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Specifying Positional Information in the Embryo: Looking Beyond Morphogens

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Concentration gradients of small diffusible molecules called morphogens are key regulators of development, specifying position during pattern formation in the embryo. It is now becoming clear that additional or alternative mechanisms involving interactions among cells are also crucial for positional specification.

A key event in development of the embryo is the allocation of cell fates according to spatial patterns—for instance cartilage in the vertebrate limb or veins in the insect wing. One mechanism for pattern formation depends on the propagation of small diffusible substances called morphogens, a term coined by Turing to describe molecules whose distribution determines patterns of gene expression as cells respond to different concentrations. In the French Flag model (Wolpert, 1969), cells are proposed to acquire, according to their distance from a boundary, a positional value that they then interpret according to their current dynamic state (see Figure 1). The fact that embryonic positional fields are probably small (<30 cell diameters) prompted the proposal that a diffusible morphogen could specify positional values (Crick, 1970). Morphogens have long been the standard framework for interpreting pattern formation experiments (see, for example, Zhu and Scott, 2004), but it is becoming clear that they are not the whole story. As Gregor et al. suggest in their new studies on the Bicoid morphogen (Gregor et al. 2007a, 2007b), even in the relatively simple membrane-free *Drosophila* blastoderm where a morphogen model seems to work, a whole layer of additional mechanisms are likely to be involved.

Can diffusible morphogens by themselves supply positional information with the necessary precision and reliability? Other, equally important, local mechanisms are known to act in position-related cell specification. Thus, while cells are evaluating their position in the embryo, they also must be acquiring directional information, that is, polarity. Most cells acquire one or several types of polarity. For example, specialized epithelia such as the sensory cells of the mammalian ear or hair cells of the fly wing may exhibit planar cell polarity, that is, a polarization in the plane of the epithelium (Lawrence et al., 2004). Planar polarity is a collective property of the whole tissue that is crucial for ensuring many of its functions, such as the proper orientation of hairs, and implies that cells distinguish among several directions, reacting differently to their neighbors according to relative position. Morphogens are presumed to be involved in planar cell polarity, but none are known for sure to specify it. Proposed mechanisms for planar polarization suggest that complex cell-cell interactions are crucial. Because the two problems of positional and directional information are clearly related, we suggest that cell-cell interactions as well as morphogens may be essential players in directing spatial patterning in the embryo.

Two conditions must be met for any mechanism specifying positional information: precision and robustness. Do morphogens on their own meet these conditions?

Precision and Robustness

Let us look first at precision, that is, the accurate positioning of boundaries between different cell types. Few studies have addressed this question explicitly at the cellular level. We do not know if the positioning of cells is determined down to the single-cell level. This may be the case during segmentation of the insect embryo as antero-posterior (A/P) parasegment boundaries are determined within a width of a single cell diameter, and this process does not result solely from single cells reading local morphogen concentrations. The requisite level of precision needs to be more fully investigated. It is not known, say, to what degree of precision the two wings of *Drosophila* are identical at the cellular level.

Precision data are available for the blastoderm of *Drosophila*, which is a syncytium containing many nuclei instead of individual cells. The morphogen Bicoid does not have to cross any cell boundaries in this syncytium, and a beautiful exponential gradient of this transcription factor is observed (Driever and Nusslein-Volhard, 1988). However, the transcriptional activation pattern of the *Hunchback* target gene in response to the Bicoid gradient reveals a problem: the precision of the location of the boundary of *Hunchback* expression is apparently greater than that of the Bicoid gradient from which it is presumably derived (Houchmandzadeh et al., 2002). It is hard to see how this

result could be explained other than by additional mechanisms beyond a simple gradient of the Bicoid morphogen. It is likely that regulatory interactions among target genes in each nucleus are involved (Jaeger et al., 2004). Indeed, two elegant studies by Gregor et al. (2007a, 2007b), recently published in *Cell*, show that establishment of the Bicoid gradient and readout by Hunchback probably involves nucleus to nucleus communication as well.

A second condition morphogens must fulfill in determining spatial patterns during embryogenesis is robustness. This means that the distribution of morphogens must be reproducible and remain reliable in spite of interfering phenomena (Kerszberg, 2004). It has proved problematic even to measure the concentration of morphogens at different sites in the embryo and hence to test various models of morphogen robustness. Experimental difficulties arise because the molecular concentrations involved are small, hence there exists an intrinsic chemical noise that also may affect the action of the morphogen. Candidate morphogen receptors expressed by embryonic cells are also "dilute," and they operate in a complex physico-chemical environment. This potentially could render morphogens unreliable.

The extracellular matrix likely presents a variety of binding sites for morphogens and hence can "trap" these molecules (Zhu and Scott, 2004). In the fly embryo, cell-surface glypicans (proteoglycans), such as Dally and Dally-like, have been proposed to shape the gradients of Decapentaplegic (DPP), a member of the bone morphogenetic protein (BMP) family, and of Wingless (the fly version of vertebrate Wnt), both of which act as morphogens in a variety of situations (Zhu and Scott, 2004). The early dorsoventral gradient of DPP interacts with factors such as Short Gastrulation, a DPP antagonist, to specify the dorsoventral axis of the fly embryo (Shimmi et al., 2005). There is also evidence during *Drosophila* embryogenesis of a cell-to-cell relay mechanism for transporting the morphogen

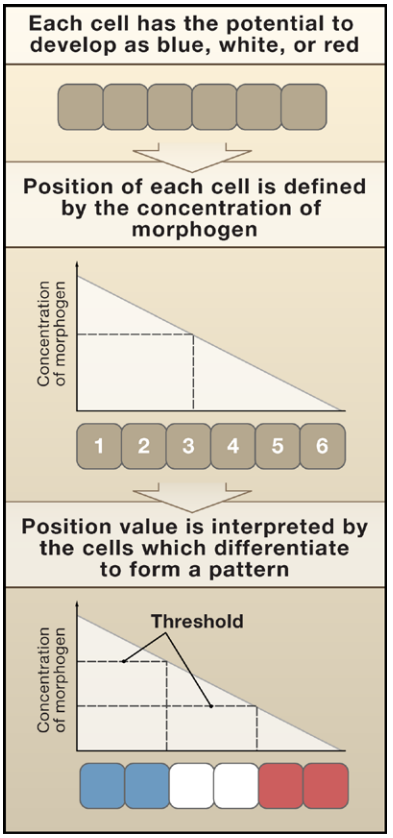

Figure 1. The French Flag Model for Positional Specification in the Embryo
All cells in an embryonic region are initially equivalent (brown cell layer) but have the potential to assume one of several differentiated fates, denoted as blue, white, or red. In the embryonic region, a space-dependent distribution of some substance (the morphogen) is established; for instance a morphogen source may be located to the left of the line, and a sink (where the morphogen is degraded) to the right. Eventually as the morphogen is propagated, this configuration leads to a stable distribution of morphogen. In the model, each cell is then able to read this morphogen concentration and compare it with a set of threshold concentrations in order to finally acquire a positional value dictated solely by the morphogen concentration at the cell's location. (Adapted from Wolpert et al., 2006.)

Hedgehog (Han et al., 2005). Yet another active mechanism based on lipoprotein particles has been proposed to transport Hedgehog and Wingless in the developing fly embryo (Panakova et al., 2005).

The extracellular space has a complex shape, and so effective diffusion times may increase as much as fivefold (Lander et al., 2002) resulting in the introduction of local variations in the morphogen gradient. The process of morphogen diffusion will of course depend on the exact three-dimensional structure of the tissue, including the density and complex topology of cell-cell contacts. Many cellular factors can also be expected to perturb or actively modulate morphogen propagation. Diffusible molecules that bind to cell-surface receptors are, for example, almost always endocytosed to some degree while remaining functional, rendering a precise determination of effective morphogen concentrations problematic. Although propagation of a molecule from a source is called "diffusion," it would be misleading to use this simple term to describe such numerous and complex processes.

How do morphogen gradients remain robust and precise despite (or perhaps with the help of) these complexities? Mathematical models have predicted morphogen gradient robustness, for example, in one-dimensional noiseless models, but such models depend on artificially homogenized properties of the propagation medium and also invoke degradation of morphogen molecules, for which there is not yet clear evidence.

Response

If morphogens are to act as graded positional cues then there must exist mechanisms for cells to perceive and interpret concentration-dependent information, and this raises problems. For example, if position is specified on a cell-by-cell basis then many more morphogen concentration thresholds (at which changes in gene activity occur) need to be established than the five or so identified in some tissues. In addition, the binding of morphogens to receptors necessary for the cellular response itself can have a very significant effect on morphogen distribution and can even prevent a gradient of receptor occupation as receptors become saturated (Kerszberg and Wolpert, 1998).

One insufficiently appreciated property of gradients is that they change over time. Freeman and Gurdon (2002) suggested that what cells do is to record the maximum concentration of the gradient at their loca-

tion. It may be that a "maximum value rule" or a time integration rule that reads the total signaling amplitude over time delivers a more stable signal for a cell's position than a simple reading of morphogen concentration at that position (Meinhardt, 1978).

Putative morphogens usually have pleiotropic effects on tissues and this will also affect patterning. Of particular interest is a study showing that the slope of a DPP gradient regulates growth during fly wing embryonic development (Rogulja and Irvine, 2005): growth and positional information are clearly interrelated and if both the concentration of DPP and its slope are involved the situation becomes very complicated.

Let us discuss in more detail some well-characterized candidate morphogens: DPP, Sonic hedgehog, and activin.

DPP, a BMP-like Morphogen

Gradients of BMPs (which belong to the TGFβ superfamily) involve a set of extracellular factors, positive feedback, and heterodimer formation to achieve peak levels of signaling in the dorsal region of the early *Drosophila* embryo (O'Connor et al., 2006). These three processes also seem to occur in the fly imaginal wing disc. The disc is divided into anterior and posterior compartments: the diffusible molecule Hedgehog is expressed in the posterior compartment, whereas diffusible DPP is expressed at the compartment boundary. Entchev et al. (2000) found that DPP is indeed distributed in a long-range concentration gradient and moves without preferential direction at a speed of more than 4 cell diameters per hr through the target tissue. Yet extracellular diffusion of DPP alone does not explain its distribution as both a stable gradient and receptor-mediated endocytosis seem to be essential for long-range regulation of DPP. Although there are some indications that at specific DPP thresholds the target genes *omb* and *spalt* are turned on (Teleman and Cohen, 2000), it is notable that even in this well-studied case there is little quantitative evidence for gradient-dependent positional gene activation. Neither this nor other studies demonstrate incontrovertibly that different local concentrations of DPP (or Hedgehog for that matter) specify positional information and gene activation with the necessary precision.

Sonic Hedgehog

Proteins of the Hedgehog family are believed to act as morphogens in a variety of tissues including *Drosophila*'s larval cuticle and wing imaginal disc, as well as the vertebrate neural tube (Ingham and McMahon, 2001; Briscoe et al., 2001). Numerous molecular interactions are involved in the propagation of Hedgehog (reviewed in Wilson and Chuang, 2006).

Patterning along the antero-posterior axis of the vertebrate limb has been suggested to be due to a graded signal from the polarizing region at the posterior margin of the bud where the gene encoding Sonic hedgehog (*Shh)* is expressed (Tickle, 2003). The best evidence that the signal is graded comes from the observation that manufacturing a reduced signal by grafting anteriorly a small number of polarizing tissue cells results in a reduced response, that is, instead of the signal specifying digits 4, 3, and 2, only digit 2 is specified. Here again, however, there is no direct evidence for a morphogen gradient determining the behavior of the cells that go to form specific digits. Indeed, there seem to be two important mechanisms: first, the total time of contact of a cell with the polarizing region and second, its residence time there, as measured by its exposure to and synthesis of Shh (Harfe et al., 2004). Similar arguments have been made regarding Shh signaling in neural tube patterning in vertebrate embryos (Stamataki et al., 2005).

Activin

In the early *Xenopus* embryo there is a presumed gradient of activin-like activity. Activin belongs to the TGFβ family (Piepenburg et al., 2004). At high concentrations of activin, the gene *goosecoid* is activated, while *Xbra* is turned on at lower activin levels (both genes encode mesodermal markers). These results have been interpreted in terms of activin binding to its cell-surface receptors (Gurdon and Bourillot, 2001). However, there is no evidence for a well-regulated, diffusion-mediated gradient of activin actually and reliably specifying cell fate by itself in the frog embryo.

Alternative Mechanisms of Patterning

What about alternative or additional patterning mechanisms? It has been suggested that a mechanism for DPP signaling could involve direct transfer of DPP receptors (not DPP ligand) from cell to cell via cytonemes. Cytonemes are actin-based filopodial extensions. In the *Drosophila* wing disc, cytonemes are oriented preferentially toward both the antero-posterior and dorsoventral organizers, and their presence and orientation correlate with DPP signaling. The DPP receptor, Thickveins, is present in punctae that move along cytonemes. These observations are claimed to be consistent with a role for cytonemes in signal transduction (Hsiung et al., 2005).

Many of the problems relating to morphogen propagation were clearly recognized by Wilson and Melton (1994) in relation to patterning of the mesoderm in *Xenopus*. They found that a variety of mesodermal markers were activated together over a wide range of activin concentrations. They concluded that the initial response to activin is relatively simple. They ascribed the further refinements observed to complex cell-cell interactions. In a study of TGFβ signaling, Reilly and Melton (1996) found evidence of a cell-to-cell relay mechanism whereby cells at high morphogen concentrations would become differentiated first and communicate information to cells that were farther away. However, when Williams and colleagues (2004) labeled the morphogen Xnr2 (a TGFβ family member) and looked in *Xenopus* embryos, they found evidence for long-range signaling by diffusion of the morphogen but not by its movement via cytonemes, filopodia, argosomes, or transcytosis as had been proposed for TGFβ morphogens.

In their two studies of the development and stability of the Bicoid gradient in fly embryos, Gregor et

al. (2007a, 2007b) suggest that some form of spatial averaging among nuclei is required to suppress noise, explaining the remarkable degree of precision they observe in the readout of Bicoid concentrations by the target gene *Hunchback*. Thus, it seems that nuclei in syncytia, like cells in multicellular embryos, may do more than merely interpret a morphogen gradient; they may actually help to establish it. As they do so, they may undergo local changes as well as long-range communication, and this process, more than the final morphogen distribution itself, imparts key positional information to them.

Specification of positional information may clearly involve a simultaneous polarization of cells and indeed of the system as a whole. Favorite model systems for studying planar cell polarity are the *Drosophila* wing and eye. It is widely assumed that one or several (some opposing) gradients are involved in wing and eye patterning, although the nature of the signal itself is unclear (even if, as many believe, it ultimately resolves into graded activation of a *Frizzled* receptor). Graded expression of the molecules Dachsous, Four-jointed, and Fat across the tissue have been reported to regulate Frizzled signaling. Remarkably, these candidate molecules are growth factors (Fat is clearly involved in tissue growth control), and all are atypical cadherins, that is, transmembrane proteins that regulate cell adhesion and communication but are unlikely to move from cell to cell and thus cannot be morphogens.

Lawrence et al. (2004) have proposed a model for establishing polarity in the abdominal epidermis of *Drosophila* based on cell-cell interactions, which may provide cells with positional information with the morphogen playing a key role. The transmembrane proteins Four-jointed, Dachsous, and Fat are involved in setting up a gradient of an unknown factor X, which determines Frizzled activity. Reading the gradient are Frizzled itself, Prickle, and VanGogh/Strabismus, enabling cells to compare the concentration of X at their position with that of their neighbors and to set their value as an average of these. On the other hand, Ma et al. (2003) proposed a model for planar cell polarity based purely on cell-cell interactions and local (cell-scale) graded distributions, where factor X is dispensed with, and hence no global positional information is imparted to cells. The model of Le Garrec et al. (2006) posits that a weak Frizzled activity gradient is read by asymmetric molecular complexes built at cell interfaces around the cadherin Flamingo. Polarization occurs robustly over hundreds of cell diameters even with noise in the Frizzled activation gradient. It is probable that these last two models have the potential, if suitably extended, to provide positional information in wing primordia of the fly embryo. In this context, we note that Baena-Lopez and Garcia-Bellido (2006) have proposed that local cell-cell interactions triggered by differences in *Vestigial* gene expression between neighboring cells could specify positional information in the fly wing.

Very different from morphogen gradients is the mechanism specifying position in the proximo-distal patterning of the vertebrate limb and in somite formation along the main body axis of vertebrate embryos (Dubrulle and Pourquié, 2004). In this last case, in particular, oscillations in time and space of molecule concentrations play a spectacular role, and cell-cell communication by signaling through the Notch receptor bound by Delta ligand is crucial for synchronizing local oscillators. It should be noted that the Notch-Delta signaling pathway figures prominently in the polarization of the *Drosophila* eye, providing a possible phylogenetic link between polarization and positional specification.

We suggest that, just like the mechanisms involved in polarization and somite formation, those for setting up positional values may involve cell-cell interactions. There might even exist an overlap among the molecular players in these seemingly independent sets of phenomena. Morphogenetic molecules do exist, but it seems improbable that their concentration alone determines the fate of cells regarding their final position in the developing embryo. Wardle and Smith (2004) reported that early in development gene expression at the single-cell level is rather variable and only later does it become more precisely linked to cell position. Thus, morphogens may represent a rather crude positional information system, which is then more finely tuned by cell-cell interactions. Clearly, the morphogen gradient does not act alone and is itself specified by a variety of complex cellular mechanisms. Morphogen propagation, signaling, and readout are only the most studied parts of an iceberg of interactions that determine positional value in the embryo.

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