Signal Processing during Developmental Multicellular Patterning

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Developing design strategies for tissue engineering and regenerative medicine is limited by our nascent understanding of how cell populations “self-organize” into multicellular structures on synthetic scaffolds. Mechanistic insights can be gleaned from the quantitative analysis of biomolecular signals that drive multicellular patterning during the natural processes of embryonic and adult development. This review describes three critical layers of signal processing that govern multicellular patterning: spatiotemporal presentation of extracellular cues, intracellular signaling networks that mediate crosstalk among extracellular cues, and finally, intranuclear signal integration at the level of transcriptional regulation. At every level in this hierarchy, the quantitative attributes of signals have a profound impact on patterning. We discuss how experiments and mathematical models are being used to uncover these quantitative features and their impact on multicellular phenotype.

Introduction

The development of a multicellular organism from an embryo is one of Nature’s most remarkable phenomena. Deciphering how this transformation occurs is a fundamental challenge in biology with profound biomedical implications. Insights into the molecular signals guiding developmental patterning may provide design strategies to promote multicellular structure formation in applications such as tissue engineering and regenerative medicine. Recently, significant attention has been given to the use of stem cells in these applications. A major challenge, however, is to engineer a cellular microenvironment that presents the right combination of cues to promote differentiation and cellular “self-organization”. This bioengineering challenge will likely benefit from a more quantitative and integrative understanding of developmental signals (1, 2). In fact, gleaning design insight from natural developmental processes may be imperative, since erroneous manipulation of developmental mechanisms can lead to pathologies. Perhaps the most striking example is that aberrations in developmental signals play a prominent role in the structural and functional regression of tissues during cancer development (3).

Development begins with asymmetric divisions of the fertilized egg, partitioning it into distinct groups of cells or “progenitor fields” that will ultimately develop into future organs (4). Each group executes a distinct gene expression program, thereby specifying it toward a unique developmental fate. The next round of specification cues further subdivides the progenitor field, with each subgroup now executing a distinct specification (i.e., gene expression) program. Thus, development involves successive rounds of asymmetry-inducing specification cues that trigger specific gene expression programs. Interspersed among these successive rounds is the cellular execution of the gene expression program. Specifically, this program dictates cell behaviors, such as division, migration, death, and extracellular matrix deposition and remodeling, events that shape and functionalize developing tissues and, ultimately, the organism.

Thus, the molecular signals at the heart of development are the specification cues and the network of biochemical reactions that process these cues and instruct the appropriate cell behavioral response. Specification cues must encode spatial information, since groups of cells must be partitioned according to a precise three-dimensional geometry. Once the specification cue has been perceived, cells must then execute the downstream gene expression program in a context-sensitive manner. Here, context has 2-fold significance. First, in any spatial context, cells are exposed to multiple cues. The specification cue must be processed in this rich backdrop of signals. Furthermore, in some cases, information from two or more specification cues must be integrated to induce the appropriate response. Second, cellular response to a specification cue must take into account the temporal context. When a cell is exposed to a specification cue at a particular time in development, its response will be biased by its developmental history. For example, the cell response will be affected by its proteomic profile, which is determined by the series of gene expression programs executed up to that time. In fact, the context-dependence of cell response is absolutely critical, since the same specification cue is often used in multiple places and times to guide development. Cells respond to the same cue in distinct ways by accounting for their spatial and temporal context through remarkably sophisticated signal integration mechanisms.

In this work, we examine these two facets, specification cues and context-dependent response, of signal processing during development. The main goal is to highlight the progress and the significant challenges in developing a quantitative, mechanistic, systems-scale understanding of developmental signaling. Specific examples of molecular networks and developmental systems are provided to crystallize certain points.

Specification Cues

Specification cues are signals that instruct cells to execute a particular gene expression program and thereby propel cells to
Figure 1. Polarization by Delta-Notch signaling. (A) In the absence of an external bias, a stochastic imbalance in the production of Delta (Dl) ligand or Notch (N) receptor can generate an initial asymmetry that is amplified by feedback loops. (B) The presence of an unequally distributed transcription factor (Numb) after division induces an initial asymmetry that is amplified. (C) An external bias (Wnt) initially polarizes Notch signaling, and differences are further amplified by Delta-Notch feedback loops. (D) An external bias (EGF) introduces differences in Delta-Notch signaling between cells, which in turn amplify the perception of the external bias.

a particular fate. These cues can be categorized according to the length scales over which they operate.

Short-Range Juxtacrine Specification. Direct cell—cell interactions or juxtacrine specification is employed during development when adjacent cells are to be induced toward distinct cell fates. The signaling pathways utilized by juxtacrine specification cues are conserved in both invertebrates and vertebrates (5). The canonical example involves the ligand, Delta, and the receptor, Notch. Both are transmembrane proteins, and upon ligand binding, several proteolytic events culminate in the release of the intracellular domain of Notch (6). This cytoplasmic domain of Notch complexes with the transcription factor Suppressor of Hairless (Su(H)) and regulates gene expression.

The Delta/Notch pathway is used in different ways during development to elicit short-range patterns of fate choices (Figure 1). During the development of gonadal cell lineages in Caenorhabditis elegans, two equipotent cells commit to divergent fates, the anchor cell (AC) and the ventral uterine (VU) cell (7). Each cell originally has an equal likelihood of becoming either AC or VU (Figure 1A). A stochastic imbalance in Delta expression initiates the binary fate choice. The “sender” cell with the slightly higher Delta expression elevates Notch signaling in its neighbor. Increased Notch signaling in the “receiver” cell is amplified by a positive feedback loop that further increases Notch expression. Concurrently, Notch signaling inhibits the production of Delta. Failing to receive a Delta signal from its neighbor, the sender continues to produce Delta, which further stimulates Notch activity in the receiver. In this manner, an intercellular positive feedback loop produces a large disparity in Notch activity between neighboring cells. The receiver cell with high Notch activity acquires the VU fate, while the sender cell commits to the AC fate.

In other situations the bias in the Delta/Notch pathway does not occur stochastically but is the result of asymmetric division (Figure 1B). During specification of the sensory precursor organ (SOP) cells in Drosophila, Delta/Notch bias is caused by an asymmetric localization of the protein Numb in one of the SOP daughter cells (8). Numb antagonizes Notch signaling by inducing its endocytosis, providing the initial imbalance that is then amplified by intercellular positive feedback (9).

Soluble extracellular factors can also provide the initial bias in Delta/Notch signaling (Figure 1C). During R3/R4 specification of photoreceptors in the Drosophila ommatidia, a higher local concentration of Wnt near the presumptive R3 cells upregulates Delta expression in this cell, thereby elevating Notch activity in its neighbor, the presumptive R4 cell. Unlike the unidirectional effect of Wnt on Delta/Notch signaling in R3/R4 specification, an intriguing bidirectional coupling occurs in the specification of vulval precursor cells in C. elegans (Figure 1D). Here, a gradient in the soluble factor LIN-3 biases the upregulation of Delta-like ligands (10) and the downregulation of Notch (11). Instead of confining the feedback loop to within the Delta/Notch interaction, Notch signaling feeds back to inhibit LIN-3 signaling. The net effect is that the wide disparity in Notch activity between neighboring cells concomitantly polarizes cell responsiveness to the soluble factor LIN-3 (12). This polarization of two signals is particularly useful in this system where six cells acquire three distinct fates as opposed to a binary fate choice based on the polarity of a single Notch signal.

Shaping Morphogen Gradients for Long-Range Induction.

The use of soluble factors is more prevalent in the context of larger cell fields. Classical models envisioned that spatial gradients in soluble cues may guide multicellular patterning over longer length scales (13). Indeed, such soluble specification cues or morphogens have been identified, including four major families: fibroblast growth factor (FGF), hedgehog (Hh), wingless (Wg/Wnt), and transforming growth factor-β (TGFβ) (14). These factors operate in a wide span of organisms including both invertebrates (Drosophila, C. elegans, and sea urchin) and vertebrates (Xenopus, zebrafish, mouse, and chicken).

Morphogens are signaling molecules that partition a field of cells into two or more fates (15). They act directly to specify fates in a concentration-dependent manner (Figure 2). Because cells respond to the local concentration, the shape of the morphogen gradient across a field of cells dictates the pattern of cell fates. Although several factors had been long thought to act as morphogens, direct experimental evidence for spatially graded, concentration-dependent action was first demonstrated for activin, a member of the TGFβ family, which guides mesoderm induction in Xenopus embryos (16).

Spatial gradients are established by localized secretion of morphogen along with its transport across the cell field (17). The secreting source may be a single cell as in the case of LIN-3
release during C. elegans vulval development (18) or an array of cells as in the case of Decapentaplegic (Dpp)-mediated anteroposterior (AP) patterning of the Drosophila wing (19). In systems where a single cell releases the morphogen, the gradient will most likely span a short distance on the order of several cell lengths. Morphogen release from several cells can lead to gradients that span hundreds of cell lengths, although short-range coverage is also possible as in Hh-initiated AP patterning in the Drosophila wing (20). Importantly, morphogens need not be extracellular factors. During early stages of Drosophila development, the embryo is a multinuclear syncytium with no cell boundaries. Gradients in maternal gene products, such as Dorsal and Bicoid, establish asymmetries in gene expression during dorsoventral (DV) and AP patterning, respectively, of the Drosophila embryo.

Secreted extracellular morphogens are generally expressed as transmembrane precursors that are later processed by proteases to release the soluble form (18, 21). The mechanisms regulating morphogen release are beginning to be uncovered. Transport between intracellular compartments and retention in the endoplasmatic reticulum (ER) play a regulatory role in the release of Spitz morphogen during Drosophila eye development (22). However, interesting questions remain: how are source cells synchronized to initiate (and to stop) the release of morphogen? Are such decisions autonomous or non-autonomous? Synchronous release may involve cell–cell communication, a plausible mechanism if the secreting source were a small group of cells. Such coordinated secretion would be more difficult if the secreting source were a large group of cells. In this case, a relay mechanism may provide synchronicity and the desired amplification. For example a long-range gradient in Dpp morphogen patterns the Drosophila wing. This gradient is set up by a shorter range gradient in Hh (23).

Once secreted, the morphogen must be transported across the cell field to establish a gradient. Several genetics studies revealed that endocytosis, the internalization of morphogen-bound receptor by the cell, plays a key role in establishing morphogen gradients (24, 25). These observations suggested that morphogen transport may occur by an active, cell-assisted process labeled transcytosis (Figure 3). In this process, morphogen-bound receptors are endocytosed and then exocytosed or brought back to the cell surface. Exocytosed complexes dissociate and release the morphogen. If the reemergence of the internalized morphogen is isotropic, transcytosis-mediated morphogen transport may be characterized by an effective diffusion coefficient (26). In fact, it has been suggested that passive diffusion may not be well-suited to establish steady-state morphogen gradients (27).

However, an integrative analysis of diffusion-mediated transport alongside key biochemical pathways revealed that diffusive transport can establish steady-state morphogen gradients, provided there are degradation sinks for the morphogen (28). In the absence of morphogen degradation, an ever-increasing morphogen concentration will result in uniform occupancy of target receptors at steady state. However, in the presence of morphogen sinks, a steady-state gradient in receptor occupancy will form in a diffusion-based transport model. Indeed, receptor-mediated endocytosis itself promotes morphogen degradation (24, 29). Thus, the requirement for endocytosis to establish morphogen gradients is entirely consistent with diffusive transport.

Furthermore, the diffusion/endocytosis model may be physically more plausible than transcytosis, since the latter may require that certain cellular processes occur at nonphysiological rates. For example, the formation of a steady-state gradient on the time scale of a typical developmental process would require that the morphogen be transported across a single cell on an average time scale of 100 s (28), seemingly unachievable considering the relatively slow kinetics of the steps involved in transcytosis: receptor association, internalization, directed transport through the cell, exocytosis, and receptor dissociation. However, it can be argued that the rates of these processes have not been measured in vivo where cells may be optimized to facilitate transcytosis (26). Furthermore, the diffusion/endocytosis model is not consistent with all experimental data (30). In cell fields where a patch of cells is deficient in endocytosis, a depression in morphogen concentration is observed adjacent to the patch on the side opposite from the secreting morphogen source. The diffusion/endocytosis model would predict that in the endocytosis-defective patch, reduced receptor degradation increases receptor expression and prevents morphogen transport, thereby producing the “shadow” effect. However, recent measurements of the receptor expression level in the endocytosis-deficient patch suggest that the increase in receptor expression is not of sufficient magnitude to explain quantitatively the shadow phenomenon (30).

In summary, an integrative analysis demonstrates that diffusion is entirely capable of establishing morphogen gradients in an endocytosis-dependent fashion, although other mechanisms such as transcytosis may play a concomitant role. However, these are not the only mechanisms involved in morphogen transport. For example, convective transport in low-flow interstitial spaces has been predicted to establish a gradient in vascular endothelial growth factor (VEGF), a chemotactic factor driving capillary formation (31).

In addition, morphogen interaction with binding agents in the extracellular matrix or on the cell surface plays a prominent role in gradient formation. On the cell surface, Sonic hedgehog (Shh) interacts not only with its receptor Patched (Ptc), but also with a non-signaling membrane glycoprotein Hip (32). In the extracellular matrix, Shh binds both heparan sulfate proteoglycans (HSPGs) (33) and vitronectin (34). The role of these multiple regulatory mechanisms in Shh morphogen gradient
formation during vertebrate neural tube development has been dissected by systems-level mathematical modeling (35). Model analysis revealed that the spatial range of Shh has a biphasic dependence on its diffusivity. Thus, diffusion-restricting mechanisms, such as sequestration by HSPGs or vitronectin, can actually extend the working range of Shh. Meanwhile, the binding and endocytosis of signaling-deficient Shh-Hip complexes may remove or shunt the morphogen from the extracellular space without altering the ability to signal via the Ptc/Smoothened(Smo) pathway.

In some cases, model predictions may be validated by comparison to direct measurements of morphogen gradients. Gradients have been directly observed in vivo using green fluorescent protein (GFP) fusion constructs. This approach has been used to study the formation of the Dpp and Wg gradients in Drosophila during the anterior-posterior patterning of wing discs and during embryonic development, respectively (24, 36, 37). Mathematical models have been constructed to explain the mechanisms of Dpp gradient formation and the role of receptors in shaping this gradient (30, 38).

However, in most cases, direct observation of the gradient has been hampered by difficulty in expressing GFP-fused morphogens or in using antibodies to detect low concentrations of morphogens (30, 39). In these cases, the intracellular expression of a gene target is measured as an indirect monitor of the morphogen gradient. For example, during C. elegans vulval development, the gradient in an EGF-like soluble factor (LIN-3) has been observed indirectly from the graded activity of a reporter of the LIN-3 target gene, egl-17 (40). Such studies offer important qualitative confirmation of the morphogen gradient but do not provide key quantitative information, such as the steepness of the steady-state gradient.

A gradient that is too shallow or too steep would not be biologically useful. A metric of gradient steepness is the ratio $L/\lambda$, where $\lambda$ is the characteristic decay length scale of the morphogen gradient and $L$ is the length of the field of cells to be patterned (41). A biologically useful gradient would have a steepness characterized by $L/\lambda = 1$. Quantitative characterization of Bicoid localization in Drosophila embryos shows that the morphogen gradient is appropriately distributed across the patterning field with $L/\lambda = 3.8$ (42).

In other systems where the morphogen is a secreted ligand, theoretical models suggest that the diffusion/endocytosis model establishes biologically useful gradients, provided ligand–receptor binding constant $k_{\text{on}}$ is less than $O(10^5) \text{ M}^{-1} \text{s}^{-1}$ and the degradation rate constant of endocytosed complexes $k_{\text{deg}}$ is $O(10^{-4}) \text{s}^{-1}$ (28). Larger values of $k_{\text{on}}$ restrict the dynamic range of the number of morphogen-receptor complexes per cell, hence limiting the system to shallow gradients. For these values, gradient steepness would be characterized by $L/\lambda \approx 4$.

The challenge remains, however, that direct observation of most morphogens is not technically feasible, and therefore quantitative characterization of the morphogen gradient remains elusive. Addressing this challenge, Shvartsman and colleagues recently described an elegant systems approach to infer quantitatively the steady-state gradient in the morphogen Gurken in the Drosophila egg chamber (43). Using molecular genetics, the expression level of the Gurken receptor (EGFR) was manipulated in order to alter the Gurken morphogen gradient. Since the gradient could not be directly measured, its effect on the expression of the target gene pipe was quantified using imaging techniques. This quantitative dataset that related EGFR expression level to the spatial boundaries of pipe expression was fit to a systems-scale model of Gurken gradient formation and signaling. This analysis revealed that the steady-state Gurken gradient is characterized by a $L/\lambda$ value of 2.7. This model-based estimation of the parameter $L/\lambda$ provides complete information about the full, nonlinear shape of the Gurken gradient and offers intriguing insight on how sensitive downstream gene expression must be to Gurken concentration. For example, a significant change in gene expression program is observed at a boundary where the Gurken gradient changes by only 3-fold, suggesting a remarkable switch-like sensitivity of these cells to Gurken concentration.

This type of ultrasensitivity to morphogen concentration would seem to suggest that even mild fluctuations in gradient formation would significantly perturb developmental patterning. Indeed, it is expected that a simple transport model involving secretion, diffusion, and degradation would yield gradients that are highly sensitive to variations in parameters, such as temperature and the secretion rate (41). In some cases, additional mechanisms are employed to buffer morphogen gradient formation against these fluctuations. For example, elevations in the morphogen secretion rate are buffered by a feedback loop during AP patterning of Drosophila wing (44). Here, the Hh morphogen induces localized expression of its receptor Ptc, which in turn...
sequesters and directs Hh to endocytic degradation. This negative feedback loop is a robust mechanism that limits the morphogen from reaching distant cells in the event of elevated morphogen secretion.

Yet, in other systems, the steady-state gradient is not robust to variations in temperature, ligand secretion rates, or geometrical variations (41, 42). The Bicoid gradient that patterns the Drosophila embryo along the anterior—posterior axis shows an embryo-to-embryo variability in its diffusion length of 5% of embryo length. Interestingly, the expression of the downstream gene target Hunchback (Hb) is robust to these fluctuations. Instead of exhibiting fluctuations in the position of Hb expression boundary of 7% of embryo length (about 7 cell diameters), the value expected on the basis of the above variability in the Bicoid gradient, embryos show only 1% variability. Thus, in some systems, robustness may be conferred not at the level of gradient formation but by the mechanisms involved in perceiving and interpreting the specification cues.

Quantitative Signal Processing and Integration

Upon receiving a specification cue, cells must choose a fate that precisely accounts for (a) the quantitative nature of the signal, (b) the spatial context that may include other environmental cues, and (c) the cell’s developmental history. Intracellular molecular networks achieve this quantitative signal processing and integration of spatial and temporal context.

The first step in processing extracellular specification cues involves their detection by cell surface receptors. The classical view has been that the number of morphogen-bound receptors determines the extent of intracellular signaling, which then dictates the gene expression program. However, in the case of the morphogen Hh, quantitative studies suggest that the ratio of the number of bound to the number of unbound receptors dictates downstream signaling (45). The canonical mode of Hh signaling involves its association with the receptor Ptc. Hh-bound Ptc is no longer able to inhibit another membrane-associated protein, Smo. In this manner, Hh promotes Smo signaling and downstream gene expression. Casali and Struhl showed that Hh-bound Ptc not only fails to inhibit Smo but also impedes free Ptc from sequestering Smo. Thus, the ratio of bound to unbound Ptc determines the degree of Smo repression.

Whether detection involves the absolute amount or the ratio of receptor states, cells must interpret this quantitative information to choose between distinct fates. In some cases, the morphogen acts over a medium-sized field of cells. An example is the fate specification of six vulval precursor cells in C. elegans where the action of the morphogen LIN-3 is coupled to signals generated by cell—cell interactions. Computational analysis of this molecular network reveals that cell—cell coupling amplifies the perception of the LIN-3 gradient (12). Thus, a gradient in LIN-3 concentration produces an even steeper difference in LIN-3-mediated intracellular signals between adjoining cells. Such gradient amplification may be particularly important in converting a shallow, graded morphogen signal into a spatial pattern of digital choices. In fact, gradient amplification may play an important role in fate specification even in larger cell fields where clear boundaries in fate choices must be established.

At the heart of this gradient amplification network is the intracellular signal MAP kinase. MAP kinase pathways are triggered in numerous developmental contexts. Agonists of EGFR, such as Gurken in Drosophila and LIN-3 in C. elegans, stimulate MAP kinase activity. During Drosophila eye development, specification of photoneurons is initiated by the expression of EGFR ligand Boss by the R8 photoneuron. Activation of the MAP kinase pathway in this cell triggers a cascade that leads to specification of the remaining R1-R7 photoneurons (46). Thus, the MAP kinase pathway is a common signaling “protocol” used across a wide range of developmental systems.

Consistent with its broad implementation, the MAP kinase pathway performs with quantitative properties that would be desirable in a developmental context. Foremost, the MAP kinase cascade has been shown to convert a graded stimulus into a digital output, a feature that has clear implications for converting a spatial morphogen gradient into a discrete pattern of cell responses (Figure 2). While some of this switch-like behavior is due to the cascade structure of the MAP kinase module, the dominant contribution is provided by positive feedback (47). In Xenopus oocyte extracts, MAP kinase activity increases the expression of Mos, an upstream element in the MAP kinase activation pathway. This positive feedback yields a switch-like response to a graded stimulus, and inhibition of protein synthesis ablates the upregulation of Mos and results in a graded output.
When the positive feedback is of sufficient magnitude, the MAP kinase pathway encodes another attractive feature for development, irreversibility (Figure 4). Upon transient stimulation with progesterone, the Xenopus oocyte irreversibly specifies from the immature to mature state (48). The MAP kinase pathway mediates this irreversible specification. The positive feedback loop in the MAP kinase module acts in concert with a second signaling module that also contains a positive feedback loop. The combined effect is that the progesterone stimulus activates maturation, and even upon removal of the stimulus, the cell remains committed to that fate.

Specification cues presented to cells during development are present only transiently, long enough for the cell to commit to a fate, but short enough to prepare the system to potentially reuse the cue for later developmental steps. For example, in C. elegans, the EGF-like morphogen LIN-3 is secreted by the anchor cell to stimulate vulval precursor cell specification (18); later, the descendants of vulval precursor cells release LIN-3 toward the anchor cell to induce vulval-uterine attachment (49). Since cues are only transient, irreversible cell commitment to execute particular fates is critical.

An important observation is that even intermediate signals, such as MAP kinase, are reused. Thus, even though intermediate signals may be irreversible on the time scale of fate commitment, they too must decay back to a basal level to be available for the next round of specification cues. We hypothesize that this reversibility of intermediate commitment signals over longer time scales is achieved by deactivating the positive feedback pathways. However, it should be noted that fate execution itself cannot be reversible. Indeed, if the fate is cell division or death, the condition of irreversibility is obviously satisfied. However, if fate execution involves the expression of specific genes, mechanisms must be put in place to maintain that expression once the specification cue and intermediate commitment signals have dissipated. Positive feedback loops at the level of gene regulation may be involved.

Notably, a conversion from an analog to an irreversible, digital signal leaves the cell with a conundrum. How do different levels of a single molecular signal, such as MAP kinase, trigger substantially distinct gene expression programs? In some systems, this quantitative decoding may occur at a point that is most proximal to gene expression: the cis-regulatory sequences that tune the level of transcriptional activity. During dorsoventral (DV) patterning of the Drosophila embryo, a gradient in the transcription factor Dorsal establishes a spatial pattern of gene expression (Figure 5). The cis-regulatory elements of gene targets like twist and snail contain low affinity binding sites for Dorsal, and these genes are expressed in the ventral-most field where Dorsal concentrations are high. Meanwhile, gene targets such as rho that are expressed more dorsally possess optimal Dorsal binding sites in their cis-regulatory elements. These higher affinity sites compensate for the lower Dorsal concentrations in that region. In fact, the number and quality of Dorsal binding sites on cis-regulatory elements strongly correlates with the pattern of dorsoventral gene expression across four divergent species of Drosophilids (50).

These findings strongly suggest a model where the binding affinity of transcriptional activators to gene targets sets the threshold of specification signal needed to initiate gene expression. Genes with low affinity transcription factor binding sites may be expressed only if the specification signal is sufficiently high. Meanwhile, genes possessing high affinity binding sites would be responsive even if the specification signal were low. However, do these high affinity binding sites remain unresponsive in regions where the specification signal is high? This additional quantitative selectivity is also prescribed by cis-regulatory elements, but via a mechanism unrelated to binding affinity for transcription factors. Cis-regulatory elements contain binding sites for multiple transcription factors, some activators and others inhibitors of gene expression. Combinatorial processing of these multiple inputs determines the net level of gene expression.

Such combinatorial processing of multiple inputs plays a key role in Drosophila DV patterning. As described above, the gene target snail contains low affinity binding sites for Dorsal and is expressed only in the ventral-most region where Dorsal concentration is highest. Interestingly, snail encodes a transcriptional repressor that binds cis-regulatory elements of other gene targets of Dorsal, including rho. Hence, Snail represses rho expression in the ventral-most region despite the high concentrations of Dorsal. Meanwhile, in the adjacent region, the concentration of Dorsal and Snail diminish. Here, rho is expressed because of its high affinity binding sites for Dorsal, while the lower concentration of Snail renders its repression less effective.

In this manner, cis-regulatory elements serve as crucial points of signal integration. Significant advances have been made in uncovering the network of transcription factors and the cis-regulatory elements on which they act. Such gene regulatory networks have been delineated for endomesoderm specification.
in sea urchin, dorsal-ventral axis patterning in *Drosophila*, vulva differentiation in *C. elegans*, and mesoderm specification in *Xenopus* (reviewed in refs 51 and 52). A key consideration, however, is that each *cis*-regulatory element or node in this network is not merely a connection for multiple inputs but is a quantitative processor. The concentrations of input transcription factors are detected by the affinity and number of sites on the node. The relative amounts of activators and repressors are also part of the calculus. These quantitative aspects of the input then determine the extent of output, i.e., gene transcriptional activity. Thus, a key challenge will be to elucidate the quantitative calculation that *cis*-regulatory elements perform when challenged by multiple concomitant transcription factors. A prototype for such quantitative analysis is the pioneering study of the gene endo-16 that is an endoderm specification marker during the development of the sea urchin embryo (53). Expression of endo-16 is regulated by a set of 6 modules, A, B, CD, E, F, and G, located in a stretch of DNA 2300 base pairs upstream of the gene promoter (Figure 6). Modules A, B, and G are responsible for gene activation, while the remaining elements are responsible for its repression (54). Both the independent and synergistic contributions of modules A and B to endo16 expression have been quantified rigorously, and a model has been developed to predict the quantitative output of the module based on the binding status of the different DNA regulatory sequences in these modules (55, 56).

The quantitative signal processing and integration that occur at the *cis*-regulatory modules of endo-16 provide not only spatial but also temporal context. During early development, this gene is expressed in the entire vegetal plate, i.e., progeny of cells derived from the *Veg 2* blastomeres. Later in development, its expression further increases in endoderm and future archenteron, while decaying back to basal level in *Veg 2* progeny cells that will become mesoderm. Throughout this time, the gene is continually repressed in micromeres or *Veg 1* progeny cells. In each of these spatial and temporal contexts, endo-16 is repressed or expressed at different levels because different panels of transcription factors act on its *cis*-regulatory modules. What is the mechanistic basis for having distinct panels of transcription factors in these various contexts? The answer is a recursive one. At an earlier stage, each panel of transcription factors was expressed as dictated by their own *cis*-regulatory elements. Thus, the history of genes that were expressed in a particular cell
encodes its temporal context, thereby priming the cell to respond appropriately to its current specification cues.

This cascade of gene expression events is not a simple linear pathway but rather a gene regulatory network. The genes and the associated cis-regulatory elements that comprise these genetic circuits have been elucidated for a wide range of developmental contexts (52). However, the quantitative calculations that occur at each cis-regulatory element remain to be elucidated. Such quantitative insight, as demonstrated in the case of endo-16, would enable the development of mathematical models of these networks. Such models can allow us to explore the capabilities of the network beyond the developmental context from which they were formulated. Indeed, development may not exhaustively cover all the contexts to which the cis-regulatory module of each gene responds. Analyzing the performance of cis-regulatory modules under developmentally unachievable contexts would offer a method to explore potential disease states or synthetic/engineering objectives.

Conclusions

There has been significant progress in uncovering the molecular signals that guide developmental processes. These studies have revealed that a relatively short list of evolutionarily conserved specification cues instruct developmental patterning over short- and long-length scales. The recurrent use of these cues to achieve remarkably diverse patterns clearly demonstrates that developmental fates are not assigned by a simple one-to-one mapping with specification cues. Rather, these cues instruct fates in a context-sensitive and dose-dependent manner.

Context and dosage are processed through two layers of sophisticated molecular networks (Figure 7). While the molecular connectivity of these networks has been elucidated for a wide range of model organisms, their quantitative processing power is only emerging. Properties such as the dose-dependent effects of morphogens such as activin, the ratiometric detection of Hh in Drosophila wing patterning, gradient amplification in morphogen perception in C. elegans, affinity-based gene regulation during DV patterning of the Drosophila embryo, and the cis-regulatory logic regulating endo-16 expression in the sea urchin demonstrate the extensive quantitative signal processing that occurs at both layers of developmental regulation.

In addition, specification cues, post-translational crosstalk, and genetic regulatory networks are intimately coupled. Post-translational crosstalk activates the appropriate panel of transcription factors that act upon the cis-regulatory elements in the gene regulatory network. Meanwhile, the action of the gene regulatory network provides the protein constituents of the post-translational networks, including the target transcription factors. In fact, some of the products of gene regulatory networks are themselves specification cues, providing the stimuli for the next round of patterning. A crucial challenge in developing predictive models of development is to account for the coupling between these regulatory layers and their quantitative attributes.

Finally, while molecular signals and networks orchestrate development, it is the cells instructed by these signals that ultimately form the remarkable multicellular patterns and structures associated with development. In most cases, developmental signals (e.g., the expression of a particular gene) have been correlated to a specific cellular fate or behavior and thus serve as markers of cell response. A major challenge is to elucidate the mechanistic relation between these molecular developmental markers and cell response. This insight will foster future mechanistic models that predict not only the spatiotemporal evolution of developmental signals but also the cellular rearrangements and turnover as development progresses.

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References and Notes

a scientific paper with numbers and references