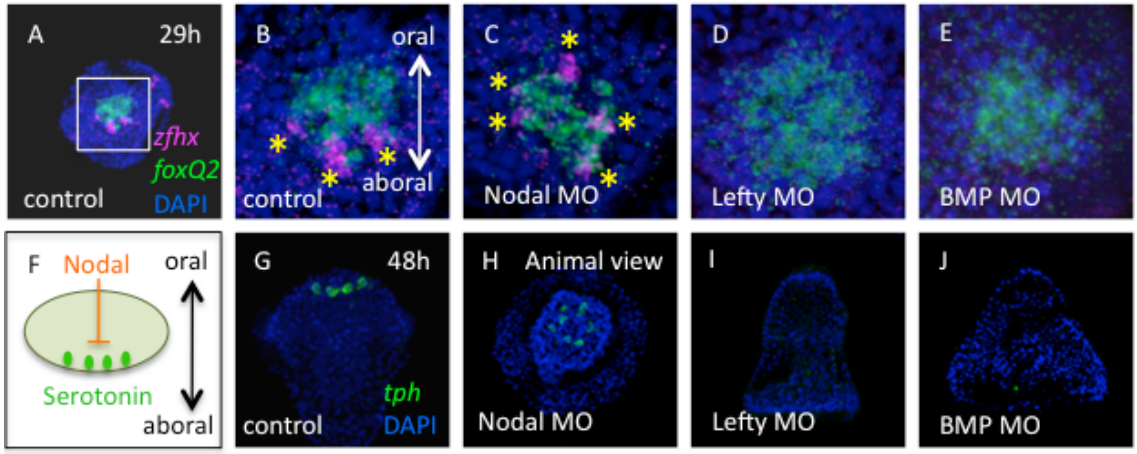

Figure 1-9. Nodal suppresses the expression of *zfhx1* on the oral side of the animal plate.

(A) The expression pattern of *zfhx1* (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* is expressed in cells along the aboral edge of the animal plate (asterisks). (C) *zfhx1* is expressed all around the circumference of the animal plate in Nodal morphants (asterisks). (D) *zfhx1* 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.

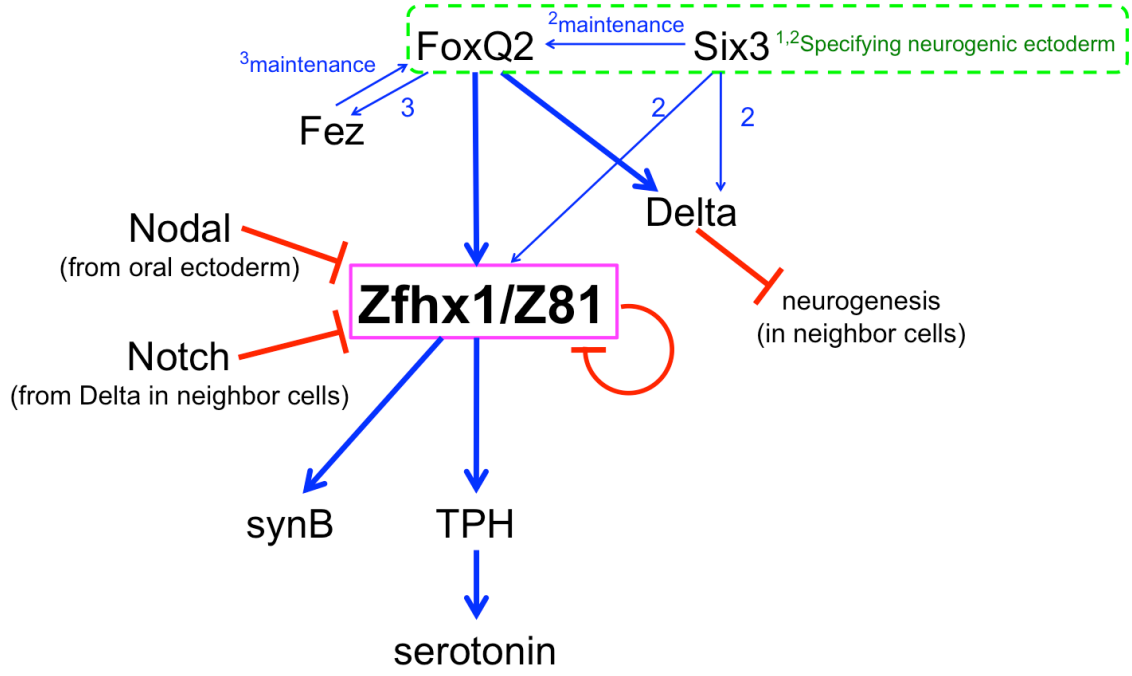

Figure 1-10. Model of the regulatory mechanisms controlling differentiation of serotonergic neurons in the sea urchin embryo.

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* and *delta* expression and Six3 supports FoxQ2 expression (2). Zfhx1 is required for the expression of *tph*, which is required for serotonin synthesis, and for *synaptotagmin B* (*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 suppresses its own expression.

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