Genetic pathways for differentiation of the peripheral nervous system in ascidians

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Ascidians belong to tunicates, the sister group of vertebrates. Peripheral nervous systems (PNSs) including epidermal sensory neurons (ESNs) in the trunk and dorsal tail regions of ascidian larvae are derived from cells adjacent to the neural plate, as in vertebrates. On the other hand, peripheral ESNs in the ventral tail region are derived from the ventral ectoderm under the control of BMP signalling, reminiscent of sensory neurons of amphioxus and protostomes. In this study, we show that two distinct mechanisms activate a common gene circuit consisting of Msx, Ascl.b, Tox, Delta.b and Pou4 in the dorsal and ventral regions to differentiate ESNs. Our results suggest that ventral ESNs of the ascidian larva are not directly homologous to vertebrate PNSs. The dorsal ESNs might have arisen via co-option of the original PNS gene circuit to the neural plate border in an ancestral chordate.
Peripheral nervous systems (PNSs) include sensory neurons. In vertebrate embryos, PNSs arise from the border of the neural plate\textsuperscript{1-4}. Cranial neural crest cells and placodes contribute to the cranial PNS, whereas the caudal PNS is thought to be derived only from neural crest cells. In anamniote embryos, additional mechanosensory neurons called Rohon-Beard sensory neurons are also formed from the neural plate border\textsuperscript{13,10}. On the other hand, in cephalochordates, the most basal chordate group, in which no neural crest cells or placodes have been identified, epidermal sensory neurons (ESNs) are found along the entire length of larval and adult bodies\textsuperscript{7,8}. These ESNs are derived from the ventral ectodermal region of neurula embryos.

*Ciona intestinalis* is a member of the tunicates, the closest relatives of vertebrates. In this species, the palps, which contain sensory neurons and differentiate at the anterior end of the larva, are derived from the anterior border of the neural plate\textsuperscript{9}. By contrast, the pigment cells, which differentiate in the brain, are derived from the anterolateral border of the neural plate\textsuperscript{10}. Indeed, the anterior and anterolateral borders of the neural plate are suggested to be a rudimentary placode and primitive neural crest, respectively\textsuperscript{11-13}. The posterior border of the neural plate gives rise to the dorsal row of the tail nerve cord and dorsal epidermis, and ESNs are differentiated from this dorsal epidermis (Supplementary Fig. 1)\textsuperscript{16}. In addition to these PNS neurons, the ascidian larva has ESNs in the ventral region of the tail. A cell lineage analysis revealed that this ventral region is not derived from the border of the neural plate\textsuperscript{16}.

The posterolateral border of the neural plate is induced at the 32-cell stage in a process coordinated by four signalling molecules\textsuperscript{17-22}. Fgf9/16/20 signalling activates *Otx* and *Nodal* in a cell pair called b6.5 (Supplementary Fig. 1). Signalling of EfnA.d (formerly EphinA-d, renamed according to a recently published nomenclature rule\textsuperscript{23}) and Gdf1/3-r (called Gdf1/3-like or Orphan TGFβ 2) and Admp (a bone morphogenetic protein (BMP)-related ligand) negatively regulates expression of *Otx* and *Nodal*. The Otx transcription factor and Nodal signalling cooperatively activate expression of Msx (formerly Msxb) and Delta.b (formerly Delta-like or Delta2)\textsuperscript{24,25}. On the other hand, the ventral ESNs are induced by Admp, which is expressed in the endoderm lining the ventral ectodermal region\textsuperscript{16,25}. Although the detailed molecular mechanisms remain to be elucidated, the ventral ESN lineage cells also express Msx and Delta.b\textsuperscript{16,26}.

In neural induction of vertebrate embryos, BMPs negatively regulate neural fate, FGFs positively induce neural fate\textsuperscript{27,31} and neural crest and placodes arise from a region at the border of the neural plate\textsuperscript{32}. On the other hand, in cephalochordate embryos, BMPs positively induce ESN formation in protostomes, including flies and annelids\textsuperscript{34,35}. Thus, in regard to the cell lineage and signalling necessary for differentiation, the anterior PNS neurons and the dorsal PNS neurons in *Ciona* larvae are reminiscent of PNS neurons in vertebrate embryos, whereas the ventral PNS neurons are reminiscent of PNS neurons in cephalochordate and protostome embryos. Given that the innovation of neural crest cells and placodes is thought to be a key event in the course of evolution from primitive invertebrate chordates to vertebrates\textsuperscript{32,36}, it is possible that the *Ciona* embryo might have both vertebrate-type and invertebrate-type PNS neurons. In the present study, we dissect a gene circuit involved in differentiation of ventral ESNs in the *Ciona* embryo, and compare it with a gene circuit involved in differentiation of dorsal ESNs for understanding evolution of PNSs in chordates.

**Results**

BMP signalling in the ventral ectodermal region. Previous studies showed that the ventral ectodermal region of the tail is induced by a BMP ligand, Admp\textsuperscript{16,25}. To determine when and where this signalling works, we used an antibody against phosphorylated Smad1/5/8 (pSmad1/5/8). To confirm that this antibody is immunoreactive against pSmad1/5/8 in ascidian embryos, we first stained early-tailbud embryos, in which BMP signalling is active in the ventral trunk region\textsuperscript{17}. As shown in Fig. 1g, we detected strong pSmad1/5/8 staining in the ventral trunk region. In addition, when *Bmp2/4* is overexpressed in the entire epidermis using the *Dlx.b* upstream region, pSmad1/5/8 staining was detected in the entire epidermis (Supplementary Fig. 2a,c). Conversely, when Noggin, which is an antagonist for BMP ligands, was overexpressed, pSmad1/5/8 staining was barely detectable (Supplementary Fig. 2b,d). Thus, this antibody successfully detected pSmad1/5/8 in ascidian embryos.

As shown in Fig. 1a-c, we observed strong pSmad1/5/8 staining in two lines of cells of the ventral ectoderm from the anterior to the posterior end at the late-gastrula stage. We labelled one of two b4.2 blastomeres of the eight-cell embryo with DIF to identify the anterior–posterior border, because the tail endoderm is derived from a pair of b4.2 cells. The tail region contained eight pSmad1/5/8-positive rows (Fig. 1c). At the neurula stage after the next cell division (10th division; most cells divide laterally), we detected pSmad1/5/8 in the anterior rows within the b4.2-line ectodermal cells and in the ventral ectodermal region of the trunk (Fig. 1d–f). The anterior five rows in the b4.2-line ectoderm contained a strong signal, and two or three additional rows contained a weaker signal. At the early-tailbud stage after the next cell division (11th division; most cells divide along the anterior–posterior axis), we detected a weak pSmad1/5/8 signal in only three anterior rows of the b4.2-line ectodermal cells (Fig. 1g–i).

Indeed, pSmad1/5/8 staining at the late-gastrula stage was lost in embryos injected with a morpholino antisense oligonucleotide (MO) against *Admp* (Fig. 2b,d), while it was not affected in control embryos injected with a control MO against *Escherichia coli lacZ* (MO) against *Admp*, and found that *Bmp2/4* is activated by Admp signalling in the ventral trunk epidermis at the tailbud stage\textsuperscript{27}, and begins to be expressed in the ventral epidermis at the late-gastrula stage\textsuperscript{26}. We examined whether the expression of *Bmp2/4* at the late-gastrula stage is also induced by Admp, and found that *Bmp2/4* expression was lost in *Admp* morphants (Fig. 2e,f). Because Smad1/5/8 may be phosphorylated and activated by Bmp2/4 in addition to Admp, we collectively call signalling of *Bmp2/4* and Admp as BMP signalling hereafter.

**Thbx2/3 is required for Msx expression in the ventral region.** Msx is important for differentiation of the dorsal ESNs\textsuperscript{24,25}, and is also expressed in the ventral tail ectoderm\textsuperscript{26}, suggesting that Msx is also important for differentiation of the ventral ESNs. To confirm this possibility, we injected a MO against Msx into fertilized eggs. In the resultant morphant embryos, *Pou4* and *Celf3.a* (*Etr-1*), which mark ESNs\textsuperscript{26,38} (see Fig. 7c,e), were not expressed (Supplementary Fig. 3a,b) in dorsal and ventral ESNs, except in a few ESNs near the tip of the tail. Because Msx is not expressed in the most posterior region, it is not surprising that ESNs near the tip of the tail of *Msx* morphants expressed *Pou4* and *Celf3.a*. We also found that *Pou4* and *Celf3.a* were ectopically expressed, when Msx was overexpressed in the entire epidermis using the *Dlx.b* upstream region (Supplementary Fig. 3c,d).

A previous study showed that expression of *Celf3.a* in the ventral ESNs is under the control of BMP signalling\textsuperscript{16}. In the present study, we found that expression of Msx in ventral tail ectoderm was similarly downstream of BMP signalling, because Msx expression in ventral tail ectoderm was upregulated in embryos overexpressed with *Bmp2/4* using the *Dlx.b*
enhancer, and downregulated in embryos overexpressed with Noggin (Fig. 3a–c).

We next investigated whether Msx began to be expressed at the same stage as Bmp2/4, which is activated by Admp signalling, as described above. Although the expression pattern of Msx has been previously described\(^26,39\), we again examined Msx expression to determine the exact timing of its expression in ventral ectodermal cells (Fig. 3d–i). Msx was not expressed in the ventral ectoderm at the late-gastrula stage, and began to be expressed in the posterior half of the ventral tail ectoderm at the neurula stage. At the initial-tailbud stage, Msx was expressed in the entire ventral region of the tail ectoderm. Thus, because its expression initiates later than that of Bmp2/4, Msx is not likely to be a direct target of Admp signalling.

To identify candidate genes that are directly regulated by Admp signalling and regulate Msx expression, we used RNA sequencing (RNA-seq) to compare gene expression in late-gastrula embryos treated with recombinant BMP4 or a BMP inhibitor, dorsomorphin. Five transcription factor genes, including Msx, were greatly upregulated in embryos treated with BMP4 and downregulated in embryos treated with dorsomorphin (Fig. 4a). Nkxtn1 (formerly Nkx-A) and NK2-3/5/6 (formerly NK4) are expressed specifically in ventral epidermis of the trunk at the tailbud stage, whereas Atoh8 (formerly Net) is not specifically expressed in the ventral epidermis\(^26\). On the other hand, Tbx2/3 is expressed in the ventral ectoderm of the tail\(^26,40,41\). We therefore determined the exact timing of the initiation and termination of Tbx2/3 expression (Fig. 4b–g). At the late-gastrula stage, Tbx2/3 was expressed in the entire ventral ectoderm (Fig. 4b,c). At the neurula stage, expression of Tbx2/3 was diminished in the posterior half of the tail ectoderm, and became weaker in the rest of the ventral ectoderm (Fig. 4d,e). At the early-tailbud stage, expression of Tbx2/3 vanished completely in the ventral tail ectoderm (Fig. 4f,g). Thus, expression of Tbx2/3 preceded Msx expression. Tbx2/3 was indeed activated by Bmp signalling, as demonstrated by the observation that it was ectopically expressed in Bmp2/4-overexpressing embryos (Fig. 4h).

Next, we injected a Tbx2/3 MO into both of the left and right b4.2 blastomeres at the eight-cell stage to confirm that Tbx2/3 regulated Msx in the tail ectoderm cell-autonomously\(^26\) (we did not inject the MO into fertilized eggs, because Tbx2/3 is also expressed in non-b-line cells). In these Tbx2/3 morphants, Msx expression was downregulated in the ventral tail ectoderm of tailbud embryos, but was unaffected in the dorsal tail ectoderm (Fig. 4i,j). For a further confirmation, the Tbx2/3 MO was injected into one of the posterior animal cells of eight-cell embryos (b4.2), Msx expression was lost in the ventral region on the injected side (Supplementary Fig. 4a,b). Thus, BMP signalling activates Tbx2/3 in the ventral tail ectoderm at the late-gastrula stage, and Tbx2/3 is required for activating Msx.

Although Tbx2/3 is required for Msx expression in the ventral tail ectoderm, it might be insufficient for Msx expression. This is because Tbx2/3 messenger RNA (mRNA) injection into fertilized eggs failed to evoke ectopic expression of Msx (Supplementary Fig. 5). BMP signalling itself or unidentified factors downstream of BMP signalling, together with Tbx2/3, might work to activate Msx.
**Figure 2** | **Admp** activates Bmp signalling and Bmp2/4 expression in the ventral ectoderm at the late-gastrula stage. (a,b) BMP signalling was detected with an antibody against phosphorylated Smad1/5/8 (green) (a) in an embryo injected with a control LacZ MO, and (b) in an embryo injected with an Admp MO. (c,d) DAPI staining shows nuclei (blue) of the embryos shown in a and b. (e,f) Expression of Bmp2/4 at the late-gastrula stage is observed in the ventral ectoderm in an embryo injected with a control LacZ MO (e), while it is lost in an embryo injected with an Admp MO (f). Scale bar, 100 μm (a). The number of embryos examined and the proportion of embryos that each panel represents are shown within the panels. DAPI, 4,6-diamidino-2-phenylindole.

**Figure 3** | Msx is under the control of Bmp signalling, and begins to be expressed in the ventral tail ectoderm at the neurula stage. (a–c) Msx expression (a) in a control early-tailbud embryo, (b) in an embryo with Bmp2/4 overexpression under the Dlx.b enhancer and (c) in an embryo with Noggin overexpression under the Dlx.b enhancer. The white arrowhead indicates the region in which Msx expression is lost. The number of embryos examined and the proportion of embryos that each panel represents are shown within the panels. (d–i) Msx expression (d,e) at the late-gastrula stage, (f,g) neurula stage and (h,i) initial-tailbud stage. (d,f,h) Ventral views and (e,g,i) lateral views are shown. Scale bar, 100 μm (a,d).
Ascl.2 and Tox are activated by Msx in the tail ectoderm. Because Ascl.2 (achaete-scute family bHLH transcription factor-b; formerly Ash2) and Tox (TOX high-mobility group box family member; formerly CAGF9) are activated downstream of Msx in the dorsal ectoderm, where dorsal ESNs differentiate, and because these two genes are also expressed in ventral tail ectoderm, we investigated the possibility that Msx regulates the expression of these two genes in the ventral tail ectoderm.
Expression of these two genes in the ventral tail ectoderm was not detectable at the late-gastrula or neurula stage, and began at the tailbud stage in normal embryos (Fig. 5a–l). These genes were strongly expressed in the ventral tail ectoderm, except for several cells near the trunk–tail border at the initial-tailbud stage. At the early-tailbud stage, both genes began to be expressed throughout the tail ventral ectoderm. Thus, expression of \( \text{Ascl.b} \) and \( \text{Tox} \) began later than \( \text{Msx} \) expression.

We first confirmed that injection of the control MO did not affect expression of \( \text{Ascl.b} \) and \( \text{Tox} \) (Fig. 6a,b). Next, we injected a MO against \( \text{Tbx2/3} \) into both of the left and right b4.2 blastomeres. In the resultant morphant embryos, expression of \( \text{Ascl.b} \) and \( \text{Tox} \) in the tail ventral ectoderm was greatly reduced, whereas expression of the two genes in the dorsal ectoderm, in which \( \text{Tbx2/3} \) was not expressed, was not affected (Fig. 6c,d). Likewise, the \( \text{Tbx2/3} \) MO was injected into one of the posterior animal cells of eight-cell embryos (b4.2); expression of \( \text{Ascl.b} \) and \( \text{Tox} \) was lost in the ventral region on the injected side (Supplementary Fig. 4c–f). In \( \text{Msx} \) morphants, the expression of \( \text{Ascl.b} \) and \( \text{Tox} \) in the dorsal and ventral ectoderm, except for a few cells near the tip of the tail, was also lost (Fig. 6e,f). Thus, the expression of \( \text{Ascl.b} \) and \( \text{Tox} \) in the dorsal ectoderm is under the control of \( \text{Msx} \) but not \( \text{Tbx2/3} \), while the expression in ventral ectoderm is under the control of \( \text{Tbx2/3} \) and \( \text{Msx} \).

We also found that the expression of \( \text{Ascl.b} \) and \( \text{Tox} \) was recovered in the tail ventral ectoderm and observed ectopically in
Figure 6 | Ascl.b and Tox are under the control of Tbx2/3, Msx and Bmp signalling. (a,b) Injection of the control LacZ MO into fertilized eggs did not affect the expression of (a) Ascl.b or (b) Tox. (c,d) When the Tbx2/3 MO was injected into the left and right posterior animal cells (b4.2) of eight-cell embryos, the expression of (c) Ascl.b and (d) Tox was greatly reduced in the ventral region (white arrowhead), but not in the dorsal region (black arrowhead). (e,f) The expression of (e) Ascl.b and (f) Tox in both the ventral and dorsal regions was lost in Msx morphants, except for a few cells in the posterior-most region (black arrowheads). (g,h) When the Tbx2/3 MO was injected into the left and right posterior animal cells (b4.2) of eight-cell embryos after electroporation of an overexpression construct for Mxs, the expression of (g) Ascl.b and (h) Tox was observed in the ventral region (arrowheads) and the lateral epidermal region. With this construct, Mxs was overexpressed in the entire epidermis under the control of the upstream sequence of Dlx.b. Note that not all epidermal cells overexpress Mxs because of mosaic incorporation of the electroporated plasmid. (i,j) The expression of (i) Ascl.b and (j) Tox was lost in the ventral region (white arrowheads), but not in the dorsal region (black arrowheads), of embryos with Noggin overexpression under the control of the upstream sequence of Dlx.b. The number of embryos examined and the proportion of embryos that each panel represents are shown. Scale bar, 100 μm (a).

Figure 7 | Ascl.b and Tox regulate Delta.b, Pou4 and Celf3.a, which mark ESNs. Expression of (a,b) Delta.b, (c,d) Pou4 and (e,f) Celf3.a marks the ESNs (black arrowheads) (a,c,e) in control embryos injected with the control LacZ MO, but is completely lost (b,d,f) in double morphants of Ascl.b and Tox. Lateral views are shown. Note that three spots seen near the tip of the tail in f are cells of the nerve cord, but not ESNs.

Finally, we examined the expression of Ascl.b and Tox in embryos with Noggin overexpression using the Dlx.b enhancer. In these embryos, the expression in the ventral tail ectoderm was specifically lost (Fig. 6i,j). This observation confirmed that BMP signalling is required for genes activated in the ventral tail ectoderm.

Tox and Ascl.b are required for differentiation of ESNs. Delta.b is expressed in dorsal and ventral ESNs, and Delta.b signalling represses Pou4 and Celf3.a (Etr-1) in the surrounding epidermal cells16,24,42. Delta.b expression in the ventral tail ectoderm begins in scattered cells of the posterior region and subsequently expands into the anterior tail region16. Through Delta/Notch-mediated lateral inhibition, the number of neurons are thought to be controlled16. We first investigated whether expression of Ascl.b and Tox preceded Delta.b expression in dorsal and ventral ESNs. Delta.b was not expressed in prospective ESNs at the neurula and initial-tailbud stages (Fig. 5m,n), and began to be expressed in prospective ESNs at the early-tailbud stage in the posterior region (Fig. 5o). At the middle-tailbud stage, prospective ESNs in the anterior tail region began to express Delta.b16 (see also Fig. 7a). Thus, initiation of Delta.b expression in prospective ESNs occurred one step later than initiation of Ascl.b and Tox expression.

When we injected MOs against either of Ascl.b or Tox, expression of Delta.b, Pou4 and Celf3.a was lost or reduced in most embryos (Table 1; Supplementary Fig. 6). In double morphants of Ascl.b and Tox, the expression of these three genes was almost completely lost (Table 1; Fig. 7a–f). Thus, Ascl.b and Tox are both required for differentiation of ESNs in the ventral and dorsal ectoderm.

Discussion
A previous study reported that there are two morphologically distinct populations among the ESNs of the ventral tail ectoderm, because posterior ESNs have longer axons than anterior ESNs43. Our analysis showed that ESNs in the anterior and posterior...
homologous to Rohon-Beard sensory neurons. It is possible that the ascidian dorsal ESNs are homologous to Rohon-Beard sensory neurons and the neural crest are overlapped considerably. We showed that Tbx2/3 is required for Msx expression in the ventral ectoderm. Although Tbx2/3 and its orthologues, Tbx2 and Tbx3, often act as a repressor, Tbx2 and Tbx3 are also known to act as activators. Therefore, it is not surprising that Tbx2/3 directly activates Msx in the ventral ectoderm, although it is also possible that Tbx2/3 acts as a repressor and indirectly activates Msx.

We also showed that ventral and dorsal tail ESNs are specified by a common genetic pathway comprising Msx, Tox/Ascl.b and Delta.b/Celf3.a/Pou4, whereas the upstream mechanism of Msx regulation differs between these two lineages (Fig. 8). In the dorsal lineage, Otx and Nodal are required for Msx expression, and these two genes are activated by a combination of the activating influence of FGF signalling and the repressive influence of Ephrin, Admp and Gdf1/3-r signalling at the 32-cell stage. In the ventral lineage, BMP signalling induces Tbx2/3 at the late-gastrula stage, and this Tbx2/3 expression is required for Msx expression. Hence, the evidence that co-option of the gene circuit downstream of Msx created a novel lineage of ESNs is persuasive. If so, which of the lineages represents the original one? We favour the hypothesis that the ventral lineage is the original one, for two reasons. First, the ventral lineage is induced by BMP signalling, and PNS neurons in amphibian and protostomes are also induced by BMP signalling. Second, although cells giving rise to the dorsal ESNs in the ascidian embryos are embedded in the epidermis and not migratory, the dorsal lineage is derived from cells located at the border of the epidermal lineage and neural plate, reminiscent of the situation in vertebrate PNSs.

In anamniote embryos, Rohon-Beard mechanosensory neurons are also formed from the neural plate border. An anatomical study suggested that Retzius bipolar cells of amphioxus are homologous to Rohon-Beard sensory neurons. It is possible that the ascidian dorsal ESNs are homologous to Rohon-Beard neurons, and this possibility is not necessarily mutually exclusive with the above possibility that the ascidian dorsal ESNs are homologous to vertebrate PNS neurons derived from neural crest cells. Indeed, the gene networks that specify Rohon-Beard neurons and the neural crest are overlapped considerably. None of these scenarios is inconsistent with our hypothesis that a novel lineage of ESNs were born by co-option of the gene circuit downstream of Msx.

In vertebrate embryos, BMP signalling negatively regulates neural fate during the initial process of neural induction, but later an intermediate level of BMP signalling is required for neural crest formation. Msx is indeed a direct target of BMP signalling, and these regulatory mechanisms upstream of Msx differ between these two lineages. These circuits are based on the results of the present and previous studies. Because Tbx2/3 alone failed to activate Msx, BMP signalling itself or unidentified factors downstream of BMP signalling, together with Tbx2/3, might work to activate Msx (a dotted arrow).

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<th>Embryos expressing the designated marker in a reduced number of prospective ESNs</th>
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Table 1 | Effects of knockdown of Ascl.b and Tox.

*Because the number of ESNs varies between individuals, we counted the number of prospective ESNs expressing Pou4 in 63 unperturbed embryos. Because the minimum number of prospective ESNs was seven, embryos with two to six cells expressing either of the markers were considered to have a reduced number of ESNs, whereas embryos with seven or more cells expressing either of the markers were considered to be normal.

Figure 8 | Summary of the regulatory gene circuits involved in differentiation of the dorsal and ventral ESNs. (a) Dorsal ESNs and ventral ESNs share a gene circuit downstream of Msx, whereas the regulatory mechanisms upstream of Msx differ between these two lineages. These circuits are based on the results of the present and previous studies. Because Tbx2/3 alone failed to activate Msx, BMP signalling itself or unidentified factors downstream of BMP signalling, together with Tbx2/3, might work to activate Msx (a dotted arrow).
vertebrate PNs, and the original lineage might have been lost in the vertebrates.

**Methods**

**In situ hybridization and immunostaining.** *C. intestinalis* adults were obtained from the National Bio-Resource Project for *Ciona*. Complementary DNA clones were obtained from our clone collection used for obtaining expressed sequence tags (ESTs)\(^1\). For whole-mount in situ hybridization, digoxigenin-RNA probes were synthesized by in vitro transcriptions with T7 RNA polymerase. Embryos were fixed in 4% paraformaldehyde in 0.1 M MOPS-NaOH (pH 7.5) and 0.5 M NaCl at 4°C overnight and then stored in 80% ethanol. After a wash with PBS containing 0.1% Tween 20 (PBST), embryos were treated with 2 μg/ml Proteinase K for 30 min at 37°C, washed again with PBST and fixed with 4% paraformaldehyde for 1 h at room temperature. Embryos were then incubated in 6 x sodium citrate buffer, 50% formamide, 5 x Denhardt’s solution, 100 μg/ml yeast tRNA and 0.1% Tween 20 for 1 h at 50°C. After this prehybridization step, specific RNA probes were added and incubated for 16 h at 50°C. Embryos were treated with RNase A, and incubated in 5 x sodium citrate buffer, 50% formamide and 0.1% tween 20 for 15 min at 50°C twice. Embryos were further incubated in 0.5% blocking reagent (Roche) in PBST for 30 min, and then in 1:2,000 alkaline-phosphatase-conjugated anti-digoxigenin antibody (Roche). After a PBST wash, embryos were further washed with 0.1 M NaCl, 50 mM MgCl\(_2\) and 0.1 M Tris-HCl (pH 9.5), and then p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) were used for detection.

To detect activation of the Brn signalling pathway, embryos were fixed with 3.7% formaldehyde in PBS, treated with 3% H\(_2\)O\(_2\) for 30 min and then incubated with a rabbit polyclonal antibody against a synthetic phosphopeptide derived from human Smad1 (Abcam, ab97689; 1:10,000; *Ciona Smad1/2/8* has the same amino acid sequence in its C-terminal end) in Can-Get-Signal Immunostain Solution B (Beyo). The signal was visualized with a TSA kit (Invitrogen) using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin-G and Alexa Fluor 488 tyramide. For RNA-seq experiments, embryos were treated with human BMP4 (100 ng/ml; HumanZyme) and dorsomorphin (100 ng/ml; Wako) continuously after fertilization, and collected at the late-gastrula stage. RNA-seq experiments were performed using the Illumina TruSeq RNA Sample Preparation kit and the Illumina HiSeq2500 sequencer. We did not take duplicates, mainly because we used this approach previously\(^{37}\). These DNA constructs were introduced by electroporation. For mRNA expression, digoxigenin-RNA probes were synthesized capped mRNA for *Tbx2/3* transcription with T7 RNA polymerase. Embryos were co-held in saline sodium citrate after injection.

**References**


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Author contributions
K.W., K.S.I. and Y.S. designed the experiments. K.W. and K.S.I. performed the experiments. Y.S. wrote the manuscript.

Additional information
Accession codes: RNA-seq data generated in this study have been deposited in the SRA database under the accession codes DRR030888–DRR030900.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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