

Gene regulatory networks for development

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The genomic program for development operates primarily by the regulated expression of genes encoding transcription factors and components of cell signaling pathways. This program is executed by cis-regulatory DNAs (e.g., enhancers and silencers) that control gene expression. The regulatory inputs and functional outputs of developmental control genes constitute network-like architectures. In this PNAS Special Feature are assembled papers on developmental gene regulatory networks governing the formation of various tissues and organs in nematodes, flies, sea urchins, frogs, and mammals. Here, we survey salient points of these networks, by using as reference those governing specification of the endomesoderm in sea urchin embryos and dorsal-ventral patterning in the *Drosophila* embryo.

Development of animal body plans proceeds by the progressive installation of transcriptional regulatory states, transiently positioned in embryonic space. The underlying mechanism is the localized expression of genes encoding sequence-specific transcription factors at specific times and places. The units of control are clusters of DNA sequence elements that serve as target sites for transcription factors, which usually correspond to enhancers, although there are other kinds of cis-regulatory DNA modules as well, such as silencers and insulators. We refer to all of these regulatory DNAs as “cis-regulatory modules.” Each module is typically 300 bp or more in length and contains on the order of 10 or more binding sites for at least four transcription factors (1, 2). In general, a particular cis-regulatory module produces a specific pattern of gene expression in space or time, and multiple modules can produce complex patterns of gene expression (3). Because each module is regulated by multiple transcription factors and each transcription factor interacts with multiple modules, it is possible to represent developmental patterns of gene expression as an interlocking network (for reviews and discussion, see refs. 2 and 4–7).

Gene regulatory networks (GRNs) are logic maps that state in detail the inputs into each cis-regulatory module, so that one can see how a given gene is fired off at a given time and place. They also provide specifically testable sets of predictions of just what target sites are hardwired into the cis-regulatory DNA sequence. The specific linkages constituting these networks provide a causal structure/function answer to the question of how any given aspect of development is ultimately controlled by heritable genomic sequence information. The architecture reveals features that can never be appreciated at any other level of analysis but that turn out to embody distinguishing and deeply significant properties of each control system.

These properties are composed of linkages of multiple genes that together perform specific operations, such as positive feedback loops, which drive stable circuits of cell differentiation (4, 7, 8).

Comparison of Diverse Developmental Systems

This Special Feature contains works that consider different aspects of GRNs in a variety of systems. The dorsal-ventral patterning of the *Drosophila* embryo is controlled by the graded distribution of a maternal transcription factor, Dorsal (reviewed in ref. 9). As we discuss below, this gradient initiates a GRN that governs the differentiation of the mesoderm, neurogenic ectoderm, and dorsal ectoderm during gastrulation. A work by Ochoa-Espinosa *et al.* (8) in this issue of PNAS describes the network underlying segmentation of the *Drosophila* embryo. There are parallels between segmentation and dorsal-ventral patterning. Both networks begin to function while the embryo is still a syncytium, so intercellular signaling plays only a limited role in the initial spatial interactions. Characteristic of these networks are dynamic and transient patterns of gene expression in which changes of regulatory state and boundaries of expression patterns are governed by multiple tiers of transcriptional repression.

Two other developmental specification systems that are considered in this collection of works rely on the early use of intercellular signals: the sea urchin embryonic endomesoderm, briefly discussed below, and the dorsal axial structures of the *Xenopus* embryo, which is discussed by Koide *et al.* in ref. 10. In both networks, specific regulatory states are placed in the proper spatial domains of the embryo in response to localized intercellular signals. Signal transduction and feedback “lockdown” loops are seen in both networks (4, 7, 10), although, at the level of morphological phenomenology, *Xenopus* and sea urchin embryos would appear to develop rather differently. Postembryonic

GRNs are the subjects of two other works in this Special Feature. Singh and Medina (11) present an analysis of mammalian B cell differentiation, and Inoue *et al.* (12) focus on vulval cell specification in larval *Caenorhabditis elegans*. Both networks are bolstered by direct cis-regulatory analysis. This Special Feature is, to our knowledge, the first time a collection of GRNs has been presented in a manner that permits direct comparisons, as we discuss below.

Two other selections in the Special Feature (13, 14) are focused on the genomic design of cis-regulatory modules such as those that compose the GRNs considered in this collection. In the first, Papatsenko and M.L. (13) demonstrate a statistical correlation between different classes of target sites and distinct transcriptional responses to higher as opposed to lower levels of the Dorsal regulator in *Drosophila* embryos. They also show how differences in the organization of binding sites can produce distinct patterns of gene expression. In the second of these works, Istrail and E.H.D. (14) make an initial effort to “parse” cis-regulatory modules into combinations drawn from a small repertoire of continuous and discrete logic functions using both *Drosophila* and sea urchin cases.

We turn now to our specific examples, the GRNs underlying specification of endomesoderm in the sea urchin embryo and dorsal-ventral patterning in *Drosophila*. Both of these networks have been derived from, and are supported by, large-scale experimental efforts.

Specification of Endomesoderm in the *Strongylocentrotus purpuratus* Embryo

The sea urchin embryo is simply constructed compared with the *Drosophila* embryo, because its role is to build only a microscopic swimming larva composed

Abbreviation: GRN, gene regulatory network.

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mainly of single layers of differentiated cells, rather than a multilayered, metameric, juvenile form of the adult body plan. In most sea urchins, the much more complex adult body plan is constructed only later, after the larva begins to feed. Consequently, the process of embryogenesis in these sea urchins is one in which the underlying GRN is organized to install differentiated cells in the right places in the embryo as directly as possible. Development begins with a stereotyped cleavage pattern. In response to intercellular signaling, between the 16- and ≈ 128 -cell stages, the lineages that will give rise to the gut of the larva, its biomineral skeleton, and a set of other distinct mesodermal cell types have largely been separated out from the lineages that will form the ectodermal wall of the larva. The endoderm, skeletal, and other mesodermal components (here endomesoderm) establish definitive regulatory states during this period and then proceed to delineate further spatial subdivisions. Within these, by 1 day after fertilization, differentiation gene batteries have begun to be expressed (for review, see ref. 15). The morphogenetic movements of gastrulation follow these embryo-wide events of territorial and cell fate specification, which include regional activation of genes required for cell motility and other gastrular functions. The network shown in Fig. 1 represents the genomic regulatory code that controls the entire endomesodermal specification process up to 24–30 h, from the initial interpretation early in development of maternal asymmetries inherited from the egg cytoarchitecture to the activation of differentiation genes.

The network consists of almost 50 genes. The large pastel areas in Fig. 1 symbolize the regulatory states of the skeletogenic (lavender) and mesoderm/endoderm (green) spatial domains, wherein genes that contribute to mesodermal specification in particular are shown on blue backgrounds and genes that execute endoderm specification are shown on yellow backgrounds. The tan area on the right represents a late-specified endodermal region that invaginates at the end of gastrulation and produces posterior portions of the gut. In all these areas, most of the named genes encode transcription factors; the remainder are differentially expressed signaling components. At the bottom of the diagram, the smaller rectangles enclose samples of the respective sets of differentiation genes, i.e., genes that encode skeletogenic, mesodermal (mainly pigment cell), and gut effector proteins. The network image shows the transcription factor inputs (vertical arrows and barred lines) that impinge on the relevant cis-regulatory modules, which are symbolized by the short horizontal lines

overlying the names of the respective genes. From each gene the color-coded thin lines display the outputs to other genes in the network (the mode of presentation and its significance are discussed in refs. 4–7 and 16; for updates, contact E.H.D. or see the web site <http://sugp.caltech.edu/endomes/webStart/bioTapestry.jnlp>, where much of the underlying experimental data also are posted). Evidence on which the network is based includes the temporal expression of all of the genes in the network (obtained by quantitative PCR); spatial expression of these genes (obtained by whole-mount *in situ* hybridization); and the results of a large-scale perturbation analysis, in which expression of each gene was taken out of the system (usually by use of antisense morpholino-substituted oligonucleotides), and the effects on all other relevant genes were measured by quantitative PCR. However, for other essential supporting information, where the causal evidence is too unique to be easily tabularized, specific publications must be consulted (also listed on the web site). For example, such evidence includes the effects of ectopic expression of given regulatory genes on spatial specification events (e.g., refs. 17 and 18).

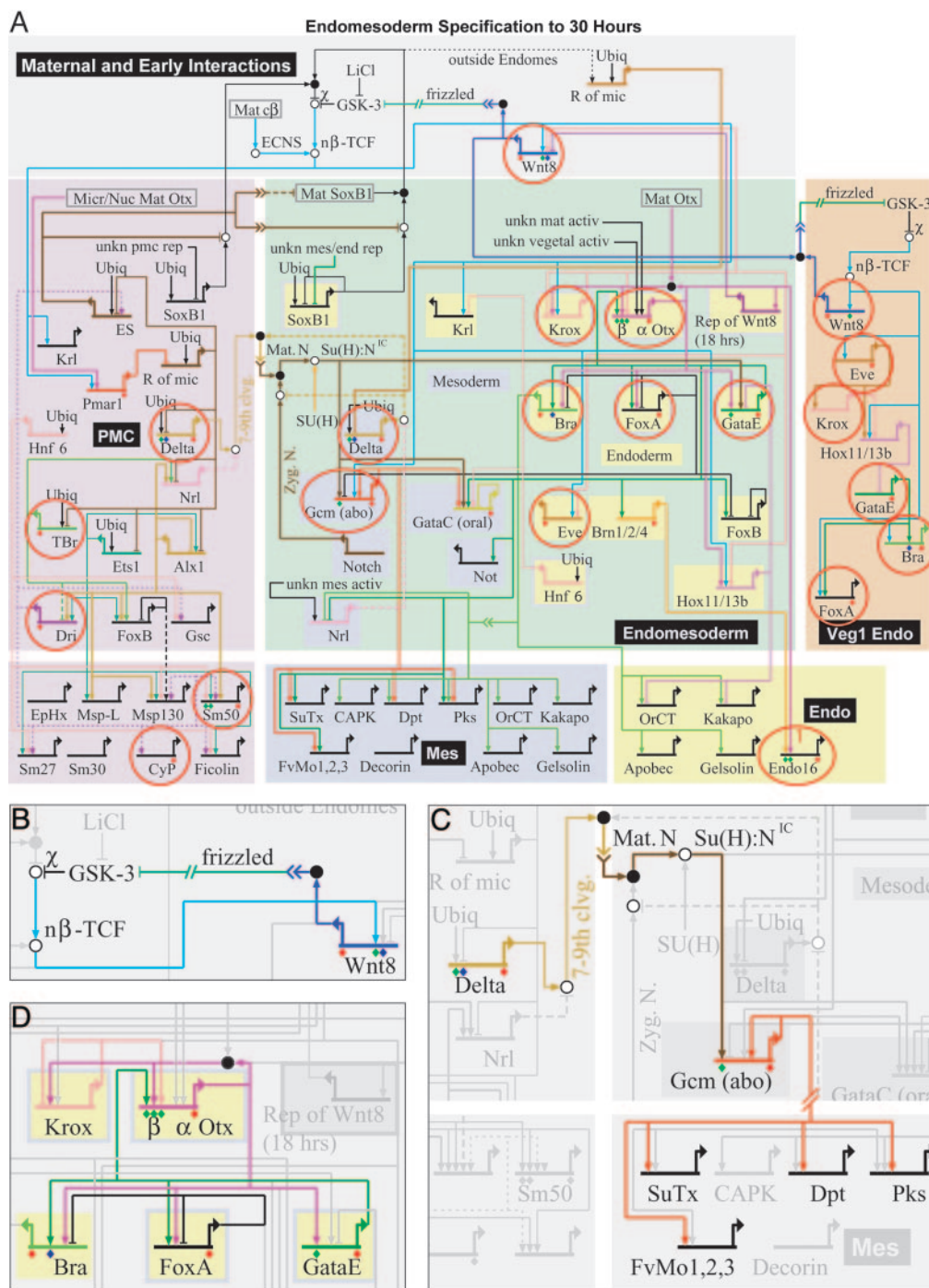
Authentication and Completeness. Completeness is the fraction of regulatory genes actually involved in the process that is included explicitly in the network as shown. Completeness can be addressed only with the aid of genomic sequence information, which provides the possibility of computational screens and the possibility of reference to the complete regulatory gene repertoire. Authenticity is the level at which the interactions pictured in the network have been demonstrated to represent cis-regulatory transactions encoded in the genomic sequence. We argue that there is only one way to authenticate a GRN, and that is by means of direct experimental manipulation of individual cis-regulatory modules. Much of the architecture of the sea urchin network in Fig. 1 is based directly on cis-regulatory experimentation, and the same is to a large extent true of all of the GRNs included in this Special Feature. Thus, with respect to authentication, these networks are among the standards in this young field.

With regard to the GRN in Fig. 1, the issue of completeness is not yet fully addressed. However, because the genome sequence of *Strongylocentrotus purpuratus* is now becoming available (obtained by the Human Genome Sequence Center of Baylor University; National Center for Biotechnology Information Trace Repository), we have been able to determine the temporal and spatial expression of hundreds of predicted regulatory genes. It has

turned out that the number of new genes that are specifically expressed in early endomesodermal domains of the embryo can be counted on the fingers of one hand for each territory. Thus, indications are that the current version of the network is not very incomplete, although we are aware of several places where linkages and gene targets are missing and of several spatial patterns of expression that are not yet completely explained. A large-scale effort has been mounted with respect to authentication of the endomesoderm network, as indicated by the thin red circles in Fig. 1. For every one of the circled genes, a small (several hundred base pairs) DNA fragment has been recovered that, when associated with a reporter gene and introduced into sea urchin eggs, reproduces the developmental pattern of expression of the endogenous gene. The circled genes include most of the key nodes of the network. Just the fact that all of the predicted cis-regulatory modules in the circles have been experimentally demonstrated to exist provides the basic level of authentication: the GRN indeed represents a genomic regulatory system, in which the DNA sequence elements contain the causal basis for the gene expression patterns that drive the developmental process. The next level of analysis is to determine whether each cis-regulatory module responds in the same way as the associated gene in the living embryo when challenged by experimental perturbations. The final level is to show by mutation that the module contains the target site(s) for the inputs predicted in the network (or, if not, to correct the network architecture). Different cis-regulatory modules circled in Fig. 1 are at different stages of experimental manipulation (see legend). But for some modules, the analysis is complete, e.g., the β -*otx* module (19), for which all of the predicted inputs were demonstrated by both perturbation and cis-regulatory mutation. Where the evidence is in, the predictions of the network shown in Fig. 1 have been substantiated at the cis-regulatory level.

GRN and How to Understand Development.

There are three ways in which the GRN provides a qualitatively different and innovative kind of understanding not otherwise accessible. First, it explains, in the causal terms of the explicit genomic regulatory code, much of the phenomenology of early embryogenesis discovered in the course of years of painstaking experimental embryology. Take, for example, the essential role of maternal β -catenin nuclearization. The work of McClay and others (20–23) has shown that nuclearization of this transcriptional cofactor is necessary to specify the future endomesoderm and that subsequent expression of the *wnt8* gene in



these same cells both requires the initial β -catenin nuclearization and is in turn required for endomesoderm development. A glance at the network tells us why this is, what functions are executed, and how these functions are programmed in the relevant genomic cis-regulatory modules. The β -catenin/Tcf transcriptional input is required for several genes that function early on in different regions of the endomesoderm, as shown by the thin blue lines emanating from the “ $\eta\beta$ -Tcf” symbol (i.e., nuclearized β -catenin-Tcf transcription factor) at the upper left of Fig. 1A. The cis-regulatory module driving the *wnt8* gene itself is among these target genes (24). This relationship sets up a positive loop, because the Wnt8 gene product acts as an intercellular ligand that in recipient cells results in further β -catenin nuclearization (Fig. 1B). Another prominent signaling phenomenon in this embryo is the dependence of mesodermal specification on a Delta/Notch interaction between the skeletogenic cells and the adjacent endomesodermal precursors (25–27). The explanation, in terms of the genomic regulatory code, is that the relevant *delta* cis-regulatory module (28) is controlled by the skeletogenic lineage regulatory system, which prevents expression of its participant genes (i.e., including *delta*) anywhere except in this domain (5, 7, 16, 17). Thereby we can account for the localization of the Delta signal. The network shows, furthermore, that in the recipient cells, the Notch receptor transcriptional complex directly activates a regulatory gene (*gcm*) that in turn locks itself stably on (A. Ransick and E.H.D., unpublished data), and *gcm* also provides positive regulatory input into downstream genes that encode pigment cell differentiation proteins (Fig. 1C; see also ref. 29). Throughout the network, the input/output regulatory relationships explain what happens in terms of the cis-regulatory DNA sequence code. Thereby the network at last closes the conceptual and evidential gap between the static genomic program for development and the dynamic progression of spatial regulatory states.

The second way in which the GRN provides a different kind of insight is in respect to its own modular structure. It is composed of multigenic assemblages of components that work together to accomplish given regulatory tasks, of which Fig. 1B and C provide specific examples. A

particularly interesting example is shown in Fig. 1D, which portrays a feedback loop that undoubtedly constitutes the driving regulatory input that initiates, and then stabilizes and promotes, the state of endoderm specification (4). We refer to a subcircuit such as this one, which performs a dedicated and specific role, as a “kernel” of the network. After early inputs activate the endomesoderm *krox* gene, the Krox transcription factor activates the *otx* gene, the products of which provide positive inputs into other endoderm regulators, including the pleiotropic, endoderm-specific *gatae* gene. But the *gatae* gene product in turn feeds back on the *otx* gene, locking the endoderm regulatory state in an “on” position (4, 19). The modular organization of the network is just becoming possible to perceive. But it is of potentially large importance for modeling its function, for reconstructing (or redesigning) it in the laboratory, and for understanding its evolution.

Dorsal–Ventral Patterning of the *Drosophila* Embryo

Dorsal–Ventral Specification and Development of Organogenic Territories. Dorsal–ventral patterning is implemented by the graded distribution of a maternal transcription factor, Dorsal, and culminates in the differentiation of specialized tissues, including cardiac mesoderm, the ventral midline of the nerve cord, and the amnioserosa (9). During a period of just 90 min, from syncytial stages to the onset of gastrulation, the dorsal–ventral axis is subdivided into three basic tissues: mesoderm, neurogenic ectoderm, and dorsal ectoderm (30). Each of these tissues generates multiple cell types during gastrulation, ≈ 3 –5 h after fertilization. Dorsal regions of the mesoderm form cardiac tissues (reviewed in ref. 31). Ventral regions of the neurogenic ectoderm form the mesectoderm or ventral midline, which is essential for the patterning of the neurons that comprise the ventral nerve cord (reviewed in ref. 32). Finally, the dorsal-most regions of the dorsal ectoderm form a contractile extraembryonic membrane called the amnioserosa, which is essential for the process of germband elongation after gastrulation (33).

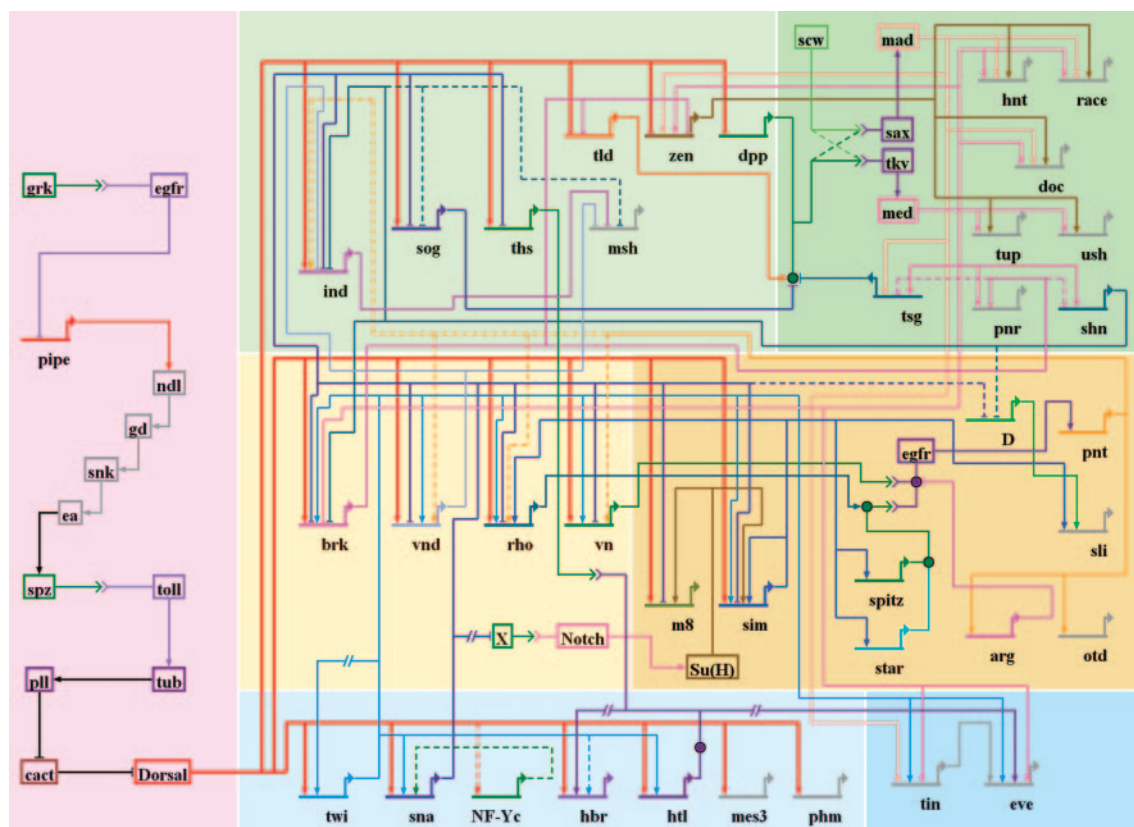
The dorsal–ventral patterning network is composed of nearly 60 genes (Fig. 2). Twelve of the genes encode proteins that are active in the perivitelline matrix surrounding the plasma membrane of the oocyte. They are responsible for creating

an activity gradient of the Spitz ligand on the surface of the unfertilized egg, with peak levels in ventral regions and lower levels in more dorsal regions (34). After fertilization, Spitz interacts with the Toll receptor, which triggers an intracellular signaling cascade that releases the Dorsal transcription factor from the Cactus inhibitor in the cytoplasm (reviewed in ref. 35). This regulated nuclear transport is restricted to ventral and lateral regions of the early embryo and is thought to mirror the extracellular gradient of the active Spitz ligand.

GRN. The Dorsal nuclear gradient leads to the differential expression of nearly 50 genes across the dorsal–ventral axis (e.g., see ref. 30). Roughly half the genes encode sequence-specific transcription factors, whereas the other half encodes components of cell signaling pathways. For example, high levels of Dorsal activate regulatory genes such as *twist* (*twi*) and *snail* (*sna*) (9) but also activate two components of the FGF signaling pathway, the FGF receptor Heartless (*Htl*) and the cytoplasmic effector *Heartbroken* (*Hbr*)/*stumps*/*Dof* (36). Cis-regulatory modules have been identified, and in many cases characterized, for about half of the zygotically expressed genes shown in the network.

Tw is an immediate early target gene of the Dorsal gradient (e.g., see ref. 37). It is activated by high levels of the gradient in ventral regions that form mesoderm. The *Tw* protein is synthesized in the presumptive mesoderm but diffuses approximately four to five “cell” diameters into ventral regions of the neurogenic ectoderm in the syncytial embryo (38, 39). *Tw* regulates nearly half of the known Dorsal target genes, including most of those activated in the mesoderm and ventral regions of the neurogenic ectoderm. Dorsal and *Tw* function in an additive fashion to activate a number of genes in the ventral mesoderm before the onset of gastrulation (e.g., see ref. 36). The *Sna* repressor is also deployed during the initial phases of the dorsal–ventral network. Like *Tw*, *Sna* regulates a large number of Dorsal target genes, particularly those expressed in the neurogenic and dorsal ectoderms (e.g., see ref. 3). In principle, these genes can be activated in the mesoderm by high levels of Dorsal but are kept off by the *Sna* repressor. Thus, dorsal–ventral patterning begins with an interlocking set

feedback loop. As discussed in the text and in more detail elsewhere (4, 19, 56), this “kernel” of the GRN is conserved without significant change in starfish, a very distantly related echinoderm. It has the functions of initially activating regulatory genes in the presumptive endoderm as a function of *krox* and β -*otx* activation; thereby activating a pleiotropic endoderm regulator, *gatae*, then locking in *gatae* expression by establishment of feedback to a β -*otx* cis-regulatory module, and stably activating the gut regulatory genes *foxa* and *brachyury*.



of three transcription factors, Dorsal, Twi, and Sna.

There are two overall features of the dorsal-ventral patterning network that we wish to emphasize, because they reveal differences and similarities with the sea urchin endomesoderm network discussed above. First, during the early phases of development, there is the extensive use in the *Drosophila* dorsal-ventral patterning network of transcriptional repressors to establish boundaries of gene expression (Fig. 2 *Left*). In the sea urchin network, although there are negative feedbacks and cross-regulations within territories, the boundaries are set by lineage and signaling interfaces. Second, during this initial phase, there are very few examples of positive autofeedback or positive cross-regulatory feedback loops such as that in Fig. 1*D*. We argue that this specific circuitry has evolved to generate dynamic and transient patterns of gene expression in syncytial embryos, which essentially lack intercellular signaling mechanisms. In contrast, after cellularization, the circuitry exhibits autoregulation and positive feedback loops involving intercellular signaling pathways (Fig. 2 *Right*). These loops implement stable networks of cellular differentiation during gastrulation, and they are essentially similar to those seen in the sea urchin endomesoderm network.

Repression and Formation of Gene Expression Boundaries. Transcriptional repression plays a pervasive role in the initial patterning of the embryo. Four of the seven regulatory genes activated by the Dorsal gradient in syncytial embryos encode sequence-specific repressors (Sna, Brinker, Vnd, and Ind). The Sna repressor creates a boundary between the presumptive mesoderm and neurogenic ectoderm by excluding the expression of at least seven different Dorsal target genes (e.g., see ref. 3). Sna is transiently expressed in the mesoderm and lost from the mesoderm during gastrulation (40). The Brinker repressor helps create a boundary between the neurogenic and dorsal ectoderms by repressing Dpp signaling components that are required for the differentiation of spe-

cialized dorsal tissues such as the amnioserosa (41, 42). Brinker maintains this boundary by repressing the Schnurri gene (43), which encodes a repressor that establishes the dorsal limits of neurogenic genes (e.g., *msh*).

Regulatory Circuitry Following Cellularization. Stable circuits of autoregulation and positive feedback loops are not seen until the onset of gastrulation. The regulatory gene *sim* coordinates a subcircuit, or kernel, within the dorsal-ventral network that is essential for the patterning of the neurogenic ectoderm. *Sim* is activated by a combination of Dorsal, Twi, and Notch signaling (44, 45). Because of this requirement for Notch, *sim* expression is not detected until the completion of cellularization. The Sna repressor keeps *sim* off in the ventral mesoderm (45) and restricts the pattern to the ventral-most cells of the neurogenic ectoderm. Sna also represses an unknown inhibitor of Notch signaling (44, 45). As a result, a Notch signal originates from the ventral mesoderm and activates the Suppressor of Hairless [Su(H)] transcription factor at the leading edge of the neurogenic ectoderm that is adjacent to the mesoderm. Once *sim* is activated in this single line of cells, it maintains its own expression through autoregulation (46). The *Sim* transcription factor also activates genes that encode components of the EGF signaling pathway, including *star*, *rhomboid* (*rho*; late expression), and *spz*, which activates the EGF receptor in ventral regions of the neurogenic ectoderm (e.g., see refs. 47–49). *Sim* and EGF signaling regulate a number of genes required for the differentiation and patterning activities of the ventral midline, including orthodenticle and slit (50).

The EGF signaling circuit “locks down” localized patterns of gene expression within the neurogenic ectoderm that are initially established by transient regulators such as Dorsal and Sna. In particular, EGF signaling maintains expression of the Pointed transcription

factor, which, in turn, sustains the expression of *vnd*, *rho*, and *vn* (51). It would appear that the EGF/Pointed pathway replaces the transient Dorsal gradient to maintain differential patterns of *vnd*, and possibly *ind*, within the neurogenic ectoderm (52). The *vnd* gene encodes a transcription factor that is required for the specification of medial neuroblasts, whereas Ind is required for intermediate neuroblasts within the ventral nerve cord (reviewed in ref. 53). Vnd functions as a repressor to form the ventral limit of the *ind* expression pattern (54), and, similarly, Ind delineates the *msh* expression pattern, which specifies the lateral neuroblasts (53). The sequential and interlocking patterns of *vnd*, *ind*, and *msh* also are seen in the vertebrate neural tube (reviewed in ref. 55).

Future Prospects

GRNs provide a means of understanding evolution of body plans. For example, after half a billion years of independent evolution, the regulatory kernel from the sea urchin endomesoderm network (Fig. 1*D*) is exactly retained in the genome of a starfish (56). It is similarly used in both systems to create the embryonic endoderm, although much else of the regulatory wiring has changed since divergence. Evolutionary changes in GRNs that control developmental processes must be the basis for morphological change. But to explicitly demonstrate this fundamental principle will require the synthesis of many more high-quality GRNs governing diverse developmental processes in a variety of animal groups. Furthermore, as even the beginnings of comparative GRN analysis suggest, such efforts will produce fascinating insights into the structure and organization of network architecture itself.

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