**Review**

# Gene regulatory systems that control gene expression in the *Ciona* embryo

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**Abstract:** Transcriptional control of gene expression is one of the most important regulatory systems in animal development. Specific gene expression is basically determined by combinatorial regulation mediated by multiple sequence-specific transcription factors. The decoding of animal genomes has provided an opportunity for us to systematically examine gene regulatory networks consisting of successive layers of control of gene expression. It remains to be determined to what extent combinatorial regulation encoded in gene regulatory networks can explain spatial and temporal gene-expression patterns. The ascidian *Ciona intestinalis* is one of the animals in which the gene regulatory network has been most extensively studied. In this species, most specific gene expression patterns in the embryo can be explained by combinations of upstream regulatory genes encoding transcription factors and signaling molecules. Systematic scrutiny of gene expression patterns and regulatory interactions at the cellular resolution have revealed incomplete parts of the network elucidated so far, and have identified novel regulatory genes and novel regulatory mechanisms.

**Keywords:** *Ciona intestinalis*, gene regulatory network

## Introduction

Transcriptional control of gene expression is one of the most important regulatory systems involved in animal development. Through combinatorial regulation mediated by multiple sequence-specific transcription factors, different cell types express different sets of genes.[1),2)] Each of transcription factors positively or negatively regulates transcription of its target genes, and there are transcription factors whose activity is modulated by cell–cell interactions. The specific set of transcription factors at a given developmental time point is activated by the specific set of transcription factors active at the preceding time point. Thus, transcriptional regulation constitutes a network. Can such gene regulatory networks causally explain the expression of every gene? Over the past two decades, the genomes of many animals have been decoded; therefore, we now have the opportunity to address this question on a genome-wide scale.

The genome of every cell is duplicated when it divides into two daughter cells, and one of the duplicated copies is inherited by each of the two daughter cells. Because networks are encoded in genomes, networks are also duplicated at the time of cell division. These networks interact with one another through cell–cell interactions. Thus, signaling molecules mediating cell–cell interactions are essential components of networks, and the spatial organization of cells within the embryo, which constrains cell–cell interactions, is important for our understanding of the developmental program in animal embryos.

In this review, we will discuss the gene regulatory network in an ascidian, *Ciona intestinalis*.[3),4)] The number of embryonic cells in this animal is small: gastrulation begins at the 112-cell stage, and a larva consists of only 2,600 cells;[5),6)] almost all cells can be identified under microscopes. Therefore, in this

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species it is relatively easy to understand how cell–cell interactions occur, and to trace gene expression temporally and spatially.

*Ciona intestinalis* is a basal chordate and a close relative of vertebrates,[7),8)] and the *Ciona* larva exhibits a typical chordate body plan. Forty notochord cells are aligned linearly along the anterior-posterior axis of the tail. The notochord is flanked dorsally by a nerve cord, ventrally by an endodermal strand, and laterally by muscle cells. The dorsal nerve cord is connected to the brain, which is located in the dorsal region of the trunk. Mesenchymal and endodermal cells are also differentiated in the trunk region. Thus, the developmental mechanism of the chordate body plan can be dissected in this simple embryo.

The draft genome sequence of this animal was determined in 2002.[9)] The genome size is approximately 160 mega-bases, and contains around 16,000 protein coding genes. After a major update,[10),11)] 68% of the genome sequences are now associated with specific chromosomes. The genome size and gene number are similar to those of non-chordate species, including protostomes, but much smaller than those of vertebrates. Therefore, the *Ciona* embryo provides an opportunity for analyzing the developmental program for the common chordate body plan in an organism with a smaller number of genes and a compact genome.

In the current assembly of the *Ciona* genome, 341 transcription factor genes of well-known classes, including bHLH, bZIP, homeobox, nuclear receptor, T-box, Ets, HMG, and zinc fingers that are annotated as transcription factors, and additional 297 zinc-finger genes that might encode transcription factors, have been comprehensively listed up[12)–19)] (http://ghost.zool.kyoto-u.ac.jp/TF_KH.html). Expression patterns up to the tailbud stage have been described for over 85% of these genes.[20)] Because cDNA clones for the remaining 15% genes were not obtained in our extensive EST collection spanning the egg through larval stages,[21)] these genes are likely to be expressed at low levels during embryonic development. Gene-expression patterns for ligands and receptors of signaling pathways of FGF, Ephrin, TGF$\beta$/BMP, Wnt, and Notch have also been examined comprehensively.[22)]

On the basis of their expression patterns, the functional interactions among these regulatory genes have been comprehensively and systematically analyzed in *Ciona* in a manner independent of particular hypotheses that could be drawn from preceding studies.[23),24)] Therefore, this network provides a unique opportunity to systematically test to what extent combinatorial regulation explains differential expression. This test may uncover incomplete parts of the network that has been elucidated so far, and may provide implications for mechanisms that cannot be explained by simple combinatorial regulation, including chromatin modification and micro RNAs. At the time of this writing, the elucidated network contains 394 edges (regulatory interactions), which interlink 113 nodes (genes) (http://ghost.zool.kyoto-u.ac.jp/201406html/). In the subsequent sections, we describe how the gene regulatory networks explain the dynamic changes in gene-expression patterns in the *Ciona* embryo. At the same time, we discuss the degree to which the network that has been elucidated explains the process of specification, which establishes specific gene-expression patterns, and highlight examples in which new regulatory mechanisms and new regulatory genes were identified through scrutiny of the elucidated gene regulatory network.

In the following sections, genes will be named according to the recent guideline for the nomenclature of tunicate genes.[25)] For the first time each gene is mentioned, its original name will be given in brackets. For genes whose new names are very different, we will show their original names as synonyms[25)] together with their new names.

## Maternal factors controlling initial states of the network

The *Ciona* embryo was historically regarded as a mosaic embryo,[26),27)] in which different blastomeres that inherit different localized maternal materials assume different developmental fates. Although this is not necessarily true, there exist at least three important maternal transcription factors and co-factors that play critical roles in fate determination: $\beta$-catenin,[28),29)] Gata.a[30),31)] (a possible ortholog of vertebrate GATA4, GATA5, and GATA6; the original name was Gata-a), and the Zic-like protein Macho-1/Zic-r.a (Macho-1 is the original name and Zic-r.a is the new name).[32),33)] The activities of $\beta$-catenin and Gata.a are restricted to the vegetal and animal halves of the early embryo, respectively, and *Macho-1/Zic-r.a* mRNA is localized at the posterior pole of the embryo. Consistently, $\beta$-catenin is required for specification of cells in the vegetal hemisphere, whereas Gata.a is required for specification of ectodermal tissues derived from the animal hemisphere. Macho-1/Zic-r.a is required for formation of muscle and mesenchyme that are

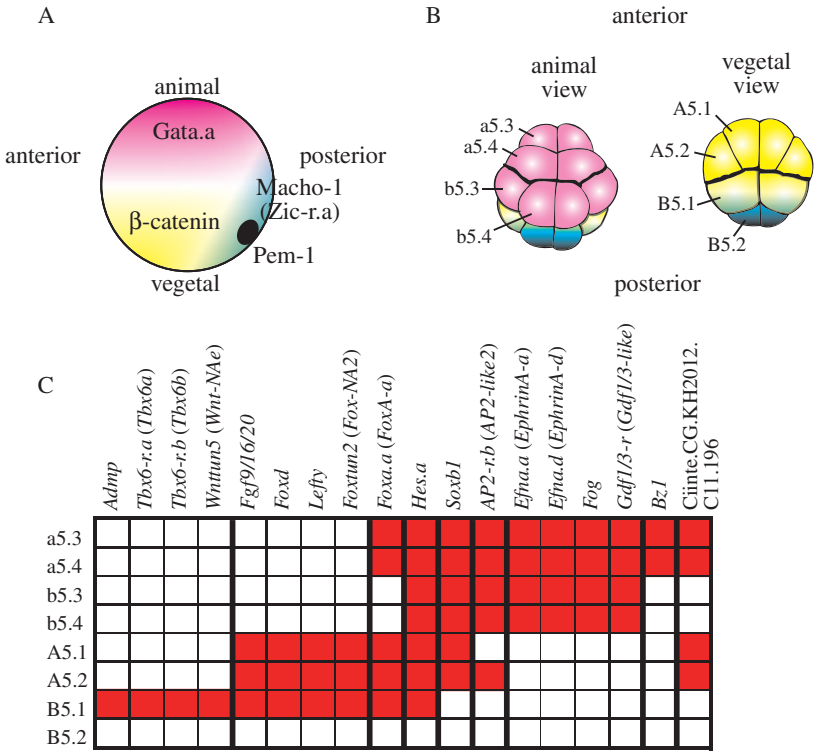


Fig. 1. Four maternal factors pre-patterns an embryo. **(A)** Localized activity of GATA-a (magenta), β-catenin (yellow), Macho-1/Zic-r.a (blue), and Pem-1 (black). Regions filled with mixed colors have mixed activities of multiple factors. **(B)** Schematic representations of the 16-cell embryo. Localized activities of the four maternal factors are indicated according to the same color code used in (A). Each cell pair of the bilaterally symmetrical embryo is designated by a unique name, indicated on the left of the illustrations. **(C)** Expression patterns of genes (*x*-axis) that are zygotically activated at the 16-cell stage in individual cells (*y*-axis). Because the expression profiles are based on comprehensive assays, we do not expect that additional regulatory genes activated from zygotic genomes at this stage will be discovered. The right-most gene is not a regulatory gene and represented by a KH-gene model identifier,[11] because there are no regulatory genes that show this ninth expression pattern. Red squares indicate expression, and each of nine expression patterns are enclosed by thick lines.

derived from the vegetal–posterior region. Thus, as shown in Fig. 1A and B, combinations of these three factors define three distinct regions in early embryos: the animal, vegetal-anterior, and vegetal-posterior regions. In addition, *Pem-1* mRNA colocalizes with *Macho-1/Zic-r.a* mRNA at the posterior pole.[34] Pem-1 protein represses transcription in the posterior–most cells (germ line cells) and also contributes to the initial setup of the gene network.[35),36]

These maternal factors initiate the zygotic developmental program. The first clear zygotic gene expression begins at the 16-cell stage, although some genes that start to be expressed at the 16-cell stage are also expressed faintly at the 8-cell stage. Seventeen regulatory genes that begin to be expressed at the 8- and 16-cell stages[22),23),30),37)–40] exhibit eight different expression patterns (Fig. 1C).

Do the combinatorial activities of the above four maternal factors explain these eight expression patterns? *Foxd*, which is expressed in the vegetal hemisphere except the posterior-most cells, is a direct target of β-catenin;[31),41] *Fog*, which is expressed in the animal hemisphere, is a direct target of Gata.a;[31] *Tbx6-r.b* (formerly called *Tbx6b*), which is expressed in the posterior vegetal cells (except the posterior-most), is a putative direct target of Macho-1/Zic-r.a.[42] Because most genes have multiple enhancers, two or more enhancers might promote expression of other genes in a more complex pattern. *Soxb1* is expressed in the animal hemisphere and the vegetal-anterior blastomeres; Gata.a might activate the expression of *Soxb1* in the animal hemisphere, and combinatorial activity of β-catenin and Macho-1/Zic-r.a might repress the expression of *Soxb1* in the vegetal-posterior blastomeres, although this possibility has not yet been experimentally tested. On the other hand, *Foxa.a* (formerly called *FoxA-a*) is expressed in the animal-anterior, vegetal-anterior

and vegetal-posterior blastomeres. This expression pattern cannot be explained by any combination of activity of the above maternal factors, suggesting the existence of unknown maternal regulatory genes or unidentified novel regulatory interactions among the four aforementioned regulatory factors. Indeed, a previous study suggested that $\beta$-catenin suppresses Gata.a activity in the vegetal hemisphere,[31] but it is not yet understood how this suppression occurs.

A microarray assay that determined gene-expression profiles in individual cells of the 16-cell embryo revealed one additional gene-expression pattern.[43] Because these are among the first zygotically activated genes, maternal factors must be responsible for their expression. To understand how maternal factors in ascidian eggs establish the initial state of the gene regulatory network, we need to understand how the four main maternal factors (along with other currently unknown factors, if any) induce these nine distinct patterns of gene expression.

### Specification of ectodermal fates in the animal hemisphere of early embryos

Once the pre-pattern for the zygotic program is established by maternal factors by the 16-cell stage, the gene regulatory network encoded in the zygotic genome begins to specify cell fates. At the 32-cell stage, two cell pairs in the animal hemisphere (the a6.5 and b6.5 cell pairs) are destined to neural fates and start to express *Otx* under the control of Gata.a.[22),30),44)] *Otx* is not activated at the 16-cell stage, because an additional activator is required for its expression: the secreted signaling molecule Fgf9/16/20, the sole ortholog of vertebrate FGF9, FGF16 and FGF20, which is expressed in the vegetal hemisphere from the 16-cell stage.[22),45)] Although this signal potentially activates *Otx* expression in all animal-hemisphere cells,[46),47)] and the areas of cell surface contact with FGF-expressing cells are correlated with *Otx* expression,[46)] suppression by Efna.d (Ephrin-A.d; formerly called EphrinA-d) restricts *Otx* expression in the neural cells. *Efna.d* is expressed in all cells in the animal hemisphere.[22)] Because this signaling molecule is anchored to the cell membrane, its signal is transmitted only to neighboring cells. As a result, inner cells, which are surrounded entirely by other animal cells, are expected to receive stronger signal than outer cells, which are surrounded by a mixture of animal cells and vegetal cells (Fig. 2A). Indeed, among the cells in the animal hemisphere, two pairs of outer cells that express *Otx* have the smallest contact surfaces with *Efna.d*–expressing cells,[47)] which was calculated with a 3D-virtual embryo.[46)] This Ephrin signaling antagonizes transduction of FGF signaling through p120RasGAP,[48)] resulting in the outer cells that express *Otx*.

A systematic and comprehensive approach also revealed the involvement of Gdf1/3-r (Gdf1.3-related; formerly called Gdf1/3-like) and Admp signaling in specific expression of *Otx*.[47)] These two factors downregulate the expression of *Otx* by direct binding of their effector transcription factor Smad (Fig. 2B). Without this signaling activity, *Otx* is occasionally expressed ectopically in presumptive epidermal cells. The antagonistic action of Efna.d cannot completely suppress the activity of Fgf9/16/20, probably because this process is intrinsically stochastic. Therefore, fluctuations in the overall activity of Fgf9/16/20 and Efna.d signaling could result in occasional ectopic expression of *Otx*. However, direct repression through the *Otx* enhancer by Gdf1/3-r and Admp signaling blocks occasional weak activating signals. Because knockdown of either *Gdf1/3-r* or *Admp* does not result in a clear phenotype (simultaneous knockdown of these genes results in a clear phenotype as described above), the function of Gdf1/3-r and Admp might be revealed only through a comprehensive analysis not based on specific hypotheses. In other words, because the antagonistic system consisting of Fgf9/16/20 and Efna.d can explain the specific expression of *Otx*, it might have been difficult to hypothesize that *Gdf1/3-r* and *Admp* cooperatively repress *Otx* expression weakly. It would not be surprising if similar hidden noise-cancelling mechanisms were used more widely in other inductive interactions.

The same double-negative regulation by Efna.d signaling and Gdf1/3-r/Admp signaling is used for specific expression of *Nodal* in the presumptive neural cells. Unlike *Otx*, *Nodal* is expressed only in the posterior pairs of the presumptive neural cells (b6.5). This is because *Nodal* expression in the anterior pair (a6.5) is repressed by *Foxa.a*,[23),47)] which is expressed in the anterior animal cells but not in the posterior animal cells.

Neural induction in *Ciona* embryos is reminiscent of neural induction in *Xenopus* embryos, in which choice between epidermal fate and neural fate is controlled by BMP and FGF signaling,[49)–51)] although FGF plays a more dominant role in the *Ciona* embryo. According to the 'default model', the default fate in *Xenopus* is neural. In the *Ciona* embryo, all ectodermal cells receive FGF signals and

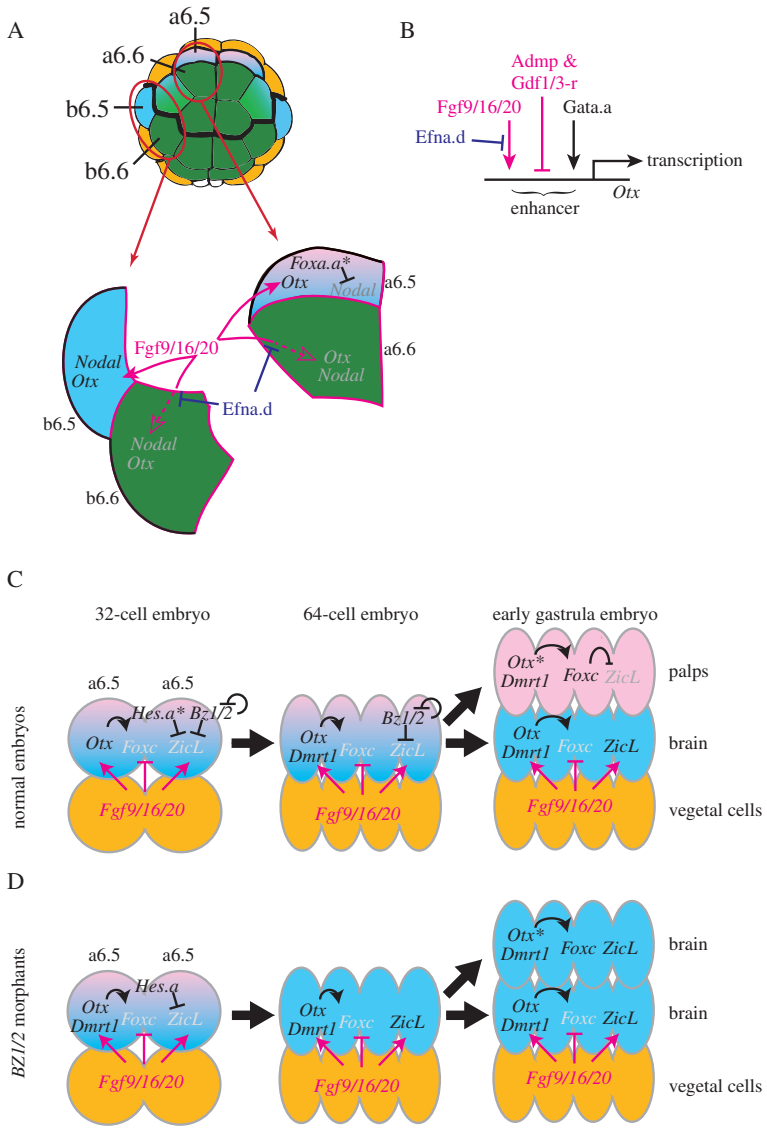


Fig. 2. Specification of neural fate. **(A)** Schematic representation of the animal hemisphere of a 32-cell embryo. Among two pairs of sister cells indicated by their cell names, a6.5 and b6.5 are specified as neural cells. Cells filled with green are epidermal cells, and cells filled with orange are vegetal cells that express *Fgf9/16/20*. Blue indicates neural fate, and pink indicates palp fate. The palps also contain neurons. Because the a6.5 cell pair gives rise to the palps and brain, this cell pair is colored blue and pink. Similarly because a pair of the cells (a6.7) between a6.5 and b6.5 gives rise to the brain and epidermis, this cell pair is colored green and blue. The border between the anterior and posterior halves is indicated by a thick line. In the lower panel, the regulatory interactions that occur in the two sister-cell pairs are shown. Cell membranes contacting cells expressing *Efna.d* are shown in magenta. Epidermal cells have larger contact surfaces with cells expressing *Efna.d*, whose protein product suppresses Fgf9/16/20 signaling. Previous studies indeed measured the contact surface areas using embryos virtually reconstructed based on a series of confocal images.[46),47)] **(B)** Schematic illustration showing how four distinct signaling molecules coordinate *Otx* expression. Effector transcription factors of Fgf9/16/20, Admp, and Gdf1/3-r signaling pathways directly control an *Otx* enhancer, whereas Efna.d signaling antagonizes Fgf9/16/20 signaling through controlling p120RasGAP activity. **(C)** Regulatory interactions for specifying brain and palp fates from the 32-cell stage to the early gastrula stage. The a6.5 lineage has brain fate (pink) and palp fate (blue). The vegetal cells abutting on either of a6.5 or its descendants with the brain fate continuously express *Fgf9/16/20* and are indicated in orange. Ovals for multiple cells, in which the same regulatory interactions are observed, are fused. **(D)** The gene regulatory networks shown in (C) cannot specify palp fate, when *Bz1* and *Bz2* are simultaneously knocked down. In (A–D), normal and flat-head arrows indicate positive and negative regulation, respectively. Dotted arrows indicate suppressed interactions. Regulatory interactions through signaling pathways are shown in magenta and dark blue. Genes that are expressed are shown by black letters, and genes that are suppressed are shown in gray letters. Genes marked by asterisks are not actively transcribed, but their protein products derived from mRNA transcribed in ancestral cells are expected.

could therefore assume neural fate in the absence of proper suppression. Epidermal cells are differentiated, even if blastomeres are continuously dissociated to prevent cell–cell interactions until the early gastrula stage.[52] This observation might suggest that the default fate is epidermal. However, it may be hard to determine whether the "true" default fate of the ascidian ectoderm is different from that of the *Xenopus* embryo. Because autocrine signaling is not completely ruled out even in dissociated cells, it is possible that autocrine signaling works differently between embryos of these two animals.

The anterior neural cells (a6.5) give rise to the brain and palp. The palp, an adhesive organ differentiated in the anterior tip of the larva, is considered to have the same origin as the vertebrate anterior placodes.[53)–55)] Brain and palp fates are specified at the gastrula stage (Fig. 2C), when brain cells start to express a Zic-like transcription factor gene, *ZicL* (or *Zic-r.b*), and palp cells start to express *Foxc*.[22),23)] *ZicL* expression is negatively regulated by *Foxc*[55)] and positively regulated by FGF signaling.[23)] *Foxc* expression is negatively regulated by Fgf9/16/20 signaling.[23),55),56)] *Fgf9/16/20* is expressed in cells abutting on the presumptive brain cells but not on the presumptive palp cells, and makes these two cell populations different. Their common ancestor cells that express *Otx* at the 32-cell stage (a6.5) abut on the *Fgf9/16/20*-expressing cells, and an antibody against a dual-phosphorylated form of the extracellular regulated kinase 1/2 (dpERK) revealed that FGF-signaling cascade is activated in these cells;[55),57)] indeed, as we described above the FGF-signaling cascade activates *Otx* expression at the 32-cell stage and *Dmrt1* is also expressed under the control of *Fgf9/16/20* in the a6.5 lineage; *Otx* and *Dmrt1* positively regulate *Foxc* expression. While the presumptive palp cells stop to express *Otx* probably because of lack of Fgf/9/16/20 signaling, *Dmrt1* continues to be expressed. It has not yet been revealed why *Dmrt1* expression continues in these cells.

Why does not FGF signaling activate *ZicL* expression in the brain cells before the gastrula stage? The *Ciona* genome encodes gene circuits that repress precocious expression of *ZicL* before the gastrula stage[40)] (Fig. 2C). Initially, Hes.a and Blimp-like zinc finger proteins (Bz1 and Bz2, or Prdm1-r.a [Pr domain containing 1, with znf domain.a] and Prdm1-r.b) repress *ZicL* at the 32-cell stage, and therefore the FGF signaling that activates *Otx* expression does not activate *ZicL* expression there. At the 64-cell stage, Bz1 and Bz2 repress *ZicL* expression, and also repress their own expression by an auto-regulatory loop. At the gastrula stage, Bz1 and Bz2 are auto-repressed and no longer repress *ZicL*; therefore, *ZicL* begins to be expressed. At this time, however, the presumptive palp cells do not abut on the Fgf9/16/20-expressing cells, and therefore these cells do not express *ZicL*. Thus, these gene circuits reasonably explain the causal mechanisms underlying gene expression in the brain and palp lineages.

The timing at which *Bz1* and *Bz2* are turned off is critical for specification of the brain and palp fates. In *Bz1* and *Bz2* double-knockdown embryos, because *ZicL* expression is not repressed at the 32-cell stage, *ZicL* expression starts precociously, and all presumptive palp cells differentiate into brain (Fig. 2D). On the other hand, overexpression of *Bz1* and *Bz2* prevents *ZicL* from being expressed in the brain lineage, because in this context *Bz1* and *Bz2* continue to repress *ZicL* expression. Gene circuits that control temporal gene expression have not yet been analyzed as extensively as those that control spatial gene expression. Systematic tests to determine whether the gene regulatory network provides a logical explanation for temporal control of gene expression may lead to identification of currently unknown gene circuits that control temporal gene expression.

## Specification of developmental fates in the vegetal hemisphere of early embryos

**Anterior vegetal cells.** As we described above, β-catenin is responsible for specifying the vegetal hemisphere, and most (possibly all) genes activated in the vegetal hemisphere of early embryos are downstream of β-catenin.[28),58)] Nerve cord, notochord, mesenchyme, or endoderm cells are derived from the anterior vegetal cells (Fig. 3A, B). A recent study revealed that transient nuclear localization of β-catenin at the 16-cell stage is required for specification of notochord and nerve cord fates in the anterior half;[59)] that is, cells in which β-catenin is not localized in the nucleus during the early stages become epidermal and neural cells, cells in which β-catenin is localized in nucleus continuously from the 16-cell stage to the 32-cell stage become cells with endodermal fate (A6.1 and A6.3), and cells in which β-catenin is localized in nucleus only at the 16-cell stage become notochord cells and nerve cord cells (A6.2 and A6.4).

At the 16-cell stage, each of the two pairs of the vegetal anterior cells has endodermal, mesodermal

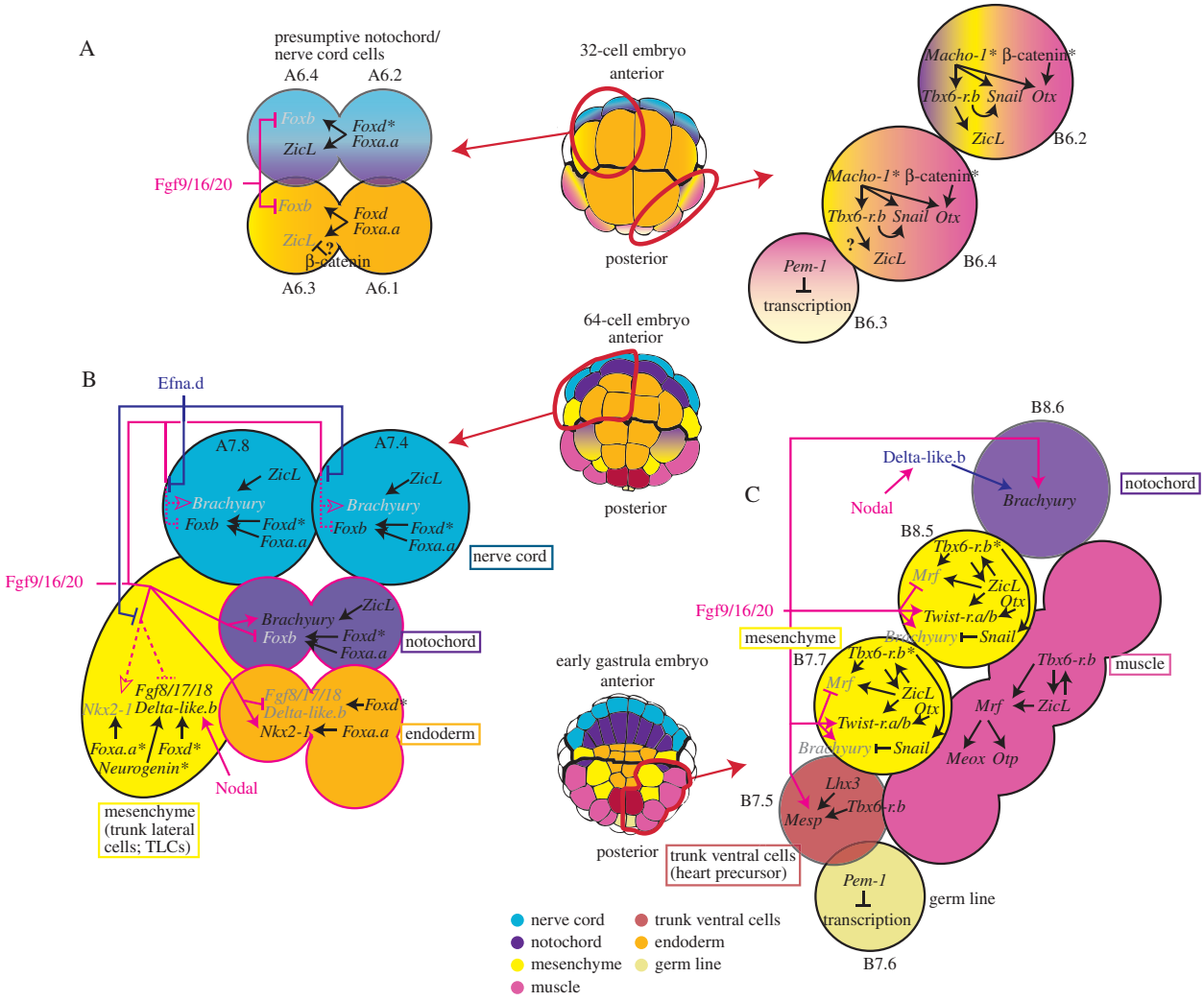


Fig. 3. Specification of endodermal and mesodermal fates. **(A–C)** The vegetal hemisphere of (A) 32-cell, (B) 64-cell, and (C) early gastrula embryos is illustrated in the center. Regulatory interactions in cells enclosed by red lines are shown on the left and right sides. Each circle represents a single cell. Circles for multiple cells in which the same regulatory interactions are observed are fused. Cells with different fates are filled with different colors shown at the bottom. The sizes of the circles are not proportional to the actual sizes of the cells. Normal and flat-head arrows indicate positive and negative regulation, respectively. Dotted arrows indicated suppressed interactions. Regulatory interactions through signaling pathways are shown in magenta and dark blue. Genes that are expressed are shown in black letters, and genes that are suppressed are shown in gray letters. Genes marked by asterisks are not actively transcribed, but their protein products derived from mRNA transcribed in ancestral cells are expected.

and nerve-cord fates. These two pairs of cells divide along the anterior-posterior axis at the 32-cell stage. The anterior descendants are presumptive notochord/nerve cord cells (A6.2 and A6.4), which express *ZicL*[22),60)] (Fig. 3A). This *ZicL* expression is positively controlled by *Foxa.a* and *Foxd*, which are expressed at the preceding 16-cell stage.[23),60)] Chromatin-immunoprecipitation assays have shown that this regulation by *Foxa.a* and *Foxd* is direct.[61)]

In the ascidian *Halocynthia roretzi*, *Not* mRNA starts to be expressed in the vegetal cells at the 16-cell stage, and it is asymmetrically distributed to the presumptive notochord/nerve cord cells.[62)] In *Ciona*, this asymmetrical distribution of mRNA is not restricted to *Not* mRNA:[59)] mRNAs expressed in the vegetal cells at the 16-cell stage, including *Fgf9/16/20*, and *Foxd*, are asymmetrically distributed to the presumptive notochord/nerve cord cells at the next division. However, soon after this division, the *Fgf9/16/20* mRNA is again observed in both of the presumptive/nerve cord cells and the cells with endodermal fate, because transcription of these genes

continues in these cells. Despite the asymmetric distribution to the presumptive notochord/nerve cord cells, *Foxd* mRNA soon becomes detectable only in the cells with endodermal fate, because transcription of *Foxd* continues only in these cells.[22)]

Currently, it is not understood how *Foxd* and *Foxa.a* specifically activate *ZicL* expression in the presumptive notochord/nerve cord cells but not in the cells with endodermal fate at the 32-cell stage. As mentioned above, because cells in which β-catenin is localized in nucleus continuously from the 16-cell stage to the 32-cell stage do not express *ZicL*, the activity of nuclear β-catenin is likely related to the specific expression of *ZicL*.[59)] However, it is not understood how nuclear localization of β-catenin is controlled, how transient nuclear localization of β-catenin activates the specific expression of *ZicL*, and how continuous nuclear localization of β-catenin represses *ZicL* expression.

ZicL directly binds to an enhancer of *Brachyury*,[63)] which encodes a key transcription factor for differentiation of the notochord, and this binding confers competence for FGF induction on this gene. Before the 64-cell stage, the presumptive notochord/nerve cord cells divide into presumptive notochord cells and presumptive nerve cord cells (purple cells and blue cells in Fig. 3B). *Fgf9/16/20* is expressed in the presumptive notochord and endoderm (encircled by magenta in Fig. 3B), and the nerve cord cells receive Efna.d signals from the animal hemisphere that inhibit FGF signaling.[64)] Thus, dpERK signals are detected in the presumptive notochord cells, but not in the presumptive nerve cord cells at the 64-cell stage. This Fgf9/16/20 signal eventually induces *Brachyury* expression only in the presumptive notochord cells. On the other hand, in the endodermal lineage, although a stronger FGF signal is expected, *Brachyury* is not expressed, because the endodermal cells lack of *ZicL* expression. Consequently, *Brachyury* is expressed exclusively in the presumptive notochord cells.[65)] *Brachyury* expression initiates the differentiation program of the notochord, and all genes known to be expressed in the notochord are downstream of *Brachyury*.[66),67)]

Because the same FGF signal represses *Foxb* expression, which is under the control of *Foxa.a* and *Foxd*, *Foxb* is expressed in presumptive nerve cord cells but not in presumptive notochord cells.[23)] *Foxb* is required for patterning of the nerve cord, which we discuss below. In *Halocynthia* embryos, *Foxb* suppresses the notochord fate in the presumptive nerve cord.[68)]

Cells with continuous nuclear β-catenin express *Lhx3/4* (formerly called *Lhx3*) at the 32-cell stage, which is an essential factor for endoderm specification in the embryo of the closely related species *Ciona savignyi*.[69)] Because this gene has the same expression pattern in *C. intestinalis* embryos,[22)] *Lhx3/4* likely has the same function in both species.

A previous study suggested that in *Halocynthia* embryos, the endodermal cells differentiated from the posterior half require FGF signaling.[70)] The same principle could be applied to *Ciona* embryos, because *Nkx2-1* (formerly called *Titf1* or *Ttf1*), which begins to be expressed specifically in the endodermal lineage at the 64-cell stage,[71),72)] is under the control of FGF signaling in *Ciona*.[23)]

The lateral cells with endoderm fate (A6.3) also contribute to mesenchyme cells. These mesenchyme cells, specifically called trunk lateral cells (TLCs), give rise to adult mesodermal cells including blood cells.[73),74)] A6.3 cells divide into presumptive endodermal cells and presumptive TLCs at the 64-cell stage. Specification of the TLCs is again controlled by antagonism between Fgf9/16/20 and Efna.d[39)] (Fig. 3B). At this stage, the presumptive endoderm cells are expected to receive more Fgf9/16/20 signal and less Efna.d signal than the presumptive TLCs. Indeed, dpERK signals were not observed in the TLCs.[39)] In the presumptive endoderm, the signaling pathway activated by Fgf9/16/20 represses expression of genes that are activated in the presumptive TLCs. Nodal signaling instructs the TLC fate by activating a specific set of genes in the TLC lineage, including *Delta-like.b* (formerly *Delta-like* or *Delta2*) and *Fgf8/17/18* (Fig. 3B). *Nodal* is expressed in the b6.5 lineage (Fig. 2A) from the 32-cell stage to the gastrula stage, and also transiently in the vegetal hemisphere (A6.1, A6.3, and B6.1) at the 32-cell stage.

**Posterior vegetal cells.** Although *Macho-1/Zic-r.a* was first identified as a maternal muscle determinant,[32),33)] it subsequently turned out to be a factor specifying the posterior part of the embryo.[75)] Indeed, mesodermal cells derived from the posterior half of the embryo, mesenchyme, muscle, and trunk ventral cells (heart precursor cells), are under the control of Macho-1/Zic-r.a. *Tbx6-r.b* and *ZicL* are important for specification of these fates. *Tbx6-r.b* is expressed in the posterior vegetal cells, except for the posterior-most cells at the 16-cell stage, and *ZicL* begins to be expressed at the 32-cell stage.[22),60),76)] At the 32-cell stage, two pairs of cells in the vegetal posterior quadrant (B6.2 and B6.4) have mesodermal

fate (Fig. 3A). The most posterior pair (B6.3) contributes to heart precursor cells, although this cell pair is kept transcriptionally silent at this stage, as we will discuss below. The expression of *ZicL* in the anterior mesodermal (presumptive muscle/mesenchyme/notochord) cells (B6.2) is under the control of *Tbx6-r.b*, but the expression of *ZicL* in the posterior mesodermal (presumptive muscle/mesenchyme) cells (B6.4) might not require control by *Tbx6-r.b*.[77] Once activated, *Tbx6-r.b* expression persists until the mid-to-late gastrula stage in the muscle lineage. The persistent expression of *Tbx6-r.b* is controlled by *ZicL*, because these two genes constitute a transient positive regulatory loop (Fig. 3C). These two transcription factors cooperate to activate *Mrf* (formerly called *MyoD* or *MDF*), the sole ortholog of vertebrate *MyoD*, *Myf5*, *Myogenin* and *MRF4*, at the early gastrula stage. This myogenic factor continues to be expressed until the tailbud stage,[78)–80)] and activates downstream transcription factor genes including *Meox* (formerly called *Mox*) and *Otp* specifically in muscle cells.[23)]

*Macho-1/Zic-r.a* and $\beta$-catenin regulate *Otx* expression in both of the mesodermal cells (B6.2 and B6.4) at the 32-cell stage[42),69)] (Fig. 3A). *Otx* cooperates with *ZicL* to activate *Twist-r.a* and *Twist-r.b* (*Twist-related.a* and *Twist-related.b*; formerly called *Twist-like1a* and *Twist-like1b*; in this review these two genes are collectively called *Twist-r.a/b*) in the mesenchyme lineage after the 64-cell stage[81)] (Fig. 3C). In addition to *Otx* and *ZicL* function, Fgf9/16/20 signaling is required for activation of *Twist-r.a/b*.[81)] Probably because the presumptive mesenchyme cells, but not the presumptive muscle cells, abut on endodermal cells expressing *Fgf9/16/20*, *Twist-r.a/b* is activated only in mesenchyme cells. Thus, the combination of Fgf9/16/20 signaling, Otx and ZicL proteins induces *Twist-r.a/b* expression, whereas the combination of Fgf9/16/20 signaling and ZicL protein induces *Brachyury*, as mentioned above. Although Fgf9/16/20 signaling and ZicL protein potentially activates *Brachyury* expression in the mesenchyme lineage, *Snail* expressed in this lineage of cells represses *Brachyury* expression.[82),83)] This *Snail* expression is under the control of *Macho-1/Zic-r.a* and *Tbx6-r.b*,[23),42)] although the positive regulatory loop of *Tbx6-r.b* and *ZicL*, which is seen in muscle cells, cannot maintain *Tbx6-r.b* expression in the presumptive mesenchyme cells at the early gastrula stage with an unknown reason.

The posterior presumptive mesenchyme cell (B7.7) begins to express *Twist-r.a/b* soon after the division of its parental cell (B6.4) at the 64-cell stage (Fig. 3A and C). On the other hand, the anterior presumptive mesenchyme cell (B8.5) begins to express *Twist-r.a/b* at the early gastrula stage. This cell is derived from B6.2 of the 32-cell embryo. B6.2 divides into a presumptive muscle cell and a cell with two developmental fates (mesenchyme and notochord) at the 64-cell stage. At the early gastrula stage, the latter cell divides into a presumptive mesenchyme cell (B8.5) and a presumptive notochord cell (B8.6) (Fig. 3C), and *Twist-r.a/b* begins to be expressed in B8.5. Thus, the posterior presumptive mesenchyme cells (B7.7 pair) express *Twist-r.a/b* earlier than the anterior presumptive mesenchyme cells (B8.5 pair). The factor that delays *Twist-r.a/b* expression in the anterior mesenchyme cells has not been revealed.

The presumptive notochord cell (B8.6), which is a sister cell of the anterior mesenchyme cell (B8.5), expresses *Brachyury*. Unlike the presumptive notochord cells in the anterior vegetal quadrant, the notochord cells in the posterior vegetal quadrant may not require *ZicL*, and instead use Notch-Delta signaling to activate *Brachyury*[23),41),60),84),85)] (Fig. 3C). The ligand gene, *Delta-like.b*, is expressed in the TLC precursors, which abut on the notochord cells in the vegetal-posterior quadrant, under the control of *Foxd*, *Neurogenin*, and Nodal signaling (Fig. 3B).

There are at least 19 maternal mRNAs localized in the posterior-most cells of the *Ciona* embryos,[34),43),86)–88)] including *Macho-1/Zic-r.a* and *Pem-1*. Pem-1, which was originally called posterior-end-mark or Pem, suppresses transcription, and the posterior-most cells are therefore always transcriptionally quiescent.[35),36)] The posterior-most cells at the 32-cell stage are called B6.3, and this pair of cells has muscle and TVC fates in addition to a germline fate. At the subsequent division, these cells divide into B7.5 and B7.6, and B7.6 becomes the new posterior-most cell. In B7.5 of the 64-cell embryo, suppression of transcription ceases, and *Lhx3/4* and *Tbx6-r.b* begin to be expressed. Subsequently, these two genes and FGF signaling activates *Mesp* in B7.5.[89)] *Mesp* is a key transcription factor in TVC specification, as all known genes expressed in TVCs are directly or indirectly regulated by *Mesp*.[89)] Indeed, in *C. savignyi* embryos, knockdown of *Mesp* resulted in loss of the heart after metamorphosis.[90)]

In the vegetal posterior quadrant, the cells with endodermal fate express *Lhx3/4* at the 32-cell stage and *Nkx2-1* at the 64-cell stage under the control of

maternal β-catenin.[22),69),71),72)] These two genes are essential for specification of the endodermal cells, as in the case of the endodermal cells in the vegetal anterior cells.

### Specification of ectodermal tissues

The endodermal and mesodermal fates are specified until the gastrula stage as in the preceding section. Cells in each of the individual tissues basically express the same set of genes, and no clear functional differences among them have been reported. On the other hand, ectodermal cells are further specified to produce at least seven territories of epidermis and a variety of neural cells. Although the networks specifying these epidermal territories and neural cell types are still far from comprehensively understood, here we focus on two sub-networks and explain their regulatory interactions.

**Nerve cord cells.** The nerve cord is a hollow tube consisting of four rows of ependymal cells. The lateral and ventral rows are derived from the vegetal hemisphere. At the 32-cell stage, as we described above, two pairs of the anterior vegetal cells have nerve cord and notochord fates. At the 64-cell stage, the fate of the anterior daughters (A7.4 and A7.8) is restricted to the nerve cord fate (Fig. 3B). Nodal signaling differentiates the lateral pair (A7.8) from the medial pair (A7.4) by activating genes including *Snail*[23),38),57)] in the descendants of A7.8 (A8.15 and A8.16) at the early gastrula stage (Fig. 4A). This Nodal signaling comes from b7.9 and b7.10, which are descendants of b6.5 of the 32-cell embryo that express *Nodal* (Fig. 2A) and contribute to the dorsal row of the nerve cord. *Snail* represses *Mnx*, which is specifically expressed in cells giving rise to the ventral row of the nerve cord, whereas *Foxb* activates *Mnx* in the medial A7.4 descendants (A9.13 and A9.15), in which *Snail* is not induced,[23)] at the late gastrula stage (Fig. 4B).

The nerve cord cells are further specified by a combination of multiple signaling pathways. Here we focus on specific expression of *Fgf8/17/18*, because this FGF regionalizes the brain of the larva. *Fgf8/17/18* expression is repressed through ERK activated by Fgf9/16/20, and induced by Nodal signaling.[57)] FGF signaling is suppressed in the nerve cord cells at the early gastrula stage as described earlier (Fig. 3), and *Foxb* and *Snail* also repress the expression of *Fgf8/17/18* (Fig. 4A). At the late gastrula stage, each nerve cord cell divides along the anterior-posterior axis, resulting in formation of two rows of nerve cord cells (Fig. 4B). FGF signaling is turned off in the anterior row, as revealed by an antibody for dpERK,[57)] probably through the action of Ephrin. *Foxb* transcription is turned off except the anterior medial cells (A9.14). Nonetheless, Foxb protein derived from mRNA transcribed at the early gastrula stage likely represses *Fgf8/17/18* expression, because knockdown of *Foxb* results in ectopic expression of *Fgf8/17/18* in all of the cells in the anterior row.[23)]

Snail repressor directs specific expression of *Fgf8/17/18* in A9.30. As described above, *Snail* expression is induced by Nodal signaling from b7.9 and b7.10 (Fig. 4A). At the late gastrula stage, *Snail* expression in A9.32 (which abuts on the descendants of b7.9 and b7.10) is enhanced by *Neurogenin* (Fig. 4B), which is activated in its parental A8.16 cell under the control of *Delta-like.b* (Fig. 4A). *Delta-like.b* is induced in b7.9 and b7.10 cells by autocrine Nodal signaling (Fig. 4A). Because the Notch ligand encoded by *Delta-like.b* is a membrane protein, its signal is transmitted exclusively to the neighbors of cells that express *Delta-like.b*, and *Neurogenin* is therefore activated only in A8.16. As a result, because Neurogenin enhances *Snail* expression, *Snail* is expressed more strongly in the A8.16 lineage than the A8.15 lineage, and Snail protein represses *Fgf8/17/18* expression more strongly in the A8.16 lineage. Thus, FGF signaling, Nodal signaling and Snail repressor activated by Delta-like.b function cooperatively to induce specific expression of *Fgf8/17/18* in A9.30.[24)]

*Fgf8/17/18* expressed in A9.30 induces the expression of Gli and Pax2/5/8-A, and represses *Otx* and *En* in neighboring cells at the tailbud stage (Fig. 4C). As a result, the neighbors of cells that express *Fgf8/17/18* give rise to a morphologically distinct structure called the 'neck' between the ganglion and the brain.

**Epidermal cells.** The expression profiles of regulatory genes indicate that there are at least seven different epidermal territories in the tailbud embryo (Fig. 4D). Specification of the ventral and lateral regions of the trunk epidermis cannot be explained simply by combinatorial regulation, because the gene expression profiles of cells that contribute to these two regions and neighboring regions did not provide plausible hypotheses to explain how the clear boundary between these two regions is established. This, along with the observation that BMP signaling is involved in specification of the ventral epidermal region,[23),91)] led to identification of a novel secreted BMP-antagonist, Pinhead.[92)] This factor specifically

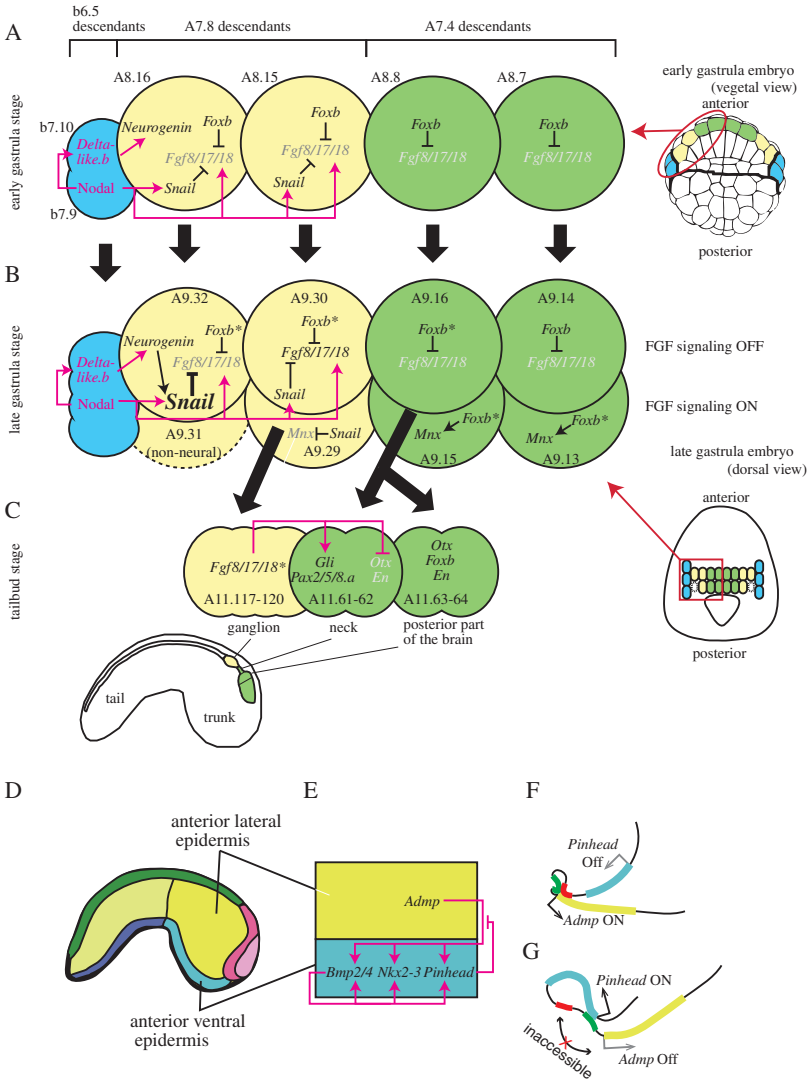


Fig. 4. Specification and patterning of the nerve cord and epidermal territories at later stages. **(A–C)** Regulatory interactions in the lineages that form the nerve cord in (A) early gastrula, (B) late gastrula, and (C) tailbud embryos. The nerve cord is derived from three lineages of cells. The A7.4 (green) and A7.8 lineages (light yellow) are derived from A7.4 and A7.8 of the vegetal hemisphere of the 64-cell embryo, and the b6.5 lineage (blue) are derived from b6.5 of the animal hemisphere of the 32-cell embryo. Fused circles indicate multiple cells with the same regulatory interactions. Only cells that form the posterior part of the brain, the neck region, and the visceral ganglion are shown at the tailbud stage. Thick black arrows indicate cell lineages. Normal and flat-head thin arrows indicate positive and negative regulation, respectively. Regulatory interactions through signaling pathways are shown in magenta. Genes that are expressed are shown in black letters, and genes that are suppressed are shown in gray letters. Genes marked by asterisks are not transcribed, but their protein products derived from mRNA transcribed in ancestral cells are expected. **(D–G)** Admp/Bmp signaling specifies the ventral epidermal region and the lateral epidermal region of the trunk. (D) Epidermal territories based on gene expression profiles. The seven distinct territories do not necessarily coincide with clonal territories of cells. (E) Regulatory interactions between the ventral epidermal region and the lateral epidermal region of the trunk. Normal and flat-head thin arrows indicate positive and negative regulation, respectively. Note that Pinhead directly binds to Admp, thereby antagonizing the activity of Admp. (F, G) In addition to the protein–protein interaction between Admp and Pinhead, *Pinhead* transcription represses *Admp* transcription. The *Pinhead* and *Admp* genes are encoded as neighbors in the genome and transcribed in the same direction. The *Admp* enhancer (red) is located near the *Pinhead* coding region (light blue), and the enhancer necessary for *Pinhead* and *Admp* transcription (green) is located near the *Admp* coding region (yellow). (F) When *Admp* is transcribed, the *Admp* promoter interacts with the *Admp* enhancer (red) and the enhancer necessary for *Pinhead* and *Admp* transcription (green). (G) *Pinhead* transcription sequesters the *Admp* enhancer in a DNA loop formed between the *Pinhead* promoter and the enhancer indicated by a green box. The *Pinhead* promoter always wins the competition for the common enhancer necessary for *Pinhead* and *Admp* transcription (green), when it is activated by signaling of Admp and Bmp2/4. Therefore, once *Pinhead* transcription is turned on, *Admp* is never transcribed.

interacts with Admp, a member of the BMP signaling molecule family, but not with Bmp2/4. Admp is expressed in the lateral epidermal region of the early tailbud embryo, and the Admp signal activates *Pinhead* and *Bmp2/4* in the ventral region (Fig. 4E) probably through the action of Chordin, which is an antagonist for Admp and Bmp2/4 and is expressed in the dorsal region of the epidermis. Once activated, Pinhead starts to antagonize Admp, and prevents the ventral region from being further expanded by Admp. Because Bmp2/4 is not antagonized by Pinhead, it activates the regulatory genes specifically expressed in the ventral epidermis, including *Nkx2-3* (formerly called *Nk4*), via autocrine signaling.

*Admp* and *Pinhead* are present as a neighboring gene pair in genomes from insects to vertebrates.[92] When *Pinhead* transcription is activated by Admp or Bmp2/4, a DNA loop is formed between the promoter and enhancer of *Pinhead*. This DNA loop sequesters the *Admp* enhancer, making it inaccessible to the *Admp* promoter (Fig. 4F and G). Thus, *Admp* transcription is turned off in the ventral cells where *Pinhead* is actively transcribed. This mechanism of transcriptional regulation provides a plausible explanation for the conservation of this bi-gene cluster among the bilaterian animals.

### Is the elucidated gene regulatory network complete?

In this section we consider how we can know to what extent combinatorial regulation encoded in the elucidated gene regulatory network explain spatial and temporal gene-expression patterns. Although this problem cannot be easily experimentally tested, it could be investigated by theoretical methods. Unfortunately, currently available methods have not yet succeed in providing solid evidence that the network is indeed sufficient for explaining every gene expression in embryos. On the other hand, there are certain minimum requirements that the network needs to satisfy,[93)–95)] and it might be easier to test whether these minimum requirements are satisfied, as discussed below.

The notion of combinatorial regulation implicitly assumes that unique combinations of regulatory inputs cause the genome to express unique combinations of genes. If this widely accepted assumption is not satisfied in the elucidated network, it implies that the network is incomplete; there might be unknown regulatory genes, novel regulatory mechanisms, or complex regulatory mechanisms that cannot be easily uncovered.

A theoretical study revealed that the dynamics of complex networks can be monitored by measuring the activity of a subset of genes, called "determining nodes".[95] In other words, measuring the activity of a subset of genes in a given cell will predict the ultimate fate of that cell. There are several key transcription factor genes. For example, *Brachyury* is essential and sufficient for differentiation of the notochord. The theory predicts that there might be determining nodes upstream of these key transcription factors. *Foxd* is one of the candidates.[95] We may test hypotheses deduced from this theory by directly manipulating the activity of determining nodes to control the network.

In this review, we have essentially treated the gene regulatory network as a Boolean network, in which gene activity is considered to be fully activated or to be fully turned off. However, this might be an over-simplification, given that differences in the expression level of *Snail* are utilized for localization of *Fgf8/17/18* expression. That said, except for this one exception, to date we identified no logical contradiction with the assumption of the Boolean network.

Intercellular interactions are critical for the functions of gene regulatory networks in animal embryos. Because geometrical relations among blastomeres are a critical factor, this information needs to be collected precisely. Virtual reconstruction of the embryo provides a practical means for accomplishing these goals. Early *Ciona* embryos have been virtually reconstructed based on a series of images captured by a confocal microscopy, and these data were then used to test the possibility that contact surface areas between cells can be used to predict the strength of inductive signals.[46] Because the ascidian embryo consists of a small number of cells, it provides an ideal system for quantitatively modeling the cell–cell interactions.

### Does the elucidated gene regulatory network explain temporal control of gene expression?

Above, we mainly argued that the gene regulatory network causally explains spatial control of gene expression. Temporal control by gene regulatory networks is important, especially for cell–cell interactions, because gene regulatory networks need to be temporarily coordinated between interacting cells. The simplest system for achieving this regulation would be one in which the progression of the network depends on the kinetics of transcription and translation. That is, after a defined period of time, a sufficient amount of mRNA and its protein product

are synthesized to activate the target genes. Such a mechanism seems to play a major role in progression of the gene regulatory network in the sea urchin embryo.[96)]

However, the kinetics of mRNA and protein production are not likely to be the only limiting factor in *Ciona* embryos. It is widely believed that the necessary levels of transcription factors differ between enhancers, depending on the numbers and properties of binding sites. Hence, even genes that are activated by the same set of transcription factors should start to be expressed at different time points, depending on characteristics of their enhancers (we will show an example of Brachyury target genes below). However, the results of comprehensive *in situ* hybridization of regulatory genes in the *Ciona* embryo[22)] did not reconcile with this prediction, because most (or possibly all) genes almost simultaneously began to be expressed soon after cell division. To date, no genes that start to be expressed in the middle of a cell cycle have yet been identified in early embryos.

Cell cycles and gene regulatory networks often regulate each other. In vertebrate skeletal muscle cells, the key transcription factors MyoD and Myf5 control cell-cycle withdrawal and induction of differentiation.[97)] In *Xenopus*, Neurogenin2 is phosphorylated in response to rising cyclin-dependent kinase (cdk) levels, and the Neurogenin2-dependent differentiation program does not begin before cells stop dividing.[98)] In both of these cases, the cell cycle halts the activities of the gene regulatory networks, and extracellular signals restart them. A similar example in *Ciona* embryos is suppression of Cdc25 activity in neural tube cells during neural tube closure; in this process, the *Cdc25* gene may be regulated transcriptionally.[99)] These components might be involved in regulation of chromatin states[100)] and poised RNA polymerase II.[101)] However, there are no reports that perturbation of components of the gene regulatory network clearly affects cell cycles in the early *Ciona* embryo, and the network likely proceeds like a cascade reaction in early embryos.

Gene expression is also temporally controlled by gene regulatory circuits. As described above, the gene circuit consisting of *Bz1* and *Bz2* temporally controls *ZicL* expression in the neural lineage.[40)] This gene circuit measures time via an auto-regulatory loop. With this time-delay circuit, the genetic program for specification of the brain starts after the split between the brain and palp fates. A recent study showed that expression of notochord-specific genes with multiple and single functional Brachyury binding sites begins around the early gastrula and neural plate stages, respectively, and expression of indirect targets of Brachyury begins around the early neurula stage.[102)] Thus, the difference of the necessary levels of Brachyury likely determines when the early- and middle-onset genes begin to be expressed, and the late-onset genes are expressed because their direct activators need to be activated under the control of *Brachyury* first. Such 'built-in' gene timer circuits have begun to be understood. However, especially in early embryos, because these gene circuits still depend on the kinetics of mRNA and protein production, there might be novel mechanisms that synchronize the gene regulatory network to the cell cycle, and thereby coordinate the independent networks in different cells.

## Does the gene regulatory network give us clues about animal evolution?

The gene regulatory networks that construct animal bodies are the products of evolution, and changes in the structure of these networks underlie evolution.[103)] Therefore, comparisons of gene regulatory networks between animals are more informative than comparisons of gene-expression patterns, which can sometimes be misleading.

It has been long debated whether the ascidian has a structure homologous to the vertebrate midbrain.[104)–106)] From a morphological and anatomical viewpoint, no such structure exists in the ascidian. On the other hand, different parts of the ascidian brain express orthologs of genes that are expressed in the vertebrate midbrain, leading to the aforementioned debate. The vertebrate midbrain is formed by the midbrain-hindbrain boundary (MHB) organizer; Fgf8 and Wnt1 are the key factors that control the boundary between the midbrain and the hindbrain.[107)–109)] Although the ascidian genome lacks an ortholog of *Wnt1*, the ascidian brain is regionalized by the regulatory gene circuit controlled by *Fgf8/17/18* expressed at the late gastrula stage,[24)] as we described above (Fig. 4A–C). This strongly suggests that the last common ancestor of *Ciona* and vertebrates used *Fgf8/17/18* and a gene circuit controlled by *Fgf8/17/18* to regionalize the brain. Although this gene is again expressed in the central nervous system of mid-tailbud embryos and this expression made the debate complicated, the gene regulatory network indicates that this later expression should not be compared with the expression of *Fgf8* in the vertebrate MHB. Thus, gene regulatory networks give us deeper insights than gene expression

patterns into the evolutionary changes that have occurred in the genome.

The brain is an ancient structure, and the anterior placode has been considered to be an innovation of vertebrates or chordates. As stated in Fig. 3D, knockdown of *Bz1* and *Bz2* results in loss of the palp, a rudimentary anterior placode, and ectopic induction of brain in its place. The *Bz1/Bz2* gene circuit delays induction of brain fate via Fgf9/16/20 signaling. During this time period, the presumptive brain cells divide, and produce a cell population that does not abut on Fgf9/16/20-expressing cells. Therefore, it is a plausible hypothesis that addition of the *Bz1/Bz2* gene circuit enabled a sub-population within the neural cell population to form the palp, and that this took place before the divergence between *Ciona* and vertebrates.

The ascidian larva has pigment cells called the ocellus and otolith. These cells are derived from the lateral border of the neural plate. In vertebrates, neural crest cells that contribute to pigment cells are also derived from the neural plate border. The gene regulatory network in the pigment cell lineage of the *Ciona* embryo is similar to that in vertebrate embryos, although the ascidian larva does not have distinct neural crest cells. The critical difference between the networks of *Ciona* and vertebrates might be the activation of *Twist* in vertebrate neural crest cells.[110] In *Ciona*, *Twist* (*Twist-r.a/b*) is specifically expressed in the mesenchyme lineage but not in the neural plate border.[81] When expression of *Twist-r.a/b* is forced in the pigment cell lineage, these pigment cells migrate in a manner that is evocative of vertebrate neural crest cells. It is therefore attractive to hypothesize that the co-option of *Twist* into the neural plate border cells occurred in the vertebrate lineage, resulting in creation of the neural crest.

Gene regulatory networks change by altering existing networks. Alterations that caused catastrophic effects on developmental programs are thought to be negatively selected. Hence, the existing networks constrain subsequent network changes, and not every alteration is possible. Elucidating the kinds of changes that are possible in extant gene regulatory networks will help us to understand how these networks have evolved. Previous studies showed that loss of a particular structure can be achieved relatively easily via a small number of changes in a gene circuit that turns on a specific developmental program. For example, vertebrate limbs can be lost by changes in expression of homeobox genes.[111),112] On the other hand, we do not yet know how the ancestral vertebrates acquired limbs. In other words, we do not yet sufficiently understand how novel structures arose, although we have begun to understand the origins of novel structures such as the anterior placodes and neural crest of vertebrates by analyzing gene regulatory networks.

## Conclusions

It is widely believed that combinatorial regulation is a major principle that explains differential gene expression in animal development. Indeed, the gene regulatory network in *Ciona* embryos causally explains the spatial expression of most genes. Incomplete parts of the elucidated network have led to identification of unknown regulatory genes, novel regulatory mechanisms, or complex regulatory mechanisms that cannot be easily uncovered.

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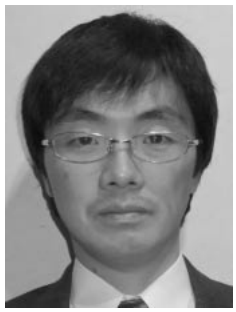
## Profile



Yutaka Satou received a Ph.D. degree in science from Kyoto University in 1999 under the supervision of Professor Noriyuki Satoh. He became an assistant professor in 1999 and then an associate professor in 2004 at Graduate School of Science, Kyoto University. He worked with Professor Michael Levine at University of California, Berkeley for one year from 2005 to 2006 as a visiting scientist. He received the JSPS Prize in 2014. He played a major role in accomplishing the genome project of an ascidian, *Ciona intestinalis*, and is studying molecular mechanisms of embryonic development by taking full advantage of his experience in the genome project.