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RESEARCH REPORT

Tfap2 and *Sox1/2/3* cooperatively specify ectodermal fates in ascidian embryos

Kaoru S. Imai[1,*], Hiroki Hikawa[1], Kenji Kobayashi[2] and Yutaka Satou[2,*]

ABSTRACT

Epidermis and neural tissues differentiate from the ectoderm in animal embryos. Although epidermal fate is thought to be induced in vertebrate embryos, embryological evidence has indicated that no intercellular interactions during early stages are required for epidermal fate in ascidian embryos. To test this hypothesis, we determined the gene regulatory circuits for epidermal and neural specification in the ascidian embryo. These circuits started with *Tfap2-r.b* and *Sox1/2/3*, which are expressed in the ectodermal lineage immediately after zygotic genome activation. *Tfap2-r.b* expression was diminished in the neural lineages upon activation of fibroblast growth factor signaling, which is known to induce neural fate, and sustained only in the epidermal lineage. *Tfap2-r.b* specified the epidermal fate cooperatively with *Dlx.b*, which was activated by *Sox1/2/3*. This *Sox1/2/3-Dlx.b* circuit was also required for specification of the anterior neural fate. In the posterior neural lineage, *Sox1/2/3* activated *Nodal*, which is required for specification of the posterior neural fate. Our findings support the hypothesis that the epidermal fate is specified autonomously in ascidian embryos.

KEY WORDS: TFAP2, Sox1/2/3, Epidermis, Ectoderm, Neural induction

INTRODUCTION

In animal embryos, the ectoderm differentiates into epidermis and neural tissues. In vertebrate embryos, inhibition of bone morphogenetic protein (BMP) and SMAD signaling is important for neural induction (Muñoz-Sanjuán and Brivanlou, 2002), whereas fibroblast growth factor (FGF) and mitogen-activated protein kinase (MAPK) signaling also plays an instructive role in neural induction (Delaune et al., 2005; Marchal et al., 2009; Streit et al., 2000; Wilson et al., 2000). In the invertebrate chordates *Ciona intestinalis* and *Ciona robusta*, neural cells are induced similarly by a combination of positive regulation by MAPK signaling and negative regulation by SMAD signaling. As a result, *Otx* expression is induced in two pairs of cells at the 32-cell stage (Fig. S1A,B) (Bertrand et al., 2003; Hudson et al., 2003; Hudson and Lemaire, 2001; Khoueiry et al., 2010; Ohta and Satou, 2013; Ohta et al., 2015). These cells are progenitors of the anterior (a-line) and posterior (b-line) neural lineages. Although *Nodal* is similarly regulated (Khoueiry et al., 2010; Ohta and Satou, 2013), *Foxa.a* additionally represses *Nodal* in the anterior lineage (Imai et al., 2006), and *Nodal* is therefore expressed only in the posterior neural lineage (Fig. S1B).

[1]Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka 560-0043, Japan. [2]Department of Zoology, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan.

*Authors for correspondence (imai@bio.sci.osaka-u.ac.jp; yutaka@ascidian.zool.kyoto-u.ac.jp)

K.S.I., 0000-0001-9113-6074; Y.S., 0000-0001-5193-0708

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In vertebrate embryos, epidermal fate is induced by BMP signaling; in frog and chick embryos, *Tfap2* is induced by BMP signaling and plays a crucial role in specifying epidermal fate (Hoffman et al., 2007; Luo et al., 2002; Qiao et al., 2012). However, in *Ciona*, *Tfap2-r.b* is activated in ectodermal lineages directly by a maternal factor immediately after zygotic genome activation (Oda-Ishii et al., 2016). As in vertebrate embryos, BMP signaling is used in dorsoventral patterning of epidermal cells in *Ciona* embryos, but this happens at a later stage (Imai et al., 2012; Pasini et al., 2006; Waki et al., 2015). Therefore, neural induction and dorsoventral patterning appear to be separable events, and epidermal fate might not be induced during neural induction in *Ciona*. In another ascidian species, *Halocynthia roretzi*, epidermal cells are differentiated from cell populations continuously dissociated from the first cleavage to the early gastrula stage (Nishida, 1992). Thus, the default ectodermal fate is likely to be epidermal cells in ascidian embryos. In the present study, we address this problem by analyzing gene regulatory pathways for the specification of epidermal and neural fates; we do not analyze nerve cord cells that are derived from the vegetal hemisphere in the present study, because these cells are specified differently (Hudson et al., 2013; Imai et al., 2006).

In addition, *Sox1/2/3* (the sole member of the *Soxb1* family in the *Ciona* genome; Yamada et al., 2003) also begins to be expressed in ectodermal lineages immediately after zygotic genome activation (Fig. S1) (Imai et al., 2004; Miya and Nishida, 2003). *Sox1/2/3* (*Sox1*, *Sox2* and *Sox3* in vertebrates) expressed in early embryos of deuterostome animals has been suggested to be involved in germ layer formation; an ortholog of *Sox1/2/3* is localized in the animal hemisphere of early embryos of frogs, lamprey, amphioxus and sea urchins (Cattell et al., 2012; Kenny et al., 1999; Penzel et al., 1997). Therefore, we also analyzed the functions of this gene in ectodermal specification in *Ciona*.

RESULTS AND DISCUSSION

Tfap2-r.b and *Sox1/2/3* are necessary for the specification of ectodermal tissues

We first knocked down *Tfap2-r.b* and *Sox1/2/3* by injecting specific morpholino antisense oligonucleotides (MOs) in *Ciona* embryos. Whereas embryos injected with the *Sox1/2/3* MO were highly disorganized after gastrulation, *Tfap2-r.b* morphant embryos yielded larvae in which the trunk and tail regions could be recognized. However, tunic, which is produced in epidermal cells, was not observed in *Tfap2-r.b* morphant larvae (Fig. S2), and some cells in the outer layer were easily dissociated. Two epidermal marker genes, *Epib* and CG.KH2012.C14.549 (Satou et al., 2001b), which were expressed normally in embryos injected with a control MO against *Escherichia coli lacZ*, were greatly reduced in *Tfap2-r.b* or *Sox1/2/3* morphants at the late gastrula stage (Fig. 1A-C; Fig. S3A-C), indicating important roles for *Tfap2-r.b* and *Sox1/2/3* in epidermal fate specification.

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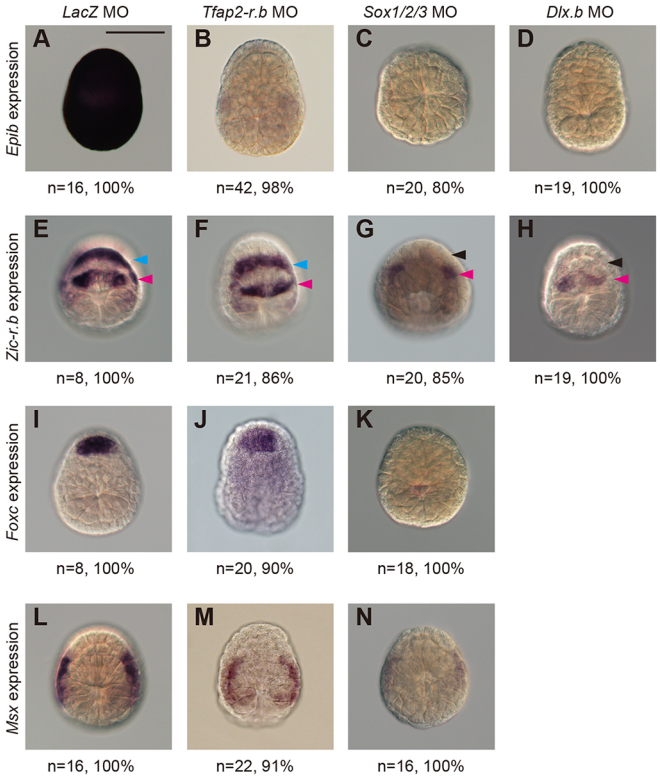

Fig. 1. Regulation of ectodermal genes by *Tfap2-r.b*, *Sox1/2/3* and *Dlx.b*. (A-N) Expression of an epidermal marker, *Epib* (A-D), which encodes a protein similar to vertebrate UDP-glucuronic acid decarboxylase, *Zic-r.b* (E-H), *Foxc* (I-K) and *Msx* (L-N) in late gastrula embryos injected with MOs against *lacZ* (control; A,E,I,L), *Tfap2-r.b* (B,F,J,M), *Sox1/2/3* (C,G,K,N) or *Dlx.b* (D,H). In E-H, magenta arrowheads indicate the expression of *Zic-r.b* in vegetal cells, which was not reduced, whereas cyan arrowheads indicate the expression of *Zic-r.b* in animal cells, which was reduced in morphants of *Sox1/2/3* or *Dlx.b* (black arrowheads). The numbers of embryos examined and the proportions of embryos that each image represents are shown beneath. Scale bar: 100 µm.

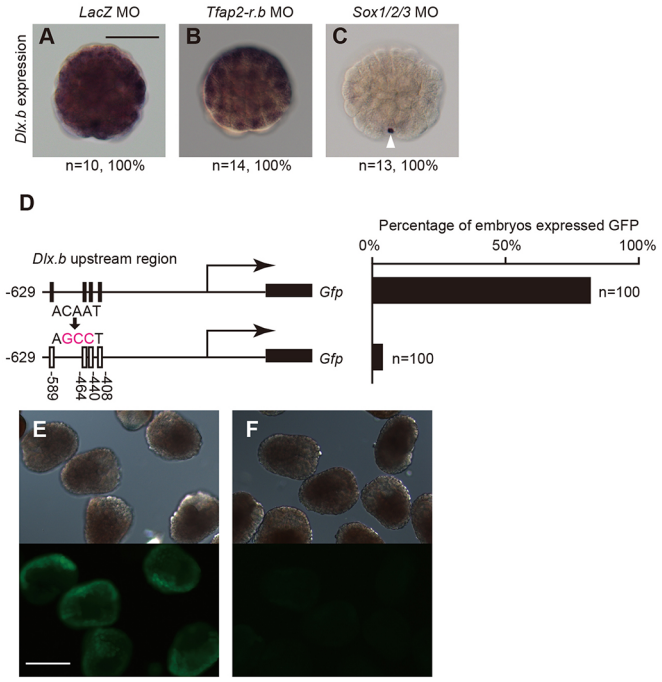

Fig. 2. *Sox1/2/3* regulates *Dlx.b*. (A-C) Expression of *Dlx.b* in embryos injected with MOs against *lacZ* (A), *Tfap2-r.b* (B) or *Sox1/2/3* (C). Arrowhead in C indicates the maternal transcript localized in the posterior pole. (D-F) A reporter assay indicating that *Dlx.b* is a direct target of *Sox1/2/3*. (D) A graph showing the percentages of embryos expressing Gfp protein. The introduced constructs are depicted on the left. Intact and mutated Sox binding sites are shown by filled and unfilled boxes, respectively, and their sequences are shown next to the boxes. (E,F) Reporter expression was examined at the late gastrula stage. Brightfield images are shown in the upper panels, and Gfp expression is shown in the lower panels. Scale bars: 100 µm.

Next, we examined the expression of neural marker genes. As explained above, there are two neural lineages in the *Ciona* embryo. The anterior neural lineage produces the brain and palps, in which sensory neurons are differentiated; *Zic-r.b* (formerly *ZicL*, renamed according to a recently published nomenclature rule; Stolfi et al., 2015b) and *Six3/6* mark the brain lineage, and *Foxc* marks the palp lineages (Ikeda et al., 2013; Imai et al., 2006; Wagner and Levine, 2012). In late gastrulae injected with the *lacZ* MO, *Zic-r.b* and *Six3/6* were expressed normally in cells with the brain fate (Fig. 1E; Fig. S3D). The expression of *Zic-r.b* and *Six3/6* was not changed in *Tfap2-r.b* morphants, and was lost in *Sox1/2/3* morphants (Fig. 1F,G; Fig. S3E,F). Similarly, *Foxc* was expressed normally in the anterior region of the neural plate in embryos injected with the *lacZ* or *Tfap2-r.b* MOs, but it was lost in *Sox1/2/3* morphants (Fig. 1I-K).

Posterior neural lineage cells express *Msx* (Fig. S1), and give rise to the dorsal row of the nerve cord, epidermal sensory neurons and epidermal cells along the nerve cord (Imai et al., 2006; Pasini et al., 2006; Roure et al., 2014; Waki et al., 2015). Whereas *Msx* was expressed normally in embryos injected with the *lacZ* or *Tfap2-r.b* MOs, it was greatly reduced in *Sox1/2/3* morphants (Fig. 1L-N). Nevertheless, because weak expression of *Msx* was detected in every experimental embryo, we confirmed this downregulation by reverse transcription followed by quantitative PCR (RT-qPCR) (Fig. S4). *Sox1/2/3* morphants showed a 71% reduction on average in *Msx* mRNA amount compared with *lacZ* MO-injected control embryos at the early gastrula stage. Thus, *Sox1/2/3* is required for specification of the anterior/posterior neural and epidermal fates, whereas *Tfap2-r.b* is required for specification of the epidermal fate, but not for the neural fates.

Sox1/2/3 regulates epidermal and anterior neural fates through *Dlx.b*

Dlx.b is expressed in the animal hemisphere at the 64-cell stage and thereafter (Fig. S1) (Imai et al., 2004), and is required for the expression of *Foxc*, *Six3/6* and epidermal regulatory genes expressed in epidermis (Imai et al., 2006). Here, we confirmed this observation by *in situ* hybridization at the late gastrula stage (Fig. S5A,B). In addition, we found that *Epib* expression in the epidermal lineage and *Zic-r.b* expression in the neural lineage were lost in *Dlx.b* morphants at the late gastrula stage (Fig. 1D,H). However, *Msx.b* expression was clearly detected in *Dlx.b* morphants (Fig. S5C). Thus, *Dlx.b* is involved in specification of the anterior neural and epidermal fates but not of the posterior neural fate.

Next, we examined whether *Tfap2-r.b* and *Sox1/2/3* would regulate *Dlx.b*. As shown in Fig. 2A-C, whereas *Dlx.b* was expressed normally in the entire animal hemisphere of embryos injected with the *lacZ* or *Tfap2-r.b* MO at the early gastrula stage, *Dlx.b* expression was abolished in *Sox1/2/3* morphants. Thus, *Sox1/2/3*, but not *Tfap2-r.b*, regulates *Dlx.b* expression.

Because the upstream sequence of *Dlx.b* that drives a reporter in ectodermal cells contains Sox binding sites (Irvine et al., 2011), we tested the possibility that Sox1/2/3 directly regulates *Dlx.b* through these sites. A reporter construct containing this upstream region

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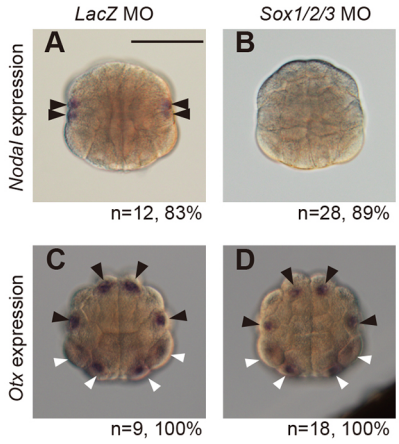

Fig. 3. *Sox1/2/3* regulates *Nodal* but not *Otx* in early embryos. (A-D) Expression of *Nodal* at the 64-cell stage (A,B) and *Otx* at the 32-cell stage (C,D) in embryos injected with MOs against *lacZ* (A,C) or *Sox1/2/3* (B,D). Arrowheads indicate the areas of expression. Note that *Otx* is also expressed in non-ectodermal cells (white arrowheads). The numbers of embryos examined and the proportions of embryos that each image represents are shown beneath. Scale bar: 100 µm.

drove reporter gene expression at the late gastrula stage (Fig. 2D,E). However, a reporter construct with mutated Sox binding sites rarely drove this reporter (Fig. 2D,F). This observation supports the hypothesis that *Dlx.b* is a direct target of Sox1/2/3.

Sox1/2/3 controls specification of the posterior neural lineage through Nodal signaling

Because *Msx* is expressed under the control of *Nodal* and *Otx* (Imai et al., 2006; Roure et al., 2014), we examined *Nodal* and *Otx* expression in *Sox1/2/3* morphants. Whereas *Nodal* was expressed normally at the 64-cell stage in embryos injected with the *lacZ* control MO, it was lost in *Sox1/2/3* morphants (Fig. 3A,B). The normal level of early *Otx* expression was observed in the anterior and posterior neural lineages of 32-cell embryos injected with the *lacZ* or *Sox1/2/3* MO (Fig. 3C,D), but late expression of *Otx* in the palp lineage of the late gastrula embryo was lost (Fig. S6A,B). Thus, it is conceivable that loss of *Nodal* expression led to downregulation of *Msx* in the posterior neural lineage cells of *Sox1/2/3* morphants. Namely, in this lineage, *Sox1/2/3* activates *Nodal*, and Nodal signaling activates *Msx*, although it is possible that *Sox1/2/3* redundantly regulates *Msx* independently of Nodal signaling.

In frog and sea urchin embryos, an ortholog of *Sox1/2/3* is involved in germ layer formation through regulation of *Nodal* expression, although *Nodal* is negatively regulated by Sox in frogs and positively regulated in sea urchins (Range et al., 2007; Zhang et al., 2003). In this respect, the ascidian *Sox1/2/3* function is similar to that of the sea urchin ortholog. On the other hand, this class of Sox genes plays an important role in maintaining a neural progenitor identity in a variety of animals (reviewed by Kamachi and Kondoh, 2013). Because *Sox1/2/3* expression is lost in the neural plate before the late gastrula stage (Imai et al., 2004) but retained in non-neuronal cells within the motor ganglion (Stolfi et al., 2011), the expression of *Sox1/2/3* in early *Ciona* embryos might also be related to this evolutionarily conserved function.

Downregulation of *Tfap2-r.b* in non-epidermal cells

At the 16-cell stage, *Fgf9/16/20* is activated in the vegetal hemisphere and induces neural fate in the animal hemisphere (Bertrand et al., 2003; Hudson et al., 2003; Hudson and Lemaire, 2001; Khoueiry et al., 2010; Ohta and Satou, 2013; Ohta et al., 2015; Roure et al., 2014). *Tfap2-r.b* is first expressed in the entire animal hemisphere, and then the expression disappears in the neural lineage at the 64-cell stage (Fig. S1) (Imai et al., 2004). Therefore, we examined whether Fgf9/16/20 signaling represses *Tfap2-r.b* expression. As shown in Fig. 4A-D, *Tfap2-r.b* expression was not downregulated in the neural lineage of embryos treated with U0126, which inhibits the FGF signaling pathway, and in *Fgf9/16/20* morphants, but it was downregulated in embryos incubated with bFGF protein. We further confirmed this downregulation by RT-qPCR (Fig. 4E); in this experiment, we isolated the anterior neural cells with a glass needle from embryos

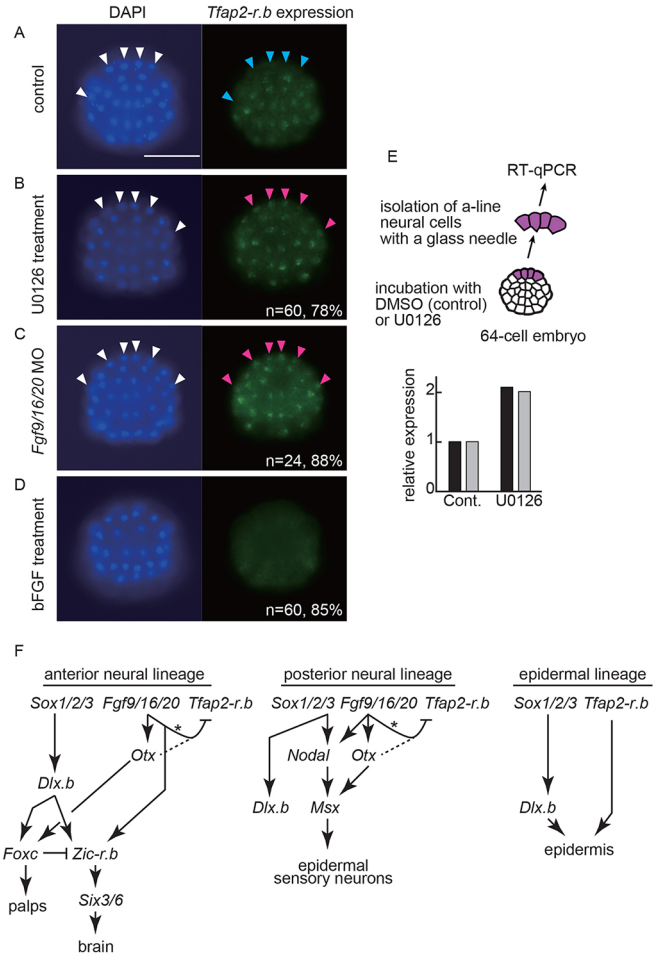

Fig. 4. *Tfap2-r.b* is regulated negatively by Fgf signaling. (A-D) Expression of *Tfap2-r.b* in normal embryos (A), embryos incubated in sea water containing U0126 (B) or basic FGF (bFGF; D), and embryos injected with *Fgf9/16/20* MO at the 64-cell stage (C). Scale bar: 100 µm. Nuclei are shown by DAPI staining on the left, and the anterior and posterior neural cells are indicated by arrowheads in A-C. Note that some nuclei of neural cells are not clearly visible. *Tfap2-r.b* expression is lost in the neural cells in A (cyan arrowheads), whereas it was not lost in B and C (magenta arrowheads). In D, the expression is completely lost. (E) The amount of *Tfap2-r.b* mRNA in the anterior neural cells of embryos treated with DMSO (control) or U0126 was measured by RT-qPCR (schematic). Two independent experimental results are shown in the graph as bars with different colors. Maternally expressed *Pouf2* mRNA was used as an endogenous control. (F) Schematics of gene regulatory circuits for specifying the anterior neural, posterior neural and epidermal lineages. These schematics were drawn on the basis of the present and previous studies. The repression of *Tfap2* by Fgf signaling (asterisks) might be indirect; *Otx* could be a candidate for a mediator of this repression (dashed lines), because it is induced by Fgf signaling (Bertrand et al., 2003; Hudson et al., 2003; Hudson and Lemaire, 2001; Khoueiry et al., 2010; Ohta and Satou, 2013; Ohta et al., 2015).

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treated with DMSO (control) or U0126, and measured the amounts of *Tfap2-r.b* mRNA. Thus, Fgf9/16/20 signaling negatively regulates *Tfap2-r.b* expression in the neural lineage.

Tfap2 is expressed in non-neural ectoderm and in the neural plate border of vertebrate embryos (Simoes-Costa and Bronner, 2015). Although *Ciona* embryos might also have a rudimentary neural crest and rudimentary placodes (Abitua et al., 2015, 2012; Ikeda et al., 2013; Manni et al., 2004; Stolfi et al., 2015a; Wagner and Levine, 2012; Waki et al., 2015), *Tfap2-r.b* expression is downregulated in these lineages by Fgf signaling. Because *Tfap2* is also expressed only in the non-neural ectoderm of embryos of another basal chordate, *Branchiostoma floridae* (Meulemans and Bronner-Fraser, 2002), the ancestral function of *Tfap2* might be to specify epidermal fate.

Conclusions

The gene regulatory pathways for specification of the epidermal and neural fates are shown in Fig. 4F. *Tfap2-r.b* and *Sox1/2/3* are the earliest genes that are expressed in the ectodermal lineage (Imai et al., 2004; Matsuoka et al., 2013; Ogura and Sasakura, 2016), and *Tfap2-r.b* is activated directly by a maternal factor, Gata.a (Oda-Ishii et al., 2016). During early stages, MAPK signaling activated by Fgf9/16/20 induces neural fate (Bertrand et al., 2003; Hudson et al., 2003; Hudson and Lemaire, 2001; Khoueiry et al., 2010; Ohta and Satou, 2013; Ohta et al., 2015). We showed that this signal also represses *Tfap2-r.b* transcription in the anterior and posterior neural linages, and that *Sox1/2/3* regulates *Nodal* and *Dlx.b*. *Nodal*, which is required for patterning and specification of the posterior neural lineage (Hudson et al., 2007; Hudson and Yasuo, 2005, 2006), activates *Msx* to specify the posterior neural lineage. *Dlx.b* is required for specification of the anterior neural lineage and the epidermal lineage (Imai et al., 2006). Cells with *Tfap2-r.b* and *Dlx.b* expression differentiate into epidermal cells. Thus, in the ascidian embryo, epidermal cells are differentiated from ectodermal cells that are not induced to become neural cells. This might represent an ancestral developmental program in a primitive chordate.

MATERIALS AND METHODS

Biological materials, gene identifiers, and in situ hybridization

C. robusta (*C. intestinalis* type A) (Brunetti et al., 2015) adults were obtained from the National Bio-Resource Project for *Ciona* (AMED, Japan). cDNA clones were obtained from our cDNA clone collection (Satou et al., 2005). Whole-mount *in situ* hybridization was performed as previously described (Ikuta and Saiga, 2007; Satou, 1999). Gene identifiers are shown in Table S1, according to the nomenclature rule of this animal (Stolfi et al., 2015b).

Gene knockdown and knockout

The sequences of the MOs (Gene Tools, LLC), which block translation, are shown in Table S2. MOs were introduced by microinjection under a microscope. All experiments were repeated at least twice independently.

To confirm the specificity of phenotypes observed in *Tfap2-r.b* morphants, we used TALEN (transcription activator-like effector nuclease) technology. The N- and C-terminal domains of TALE and the *Fok*I nuclease domain were taken from the Platinum Gate TALEN Kit (Sakuma et al., 2013). *Epib* expression was similarly downregulated in experimental embryos (Fig. S7A,B). For confirmation of the specificity of the *Sox1/2/3* MO, we also injected a second MO (Table S2). In embryos injected with this second MO, *Dlx.b* and *Nodal* were downregulated in the neural lineage (Fig. S7C,D). The MOs against *Dlx.b*, *Fgf9/16/20* and *E. coli lacZ* were used previously (Imai et al., 2006; Satou et al., 2001a).

Reporter assay and RT-qPCR

DNA constructs for analyzing cis-regulatory elements were introduced by electroporation (Corbo et al., 1997). The constructs contain the chromosomal region KhC7:631350–630500.

Reverse transcription was performed using the Cell-To-Ct kit (Thermo Fisher Scientific); qPCR was performed with the Taqman method using the primer and probe sets shown in Table S3.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.S.I. and Y.S. conceived the project and wrote the paper. K.S.I, H.H., K.K., and Y.S. performed experiments.

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Supplementary information

Supplementary information available online at
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