The genetic program to specify ectodermal cells in ascidian embryos

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Abstract

The ascidian belongs to the sister group of vertebrates and shares many features with them. The gene regulatory network (GRN) controlling gene expression in ascidian embryonic development leading to the tadpole larva has revealed evolutionarily conserved gene circuits between ascidians and vertebrates. These conserved mechanisms are indeed useful to infer the original developmental programs of the ancestral chordates. Simultaneously, these studies have revealed which gene circuits are missing in the ascidian GRN; these gene circuits may have been acquired in the vertebrate lineage. In particular, the GRN responsible for gene expression in ectodermal cells of ascidian embryos has revealed the genetic programs that regulate the regionalization of the brain, formation of palps derived from placode-like cells, and differentiation of sensory neurons derived from neural crest-like cells. We here discuss how these studies have given insights into the evolution of these traits.

Keywords: Ascidians, midbrain-hindbrain boundary organizer, neural crest, placodes, telencephalon, gene regulatory network

Introduction

The ascidian is the closest relative of vertebrates, and they share the same basic body plan (Lemaire, 2011, Satoh, 2003). This shared body plan includes the dorsal nerve cord, which anteriorly ends with the brain. Neurogenic placodes and neural crest are often considered to be specific to vertebrate embryos (Gans & Northcutt, 1983). However, recent studies have revealed that the ascidian embryo has neurogenic placode-like cells and neural crest-like cells, although it has been suggested that they may be primitive or vestigial (Abitua *et al.*, 2015, Abitua *et al.*, 2012, Cao *et al.*, 2019, Horie *et al.*, 2018, Ikeda *et al.*, 2013, Liu & Satou, 2019, Manni *et al.*, 2005, Mazet *et al.*, 2005, Stolfi *et al.*, 2010, Wagner & Levine, 2012, Waki *et al.*, 2015). Evolutionary changes occur through alterations of gene regulatory networks (GRNs) for the specification of cell types and tissues over time. Therefore, the analysis of the structure and functions of the GRNs responsible for embryonic structures is essential for understanding how such embryonic structures evolved.

The ascidian genome is much simpler than those of vertebrates. The genome of the most widely used ascidian, *Ciona intestinalis* (type A or *C. robusta*), has been almost completely sequenced (Dehal *et al.*, 2002, Satou *et al.*, 2008a, Satou *et al.*, 2019). The genome size is approximately 125 Mb, containing about 16,000 genes. Among them, only about 600 genes encode transcription factors and signaling ligands, which represent the main GRN nodes. However, not all regulatory genes are expressed during embryonic development. Among maternally expressed regulatory genes, Gata.a, β-catenin, Tcf7, and Macho-1 (Zic-r.a) are particularly important, because these factors combinatorially set up the initial pattern of zygotic gene expression in the 16-cell embryo (Bertrand *et al.*, 2003, Hudson *et al.*, 2013, Imai *et al.*, 2000, Nishida & Sawada, 2001, Oda-Ishii *et al.*, 2016, Oda-Ishii & Satou, 2018, Rothbächer *et al.*, 2007, Satou *et al.*, 2002, Yagi *et al.*, 2004, Imai *et al.*, 2019). Only about 90 genes are zygotically expressed between the 16-cell stage

54 and the early gastrula stage (Imai *et al.*, 2004, Miwata *et al.*, 2006). At the early gastrula stage, the
55 developmental fate of individual cells is largely restricted to one tissue (Nishida, 1987), although
56 some tissues further differentiate into multiple cell types in later embryos (Tokuoka *et al.*, 2004,
57 Tokuoka *et al.*, 2005, Satou *et al.*, 2001, Cao *et al.*, 2019, Ryan *et al.*, 2016, Kusakabe *et al.*, 2002).
58 Thus, the GRN responsible for the cell fate specification in the ascidian embryo is constituted with
59 the limited number of regulatory genes. Therefore, because of the limited number of genes involved,
60 a comprehensive genome-wide analysis in this experimental system is particularly feasible and could
61 lead to a complete understanding of the structure and functions of the GRN involved in the cell fate
62 specification (Imai *et al.*, 2006, Satou & Imai, 2015).

63 Another advantage is that the number of cells that constitute an ascidian embryo is small.
64 Even a larva contains only 2,600 cells (Satoh *et al.*, 2003). Because of this embryonic simplicity, the
65 identity of cells expressing a specific gene of interest can be easily determined by *in situ*
66 hybridization and single-cell transcriptome analysis in normal and experimental embryos (Cao *et al.*,
67 2019, Horie *et al.*, 2018, Imai *et al.*, 2004, Imai *et al.*, 2006).

68

69 **Specification of ectodermal fate**

70 After fertilization, ascidian embryos divide twice along the animal–vegetal axis, while the
71 third division occurs latitudinally. Most ectodermal cells are derived from the animal hemisphere
72 cells, although a small number of nerve cord cells are derived from the vegetal hemisphere (Nishida,
73 1987). In the animal hemisphere, a maternal transcription factor, Gata.a, directly activates *Tfap2-r.b*
74 (encoding a TFAP2-like transcription factor), *Efna.d* (encoding type-A ephrin), and *Gdf1/3-r*
75 (encoding a TGFβ signaling molecule) between the 8-cell and 16-cell stages (Oda-Ishii *et al.*, 2016,

Imai *et al.*, 2004, Oda-Ishii *et al.*, 2018, Rothbächer *et al.*, 2007, Imai *et al.*, 2019) (Figure 1a, b). *Sox1/2/3* is also activated between the 8-cell and 16-cell stages (Miya & Nishida, 2003, Imai *et al.*, 2004), although it is still unclear how this gene is activated. However, *Sox1/2/3* expression begins immediately after the zygotic genome activation, and therefore it has been hypothesized that it is directly activated by maternal factors. *Sox1/2/3* activates *Dlx.b*, which specifies the epidermal fate in cooperation with *Tfap2-r.b* at a later stage (Imai *et al.*, 2017). Efna.d plays a key role in restricting the neural fate to two pairs of cells within the ectodermal territory by suppressing the MAPK pathway activity in epidermal cells, while Gdf1/3, as well as Admp, which is secreted from the vegetal hemisphere, also weakly suppress the expression of neural genes in the animal hemisphere (Ohta & Satou, 2013, Ohta *et al.*, 2015). In opposition to the secreted proteins mentioned before, Fgf9/16/20, which is secreted from the vegetal hemisphere, induces the neural fate through the activation of the MAPK pathway (the interaction of these signaling factors is further discussed in the next section). Interestingly, the presumptive epidermal cells do not express signaling molecules capable of inducing the epidermal fate in an instructive way. Thus, no instructive cell-cell interactions are required for specification of the epidermal fate, meaning that the 'default' fate, in the absence of instructive signals, is epidermal. This conclusion is consistent with the result of an embryological experiment, in which continuously dissociated cells (no cell-cell interactions occur) become epidermal cells (Nishida, 1992).

Neural induction

The ascidian embryo differentiates the neural plate in the ectoderm, as vertebrate embryos do. In vertebrate embryos, the anterior boundary of the neural plate produces neurogenic placodes, which are often considered to be a vertebrate-specific feature (Gans & Northcutt, 1983). It has been

hypothesized that the anterior neural plate boundary (ANB) of the ascidian embryo has the same evolutionary origin as the vertebrate cranial placodes, and accumulating evidence has indeed supported this hypothesis (Abitua *et al.*, 2015, Wagner & Levine, 2012, Liu & Satou, 2019, Manni *et al.*, 2005, Mazet *et al.*, 2005, Horie *et al.*, 2018). Similarly, it has been considered that cells in the lateral neural plate boundary (LNB) of ascidian embryos share the same evolutionary origin with the vertebrate neural crest (Abitua *et al.*, 2012, Waki *et al.*, 2015, Stolfi *et al.*, 2015). Details for the ANB and LNB cells are discussed in the subsequent sections. The ANB and LNB cells, together with the anterior and lateral parts of the neural plate, are derived from two pairs of cells in which *Otx* is expressed at the 32-cell stage (Figure 2a); cell-cell interactions are necessary for *Otx* expression, and these interactions are considered to represent neural induction in the ascidian embryo (Hudson & Lemaire, 2001).

The *Otx* expression is induced by a secreted signaling molecule, Fgf9/16/20, while three other signaling molecules, Ephrin (Efna.d), Gdf1/3-r, and Admp, are negative regulators of *Otx* expression (Figure 2b) (Bertrand *et al.*, 2003, Ohta & Satou, 2013, Ohta *et al.*, 2015). *Fgf9/16/20* is expressed in all vegetal hemisphere cells except the most posterior germline cells and potentially activates the downstream intracellular MAPK pathway in all cells in the animal hemisphere. However, in all the animal cells, except the neural cells, the activity of the MAPK pathway is suppressed by Efna.d, which is a signaling molecule anchored to the cell membrane and expressed in all animal cells. The MAPK pathway activity that is not suppressed by the Efna.d activity in the presumptive neural cells differentiates the neural fate from the naive ectodermal fate. In the 32-cell embryo, the presumptive epidermal cells are expected to receive more Efna.d molecules than the presumptive neural cells, because the epidermal cells have a larger surface contact areas with *Efna.d*-expressing cells than the neural cells (Tassy *et al.*, 2006, Ohta & Satou, 2013, Ohta *et al.*, 2015). This antagonism between Fgf9/16/20 and Efna.d is repeatedly used during the ascidian development

123 (Haupaix *et al.*, 2014, Haupaix *et al.*, 2013, Picco *et al.*, 2007, Shi & Levine, 2008). The MAPK
124 pathway, which is activated specifically in the neural lineage, directly regulates the activity of the
125 Ets and Gata.a transcription factors, which in turn regulate *Otx* (Bertrand *et al.*, 2003).

126 Admp is a signaling molecule closely related to the BMP protein family, and Gdf1/3-r is a
127 TGFβ signaling factor related to the mammalian Gdf1 and Gdf3 proteins and the *Xenopus* Vg1
128 factor. These two signaling molecules weakly repress *Otx* expression. In the absence of these factors,
129 *Otx* is ectopically expressed in all animal cells, because the activity of the MAPK pathway is not
130 completely suppressed by Efna.d (Ohta & Satou, 2013). The *Otx* expression in the anterior and
131 posterior neural cells contributes to the formation of the ANB and LNB cells. In addition, in the
132 posterior neural cells, *Nodal* is expressed and plays an important role (see below). Because *Nodal* is
133 repressed in the anterior cells by Foxa.a, *Nodal* is expressed specifically in the posterior lineage
134 (Ohta & Satou, 2013). In this way, the neural induction occurs through the multiple signaling
135 pathways, and this mechanism may represent a conserved mechanism for the neural induction
136 among tunicates and vertebrates, because the FGF signaling pathway and BMP signaling pathway
137 regulate positively and negatively neural fate similarly in tunicate and vertebrate embryos (Delaune
138 *et al.*, 2005, Marchal *et al.*, 2009, Munoz-Sanjuan & Brivanlou, 2002, Streit *et al.*, 2000, Wilson *et*
139 *al.*, 2000).

140 Meanwhile, in amphioxus, the BMP signaling pathway negatively regulates neural fate,
141 while the FGF signaling pathway is not essential for neural induction (Yu *et al.*, 2007, Bertrand *et*
142 *al.*, 2011). It is not clear whether these two signaling pathways were used in common ancestral
143 chordates of vertebrates, tunicates, and amphioxus. A recent study also suggested that Nodal
144 signaling is important for neural induction in amphioxus and *Xenopus* (Le Petillon *et al.*, 2017).
145 However, in ascidian embryos, Nodal is not expressed before *Otx* expression, and Gdf1/3-r, which

146 probably shares the intracellular signal transduction pathway with Nodal, regulates neural fate
147 negatively. It remains to be determined whether ancestral chordates used Nodal signaling for the
148 neural induction.

149 The necessity of inhibiting the BMP activity for the neural induction in the ascidian
150 embryo had been debated (Darras & Nishida, 2001, Passamaneck & Di Gregorio, 2005, Kourakis &
151 Smith, 2005). However, recent studies have revealed that BMP signaling negatively regulates the
152 expression of *Otx* and *Nodal* (Ohta & Satou, 2013, Ohta *et al.*, 2015). In their upstream regulatory
153 regions, cis-regulatory elements responsive to Fgf signaling may play a more dominant role than
154 those responsive to BMP signaling, and therefore strong inhibition of BMP activity may not be
155 necessary. Consistent with this view, no BMP antagonists have not been found to be expressed at the
156 32-cell stage even in a comprehensive expression assay (Imai *et al.*, 2004)

157

158 **Anterior neurogenic placodes and the anterior part of the neural plate**

159 The anterior part of the brain is derived from the anterior neural cell pair with *Otx*
160 expression in the 32-cell embryo (cyan cells in Figure 2a), and the brain fate specification requires
161 *Zic-r.b* (Imai *et al.*, 2002b). Although the expression of *Zic-r.b* is regulated positively by Fgf9/16/20
162 (Wagner & Levine, 2012), the expression of *Zic-r.b* is repressed by Hes.a and Prdm1-r
163 transcriptional factors at the 32-cell stage (Ikeda *et al.*, 2013, Ikeda & Satou, 2017). Between the 32-
164 and 64-cell stages, the neural cells divide in the mediolateral direction (Figure 3a). Although all of
165 the daughter cells are in extensive contact with vegetal cells expressing *Fgf9/16/20*, the expression
166 of *Zic-r.b* is still repressed by Prdm1-r at the 64-cell stage. Because Prdm1-r also represses its own
167 gene expression, this repression is relieved after the next division. As this cell division occurs in the

168 animal–vegetal direction, the daughter cells closer to the vegetal pole, but not the remaining
169 daughter cells, extensively contact vegetal cells expressing *Fgf9/16/20* at the 112-cell stage (the right
170 panel in Figure 3a). Therefore, Fgf9/16/20 only acts on the daughters closer to the vegetal pole to
171 induce the expression of *Zic-r.b*, while the remaining daughters express *Foxc* instead, which is
172 negatively regulated by Fgf9/16/20 in the cells with *Zic-r.b* expression (Wagner & Levine, 2012).
173 The cells expressing *Foxc* give rise to the ANB cells. Thus, in the ascidian embryos, the ANB cells
174 and the anterior neural plate cells are derived from a pair of common precursor cells.

175 In vertebrates, it is controversial whether placodes are derived from cells specified first as
176 neural plate border cells that have potentials to become neural crest and placode cells or whether
177 placodes are induced from the non-neural ectoderm (Meulemans & Bronner-Fraser, 2004, Pieper *et*
178 *al.*, 2012, Schlosser *et al.*, 2014). On the other hand, in the ascidian embryo, the ANB cells are
179 derived from the anterior neural cells with *Otx* expression but not from the non-neural ectoderm,
180 while the ANB and LNB are derived from distinct lineages. Hes.a and Prdm1-r repressors play an
181 important role in specifying the ANB region of the ascidian embryo by delaying *Zic-r.b* expression
182 until the cells divide along the anterior-posterior axis; if *Hes.a* and *Prdm1-r* are knocked down, all
183 cells in these lineages are specified to the brain fate (Ikeda *et al.*, 2013). Therefore, this delay
184 mechanism enables the specification of the ANB within the neural lineage. It is possible that the
185 addition of this delay mechanism might represent the initial event in the evolutionary acquisition of
186 the ANB cells; it is likely that emergence of the neural plate predates emergence of placodes,
187 because amphioxus embryos have the neural plate but not a clear counterpart for the pre-placodal
188 region or placodes (Schlosser *et al.*, 2014). More specifically, the appearance of binding sites for
189 Hes.a and Prdm1-r in the regulatory region of *Zic-r.b* might have been an evolutionary important
190 event.

The ANB cells divide twice along the anterior-posterior axis to make four cell rows between the 112-cell stage and the neurula stage (Figure 3b). The central two rows express *Emx*, while the anterior and posterior rows express *Foxg* (*Foxg1)* (Horie *et al.*, 2018, Imai *et al.*, 2004, Liu & Satou, 2019, Wagner *et al.*, 2014). The *Foxg* expression is induced by the MAPK signaling pathway, which is active in the anterior and posterior rows (Liu & Satou, 2019). Because *Foxg* represses *Emx*, *Emx* expression is restricted to the central two rows. The anterior row expressing *Foxg* contributes to the protrusive structures with sensory neurons within the palps, while the second and third rows expressing *Emx* contribute to the basal structures supporting the palp protrusions. The posterior row expressing *Foxg* gives rise to the oral siphon primordium, which contains neurons with chemosensory and GnRH-releasing functions (Abitua *et al.*, 2015). Thus, this primitive placode-like structure produces multiple tissues. This is reminiscent of the anterior pre-placodal ectoderm of the vertebrate embryo, which produces multiple placodes including the adenohypophyseal and olfactory placodes. GRN structures responsible for specification of cell types in the ascidian ANB and the vertebrate pre-placodal ectoderm should be revealed more precisely and thoroughly to determine whether these cell type specification mechanisms share the evolutionary origin.

Brain regionalization

The ascidian larval central nervous system (CNS) contains several morphologically distinct structures (Figure 4a). The most anterior structure is the sensory vesicle, which is also called the brain. The next distinct structure is called the visceral ganglion, which contains motor neurons, followed by the nerve cord. There is a narrow region between the brain and the visceral ganglion, called the neck region. It has been long debated whether these morphologically distinct regions correspond to the vertebrate brain parts such as the forebrain, midbrain, and hindbrain (Wada *et al.*,

214 1998, Takahashi & Holland, 2004, Dufour *et al.*, 2006, Meinertzhagen *et al.*, 2004).

215 The midbrain-hindbrain boundary (MHB) organizer plays a key role in regionalizing the
216 vertebrate brain. In this region, *Otx* and *Gbx* are expressed anteriorly and posteriorly, respectively,
217 and Fgf8 plays a key role in the boundary formation (Liu & Joyner, 2001). Although the ascidian
218 genome lacks *Gbx* (Wada *et al.*, 2003, Satou *et al.*, 2008b), *Otx* is expressed only in the sensory
219 vesicles but not in the neck or visceral ganglion (Figure 4a). The neck region expresses *Hox1,*
220 *Pax2/5/8*, and *Phox2*, while the visceral ganglion expresses *Hox1*, *En*, and *Dmbx* (Wada *et al.*, 1998,
221 Takahashi & Holland, 2004, Dufour *et al.*, 2006, Ikuta & Saiga, 2007, Imai *et al.*, 2009). Although
222 these genes are expressed in specific domains in vertebrate brains, there is no perfect correspondence
223 between the ascidian and vertebrate gene expression patterns in the brain. Nevertheless, these studies
224 have established that the ascidian CNS consists of several distinct domains with different gene
225 expression profiles (Figure 4a) (Wada *et al.*, 1998, Takahashi & Holland, 2004, Dufour *et al.*, 2006,
226 Meinertzhagen *et al.*, 2004, Ikuta & Saiga, 2007).

227 The ascidian ortholog for vertebrate *Fgf8*, *Fgf8/17/18*, is repeatedly expressed in different
228 CNS cells at different stages (Imai *et al.*, 2002a, Imai *et al.*, 2009). The GRN analysis revealed that
229 *Fgf8/17/18* expression in the gastrula embryo is responsible for the establishment of different gene
230 expression patterns between the sensory vesicle and the neck. In particular, in embryos in which
231 *Fgf8/17/18* is knocked down, *Pax2/5/8* expression is lost and the expression of *Otx* and *En* is
232 observed in the neck region (see Figure 4a). This phenotype is reminiscent of that seen in the
233 zebrafish *ace* (*Fgf8a*) mutants, in which *Otx2* expression (midbrain) is expanded and *Pax8*
234 expression (hindbrain) is lost (Jaszai *et al.*, 2003). A similar phenotype is also found in *Fgf8* mutant
235 mice (Chi *et al.*, 2003). In ascidian embryos, *Fgf8/17/18* plays a role in patterning the brain at the
236 gastrula stage, which is a much earlier stage than the one observed in vertebrate embryos. This

unexpected difference in the induction timing emphasizes the importance of analyzing the whole development process.

The origin of the mutually exclusive expression of *Otx* and *Gbx*, which is seen in the MHB of vertebrate embryos, may be traced back to the appearance of bilaterians as it is observed in various bilaterian animals, including *Drosophila* (Urbach, 2007, Hirth et al., 2003). However, whether Fgf is used either for formation or maintenance of the *Otx*/*Gbx* expression pattern in ancestral animals of all bilaterians is not clear, although it is likely that Fgf was involved in the *Otx*/*Gbx* system in the ancestral deuterostome animal (Pani *et al.*, 2012). It is also not clear when this mechanism began to be used for regionalizing the CNS. Furthermore, it is still debated when the centralized nervous system emerged, and whether the nervous systems seen in the bilaterian animals share a common evolutionary origin (Martin-Duran *et al.*, 2018, Holland *et al.*, 2013, Arendt *et al.*, 2016). Nevertheless, the GRN in the ascidian embryo indicated that the last common ancestor of vertebrates and ascidians had an Fgf8/17/18-dependent system to regionalize the CNS.

In vertebrate embryos, the forebrain is further regionalized into the telencephalon and diencephalon. For this regionalization, a structure called the anterior neural ridge (ANR) is required (Shimamura & Rubenstein, 1997, Houart *et al.*, 1998). The ANR is formed in the anterior boundary of the neural plate of vertebrate embryos, and contribute to the anterior neurogenic placodes. The ANR and the anterior part of the forebrain express *Fgf8*, which plays a key role in the development of the telencephalon within the forebrain (Shimamura & Rubenstein, 1997). Here, *Fgf8* constitutes a positive feedback loop with *Foxg* (Figure 4b) (Tao & Lai, 1992, Martynoga *et al.*, 2005, Kawauchi *et al.*, 2009). As already mentioned before, *Foxg* is expressed in the anterior and posterior rows of the four rows of the ascidian placode-like ANB cells, although the ascidian larva does not have any morphologically similar structure to the vertebrate telencephalon. In the ascidian embryos, *Foxg* is

activated under the control of the MAPK pathway, while *Fgf8/17/18* is not expressed in the ANB or nearby cells, including cells in the brain (Liu & Satou, 2019). Thus, in the ascidian, while *Foxg* is a potential target of Fgf8/17/18, which has the ability to activate the MAPK pathway, *Fgf8/17/18* is not a target of Foxg. Currently, the factor that activates the MAPK pathway in the ascidian ANB has not been identified.

If the last common ancestor of ascidians and vertebrates had the telencephalon, the *Fgf8* ortholog would have been regulated directly or indirectly by Foxg, in order to constitute the regulatory *Foxg-Fgf8* loop. Then this loop would have been lost in the ascidian lineage. Alternatively, if the last common ancestor did not have the telencephalon, *Foxg* would have been expressed in the ANB cells without regulating *Fgf* genes. In this latter case, after the split of vertebrates and ascidians, *Fgf8* (or *Fgf8/17/18)* would have acquired a cis-regulatory region regulated directly or indirectly by Foxg. In either case, a simple loss or acquisition of a cis-regulatory element could explain the difference in *Foxg* expression between current vertebrates and ascidians. Because amphioxus does not have a distinct telencephalon-like structure or distinct brain cells with *Foxg1* expression during embryonic stages (Toresson *et al.*, 1998), the latter hypothesis is more likely. On the other hand, the adult amphioxus brain expresses *Foxg1* (Benito-Gutiérrez *et al.*, 2018) and therefore it is possible that this gene plays a similar role in the amphioxus adult brain but not in the embryonic brain (Briscoe & Ragsdale, 2019). With this reason, the former hypothesis cannot be ruled out. Nevertheless, the ascidian GRN structure suggests that the last common ancestor of vertebrates and ascidians had the gene circuit, at least partially.

Another study has proposed a similar model in which the incorporation of gene regulatory circuits in the ANB into forebrain regions might be critical for the evolution of the telencephalon, on the basis of the observation that the ascidian ANB expresses a gene set that has been implicated in

specification of the vertebrate telencephalon (Cao *et al.*, 2019).

Sensory neurons in the tail

The ascidian larvae have sensory neurons (SNs) along the dorsal and ventral midlines of the tail (Figure 5a) (Takamura, 1998). These neurons are likely to be mechanosensors and control swimming. The dorsal SNs are derived from the LNB cells, while the ventral SNs are differentiated from non-neural ectodermal cells (Pasini *et al.*, 2006). From this point of view, the dorsal SNs are similar to the vertebrate peripheral neurons, which are derived from the neural crest formed at the lateral border regions of the neural plate. On the other hand, the ventral SNs are similar to the peripheral neurons of other invertebrate species including amphioxus, annelids, and insects, in which peripheral neurons are formed within the epidermal region (Lu *et al.*, 2012, Rusten *et al.*, 2002, Denes *et al.*, 2007, Zhao *et al.*, 2019).

The GRN analysis for the ventral SNs in ascidian embryos has shown that BMP signaling induces *Tbx2/3* and subsequently *Msx*, which then activates a gene circuit promoting the SNs differentiation (Figure 5b) (Waki *et al.*, 2015). Neurons in the peripheral nervous system (PNS) of amphioxus and protostomes are also induced by BMP signaling, which is another similarity between the ventral epidermal SNs of the ascidian embryo and the PNS neurons of other invertebrates (Lu *et al.*, 2012, Rusten *et al.*, 2002, Denes *et al.*, 2007, Zhao *et al.*, 2019, Holland, 2009). Interestingly, the ascidian dorsal SNs are differentiated using the same gene circuit beginning with *Msx* (Figure 5b) (Waki *et al.*, 2015, Roure & Darras, 2016, Roure *et al.*, 2014). As already mentioned, these neurons are derived from the LNB cells, and these LNB cells are derived from cells expressing *Nodal* and *Otx* at the 32-cell stage. *Msx* is indeed regulated under the control of *Nodal* and *Otx*, which are activated by Fgf signaling and repressed by BMP signaling, as discussed above. Thus, from this viewpoint, the dorsal SNs are similar to vertebrate peripheral neurons. These observations indicate

306 that the ventral SNs of ascidian larvae share the evolutionary origin with those of invertebrates, and
307 that the dorsal SNs share the evolutionary origin with the vertebrates.

308 The most likely scenario of the evolution of the dorsal SNs is that the dorsal SNs were
309 born by co-option of the gene circuit activated by *Msx* expression, because this circuit is shared
310 between the dorsal and ventral lineages and the ventral one is likely to be ancient. A change in an
311 upstream regulatory element of *Msx* may have been enough to activate the gene circuit in the LNB.

312

313 **Conclusions**

314 GRNs control gene expression temporally and spatially during animal development.
315 Changes in the gene expression patterns are caused by changes in GRNs. Therefore, GRN-based
316 comparisons have given more direct and precise insights into how various traits have been acquired
317 during evolution than gene expression pattern-based comparisons. For example, in the epidermal
318 SNs of the ascidian larval tail, the precise point of the co-option of the gene circuit for SN
319 differentiation between the two distinct PNS lineages has been identified. It is highly likely that this
320 co-option is related to the evolutionary emergence of the neural crest cells. The analyses of the
321 regulatory mechanisms of *Foxc* and *Foxg* expression indicated the possible evolutionary changes
322 that might have led to the acquisition of the placodes and telencephalon. Many changes other than
323 such possible critical changes are expected to have occurred during evolution, and therefore it may
324 be difficult to directly test such possibilities using extant animals in most cases. Nevertheless,
325 several pioneering studies have succeeded in testing such possibilities (Abitua *et al.*, 2012, Horie *et*
326 *al.*, 2018, Lu *et al.*, 2012). For example, misexpression of *Twist-r* converts non-migratory neural-
327 crest like cells to migratory cells (Abitua *et al.*, 2012). Thus, GRN studies have revealed which type

328 of changes have the potential to introduce new features during evolution, and that the ascidian
329 embryos and larvae share more features with vertebrates than previously thought.
330

References

Abitua, P. B., Gainous, T. B., Kaczmarczyk, A. N. et al. 2015. The pre-vertebrate origins of neurogenic placodes. *Nature,* **524,** 462-465.

Abitua, P. B., Wagner, E., Navarrete, I. A. & Levine, M. 2012. Identification of a rudimentary neural crest in a non-vertebrate chordate. *Nature,* **492,** 104-107.

Arendt, D., Tosches, M. A. & Marlow, H. 2016. From nerve net to nerve ring, nerve cord and brain - evolution of the nervous system. *Nature Reviews Neuroscience,* **17,** 61-72.

Benito-Gutiérrez, È., Stemmer, M., Rohr, S. D. et al. 2018. Patterning of a telencephalon-like region in the adult brain of amphioxus. *Preprint at https://www.biorxiv.org/content/10.1101/307629v1.full*.

Bertrand, S., Camasses, A., Somorjai, I. et al. 2011. Amphioxus FGF signaling predicts the acquisition of vertebrate morphological traits. *Proc Natl Acad Sci U S A,* **108,** 9160-9165.

Bertrand, V., Hudson, C., Caillol, D., Popovici, C. & Lemaire, P. 2003. Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. *Cell,* **115,** 615-627.

Briscoe, S. D. & Ragsdale, C. W. 2019. Evolution of the Chordate Telencephalon. *Current Biology,* **29,** R647-R662.

Cao, C., Lemaire, L. A., Wang, W. et al. 2019. Comprehensive single-cell transcriptome lineages of a proto-vertebrate. *Nature,* **571,** 349-354.

Chi, C. L., Martinez, S., Wurst, W. & Martin, G. R. 2003. The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. *Development,* **130,** 2633-2644.

Darras, S. & Nishida, H. 2001. The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. *Dev Biol,* **236,** 271-288.

Dehal, P., Satou, Y., Campbell, R. K. et al. 2002. The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. *Science,* **298,** 2157-2167.

Delaune, E., Lemaire, P. & Kodjabachian, L. 2005. Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. *Development,* **132,** 299-310.

Denes, A. S., Jekely, G., Steinmetz, P. R. et al. 2007. Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. *Cell,* **129,** 277-288.

Dufour, H. D., Chettouh, Z., Deyts, C. et al. 2006. Precraniate origin of cranial motoneurons. *Proc Natl Acad Sci U S A,* **103,** 8727-8732.

Gans, C. & Northcutt, R. G. 1983. Neural Crest and the Origin of Vertebrates - a New Head. *Science,* **220,** 268-273.

Haupaix, N., Abitua, P. B., Sirour, C., Yasuo, H., Levine, M. & Hudson, C. 2014. Ephrin-mediated restriction of ERK1/2 activity delimits the number of pigment cells in the Ciona CNS.

366 *Developmental Biology,* **394,** 170-180.
367 Haupaix, N., Stolfi, A., Sirour, C. et al. 2013. p120RasGAP mediates ephrin/Eph-dependent attenuation of
368 FGF/ERK signals during cell fate specification in ascidian embryos. *Development,* **140,** 4347-
369 4352.
370 Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M. & Reichert, H. 2003. An urbilaterian origin of
371 the tripartite brain: developmental genetic insights from Drosophila. *Development,* **130,** 2365-
372 2373.
373 Holland, L. Z. 2009. Chordate roots of the vertebrate nervous system: expanding the molecular toolkit.
374 *Nature Reviews Neuroscience,* **10,** 736-746.
375 Holland, L. Z., Carvalho, J. E., Escriva, H. et al. 2013. Evolution of bilaterian central nervous systems: a
376 single origin? *Evodevo,* **4**.
377 Horie, R., Hazbun, A., Chen, K., Cao, C., Levine, M. & Horie, T. 2018. Shared evolutionary origin of
378 vertebrate neural crest and cranial placodes. *Nature,* **560,** 228-232.
379 Houart, C., Westerfield, M. & Wilson, S. W. 1998. A small population of anterior cells patterns for
380 forebrain during zebrafish gastrulation. *Nature,* **391,** 788-792.
381 Hudson, C., Kawai, N., Negishi, T. & Yasuo, H. 2013. β-catenin-driven binary fate specification
382 segregates germ layers in ascidian embryos. *Curr Biol,* **23,** 491-495.
383 Hudson, C. & Lemaire, P. 2001. Induction of anterior neural fates in the ascidian Ciona intestinalis. *Mech*
384 *Dev,* **100,** 189-203.
385 Ikeda, T., Matsuoka, T. & Satou, Y. 2013. A time delay gene circuit is required for palp formation in the
386 ascidian embryo. *Development,* **140,** 4703-4708.
387 Ikeda, T. & Satou, Y. 2017. Differential temporal control of Foxa.a and Zic-r.b specifies brain versus
388 notochord fate in the ascidian embryo. *Development,* **144,** 38-43.
389 Ikuta, T. & Saiga, H. 2007. Dynamic change in the expression of developmental genes in the ascidian
390 central nervous system: revisit to the tripartite model and the origin of the midbrain-hindbrain
391 boundary region. *Dev Biol,* **312,** 631-643.
392 Imai, K., Takada, N., Satoh, N. & Satou, Y. 2000. β-catenin mediates the specification of endoderm cells
393 in ascidian embryos. *Development,* **127,** 3009-3020.
394 Imai, K. S., Hikawa, H., Kobayashi, K. & Satou, Y. 2017. Tfap2 and Sox1/2/3 cooperatively specify
395 ectodermal fates in ascidian embryos. *Development,* **144,** 33-37.
396 Imai, K. S., Hino, K., Yagi, K., Satoh, N. & Satou, Y. 2004. Gene expression profiles of transcription
397 factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding
398 of gene networks. *Development,* **131,** 4047-4058.
399 Imai, K. S., Kobayashi, K., Kari, W. et al. 2019. Gata is ubiquitously required for the earliest zygotic gene
400 transcription in the ascidian embryo. *Dev Biol*.
401 Imai, K. S., Levine, M., Satoh, N. & Satou, Y. 2006. Regulatory blueprint for a chordate embryo. *Science,*

312, 1183-1187.

Imai, K. S., Satoh, N. & Satou, Y. 2002a. Region specific gene expressions in the central nervous system of the ascidian embryo. *Mech Dev,* **119,** S275-S277.

Imai, K. S., Satou, Y. & Satoh, N. 2002b. Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in Ciona savignyi embryos. *Development,* **129,** 2723-2732.

Imai, K. S., Stolfi, A., Levine, M. & Satou, Y. 2009. Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system. *Development,* **136,** 285-293.

Jaszai, J., Reifers, F., Picker, A., Langenberg, T. & Brand, M. 2003. Isthmus-to-midbrain transformation in the absence of midbrain-hindbrain organizer activity. *Development,* **130,** 6611-6623.

Kawauchi, S., Kim, J., Santos, R., Wu, H. H., Lander, A. D. & Calof, A. L. 2009. Foxg1 promotes olfactory neurogenesis by antagonizing Gdf11. *Development,* **136,** 1453-1464.

Kourakis, M. J. & Smith, W. C. 2005. Did the first chordates organize without the organizer? *Trends Genet,* **21,** 506-510.

Kusakabe, T., Yoshida, R., Kawakami, I. et al. 2002. Gene expression profiles in tadpole larvae of Ciona intestinalis. *Dev Biol,* **242,** 188-203.

Le Petillon, Y., Luxardi, G., Scerbo, P. et al. 2017. Nodal-Activin pathway is a conserved neural induction signal in chordates. *Nat Ecol Evol,* **1,** 1192-1200.

Lemaire, P. 2011. Evolutionary crossroads in developmental biology: the tunicates. *Development,* **138,** 2143-2152.

Liu, A. & Joyner, A. L. 2001. Early anterior/posterior patterning of the midbrain and cerebellum. *Annu Rev Neurosci,* **24,** 869-896.

Liu, B. & Satou, Y. 2019. Foxg specifies sensory neurons in the anterior neural plate border of the ascidian embryo. *Nat Commun,* **10,** 4911.

Lu, T. M., Luo, Y. J. & Yu, J. K. 2012. BMP and Delta/Notch signaling control the development of amphioxus epidermal sensory neurons: insights into the evolution of the peripheral sensory system. *Development,* **139,** 2020-2030.

Manni, L., Agnoletto, A., Zaniolo, G. & Burighel, P. 2005. Stomodeal and neurohypophysial placodes in Ciona intestinalis: insights into the origin of the pituitary gland. *Journal of experimental zoology. Part B, Molecular and developmental evolution,* **304,** 324-339.

Marchal, L., Luxardi, G., Thome, V. & Kodjabachian, L. 2009. BMP inhibition initiates neural induction via FGF signaling and Zic genes. *Proc Natl Acad Sci U S A,* **106,** 17437-17442.

Martin-Duran, J. M., Pang, K., Borve, A. et al. 2018. Convergent evolution of bilaterian nerve cords. *Nature,* **553,** 45-+.

Martynoga, B., Morrison, H., Price, D. J. & Mason, J. O. 2005. Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor

438 proliferation and apoptosis. *Developmental Biology,* **283,** 113-127.
439 Mazet, F., Hutt, J. A., Milloz, J., Millard, J., Graham, A. & Shimeld, S. M. 2005. Molecular evidence
440 from Ciona intestinalis for the evolutionary origin of vertebrate sensory placodes. *Dev Biol,* **282,**
441 494-508.
442 Meinertzhagen, I. A., Lemaire, P. & Okamura, Y. 2004. The neurobiology of the ascidian tadpole larva:
443 recent developments in an ancient chordate. *Annu Rev Neurosci,* **27,** 453-485.
444 Meulemans, D. & Bronner-Fraser, M. 2004. Gene-regulatory interactions in neural crest evolution and
445 development. *Dev Cell,* **7,** 291-299.
446 Miwata, K., Chiba, T., Horii, R. et al. 2006. Systematic analysis of embryonic expression profiles of zinc
447 finger genes in Ciona intestinalis. *Dev Biol,* **292,** 546-554.
448 Miya, T. & Nishida, H. 2003. Expression pattern and transcriptional control of SoxB1 in embryos of the
449 ascidian Halocynthia roretzi. *Zool Sci,* **20,** 59-67.
450 Munoz-Sanjuan, I. & Brivanlou, A. H. 2002. Neural induction, the default model and embryonic stem
451 cells. *Nat Rev Neurosci,* **3,** 271-280.
452 Nishida, H. 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme.
453 III. Up to the tissue restricted stage. *Dev Biol,* **121,** 526-541.
454 Nishida, H. 1992. Developmental Potential for Tissue Differentiation of Fully Dissociated Cells of the
455 Ascidian Embryo. *Roux Arch Dev Biol,* **201,** 81-87.
456 Nishida, H. & Sawada, K. 2001. macho-1 encodes a localized mRNA in ascidian eggs that specifies
457 muscle fate during embryogenesis. *Nature,* **409,** 724-729.
458 Oda-Ishii, I., Abe, T. & Satou, Y. 2018. Dynamics of two key maternal factors that initiate zygotic
459 regulatory programs in ascidian embryos. *Dev Biol,* **437,** 50-59.
460 Oda-Ishii, I., Kubo, A., Kari, W., Suzuki, N., Rothbacher, U. & Satou, Y. 2016. A maternal system
461 initiating the zygotic developmental program through combinatorial repression in the ascidian
462 embryo. *PLoS genetics,* **12,** e1006045.
463 Oda-Ishii, I. & Satou, Y. 2018. Initiation of the zygotic genetic program in the ascidian embryo. *Semin*
464 *Cell Dev Biol*.
465 Ohta, N. & Satou, Y. 2013. Multiple signaling pathways coordinate to induce a threshold response in a
466 chordate embryo. *PLoS genetics,* **9,** e1003818.
467 Ohta, N., Waki, K., Mochizuki, A. & Satou, Y. 2015. A Boolean function for neural induction reveals a
468 critical role of direct intercellular interactions in patterning the ectoderm of the ascidian embryo.
469 *PLoS Comput Biol,* **11,** e1004687.
470 Pani, A. M., Mullarkey, E. E., Aronowicz, J., Assimacopoulos, S., Grove, E. A. & Lowe, C. J. 2012.
471 Ancient deuterostome origins of vertebrate brain signalling centres. *Nature,* **483,** 289-294.
472 Pasini, A., Amiel, A., Rothbacher, U., Roure, A., Lemaire, P. & Darras, S. 2006. Formation of the ascidian
473 epidermal sensory neurons: insights into the origin of the chordate peripheral nervous system.

PLoS Biol, **4,** e225.

Passamaneck, Y. J. & Di Gregorio, A. 2005. Ciona intestinalis: Chordate development made simple. *Developmental Dynamics,* **233,** 1-19.

Picco, V., Hudson, C. & Yasuo, H. 2007. Ephrin-Eph signalling drives the asymmetric division of notochord/neural precursors in Ciona embryos. *Development,* **134,** 1491-1497.

Pieper, M., Ahrens, K., Rink, E., Peter, A. & Schlosser, G. 2012. Differential distribution of competence for panplacodal and neural crest induction to non-neural and neural ectoderm. *Development,* **139,** 1175-1187.

Rothbächer, U., Bertrand, V., Lamy, C. & Lemaire, P. 2007. A combinatorial code of maternal GATA, Ets and β-catenin-TCF transcription factors specifies and patterns the early ascidian ectoderm. *Development,* **134,** 4023-4032.

Roure, A. & Darras, S. 2016. Msxb is a core component of the genetic circuitry specifying the dorsal and ventral neurogenic midlines in the ascidian embryo. *Developmental Biology,* **409,** 277-287.

Roure, A., Lemaire, P. & Darras, S. 2014. An otx/nodal regulatory signature for posterior neural development in ascidians. *PLoS genetics,* **10,** e1004548.

Rusten, T. E., Cantera, R., Kafatos, F. C. & Barrio, R. 2002. The role of TGF beta signaling in the formation of the dorsal nervous system is conserved between Drosophila and chordates. *Development,* **129,** 3575-3584.

Ryan, K., Lu, Z. Y. & Meinertzhagen, I. A. 2016. The CNS connectome of a tadpole larva of Ciona intestinalis (L.) highlights sidedness in the brain of a chordate sibling. *Elife,* **5**.

Satoh, N. 2003. The ascidian tadpole larva: comparative molecular development and genomics. *Nat Rev Genet,* **4,** 285-295.

Satoh, N., Satou, Y., Davidson, B. & Levine, M. 2003. Ciona intestinalis: an emerging model for whole-genome analyses. *Trends Genet,* **19,** 376-381.

Satou, Y. & Imai, K. S. 2015. Gene regulatory systems that control gene expression in the Ciona embryo. *Proc Jpn Acad Ser B Phys Biol Sci,* **91,** 33-51.

Satou, Y., Mineta, K., Ogasawara, M. et al. 2008a. Improved genome assembly and evidence-based global gene model set for the chordate Ciona intestinalis: new insight into intron and operon populations. *Genome Biol,* **9,** R152.

Satou, Y., Nakamura, R., Yu, D. et al. 2019. A Nearly Complete Genome of Ciona intestinalis Type A (C. robusta) Reveals the Contribution of Inversion to Chromosomal Evolution in the Genus Ciona. *Genome biology and evolution,* **11,** 3144-3157.

Satou, Y., Takatori, N., Yamada, L. et al. 2001. Gene expression profiles in Ciona intestinalis tailbud embryos. *Development,* **128,** 2893-2904.

Satou, Y., Wada, S., Sasakura, Y. & Satoh, N. 2008b. Regulatory genes in the ancestral chordate genomes. *Dev Genes Evol,* **218,** 715-721.

510 Satou, Y., Yagi, K., Imai, K. S., Yamada, L., Nishida, H. & Satoh, N. 2002. macho-1-Related genes in
511 Ciona embryos. *Dev Genes Evol,* **212,** 87-92.
512 Schlosser, G., Patthey, C. & Shimeld, S. M. 2014. The evolutionary history of vertebrate cranial placodes
513 II. Evolution of ectodermal patterning. *Developmental Biology,* **389,** 98-119.
514 Shi, W. & Levine, M. 2008. Ephrin signaling establishes asymmetric cell fates in an endomesoderm
515 lineage of the Ciona embryo. *Development,* **135,** 931-940.
516 Shimamura, K. & Rubenstein, J. L. R. 1997. Inductive interactions direct early regionalization of the
517 mouse forebrain. *Development,* **124,** 2709-2718.
518 Stolfi, A., Gainous, T. B., Young, J. J., Mori, A., Levine, M. & Christiaen, L. 2010. Early chordate origins
519 of the vertebrate second heart field. *Science,* **329,** 565-568.
520 Stolfi, A., Ryan, K., Meinertzhagen, I. A. & Christiaen, L. 2015. Migratory neuronal progenitors arise
521 from the neural plate borders in tunicates. *Nature,* **527,** 371-374.
522 Streit, A., Berliner, A. J., Papanayotou, C., Sirulnik, A. & Stern, C. D. 2000. Initiation of neural induction
523 by FGF signalling before gastrulation. *Nature,* **406,** 74-78.
524 Takahashi, T. & Holland, P. W. 2004. Amphioxus and ascidian Dmbx homeobox genes give clues to the
525 vertebrate origins of midbrain development. *Development,* **131,** 3285-3294.
526 Takamura, K. 1998. Nervous network in larvae of the ascidian Ciona intestinalis. *Dev Genes Evol,* **208,** 1-
527 8.
528 Tao, W. & Lai, E. 1992. Telencephalon-Restricted Expression of Bf-1, a New Member of the Hnf-3 Fork
529 Head Gene Family, in the Developing Rat-Brain. *Neuron,* **8,** 957-966.
530 Tassy, O., Daian, F., Hudson, C., Bertrand, V. & Lemaire, P. 2006. A quantitative approach to the study of
531 cell shapes and interactions during early chordate embryogenesis. *Curr Biol,* **16,** 345-358.
532 Tokuoka, M., Imai, K. S., Satou, Y. & Satoh, N. 2004. Three distinct lineages of mesenchymal cells in
533 Ciona intestinalis embryos demonstrated by specific gene expression. *Dev Biol,* **274,** 211-224.
534 Tokuoka, M., Satoh, N. & Satou, Y. 2005. A bHLH transcription factor gene, Twist-like1, is essential for
535 the formation of mesodermal tissues of Ciona juveniles. *Dev Biol,* **288,** 387-396.
536 Toresson, H., Martinez-Barbera, J. P., Bardsley, A., Caubit, X. & Krauss, S. 1998. Conservation of BF-1
537 expression in amphioxus and zebrafish suggests evolutionary ancestry of anterior cell types that
538 contribute to the vertebrate telencephalon. *Development Genes and Evolution,* **208,** 431-439.
539 Urbach, R. 2007. A procephalic territory in Drosophila exhibiting similarities and dissimilarities
540 compared to the vertebrate midbrain/hindbrain boundary region. *Neural Dev,* **2**.
541 Wada, H., Saiga, H., Satoh, N. & Holland, P. W. 1998. Tripartite organization of the ancestral chordate
542 brain and the antiquity of placodes: insights from ascidian Pax-2/5/8, Hox and Otx genes.
543 *Development,* **125,** 1113-1122.
544 Wada, S., Tokuoka, M., Shoguchi, E. et al. 2003. A genomewide survey of developmentally relevant
545 genes in Ciona intestinalis. II. Genes for homeobox transcription factors. *Dev Genes Evol,* **213,**

222-234.

Wagner, E. & Levine, M. 2012. FGF signaling establishes the anterior border of the Ciona neural tube. *Development,* **139,** 2351-2359.

Wagner, E., Stolfi, A., Choi, Y. G. & Levine, M. 2014. Islet is a key determinant of ascidian palp morphogenesis. *Development,* **141,** 3084-3092.

Waki, K., Imai, K. S. & Satou, Y. 2015. Genetic pathways for differentiation of the peripheral nervous system in ascidians. *Nat Commun,* **6,** 8719.

Wilson, S. I., Graziano, E., Harland, R., Jessell, T. M. & Edlund, T. 2000. An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. *Curr Biol,* **10,** 421-429.

Yagi, K., Satoh, N. & Satou, Y. 2004. Identification of downstream genes of the ascidian muscle determinant gene Ci-macho1. *Dev Biol,* **274,** 478-489.

Yu, J. K., Satou, Y., Holland, N. D. et al. 2007. Axial patterning in cephalochordates and the evolution of the organizer. *Nature,* **445,** 613-617.

Zhao, D., Chen, S. Y. & Liu, X. 2019. Lateral neural borders as precursors of peripheral nervous systems: A comparative view across bilaterians. *Development Growth & Differentiation,* **61,** 58-72.

563 **Figure legends**

564 **Figure 1. The animal hemisphere cells are ectodermal.** (a) The bilateral 16-cell embryos. Cells
565 in the animal hemisphere are colored. (b) The gene circuits specifying the epidermal and neural fate.
566 At the 16-cell stage, *Sox1/2/3*, *Tfap2-r.b*, *Gdf1/3-r*, and *Efna.d* are expressed in the animal
567 hemisphere and initiate the genetic program for the development of ectodermal tissues.

568 **Figure 2. Neural induction in the ascidian embryo.** (a) Among the animal cells, two anterior cells
569 (cyan) and two lateral cells (green) are induced to express *Otx* (indicated by dots). The cells colored
570 in orange have the epidermal fate. (b) The regulatory mechanism that drives *Otx* expression in the
571 neural cells. Fgf9/16/20, which comes from the vegetal hemisphere, induces *Otx* expression. This
572 signaling molecule activates Ets1/2.b and Gata.a through the MAPK signaling pathway. However, in
573 non-neural cells, Ephrin signaling downregulates the MAPK signaling pathway, and thereby *Otx* is
574 not activated. In both lineages, Admp and Gdf1/3 weakly suppress *Otx* expression.

575 **Figure 3. Gene regulatory mechanisms specifying the anterior border of the neural plate.** (a) In
576 the anterior neural lineage, Fgf9/16/20 signaling positively regulates *Zic-r.b*, although this action is
577 antagonized by two transcriptional repressors, Hes.a and Prdm1-r. At the 112-cell stage, these
578 repressors disappear, and only a subset of the anterior neural cells are adjacent to cells expressing
579 *Fgf9/16/20*. Therefore, at the 112-cell stage, this subset of cells (yellow) expresses *Zic-r.b*, while the
580 remaining cells, which will give rise to the anterior neural plate border (ANB; blue), express *Foxc*.
581 Sister cell relationships are indicated by connecting lines. (b) The neural plate and the anterior and
582 lateral border regions of cells at the gastrula stage and the ANB at the neurula stage.

583 **Figure 4. The central nervous system (CNS) of the ascidian larva.** Four morphologically distinct
584 regions are recognizable in the CNS; the brain, neck, visceral ganglion, and nerve cord. The

585 expression patterns of several important genes in the CNS is shown. For simplicity, gene expressions
586 between the gastrula and tailbud stages are summarized according to the cell lineages. Fgf8/17/18
587 positively and negatively regulates the fate of the neck and brain, respectively. (b) A regulatory loop
588 between *Fgf8* and *Foxg1* acting in the anterior neural ridge and telencephalon of vertebrate embryos
589 is only partially conserved in the ascidian embryos.

590 **Figure 5. Specification of the dorsal and ventral sensory neurons (SNs) of the ascidian larva.**
591 (a) The ascidian tadpole larva develops the epidermal SNs in the dorsal and ventral sides of the tail
592 (green and orange, respectively). Note that the number of SNs varies among individuals (Pasini *et*
593 *al.*, 2006, Waki *et al.*, 2015). (b) Gene regulatory circuits specifying the dorsal and ventral SNs. Both
594 SNs share the same gene circuit beginning with *Msx* expression.

Fig1

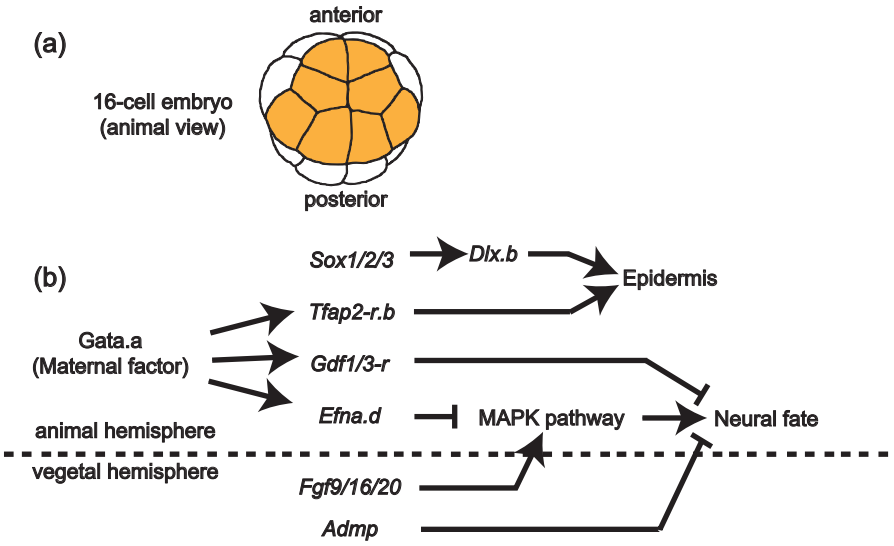

(a)
anterior
16-cell embryo
(animal view)
posterior
(b)
Sox1/2/3
Dlx.b
Epidermis
Tfap2-r.b
Gata.a
(Maternal factor)
Gdf1/3-r
Efna.d
MAPK pathway
Neural fate
animal hemisphere
vegetal hemisphere
Fgf9/16/20
Admp

Fig2

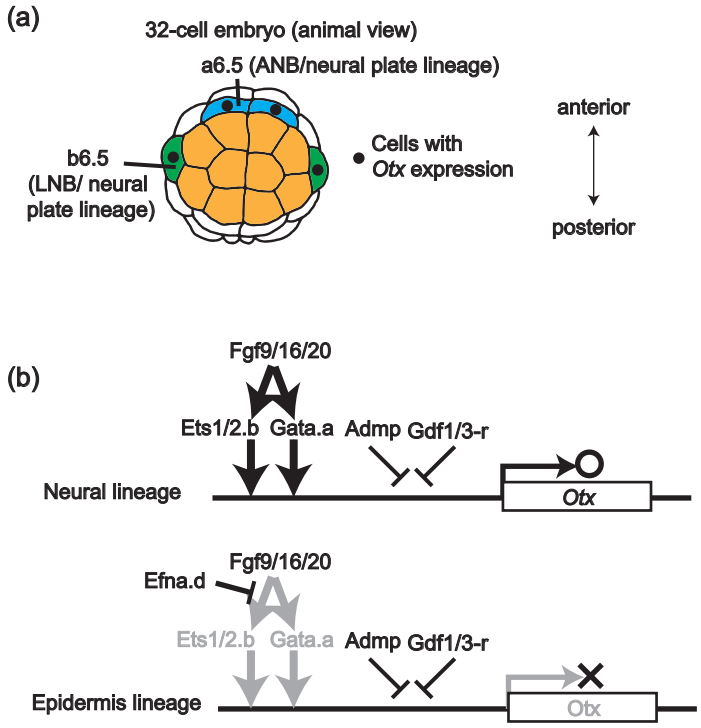

(a)
32-cell embryo (animal view)
a6.5 (ANB/neural plate lineage)
b6.5
(LNB/ neural
plate lineage)
Cells with
Otx expression
anterior
posterior
(b)
Fgf9/16/20
Ets1/2.b
Gata.a
Admp
Gdf1/3-r
Neural lineage
Otx
Fgf9/16/20
Efna.d
Ets1/2.b
Gata.a
Admp
Gdf1/3-r
Epidermis lineage
Otx

Fig3

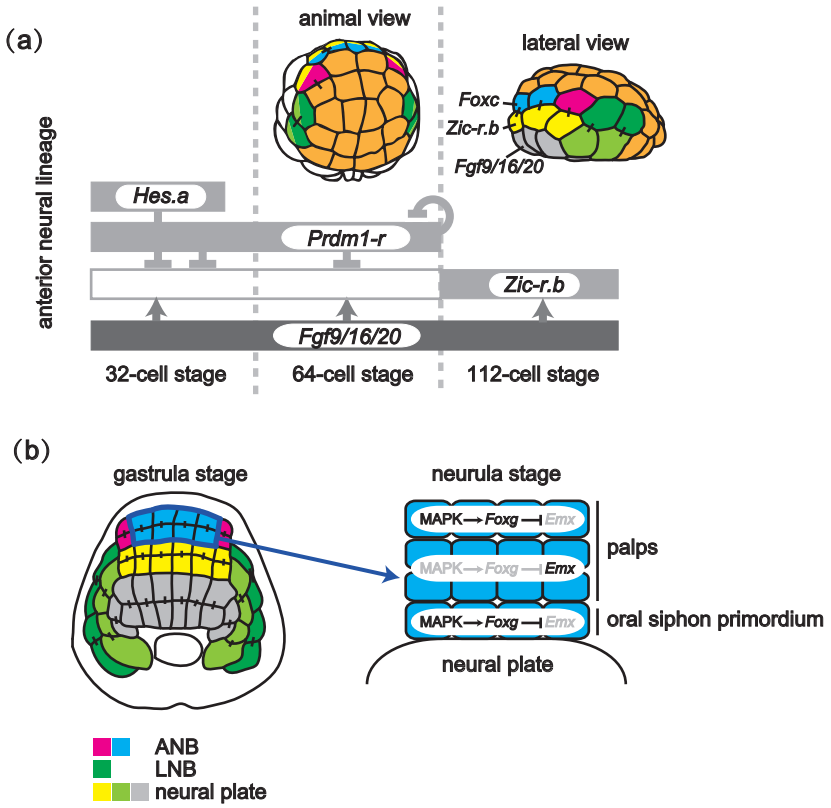

(a)
animal view
lateral view
Foxc
Zic-r.b
Fgf9/16/20
anterior neural lineage
Hes.a
Prdm1-r
Zic-r.b
Fgf9/16/20
32-cell stage
64-cell stage
112-cell stage
(b)
gastrula stage
neurula stage
MAPK → Foxg ⊣ Emx
MAPK → Foxg ⊣ Emx
MAPK → Foxg ⊣ Emx
palps
oral siphon primordium
neural plate
ANB
LNB
neural plate

Fig4

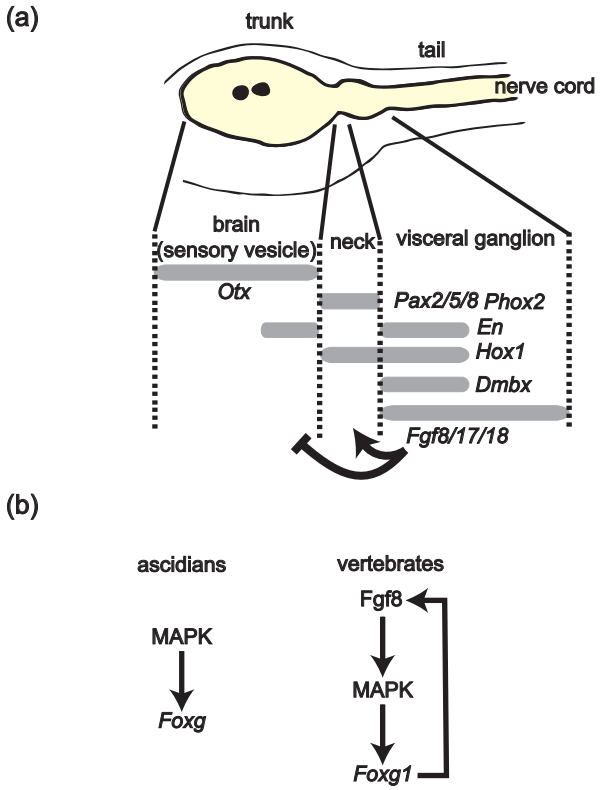
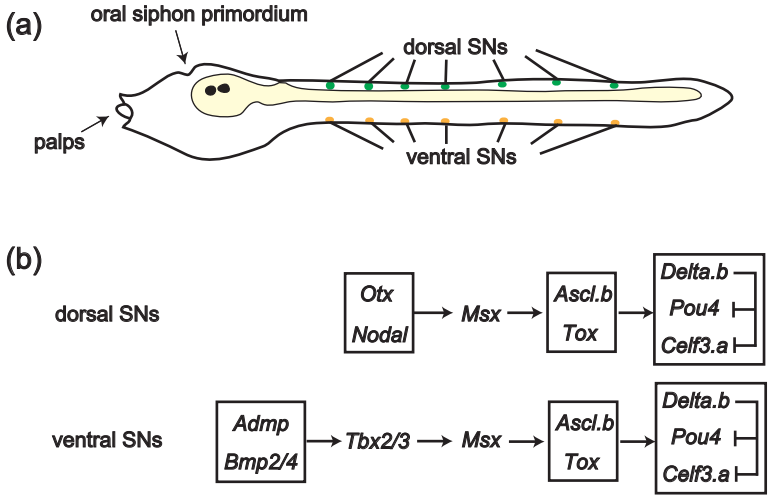

(a)
trunk
tail
nerve cord
brain
(sensory vesicle)
neck
visceral ganglion
Otx
Pax2/5/8 Phox2
En
Hox1
Dmbx
Fgf8/17/18
(b)
ascidians
MAPK
Foxg
vertebrates
Fgf8
MAPK
Foxg1

Fig5


(a)
oral siphon primordium
dorsal SNs
palps
ventral SNs
(b)
dorsal SNs
Otx
Nodal
Msx
Ascl.b
Tox
Delta.b
Pou4
Celf3.a
ventral SNs
Admp
Bmp2/4
Tbx2/3
Msx
Ascl.b
Tox
Delta.b
Pou4
Celf3.a