Gene Regulation: Gene Control Network in Development

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Abstract

Controlling the differential expression of many thousands of genes is the most fundamental task of a developing organism. It requires an enormous computational device that has the capacity to process in parallel a vast number of regulatory inputs in the various cells of the embryo and come out with regulatory outputs that are tissue specific. The regulatory genome constitutes this computational device, comprising many thousands of processing units in the form of *cis*-regulatory modules. The interconnected *cis*-regulatory modules that control regulatory gene expression create a network that is the underlying mechanism of specification. In this review we use the gene regulatory network that governs endomesoderm specification in the sea urchin embryo to demonstrate the salient features of developmental gene regulatory networks and illustrate the information processing that is done by the regulatory sequences.

Contents

INTRODUCTION

A single cell, the fertilized egg, gives rise to dozens or even hundreds of cell types that have various cellular functions and that self-organize to form the adult body plan. That means the egg must contain both the initial conditions and the program that processes these initial conditions and translates them into specification and differentiation. While the initial conditions have the form of particular proteins or mRNA anisotropies, the hardware and the software of the developmental program are one, the genome. The genome contains all the instructions necessary to process the maternal anisotropies and transform them into differential gene expression. Because the egg and its descendants contain the same genome, a fundamental question emerges: How does a similar static code enable the dynamic differential gene expression? What is the program that regulates specification?

Regulatory genes: transcription factors and signaling molecules

Transcription factors: proteins that bind to specific sequences in the DNA and activate or repress the transcription of a gene

The regulatory apparatus contains two complementary components. One component is the regulatory genes, i.e., transcription factors and signaling molecules. Transcription factors bind to specific sequences in the DNA and activate or repress the transcription of a gene. Signaling molecules carry out the communication between the cells and initiate the activation of certain transcription factors in the cells that receive the signal. The regulatory state is defined as the total set of active transcription factors in a cell nucleus. The complementary part of the regulatory apparatus and the one that is similar for all the cells in the organism is the regulatory genome. Every gene contains regulatory sequences that control when and where it is expressed. The regulatory sequences are arranged in units that are termed *cis*-regulatory modules. Every *cis*-regulatory module contains a cluster of different transcription factor binding sites (**Figure 1*a***). A *cis*-regulatory module acts like an information processor, the input that it reads is the regulatory state of the cell and the output is either activation or repression of the gene that it controls. According to the data on *cis*-regulatory modules in bilaterian species, genes can have 5 to 20 *cis*-regulatory modules, each responsible for activating the gene in a particular time and domain in the organism (12) (**Figure 1*b***).

Specification is the process by which cells acquire identities or fates that they and their progeny will adopt. On the mechanism level that means the process by which cells reach the specific regulatory state that defines their identity and the differentiation genes that they express. To reach a given specification state the cells go through various regulatory states and one leads to the next. An initial set of transcription factors together with signaling cues

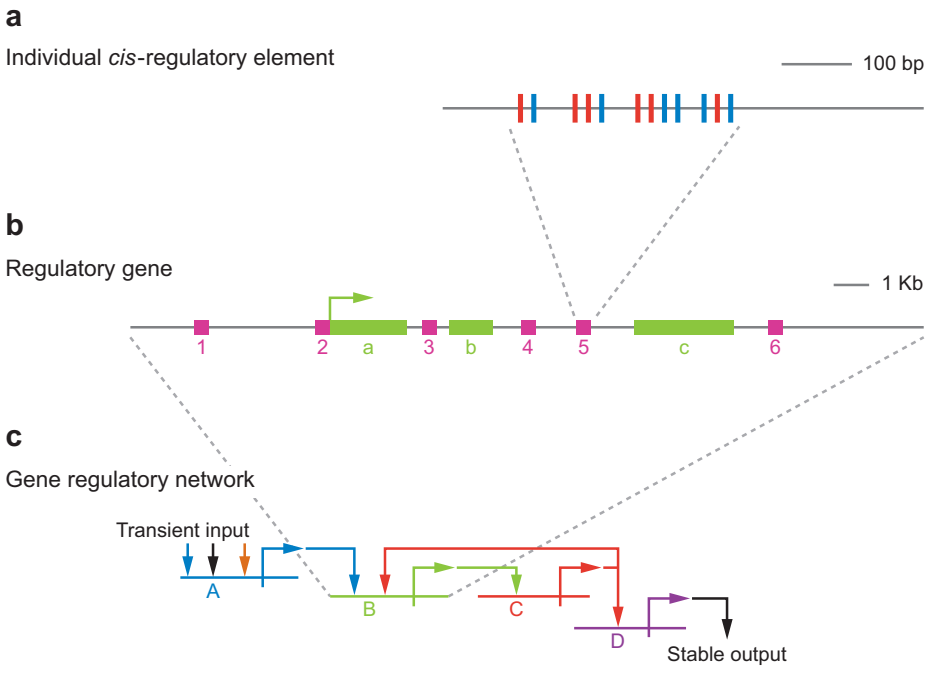


Figure 1

The gene regulatory hierarchy. (*a*) An individual *cis*-regulatory module contains a cluster of several transcription factor binding sites, indicated in red and blue boxes. (*b*) A gene contains a number of *cis*-regulatory modules (*pink boxes*) that control its expression at different times and lineages in the developing embryo. The exons are indicated in light green boxes. (*c*) The inter-regulating transcription factors and signaling molecules form a network that is essentially the genomic program for specification. (Reprinted from Reference 12.) In this diagram the color codes of the three levels match, so panel *a* presents the *cis*-regulatory module of gene B (see panels *b* and *c*), which has binding sites of transcription factors A (*blue*) and C (*red*).

from the neighboring cells activates a number of *cis*-regulatory modules. The active modules turn on the expression of regulatory genes that construct the next regulatory state of the cell until specification and differentiation are achieved. These interregulating genes form a regulatory network that is essentially the genomic program for development. A node in the network is a regulatory gene and its multiple *cis*-regulatory modules that receive input from elsewhere in the network and provide output that is destined to targets elsewhere in the network (**Figure 1*c***). Studying gene regulatory networks enables the understanding of the mechanism underlying the developmental process at the most fundamental level (12).

The construction of a map of a gene regulatory network is based on extensive experimental data of all the genes the network comprises (38). The identification of regulatory genes is becoming more feasible with the sequencing of the genomes of diverse species. Candidate regulatory genes can be found by a computational analysis that identifies transcription factors and signaling molecules in the genome (6, 16, 17, 31). The spatial and temporal pattern of gene expression can be obtained by whole mount in situ hybridization (WMISH) and quantitative PCR, respectively (4, 18, 24, 37). The interconnection between the regulatory genes is based on a perturbation analysis in which the expression of each gene is blocked and the effect on the expression level

Signaling molecules: the molecules that carry out the communication between the cells

Regulatory state: the total set of active transcription factors in a cell nucleus at a given time and domain of the embryo

Table 1 Some models of gene regulatory networks for specification and differentiation[a]

Organism	Domain specification	Reference(s)
Sea urchin (Sp)	Endomesoderm	12, 14, 39
Starfish (Am)	Endoderm	15
Mouse (Mm)	Pancreatic β-cells	12, 47, and references therein
Mouse (Mm)	Hematopoietic stem cells and erythroid lineage specification	60
Mammals	B-cell specification	33, 52
Mammals	T-cell specification	1, 52
Vertebrates	Heart field specification	12, 13, and references therein
Frog (Xl)	Mesoderm	23
Ascidian (Ci)	Notochord	9, 10, 19, 56
Ascidian (Ci)	Different territories	19, 51
Fruit fly (Dm)	Dorsal-Ventral axis	25, 55–57
Fruit fly (Dm)	Heart field specification	12, 13, and references therein
Nematode (Ce)	Left-right ASE taste neurons	12, 21
Nematode (Ce)	Vulva	20
Nematode (Ce)	C cell lineage	2

[a]We include only networks for which a *cis*-regulatory analysis for some of the key regulatory genes is available.

Abbreviations: Am, *Asterina miniata*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Sp, *Strongylocentrotus purpuratus*; Xl, *Xenopus laevis*.

of all the other genes in the network is measured by WMISH (4, 19, 37, 44), quantitative PCR (39, 67, 68), or microarray examination (2, 23, 35, 57). The final and most reliable authentication of a network connection is done by a *cis*-regulatory analysis at the key nodes of the network (7, 8, 22, 27, 34, 36, 46, 63–66, 68–70). This is the only way to test whether a connection between two regulatory genes is direct.

Genome sequencing enables the application of comparative computational tools that help detect regulatory genes and their *cis*-regulatory modules (3, 57, 65). As a result there are an increasing number of gene regulatory network models that describe domain specification of various organisms. A list of some of the gene regulatory network models is given in **Table 1**.

At first glance the various gene regulatory networks for development look diverse in both structure and function. However, there are some general features that are universal to all developmental networks. (*a*) The specific combination of transcription factors is what leads to activation or repression of a particular *cis*-regulatory module, and not just the action of a single gene. This allows the recurring use of regulatory genes in diverse domains to create various combinations in different specification states. (*b*) The networks are modular and can be resolved into subcircuits in which every subcircuit is responsible for a specific developmental task. Different subcircuits are active in different domains and times in the embryo. (*c*) The network subcircuits are composed of typical functional elements: regulatory state turn-on by inherited anisotropy or signaling; specification establishment and persistence by, for example, positive-feedback loops; alternative fate exclusion and boundary formation by repressors; and subcircuit that

***Cis*-regulatory module:** a short DNA region that contains a cluster of different transcription factors binding sites that controls the activation or repression of a gene

Specification state: regulatory state that is cell type specific so it defines the cell identity and the differentiation genes that it expresses

constitutes a transient regulatory state may contain an internal turn-off element (e.g., autorepression).

In this review we explore the gene regulatory network that governs the specification and differentiation of the endomesoderm lineages in the first 30 h of the sea urchin embryo (2a, 14, 39). We analyze some of the network principle subcircuits, dissect the functional elements they contain, show how the wiring of these elements is encoded in the genome, and illustrate how the network subcircuits collaborate to execute the specification program over time.

GENE REGULATORY NETWORK FOR ENDOMESODERM SPECIFICATION IN THE SEA URCHIN EMBRYO

A gene regulatory network describes a process that occurs in several spatial domains in the developing embryo. The domains vary with time as the process progresses: Cells divide, change their volume, change their shape, and move relative to one another. Before we examine the specification process at the molecular level, we have to get acquainted with the different territories and the way they evolve. In **Figure 2*a*** we present a schematic diagram of the developing sea urchin embryo up to 55 h after fertilization (see legend for details). The endomesoderm lineages emerge from the vegetal plate. The fourth cleavage is uneven and results in small and large tiers of cells, the micromeres and the macromeres, respectively. At the fifth cleavage the micromeres divide into small and large micromeres. The large micromeres form a signaling center that initiates the specification of the vegetal plate. Following cleavage stage the 16 descendants of the large micromeres ingress into the blastocoel and later fuse to form the skeleton. These cells are called primary mesenchyme cells (PMCs) because they are the first to ingress into the blastocoel. Macromere descendants form the mesoderm and the endoderm. The tier of cells closer to the micromere descendants becomes mesoderm and gives rise to several cell lineages, e.g., pigment cells. These cells are termed secondary mesenchyme cells (SMCs). Gastrulation begins at about 30 h with the invagination of the cells that become the endoderm and with the formation of a gut.

The gene regulatory network for the sea urchin embryo development is presented in **Figure 2*b***. This is a static representation of all the known interactions between the regulatory genes that participate in the endomesoderm specification up to 30 h after fertilization. All these interactions are encoded in the DNA in the form of *cis*-regulatory elements (30); therefore we refer to this representation as a view from the genome. The developmental process is nevertheless dynamic. In every step and domain only some of the network nodes are active in a given cell, depending on its regulatory state. In order to understand the sequential establishment of regulatory states, we have to follow the regulatory states that are present in different domains and see which nodes are activated by them, that is, to observe from the point of view of the nucleus. This reduces the complex overall wiring to the functional subcircuits that compose the network and elucidates how these subcircuits give rise and maintain specification states.

The early stage is dominated by the onset of new regulatory states initiated by maternal anisotropy in the vegetal pole. In the next section we portray the subcircuits that contribute to this regulatory activity. The later stage the network describes is that of establishment and maintenance of specification states and activation of differentiation gene batteries. We describe some of the subcircuits that regulate this stage (see below).

EARLY STAGE: MATERNAL ANISOTROPY AND SIGNALING INITIATE DOMAIN SPECIFICATION

Maternal anisotropies are asymmetric distributions of particular proteins, mRNA, or even

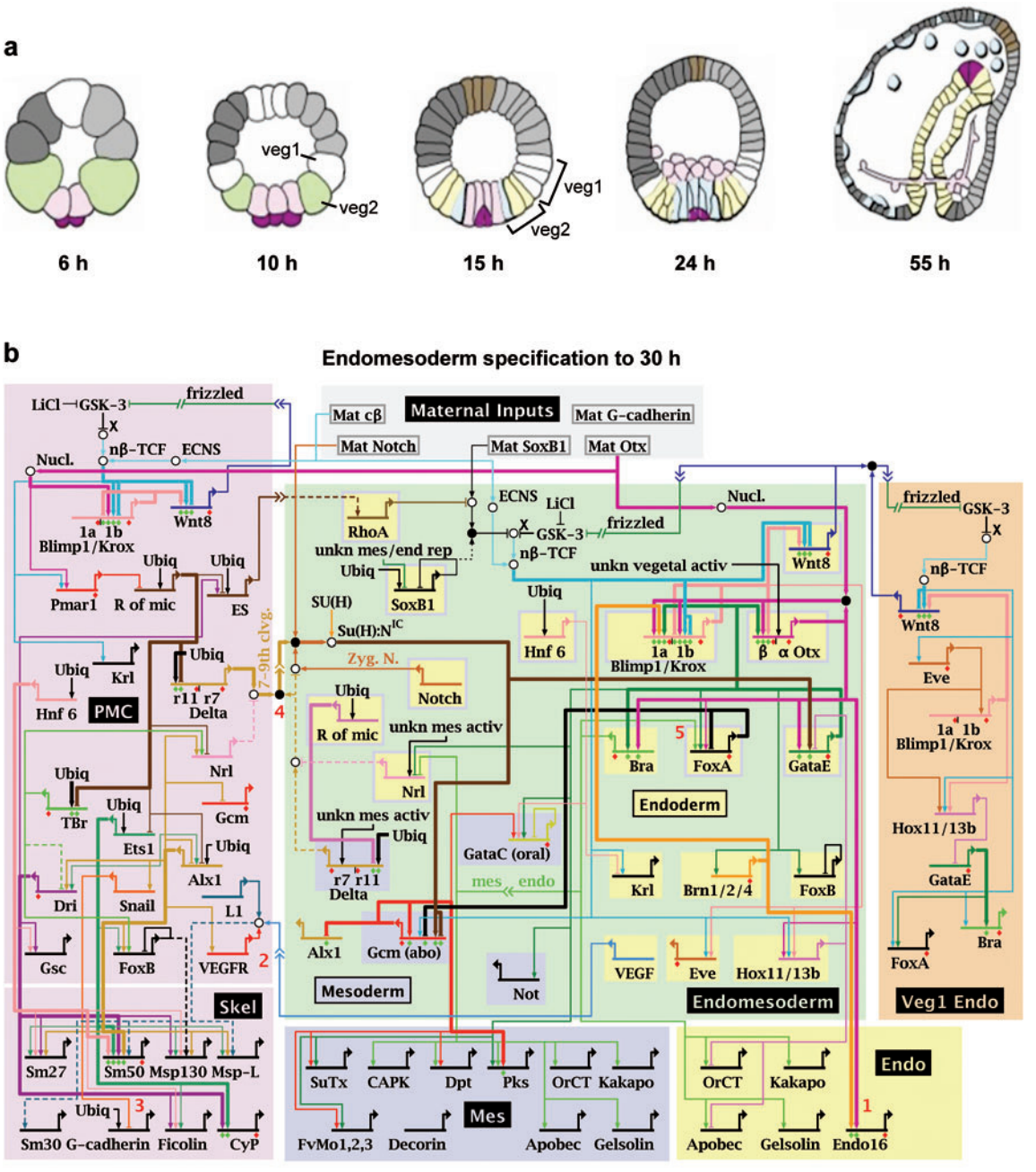
A node in the network: gene and relevant *cis*-regulatory modules that receive inputs from elsewhere in the network and provide regulatory output destined to targets elsewhere in the network

WMISH: whole mount in situ hybridization

PMCs: primary mesenchyme cells

SMCs: secondary mesenchyme cells

Differentiation gene batteries: functionally related gene sets that are controlled by the same transcription factors and that encode at the protein level the functional and structural properties of a cell type



cellular components in the egg. The localization of these components in the egg gives rise to localized regulatory activity in the embryo, which leads to axis formation and specification initiation (12). In early cleavage in sea urchin an inherited maternal anisotropy leads to the activation of particular regulatory genes in the micromeres that turn on three primary specification subcircuits. Two of the subcircuits include signaling elements that promote the advancement of the regulatory activity to the next tier of cells. The three subcircuits, named by the principal genes that compose them, are *β-catenin-wnt8-blimp1*, *Pmar1-Repressor of the micromeres*, and *delta-notch* signaling.

Dynamic Spatial Patterning by *β-catenin-wnt8-blimp1* Subcircuit

In **Figure 3*a*** we present a diagram that illustrates the *β-catenin-wnt8-blimp1* subcircuit. When β-catenin is stabilized so it enters the nucleus, it binds to the transcription factor TCF1 and forms a permissive complex that allows transcription. In cells where β-catenin is degraded by GSK-3, Groucho binds to TCF1 and together they form a dominant-repressive complex (43). Thus, stabilization of β-catenin leads to its entrance to the nucleus, where it removes the repression that is induced by Groucho so that *cis*-regulatory modules that have TCF1 binding sites are activated.

The degradation activity of GSK-3 is blocked by activated Disheveled protein. The Disheveled protein is distributed asymmetrically in the egg and is localized in the future vegetal pole, as was shown by Weitzel et al. (61) (**Figure 3*b***). Owing to this maternal anisotropy, β-catenin is initially stabilized only in the vegetal plate of the embryo, and mostly in the micromeres (29, 61) (**Figure 3*c***). In these cells nuclear localized β-catenin binds to TCF1 and removes the repression induced by the globally expressed Groucho (43) (**Figure 3*d***) so the gene *blimp1* is activated (**Figure 3*a***) (J. Smith & E.H. Davidson, unpublished data). The β-catenin-TCF1 permissive complex together with Blimp1 activates the expression of Wnt8 signaling molecule (34) (**Figure 3*a***). Wnt8 signal is received by the neighboring cells and induces further stabilization of β-catenin by activating the Disheveled protein. Therefore Wnt8 signaling has a positive feedback into Blimp1 and Wnt8 in the same ring of cells in a community effect and also turns on

Figure 2

(*a*) Schematic diagrams of the sea urchin (*Strongylocentrotus purpuratus*) embryo development. Light-purple cells at 6 h are the large micromeres, from their descendants the skeleton forms. The light-green tier of cells at 6 h is the macromeres. Their descendants form mesoderm and endoderm cells. The division of macromeres gives rise to two tiers of cells, veg1 and veg2. The veg2 ring of cells is colored light green at 10 h. Veg2 gives rise to several cell types, for example, pigment cells (*light blue*) and the endodermal domain that produces the gut (*yellow*). Gastrulation begins at approximately 30 h. (Modified from Reference 45.) (*b*) Gene regulatory network for endomesoderm specification in the sea urchin embryo, up to 30 h after fertilization. The network was initially presented by Davidson et al. (14) and is constantly updated at **http://sugp.caltech.edu/endomes/**, where the time course in the different domains and much of the underlying experimental data as a current list of supporting references are maintained. This version of the network was last updated November 30, 2006. The upper part of the network contains regulatory genes while the bottom rectangles include differentiation genes. Time proceeds from top to bottom, so that earlier events are depicted in the upper part of the regulatory network diagram. The network is separated into blocks that correspond to the three lineages, primary mesenchyme cells (PMCs), secondary mesenchyme cells (SMCs), and endoderm. Accordingly, the colors of the boxes in the diagram correspond with the colors of embryo domains in panel *a*. The genes that are active in veg2 cells and their descendants are shown in the light-green box. Genes that are active only in the SMCs are indicated in light-blue boxes, genes that are active only in endoderm cells are indicated in yellow boxes, and genes that are active in both cell lineages are indicated in yellow boxes framed by light blue.

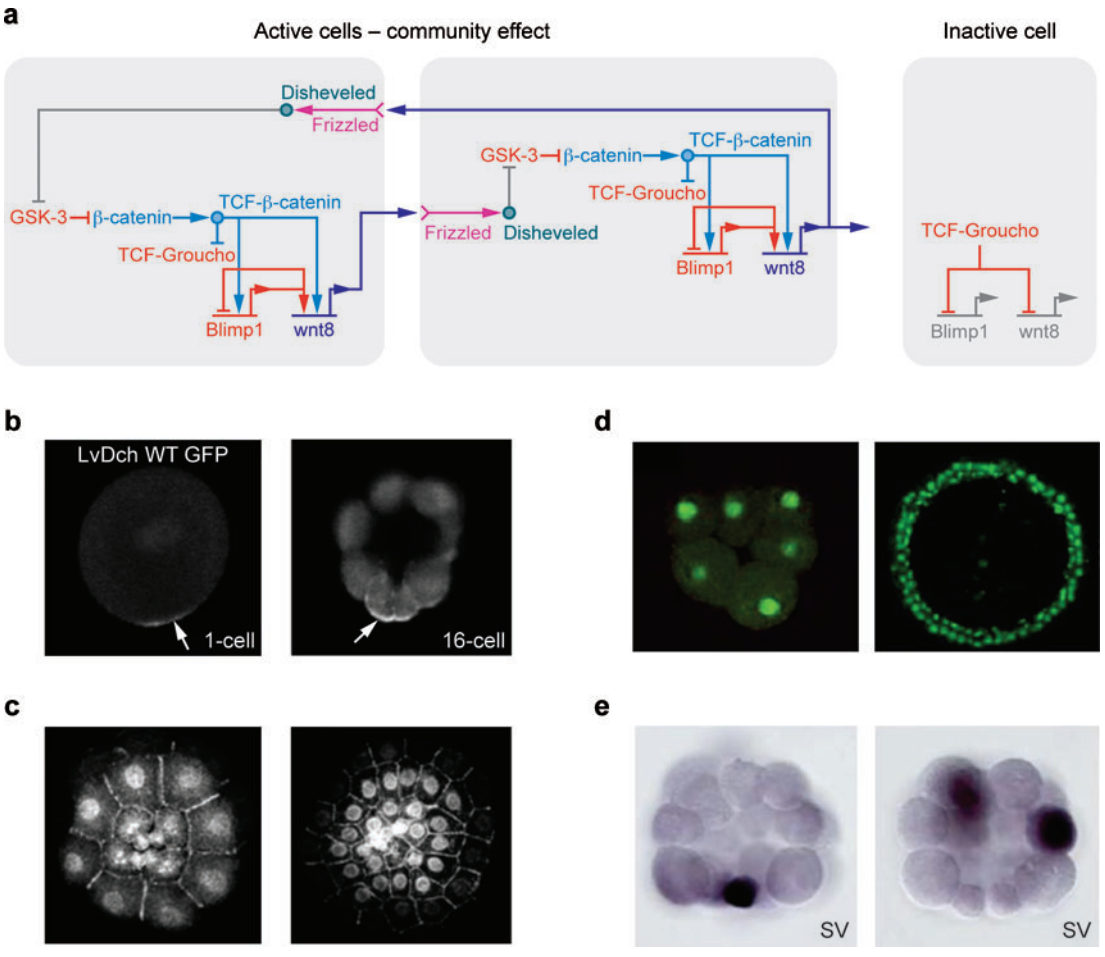


Figure 3

β-catenin-wnt8-blimp1 subcircuit. (*a*) Left: An illustration of the *β-catenin-wnt8-blimp1* subcircuit in two neighboring cells. In the two cells activated Disheveled blocks GSK-3 from degrading β-catenin. Stabilized β-catenin enters the nucleus and forms a permissive complex with TCF1 that turns on *blimp1*. Blimp1 and β-catenin-TCF1 complex together activate the expression of *wnt8*, a signaling molecule. Wnt8 reception by the Frizzled receptor of the neighboring cell further activates the Disheveled protein in a community effect. The subcircuit is turned off by Blimp1 autorepression and the successive shutoff of the *wnt8* gene. The reception of the Wnt8 signal by the next tier of cells turns on this subcircuit there, so the expression of *wnt8* and *blimp1* forms a ring pattern that advances from the vegetal plate toward the animal pole (J. Smith & E.H. Davidson, unpublished data). Right: In cells where β-catenin is unstable, Groucho binds to TCF1 and represses *blimp1* and *wnt8*. (*b*) Anisotropic distribution of the Disheveled-GFP fusion protein that illustrates the endogenous protein distribution, in the egg (*left*) and at the 16-cell stage (*right*). (Reprinted from Reference 61.) (*c*) Nuclearization of β-catenin in the vegetal plate of the sea urchin blastula at the fifth and the seventh cleavage in *Lytechinus variegatus*. (Reprinted from Reference 29.) (*d*) Groucho is present ubiquitously in all the nuclei of the sea urchin embryo at the 16-cell stage and at hatched blastula stage. (Reprinted from Reference 43.) (*e*) *wnt8* regulation by the β-catenin-TCF1 permissive complex is encoded in the *cis*-regulatory modules of the *wnt8* gene. WMISH of a reporter construct that contains a region of Wnt8 *cis*-regulatory module shows correct expression similar to that of the *wnt8* gene (*left*). Mutation of two TCF1 binding sites results in ectopic expression in the ectoderm, since Groucho cannot repress transcription when TCF1 does not bind to the DNA (*right*). (Reprinted from Reference 34.)

this subcircuit in the next tier of cells (62) (**Figure 3*a***).

While Wnt8 signaling is responsible for the expansion of this subcircuit toward to animal pole, Blimp1 is responsible for shutting it off in the domains it has already passed (28; J. Smith & E.H. Davidson, unpublished data). Blimp1 autorepresses itself, and because its input is required for the activation of *wnt8*, *wnt8* expression is also stopped (**Figure 3*a***). The expression of these two genes and of other particular genes regulated by β-catenin-TCF1 forms a ring that moves from the vegetal pole toward the animal pole (J. Smith & E.H. Davidson, unpublished data). The use of one of the subcircuit internal components to sequentially shut it off is a parsimonious and elegant way to control well-timed spatial expression pattern.

The anisotropic distribution of the Dishevelled protein that leads to the onset of this subcircuit is inherited from the egg. Yet the processing of this information and the induction of the expression output are performed by the *cis*-regulatory modules of *blimp1* and *wnt8* (34; J. Smith & E.H. Davidson, unpublished data). In **Figure 3*e*** we present some of the experimental evidence for the wiring of this subcircuit that was obtained by studying the *wnt8 cis*-regulatory elements (34). The *cis*-regulatory module that controls the early expression of *wnt8* has both Blimp1 and TCF1 binding sites, and both inputs are necessary for the activation of the gene. As expected, TCF1 binding sites are also responsible for the repression of the *wnt8* gene outside the vegetal plate and for the spatial localization of this subcircuit (34).

Autonomous Micromere Specification by *Pmar1* Subcircuit

β-catenin entrance to the nucleus enables the expression of key regulatory genes. In the micromeres, nuclearized β-catenin together with the maternal transcription factor Otx (26) activates the expression of a repressor, Pmar1, leading to the onset of the micromere regulatory state (37). Pmar1 represses a ubiquitous repressor, Repressor of micromeres, allowing transcription of micromere-specific genes such as the signaling molecule Delta and the transcription factors Ets1, Alx1, and Tbr (37, 41) (**Figure 4*a***). It is also required for the secretion of an early signal from the micromeres to the neighboring macromeres that is necessary for the endomesoderm specification (41). Ets1, Alx1, and Tbr activate the expression of other regulatory genes and also the expression of the skeletogenic differentiation gene batteries. Delta reception by the next tier of cells induces the activation of essential lineage-specific regulatory genes.

In **Figure 4*b*** we show perturbation data that demonstrate Pmar1 regulation of *delta* and *tbr*. Embryos injected with *pmar1* mRNA, so the Pmar1 protein is expressed globally, show extensive ectopic expression of *delta* and

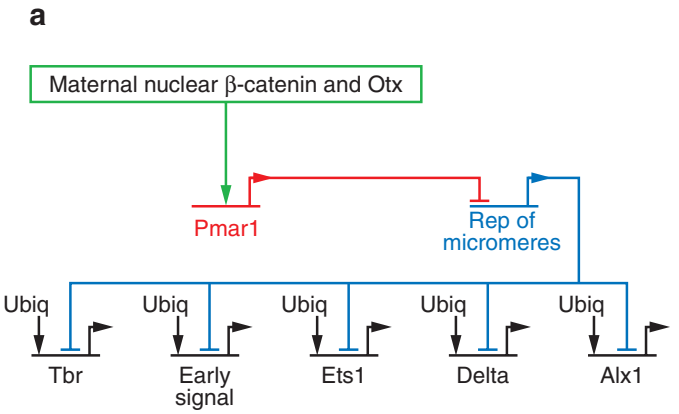


Figure 4

Pmar1-repressor of micromere subcircuit. (*a*) Network diagram of this subcircuit. Nuclearized β-catenin together with maternal Otx activates the repressor Pmar1 in the micromeres. Pmar1 represses an otherwise globally active repressor, Repressor of micromeres, allowing the expression of downstream micromere-specific regulatory genes. (*b*) WMISH of *delta* and *tbr*, which are downstream of the Pmar1 activity. The normal expression of these genes is localized to the micromeres (*left*). When Pmar1 is expressed globally because of *pmar1* mRNA injection, the genes are expressed ectopically in all the cells of the embryo, probably owing to the activation by a ubiquitous activator (*right*). (Reprinted from Reference 37.) (*c*) GFP reporter construct of a *cis*-regulatory module that controls the expression of the gene *delta* in the micromere lineage shows expression similar to that of the endogenous gene (*left*). When the reporter construct was coinjected with *pmar1* mRNA, GFP was expressed globally. (Reprinted from Reference 46.)

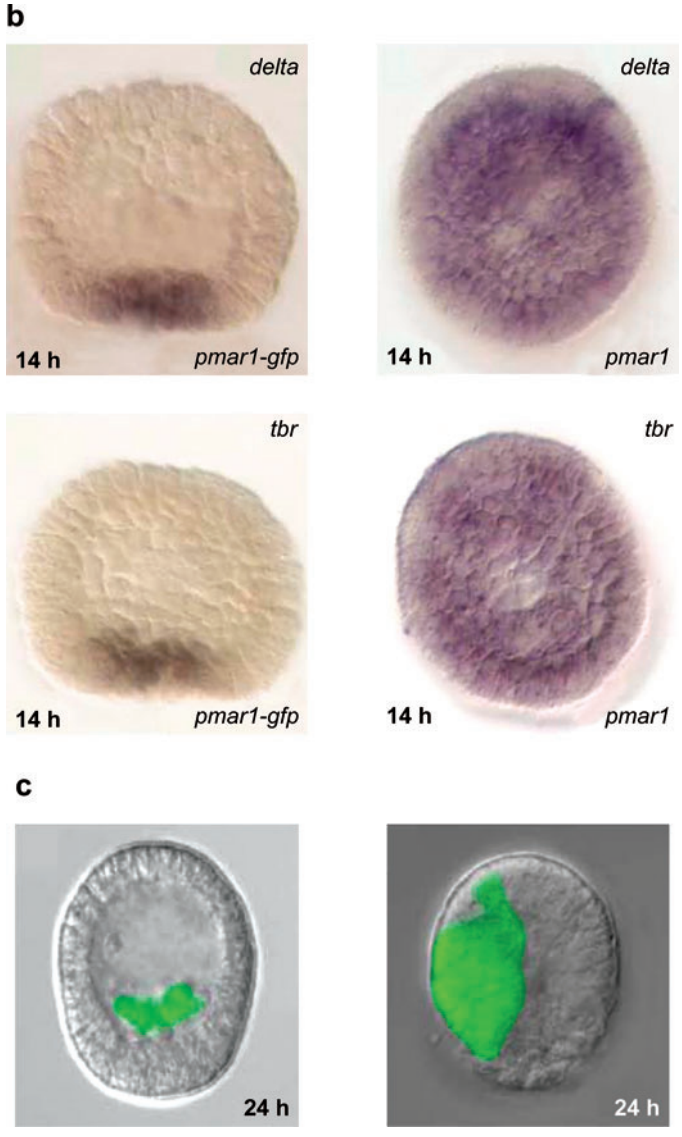


Figure 4
(*Continued*)

tbr: Instead of a localized expression in the primary mesenchyme cells (PMCs), *delta* and *tbr* are expressed in all the cells of the embryo, probably owing to a ubiquitous activator (37). To study the genomic code for this subcircuit a *cis*-regulatory module that controls *delta* expression in the PMCs was isolated (46). **Figure 4*c*** shows that the expression of a reporter construct controlled by this module is similar to that of *delta* in a normal embryo. When the construct was coinjected to fertilized eggs together with *pmar1* mRNA, the GFP is expressed globally, similarly to the endogenous gene.

NIC: Notch intracellular domain

Su(H): Suppressor of Hairless

SMC Specification Induced by *delta-notch* Signaling

One of the regulatory genes downstream of Pmar1 regulation is the signaling molecule Delta (58). Delta binds to Notch receptor in the neighboring tier of cells (32, 48–50, 59) (**Figure 5*a***). As a result the Notch intracellular domain (NIC) enters the cell nucleus and competes with the corepressor, Groucho, on binding to the transcription factor Suppressor of Hairless [Su(H)] (7). Similar to the β-catenin-TCF1 interaction, NIC and Su(H) form an activating complex so the repression that was induced by the corepressor is removed. The Su(H)-NIC complex activates the expression of the gene *gcm*, which initiates the pigment cell specification state establishment (43a). In **Figure 5*b*** we can see how *gcm* is expressed only in the ring of cells that directly contacts the micromere descendants (44).

The Delta-Notch pathway changes the regulatory state of the receiving cells. The code that transforms this change into the onset of gene expression is encrypted in the *cis*-regulatory element of the downstream genes. **Figure 5*c*** portrays how the *gcm* response to the Delta-Notch pathway is encoded in this gene's *cis*-regulatory module (43a). A GFP reporter construct of a *cis*-regulatory module of the *gcm* gene expresses correctly when injected into sea urchin fertilized egg. However, when two Su(H) sites are mutated, extensive ectopic expression is observed. When the intact construct is injected into eggs expressing a dominant negative of Su(H) that prevents Su(H) regulatory activity, a similar ectopic expression is detected. These two complementary experiments show that indeed Su(H) acts as a *gcm* repressor in the domains that do not receive the Delta-Notch signal.

The main developmental task in the early hours is to turn on new specification states in

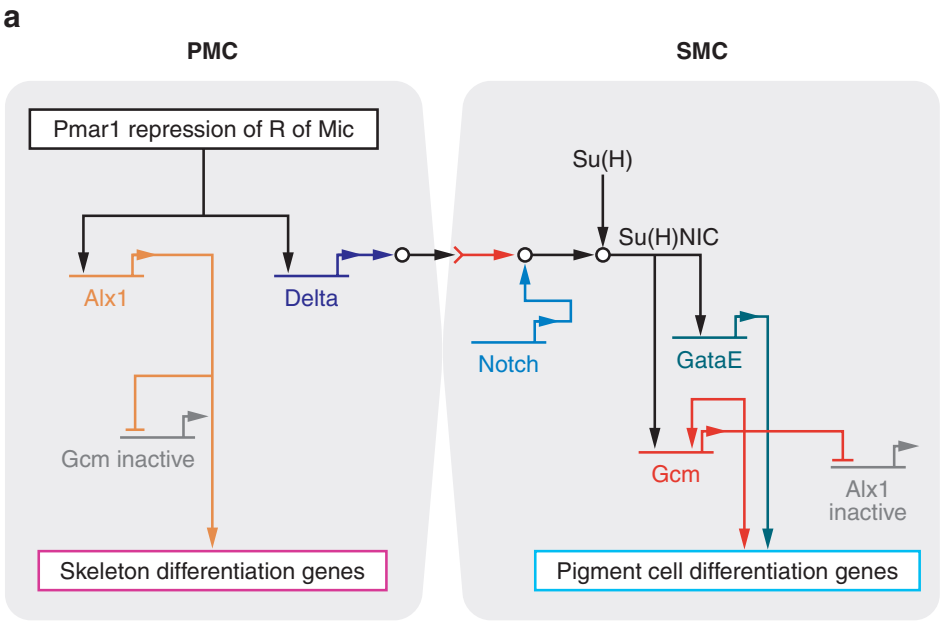


Figure 5

PMC and SMC specification subcircuits. (*a*) Pmar1 activity in the micromeres activates *alx1* and *delta*. Delta is secreted and received by Notch receptors in the neighboring SMC. Following the Delta reception, NIC enters the nucleus and competes with a dominant corepressor on binding to Su(H). Su(H) and NIC form an activation complex that turns on the genes *gcm* and *gatae*. *gcm* locks itself on and activates a pigment cell differentiation gene battery. *gcm* and *alx1* mutually repress each other to prevent regulatory state ambiguity in the SMC and the PMC. (*b*) WMISH of the *gcm* gene shows that at 12 h the gene expression is confined to the ring of cells that receive the Delta signal (*top*). These cells ingress into the blastocoel, later in development (*bottom*). (Reprinted from Reference 44.) (*c*) *cis*-regulatory control of the *gcm* response to Delta-Notch signaling (43a). Scoring of a GFP reporter construct regulated by *cis*-regulatory modules of *gcm*. The graphs indicate the percent of embryos that express correctly in the SMC, ectopically in other embryo domains, or in both. The reporter construct expresses correctly by 80% of the embryos. A modified construct in which Su(H) binding sites were mutated expresses mostly ectopically. Similar percents of ectopic expression are observed when the intact construct is injected into embryos that express a dominant negative of Su(H) (DnSuH). Both the mutation of Su(H) binding sites and the presence of DnSuH eliminate the repression activity of Su(H) and therefore the ectopic expression. (Reprinted from Reference 12.)

the different domains. The subcircuits that we present in this section demonstrate how maternal anisotropic input is processed by the *cis*-regulatory modules of regulatory genes into the onset of new spatially localized regulatory states. The anisotropic distribution of Disheveled gives rise to localized nuclearization of β-catenin that turns on a *β-catenin-blimp1-wnt8* subcircuit and activates *pmar1* expression in the micromeres. The Pmar1 activity sets off the micromere lineage specification state and activates Delta-Notch signaling. The reception of the Delta signal by the SMCs instigates the pigment cell lineage specification state. The activation of the *β-catenin-blimp1-wnt8* subcircuit in the endoderm turns on the endoderm specification subcircuit (see below).

These three turn-on subcircuits utilize a common mechanism. They cause activation in particular domains and preclude activation elsewhere. In the case of Wnt8-β-catenin and Delta-Notch the globally expressed corepressor Groucho is replaced by coactivator

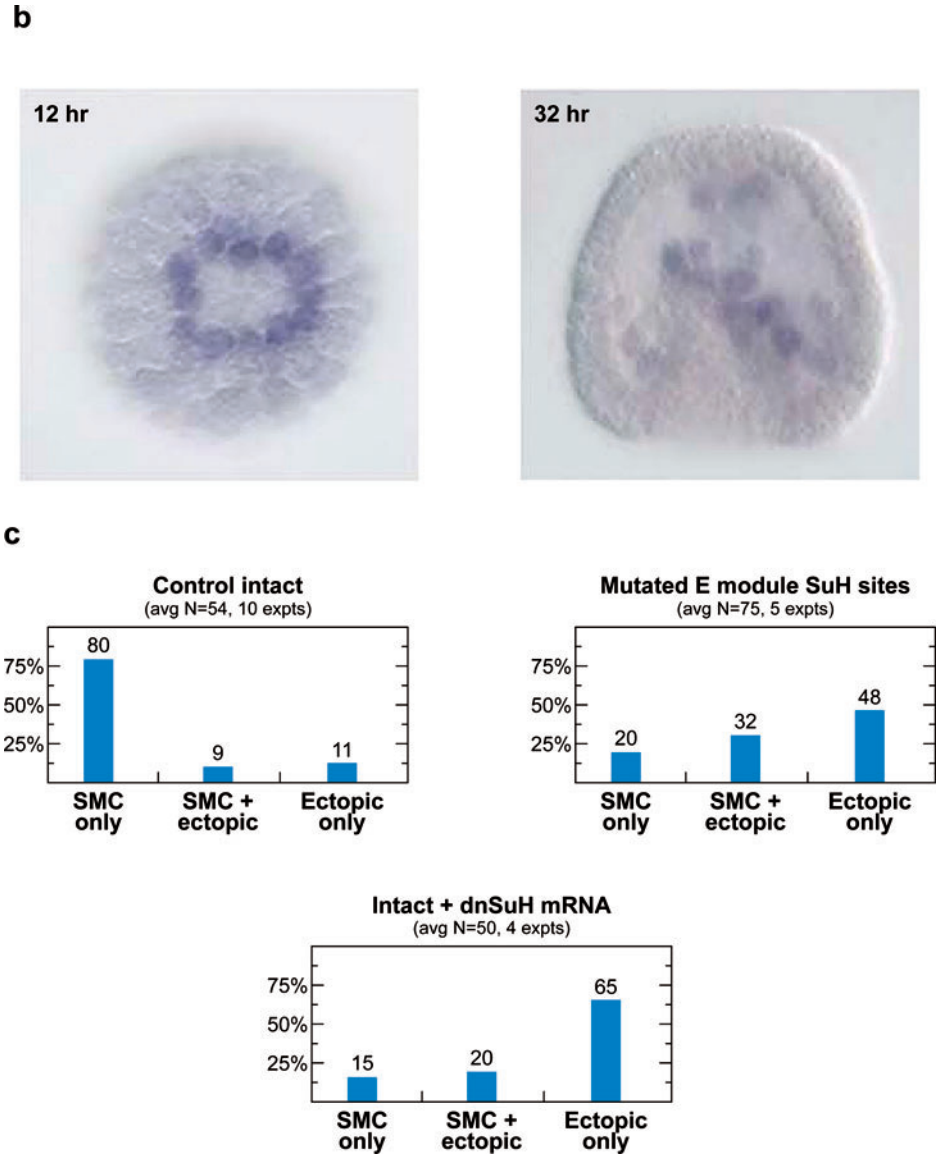


Figure 5
(*Continued*)

in a restricted domain. In the case of Pmar1 the globally expressed Repressor of the micromeres is repressed only in the micromeres so the downstream genes are activated there. The use of global repression and local activation to define the boundaries of the new domains is a feature of the earlier stages in which the emerging territories are few. Later in development a fine-tuning of the activation and repression takes place. As specification states are established localized repressors are turned on to prevent specific alternative fates.

LATER STAGE: SPECIFICATION STATE ESTABLISHMENT AND PERSISTENCE

The later stage of the blastula is governed by positive-feedback loops that promote the establishment of specification states and by

turn-on of localized repressors that exclude alternative fates. In this section we describe the subcircuits involved in the specification of two mesoderm lineages and the endoderm lineage. In the first subsection we present the subcircuits that are active in the early mesoderm specification. In the second subsection we present the subcircuits responsible for the subdivision of SMC and endoderm lineages and the formation of the gut.

Early Mesoderm Subcircuits: PMC and SMC Specification

The mesoderm territory consists of the cells between the outer cell territory, the ectoderm, and the inner cell territory, the endoderm. The network describes the specification of two mesoderm cell lineages, the skeletogenic lineage and the pigment cell lineage. The skeletogenic lineage is formed from the large micromere descendants, the PMCs. Once they ingress into the blastocoel they form a ring around the invaginating endoderm and later fuse to form the skeleton (5) (**Figure 2*a***). The pigment cell lineage is formed from the SMCs, the descendants of the veg2 ring of cells that are the immediate neighbors of the PMCs. These cells enter individually into the blastocoel, migrate toward the aboral ectoderm territory, and bind there (5) (**Figure 2*a***).

PMC ingression happens as early as 20 h after fertilization, and some differentiation genes are turned on even sooner. To allow this, the differentiation process starts as soon as specification states are established in the mesoderm territory. In the skeletogenic lineage, Ets1, Alx1, and other regulatory genes (**Figure 4*a***) activate a battery of skeletogenic differentiation genes, e.g., biomineralization proteins (*sm27* and *sm50*), glycoproteins (*msp130* and *msp130L*), and *cyclophilin* and *ficolin* (**Figure 2*b***). In the pigment cell lineage Gcm drives the expression of the differentiation gene battery, which encodes pigment synthesis enzymes such as *SuTx* (sulfotransferase), *Dpt* (dopachrome tautomerase), *Pks* (polyketide synthetase), and *FvMo* (flavine-containing monooxygenase) (4) (**Figure 2*b***). The network describes the entire developmental process, from the response to the initial anisotropic cues, through the activation of lineage-specific regulatory genes, to the expression of differentiation genes. The network architecture is shallow in that few specification stages lead to differentiation, which is typical of Type 1 embryos (12).

gcm expression continues long after the Delta-Notch signal turns off. The expression of this key specification gene is maintained by a simple positive-feedback loop of the *gcm* onto itself (**Figure 5*a***). A positive-feedback loop is a typical functional element that gene regulatory networks use to stabilize and lock cells into a specification state. This network element characterizes the stage of specification state establishment. Another feature of this stage is a local exclusion of alternative fate, i.e., local repression of genes that do not belong to the specified domain. Gcm contributes to this function by downregulating the expression of the PMC transcription factor, *alx1*, in the SMC (**Figure 5*a***) (S. Damle & E.H. Davidson, unpublished data).

On the other hand, exclusion of SMC fate takes place in the PMC lineage. *delta* is expressed in the large micromeres and their descendants from ~8 to 20 h after fertilization. The micromeres have cell-cell contact with other micromeres as well as with the SMC. This raises the question, How is the Delta signal prevented from turning on SMC genes in the micromeres and their descendants, the PMC? The answer seems to lie in the timely activation of the different regulatory genes. *Pmar1* activation upregulates *delta* and *alx1*. Whereas the reception of the Delta signal activates *gcm*, Alx1 represses *gcm* expression in the PMC to exclude SMC fate (40; P. Oliveri, unpublished data) (**Figure 5*a***). The PMCs present a synchronized regulatory architecture that eliminates regulatory mistakes or signaling noise that the system can face.

Interestingly, when *gcm* expression is turned on specifically in the PMCs they cease

the expression of some skeletogenic genes and acquire pigment cell properties (S. Damle & E.H. Davidson, unpublished data). The opposite is also true; when skeletogenic regulators are activated in the SMCs they turn off the pigment cell program (P. Oliveri & E.H. Davidson, unpublished data). That a single gene, in this case *gcm*, is sufficient to change the PMC into SMC demonstrates how crucial exclusion control is for the normal development of the embryo.

Mesoderm and Endoderm Specification Subcircuits: veg2 Subdivision into SMC and Gut

The endoderm territory in the sea urchin larva constitutes the gut. At the eighth cleavage the veg2 ring of cells is dividing axially to form two rings of cells, an inner ring where the cells are touching the micromeres and an outer ring that is farther away. The inner ring of cells continues to receive the Delta-Notch signal from the micromeres and eventually becomes

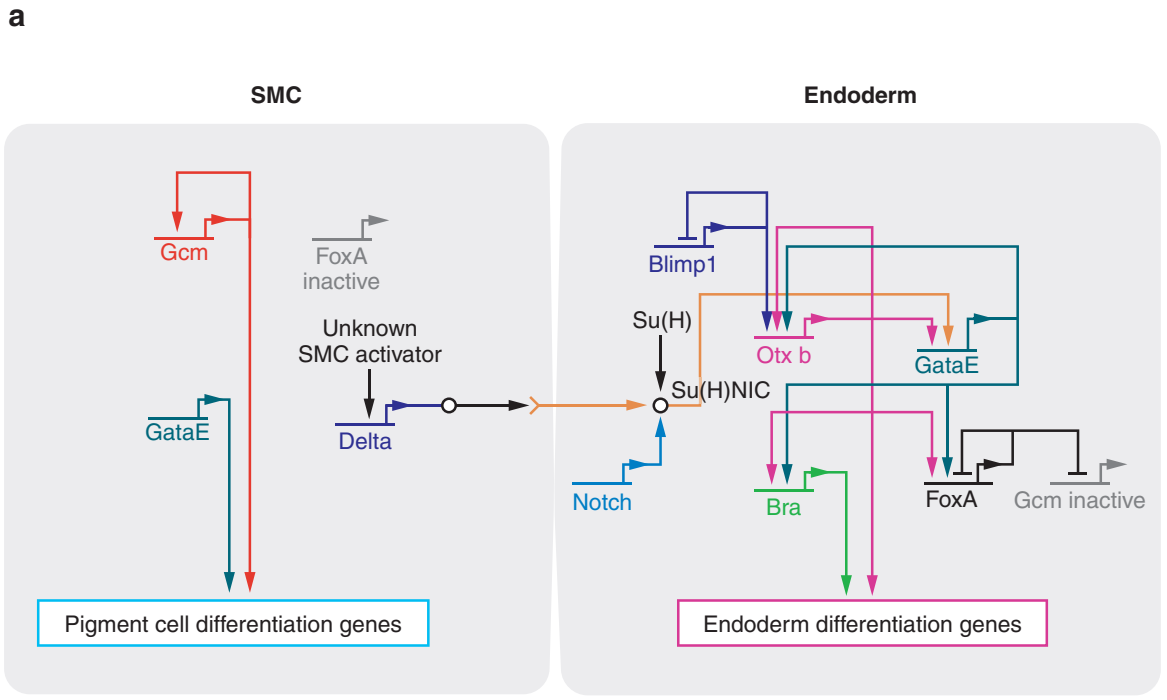


Figure 6

(*a*) SMC and endoderm maintenance and mutual exclusion subcircuits. Gcm positively regulates itself to maintain the pigment cell specification state. The Otxβ-Gatae positive-feedback loop maintains the expression of key endodermal regulatory genes, e.g., FoxA that represses Gcm to exclude pigment cell fate. (*b*) A *cis*-regulatory module that controls the expression of the *otxβ1/2* isoform in the endoderm. The module contains a cluster of Otx, GataE, and Blimp1 binding sites. Blocking translation by injecting morpholino-antisense oligonucleotide of *blimp1*, *gataE*, and *otxβ* significantly reduces the expression of *otxβ1/2* transcript (*bottom*, *left*). Interfering with the *cis*-regulatory processing by mutating binding sites of a reporter construct reduces the reporter expression in a manner similar to that of antisense injection. (Reprinted from Reference 68.) (*c*) Conservation of the endoderm specification subcircuit in the sea urchin and in the starfish (13, 15). The subcircuit is conserved except for the following differences: *tbr* takes part in the endoderm specification in starfish, and in sea urchin it participates in the micromere lineage specification and not in the endoderm. Foxa represses *gatae* in the starfish and not in the sea urchin, whereas Blimp1 represses itself in the sea urchin and not in the starfish. All the other linkages are conserved. (Reprinted from Reference 13.)

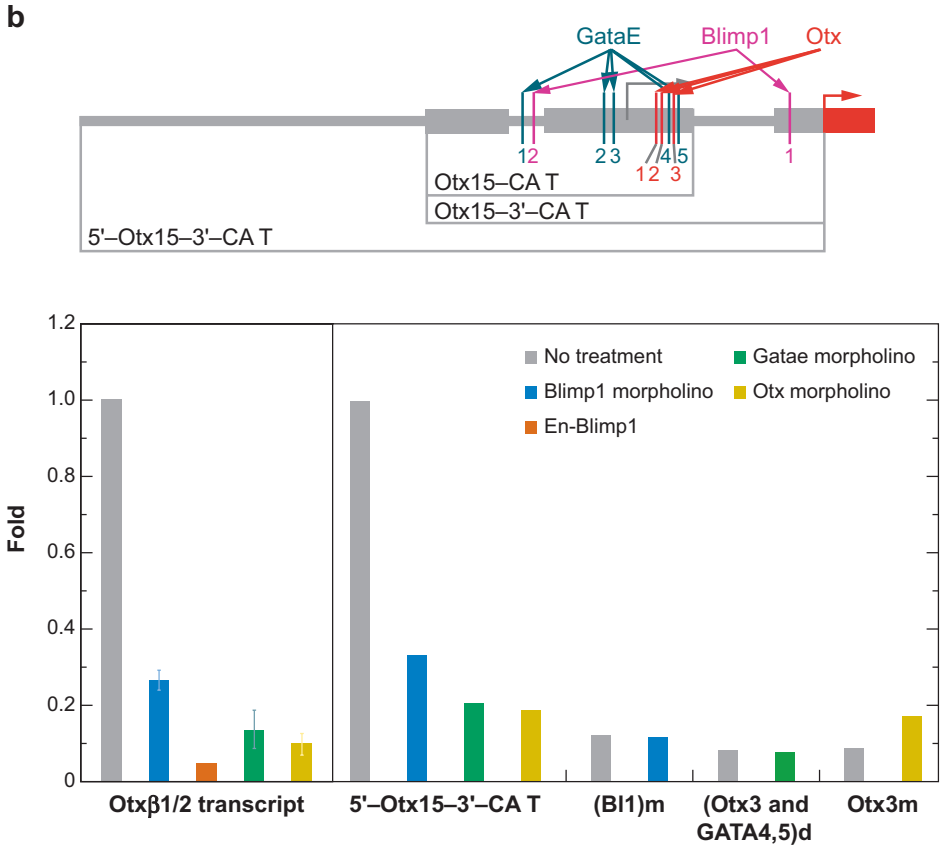


Figure 6
(*Continued*)

the SMC. The descendants of the outer ring of cells form most of the endoderm. Therefore, following the eighth cleavage differential signaling inputs and accumulating regulatory differences eventually lead to the establishment of different stabilizing feedback loops and mutual exclusion regulation in the new subdomains.

The regulatory state in the veg2 ring of cells just before the eighth cleavage is such that the *β-catenin-blimp1-wnt8* subcircuit is active, Delta reception activates *gcm*, and maternal Otx activates *foxa*. Once the cells divide, Delta is not received by the cells that do not have direct contact with the micromeres and therefore *gcm* is shut off in these cells. The cells that touch the micromeres still receive the Delta signal and continue to express *gcm* while FoxA clears, probably owing to indirect repression by *gcm*. From now on, *gcm* is expressed only in the inner ring of cells, and *foxa* is expressed only in the outer ring of cells (**Figure 6*a***).

Once the PMCs ingress into the blastocoel and cease to have cell-cell contact with the SMCs, they stop expressing *delta*. At about this time *delta* expression is activated in the SMCs. The use of the same regulatory gene at various times and domains in the embryo is a general feature of gene regulatory networks. The SMC Delta and the PMC Delta are received by different cells with different histories and hence different regulatory states. Therefore, there are differences as well as similarities in the activation of the Delta-Notch target genes in the receiving cells. An example of a difference is the activation of the *gcm* gene. Reception of the PMC Delta activates *gcm* in

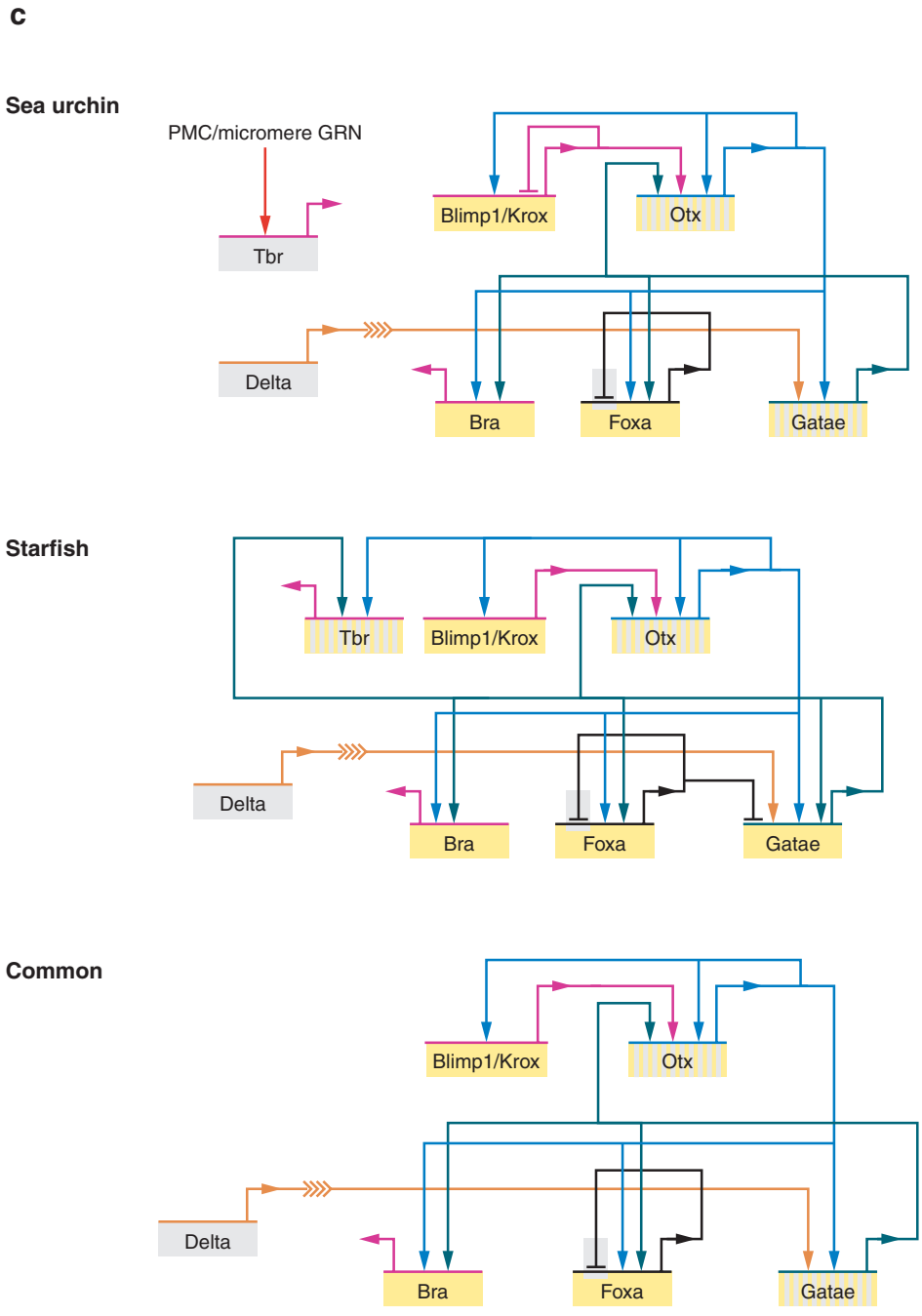


Figure 6
(*Continued*)

the SMC. However, the Delta-receiving endoderm cells express *foxa*, which downregulates *gcm* expression there (41a) (**Figure 6*a***). This is another instance of direct exclusion of *gcm* expression outside the pigment cell lineage. An example of a similarity is the *gatae* expression that has a positive input from Delta-Notch signaling in both the SMC and the

endoderm (24) (**Figure 6*a***). The maintenance of *gatae* expression in the endoderm is unique to this cell lineage.

Approximately 18 h after fertilization the *β-catenin-Blimp1-wnt8* subcircuit is active in the future endoderm territory. Blimp1 activates a later isoform of the *otx* gene, *otxβ*. Otxβ positively regulates itself and *gatae* expression. GataE activates further expression of *otxβ*, and a stabilizing feedback loop is established between *otxβ* and *gatae*, so that the positive input of Blimp1 is no longer required to maintain their expression (68) (**Figure 6*a***). Indeed, Blimp1 expression moves forward to the next tier of cells and clears from the veg2 ring by autorepression (**Figure 6*a***). Otxβ and GataE regulate the expression of essential endoderm specification genes, *bra* and *foxa*. *otxβ*, *gatae*, *foxa*, and *bra* control the endoderm specification and differentiation and are necessary for gastrulation. Perturbing the expression of either *blimp1* or the four endoderm genes leads to loss of gut formation.

The key DNA components that control the execution of this subcircuit are the *cis*-regulatory modules of these five regulatory genes. In **Figure 6*b*** we present the result of the study of the *otxβ cis*-regulatory module (68). As expected, the module contains binding sites of Blimp1, GataE, and Otxβ. Interfering with either the *trans* input, by blocking the translation of a transcription factor, or the *cis* processing, by mutating the relevant binding sites, results in the similar decrease of expression. These results show that the GataE, Otxβ, and Blimp1 inputs are necessary for Otxβ expression in the endoderm.

The endoderm specification subcircuit formed by *blimp1*, *otxβ*, *gatae*, *foxa*, and *bra* is an example of a network kernel. A kernel is an evolutionary conserved subcircuit dedicated to a specific developmental function, such as endoderm specification and gastrulation. Comparing the sea urchin kernel with its orthologue in the starfish shows that all key linkages in the sea urchin kernel are present in the starfish (15) (**Figure 6*c***). The last common ancestor of the sea urchin and the starfish lived ~500 mya (42). This remarkable evolutionary conservation is characteristic of kernels and it indicates the importance and inflexibility of this subcircuit in the network.

CONCLUSIONS

The most fundamental level of understanding the developmental process is the regulation of differential gene expression. The basic processing unit of the genomic regulatory code is that of the single *cis*-regulatory module, which contains a cluster of binding sites that defines its conditional activation. The developmental process is mediated by networks of such units, especially those that execute the expression of regulatory genes. The network connections define a program that runs in every cell of the embryo. In each cell of the embryo the units of the regulatory genome process the input that is the existing regulatory state. Some units are activated and some are inactivated, and the process proceeds to the next regulatory state, to a fine-tuning of specification states, to the onset of new subdomains, and to morphogenesis.

The regulatory genome of an organism consists of many networks such as the one considered here. It is the underlying computational device that facilitates animal development by controlling the spatial and temporal differential expression of many thousands of genes. Both similarities and differences between the regulatory genomes of different organisms carry significant information. The similarities teach us about the essential evolutionary conserved components of developmental networks and about the networks' design principles. The differences provide the key to understanding the mechanism underlying phenotypic differences between organisms and to account for their different body plans.

SUMMARY POINTS

1. The instructions for specification and differentiation are encoded in the regulatory sequences of the genomic DNA.
2. The basic processing units of the regulatory genome are single *cis*-regulatory modules.
3. The regulatory genes are interconnected by their *cis*-regulatory modules to form a network that is essentially the genomic program for development.
4. Although the overall network wiring is dense and complex, the network can be reduced into functional subcircuits, each responsible for a specific developmental task.
5. The typical functional elements that subcircuits are made of are regulatory state turn-on, regulatory state maintenance, exclusion of alternative fates, and subcircuit shutoff.
6. Subcircuits that involve the transformation of maternal anisotropies into new regulatory states and the activation of signaling pathways dominate early cleavage stage.
7. Subcircuits that control specification state establishment are the next to turn on. They are characterized by stabilizing positive-feedback loops and turn-on of local repressors to prevent alternative fates.
8. The similarities between networks teach us of the essential evolutionary conserved network components, and the differences account for the diversity in organisms' body plans.

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15. Innovative study of the evolution of different body plans by comparing gene regulatory networks of two species, the starfish and the sea urchin.

19. Combination of comprehensive expression pattern study and perturbation analysis results with a provisional gene regulatory network for the specification of several tissues of *Ciona* tadpole.

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