Morphogen Gradients: From Generation to Interpretation

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Abstract

Morphogens are long-range signaling molecules that pattern developing tissues in a concentration-dependent manner. The graded activity of morphogens within tissues exposes cells to different signal levels and leads to region-specific transcriptional responses and cell fates. In its simplest incarnation, a morphogen signal forms a gradient by diffusion from a local source and clearance in surrounding tissues. Responding cells often transduce morphogen levels in a linear fashion, which results in the graded activation of transcriptional effectors. The concentration-dependent expression of morphogen target genes is achieved by their different binding affinities for transcriptional effectors as well as inputs from other transcriptional regulators. Morphogen distribution and interpretation are the result of complex interactions between the morphogen and responding tissues. The response to a morphogen is dependent not simply on morphogen concentration but also on the duration of morphogen exposure and the state of the target cells. In this review, we describe the morphogen concept and discuss the mechanisms that underlie the generation, modulation, and interpretation of morphogen gradients.

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INTRODUCTION

Multicellular organisms come in many forms and shapes, but all of them face the same fundamental challenge during development: generation of distinct cell types and organs from a single cell. One way that this patterning process can be achieved is through morphogen gradients (Briscoe et al. 2010). Morphogens are long-range signaling molecules that act over a few to several dozen cell diameters to induce concentration-dependent cellular responses. The graded distribution of a morphogen within a tissue exposes cells to different morphogen concentrations. Cells exposed to high levels of morphogen signaling activate different transcriptional programs and adopt different fates than cells exposed to lower levels. Graded morphogen distribution thereby subdivides tissues into distinct cell types that are arranged as a function of their distance from the source (Briscoe et al. 2010).

This review discusses the mechanisms underlying morphogen-mediated fate specification. We refer the reader to recent reviews and studies that discuss other aspects of morphogen signaling, such as axon guidance, cell and tissue polarity, growth control, regeneration, evolution, and the application of morphogens in regenerative medicine (Brockes & Kumar 2008, Crickmore & Mann 2008, Lynch & Roth 2011, Meinhardt 2009, Schwank et al. 2011, Strutt 2009, Swaney et al. 2010, Umulis et al. 2009, Wichterle et al. 2002). We begin by describing the history of the morphogen concept and the molecular characterization of morphogens. Next, we address how morphogen gradients form and how morphogens move through tissues. We describe how interactions between morphogens and target tissues modify the shape of morphogen gradients. We then discuss how morphogen signals are transduced and how cells interpret different concentrations and durations of morphogen exposure to generate distinct transcriptional responses. Finally, we discuss putative mechanisms that allow morphogen gradients to pattern tissues precisely and robustly. We illustrate general principles

by focusing on a few key morphogen systems. Many other reviews provide more detailed information about individual morphogens (Affolter & Basler 2007, Dessaud et al. 2008, Gallet 2011, Grimm et al. 2010, Moussian & Roth 2005, Porcher & Dostatni 2010, Reeves & Stathopoulos 2009, Schier 2009, Shilo & Barkai 2007, Umulis et al. 2009, Wartlick et al. 2009, Yan & Lin 2009, Zakin & De Robertis 2010).

HISTORY OF THE MORPHOGEN CONCEPT

Embryological and Theoretical Studies

The current concept of morphogen-regulated development is a synthesis of several ideas—induction, gradients, thresholds, and diffusion—that emerged from embryological and theoretical studies starting in the early twentieth century. The idea of induction, whereby a cell or tissue instructs neighboring cells to adopt a particular fate, was suggested as a mechanism underlying embryonic patterning. Other studies proposed that concentration gradients of molecules could provide cells with information about their position within a tissue, and concentrations above or below a certain threshold might elicit different responses. Finally, the idea of diffusion was suggested as a mechanism that could generate gradients by the spreading of molecules throughout target fields.

The first examples of induction came from experiments in amphibians by Warren H. Lewis, Hans Spemann, and Hilde Mangold. Lewis found that transplantation of the optic cup into the epidermis caused ectopic lens formation (Lewis 1904, Wolpert 1986). Spemann and Mangold found that transplanting the dorsal pole of a gastrula embryo to the ventral side of a host embryo could induce a second embryonic axis (Spemann & Mangold 1924, Wolpert 1986). These studies revealed the inductive abilities of certain tissues but raised the question of how a single tissue could induce multiple cell types.

In the early twentieth century, Thomas Hunt Morgan and others proposed the idea that gradients might coordinate development (Boveri 1901, Morgan 1901). Morgan suggested that a gradually decreasing distribution of "material" from the animal to the vegetal pole of the developing sea urchin embryo could control gastrulation and generate patterns within developing tissues. This idea did not gain immediate acceptance, however, because it was unclear how the continuously graded distribution of a material could generate discrete regions of specification. Albert Dalcq and Jean Pasteels introduced the idea of thresholds in morphogenesis (Dalcq 1938), and Klaus Sander provided experimental evidence supporting the idea that exposure to a range of concentrations might be sufficient to induce a particular fate, as long as signaling levels were within certain boundaries (Sander 1960). Signaling levels below or above these boundaries would result in adoption of different fates. Leopold von Ubisch connected the concept of morphogen gradients to differential gene activation and thus was able to provide a molecular framework for morphogen gradient interpretation (von Ubisch 1953).

Hildegard Stumpf made the link between gradients and induction in the 1960s. On the basis of transplantation studies in moth pupae, she suggested that the graded distribution of inductive molecules within tissues could account for developmental patterning (Stumpf 1966). Stumpf proposed that an inductive signal was released from a localized source—i.e., an inductive tissue—and that it instructed the concentration-dependent generation of several distinct cell types in surrounding tissues. In the related positional information theory, Lewis Wolpert and others proposed that each cell within a field is assigned a "positional value" that provides information about the cell's position with respect to other cells (Lawrence 1966, Stumpf 1966, Wolpert 1969). Cells with different positional values could adopt different fates, and positional values could be assigned on the basis of morphogen exposure. Importantly, a signal providing positional information

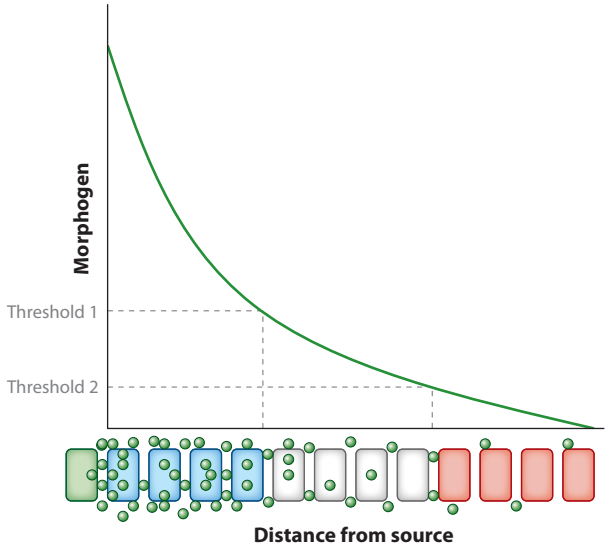

Figure 1

The French flag model. Morphogen is secreted from a source cell (*green*) and forms a concentration gradient within the tissue. Cells exposed to morphogen concentrations above threshold 1 exhibit a distinct response (*blue*). Cells exposed to intermediate morphogen concentrations (between thresholds 1 and 2) exhibit the "white" response, whereas cells exposed to levels below threshold 2 exhibit the "red" response. In this way, a concentration gradient of a single, diffusing substance could give rise to multiple cell fates and assign positional values to cells. Figure based on Kicheva & González-Gaitán (2008).

would not give specific instructions about the particular fate a cell should adopt. Instead, a combination of genotype, prior exposure to developmental signals, and positional information would control cell fate decisions. Thus, the same morphogen could be used in multiple tissue types or animals to provide positional information without dictating a particular cell fate (Wolpert 1969). The influential "French flag" model synthesized the concepts of induction, thresholds, and positional information and illustrated how gradients of inductive molecules could subdivide developing tissues into discrete regions of differentiation (**Figure 1**).

The French flag model provided one explanation for how gradients could pattern developing tissues, but how would these gradients be formed? As early as 1952, Alan Turing, who coined the term morphogen ("form producer"), and later Alfred Gierer and Hans Meinhardt, developed mathematical models describing how gradients could form in tissues (Gierer & Meinhardt 1972, Turing 1952). Their models described how interactions between diffusing molecules could lead to the formation of gradients (and other patterns) across a field of cells. In such models, a locally acting activator induces both its own synthesis and the synthesis of a long-range inhibitor. This can result in self-organization that generates patterns in an initially homogenous field of cells (Kondo & Miura 2010).

In 1970, Francis Crick proposed a simple "source-sink" model for the generation of morphogen gradients (Crick 1970). He suggested that localized cells produce a morphogen and secrete it into surrounding tissue. Morphogen molecules then diffuse and are destroyed by "sink" cells that are located at the opposite end of the tissue. These processes of diffusion and destruction together would result in a stable concentration gradient of morphogen, with the highest concentration located near the source cells and the lowest near the sink.

By the beginning of the 1970s, theoretical models and further embryological studies (Summerbell et al. 1973, Tickle et al. 1975, Wolpert et al. 1971) provided possible explanations of how morphogen gradient–mediated development could occur, but the discovery and characterization of morphogens had to await the advent of developmental genetics and molecular biology.

Molecular Studies

Despite the wealth of theories on gradient-mediated biological patterning, direct evidence that gradients control pattern formation in vivo was lacking until the late twentieth century. For example, molecular studies in the 1980s identified molecules that were distributed in gradients within developing tissues but did not function as morphogens. The small molecule retinoic acid (RA), for example, was found to form a shallow gradient within developing limb buds, but an essential patterning role for this gradient has not been demonstrated (Thaller & Eichele 1987). Graded distribution of the homeodomain protein Caudal was found along the anterior-posterior axis in *Drosophila*

embryos (Macdonald & Struhl 1986, Mlodzik & Gehring 1987, Mlodzik et al. 1985), but the patterning defects in *caudal* mutants could be rescued by nongraded expression of Caudal protein, which argues against a requirement for the gradient (Macdonald & Struhl 1986).

The graded distribution and function of the Bicoid protein in *Drosophila* embryos provided the first clear connection between a molecular gradient and pattern formation. The Bicoid transcriptional regulator forms an anterior-to-posterior gradient in the syncytial blastoderm (Driever & Nüsslein-Volhard 1988a, Struhl et al. 1989) (**Supplemental Figure 1**; follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org/**). High levels of Bicoid are required for expression of anterior marker genes, whereas genes with more posterior expression domain boundaries are expressed at lower levels of Bicoid (Driever & Nüsslein-Volhard 1988b, Struhl et al. 1989). Dampening of the Bicoid gradient resulted in the loss of anterior markers and the anterior shift of posterior markers. Although Bicoid was the first morphogen to be discovered, its molecular nature (transcriptional regulator) and environment (syncytium) make it unusual compared with most other morphogens, which are extracellular ligands that act in cellularized tissues. However, Bicoid does provide a valuable system for the study of how graded transcriptional effector activity, the ultimate outcome of morphogen gradient activity, leads to differential gene expression. Shortly after the discovery of the anterior-posterior Bicoid gradient, a ventral-to-dorsal nuclear gradient of the transcriptional regulator Dorsal was discovered and found to be required for dorsal-ventral patterning in *Drosophila* (Roth et al. 1989, Rushlow et al. 1989, Steward 1989). Thus, Bicoid and Dorsal were the first examples of transcriptional regulator gradients that control embryonic patterning.

Members of the transforming growth factor β (TGFβ) family were the first extracellular morphogens to be identified. Graded and long-range activity of *Drosophila* Decapentaplegic (Dpp) was found to pattern multiple tissues, including the dorsal-ventral embryonic axis and the wing imaginal disc (Affolter & Basler 2007, Ferguson & Anderson 1992, Lecuit et al. 1996, Nellen et al. 1996, Umulis et al. 2009). Exposure of *Xenopus* cells to different concentrations of the TGFβ signal Activin induced different mesodermal and endodermal cell types (Green & Smith 1990, Green et al. 1992, Gurdon et al. 1994). In this assay, Activin mimics the effects of TGFβ signals of the Nodal family, which are the endogenous morphogens that pattern the germ layers (Chen & Schier 2001, Schier 2009, Shen 2007). The 1990s also saw the identification of several additional extracellular morphogen ligands ranging from Wingless (Wg)/Wnt to Hedgehog (Hh) (Briscoe et al. 2001, Heemskerk & DiNardo 1994, Katz et al. 1995, Kiecker & Niehrs 2001, Morisato & Anderson 1994, Neumann & Cohen 1997, Tabata & Kornberg 1994, Zecca et al. 1996). Following the discovery of bona fide morphogens, research began to address how morphogen gradients form, how morphogen signals are transduced, and how responding cells interpret graded signals and modulate the formation and interpretation of morphogen gradients. We discuss these aspects of morphogen biology in the following sections.

Supplemental Material

GENERATION OF MORPHOGEN GRADIENTS

How do morphogens move through target fields? Do they diffuse passively, or are they actively transported? Do morphogens move as individual molecules or as higher-order aggregates? Do they travel through extracellular spaces, or do they move through cells? Here we discuss our current knowledge of the mechanisms by which morphogen gradients are formed.

The Synthesis-Diffusion-Clearance Model of Gradient Formation

The prevailing model of morphogen gradient formation is the synthesis, diffusion,

and clearance (SDC) model [also called the synthesis-diffusion-degradation (SDD) model]. According to this model, morphogen is produced from a localized source, diffuses through tissues, and is cleared (Crick 1970, Wartlick et al. 2009). In the simplest scenario, a group of secreting cells creates a morphogen flux into an initially homogeneous field of cells. Morphogen molecules then move through the target field by diffusion. Clearance results in the removal of morphogen from the diffusible pool, for example, by immobilization, degradation, or endocytosis (Lander et al. 2009). The combination of constant flux from a localized source, diffusion, and uniform clearance results in a decaying concentration gradient. The distance over which the gradient decays depends on the diffusivity and clearance rates: the higher the diffusivity and lower the clearance rate are, the longer the morphogen's range (**Supplemental Figure 2**) (for a detailed mathematical description, see Wartlick et al. 2009).

Recent studies have begun to test these theoretical predictions by measuring morphogen gradient profiles using fluorescent protein fusions or immunohistochemistry (e.g., Callejo et al. 2006; Chamberlain et al. 2008; Driever & Nüsslein-Volhard 1988a; Gregor et al. 2007a,b; Gritli-Linde et al. 2001; Kicheva et al. 2007; Roth et al. 1989; Rushlow et al. 1989; Steward 1989; Strigini & Cohen 2000; Teleman & Cohen 2000; Yu et al. 2009). These studies show that most morphogen gradients approximate an exponentially decaying curve within developing tissues, which supports the SDC model. The exponential profiles of morphogen gradients suggest that clearance of most morphogens occurs throughout tissues rather than at a localized sink far from the source, as proposed by Crick (1970).

Biophysics of Gradient Formation

Recent biophysical measurements further support the SDC model and suggest that morphogen gradients are formed via diffusion (Abu-Arish et al. 2010, Kicheva et al. 2007, Lander 2007, Yu et al. 2009). For example, the spatial profile and biophysical properties of fluorescently labeled FGF8 (fibroblast growth factor 8) in zebrafish embryos and Dpp in imaginal discs are consistent with the idea that these morphogens spread nondirectionally via a diffusive process (Kicheva et al. 2007, Yu et al. 2009). Surprisingly, measured diffusion coefficients vary dramatically depending on the morphogen, tissue context, and experimental approach. The effective diffusion coefficients of Wg-GFP (green fluorescent protein) and Dpp-GFP in the *Drosophila* wing disc were found to be 0.05 $\mu m^2\ s^{-1}$ and 0.1 $\mu m^2\ s^{-1}$, respectively (Kicheva et al. 2007). In the imaginal disc that gives rise to the small wing-like organ called the haltere, the effective diffusion coefficient of Dpp-GFP is much lower, less than 0.003 $\mu m^2\ s^{-1}$ (Wartlick et al. 2011). These effective diffusion coefficients are more than 1,000 times smaller than the diffusion coefficient of GFP in solution or the diffusion coefficient of FGF8-GFP in extracellular spaces within zebrafish embryos [50 $\mu m^2\ s^{-1}$ (Yu et al. 2009)]. Analysis of Bicoid-GFP in the cortical cytoplasm has yielded diffusion coefficients that range from 0.3 $\mu m^2\ s^{-1}$ (Gregor et al. 2007b) to ~7 $\mu m^2\ s^{-1}$ (Abu-Arish et al. 2010, Porcher et al. 2010) at mitotic cycle 14. In the nucleus, two diffusion processes of Bicoid-GFP have been detected with diffusion coefficients of ~0.2 $\mu m^2\ s^{-1}$ and ~8 $\mu m^2\ s^{-1}$, respectively (Porcher et al. 2010).

Why do the measured diffusion coefficients of morphogens differ by four orders of magnitude? The movement of some but not other morphogens may be hindered by obstructions in the cellular environment and by interactions with other molecules (Rusakov & Kullmann 1998, Thorne et al. 2008). However, there is a striking correlation between diffusion coefficients and experimental approaches. The high diffusion coefficients were deduced from fluorescence correlation spectroscopy (FCS) measurements, whereas the lower diffusion coefficients were determined by fluorescence recovery after photobleaching (FRAP). These two techniques measure diffusion within different environmental contexts, time windows, and length scales (**Supplemental Figure 3**) (see

Grimm et al. 2010 for a detailed discussion). The FCS experiments observed diffusion in small volumes (<0.5 μm^3) and over short timescales (<100 s). In contrast, the FRAP experiments observed diffusion over multiple cells or nuclei (>1,000 μm^3) and long time windows (~1 h). Considering that morphogen gradients range from 10–200 μm and are formed within 30–300 min, Grimm et al. (2010) have argued that FRAP is a better measure of overall morphogen movement than FCS. For example, FCS might detect mainly the short-term diffusion of mobile molecules in a subenvironment within a tissue. In contrast, FRAP examines a cohort of molecules in different environments. The FRAP-based measurements thus provide effective diffusion coefficients that potentially reflect long-term movement through multiple environments (e.g., apical and basolateral, extracellular and intracellular, matrix and membrane) or that might be dominated by processes that trap molecules and cause them to accumulate locally (e.g., binding to extracellular matrix, endocytosis) (Bergmann et al. 2007). In contrast, the high diffusion coefficient of FGF8 measured by FCS (~50 $\mu m^2\ s^{-1}$) might correspond to free diffusion in extracellular spaces located at a distance from cell membranes. Indeed, the observation that a fraction of FGF8 signals has a diffusion coefficient of ~5 $\mu m^2\ s^{-1}$ (Yu et al. 2009) suggests that the overall effective diffusion of morphogens is a composite of interactions with multiple environments. Additional studies are needed to determine whether FRAP is the more accurate measure of long-range diffusivity, whereas FCS provides a potential means to dissect movements and interactions at smaller scales.

Clearance rate coefficients provide an additional test of SDC models but have been determined directly only for recombinant, bacterially produced FGF8 in zebrafish. Consistent with an SDC mechanism, FGF8 has a half-life between 9 and 18 min (Yu et al. 2009). On the basis of the shape of the gradient and the diffusion coefficients, the half-lives of Dpp, Wg, and Bicoid have been estimated as 45, 8, and 40 min, respectively (Abu-Arish et al. 2010, Kicheva et al. 2007). The short half-life of Wg may contribute to the comparatively short range of the Wg gradient. Proteasome-mediated degradation of Bicoid is crucial for normal gradient formation (Liu & Ma 2011). Interestingly, Wartlick et al. (2011) suggested that clearance rates for Dpp decrease as the wing disc grows, which allows the formation of a longer-range gradient in larger tissues.

For clarity, we have thus far made some simplifying assumptions when discussing the SDC model (e.g., the tissue being patterned is flat and completely uniform in composition, and clearance rates are equal throughout the tissue). In reality, gradient formation takes place in a dynamic 3D tissue. Therefore, although the basic concept of synthesis, diffusion, and clearance remains valid, several additional factors must be considered when modeling gradient formation in vivo. For example, target tissues are often heterogeneous, and feedback mechanisms can modify diffusion and clearance (Bollenbach et al. 2008, Cadigan et al. 1998, Chen & Struhl 1996, Dessaud et al. 2007, Lecuit & Cohen 1998). In addition, the sources of some morphogens are dynamic and not tightly localized. For example, Bicoid mRNA is not localized solely to the anterior-most pole; an extended SDC model is required to account for Bicoid protein distribution (Little et al. 2011, Spirov et al. 2009, St. Johnston et al. 1989).

Some gradients form by mechanisms other than SDC. For example, a gradient of FGF8 mRNA in the embryonic mouse tail bud results from localized transcription followed by cell division and transcript degradation (Dubrulle & Pourquié 2004). Thus, the FGF8 mRNA and protein gradients in the tail bud form by a cell lineage transport mechanism rather than by diffusion from a localized source. Other variations in gradient formation are exemplified by RA and bone morphogenetic protein (BMP). The RA gradient in the zebrafish hindbrain may be shaped mainly by spatially regulated expression of enzymes that degrade RA, such that a localized source of RA is not strictly necessary (White et al. 2007). As discussed below, gradients of BMP antagonists help

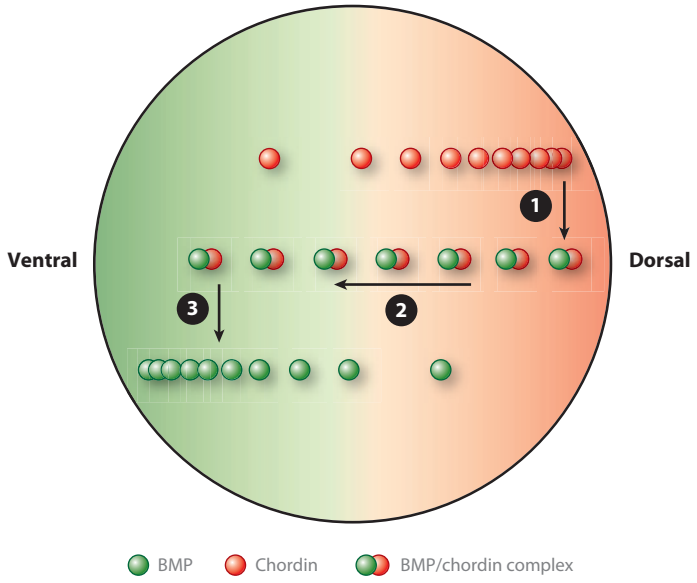

Figure 2

The bone morphogenetic protein (BMP) shuttling mechanism. In *Xenopus* embryos, Chordin (*red*) is secreted from the dorsal region, whereas BMP (*green*) is initially uniformly expressed. ① Chordin, upon secretion from the dorsal region, forms a complex with and antagonizes BMP. ② This interaction mobilizes BMP as complexes diffuse in the extracellular space. ③ Chordin is cleaved by an extracellular protease, which causes it to release and deposit BMP at the site of cleavage. This shuttling generates a ventral-to-dorsal gradient. Figure based on Lewis (2008).

redistribute initially uniform BMP molecules into a gradient (Ben-Zvi et al. 2008, Eldar et al. 2002, Holley et al. 1996, Marqués et al. 1997, Shimmi & O'Connor 2003, Shimmi et al. 2005, Umulis et al. 2009, Wang & Ferguson 2005, Zakin & De Robertis 2010).

Morphogen Vehicles

In the simplest case, morphogens diffuse through tissues as individual monomers or dimers. Beads soaked with RA, Activin, or FGF8 can serve as ectopic signal sources and induce long-range signaling (Gurdon et al. 1994, Nowak et al. 2011, White et al. 2007). In these cases, there is no apparent requirement for modification of signaling molecules before release. It is becoming clear, however, that most morphogens oligomerize, interact with other diffusible proteins, or are lipid modified (**Supplemental Figure 4**). For example, formation of the Dpp/BMP gradients in *Drosophila*, *Tribolium*, and *Xenopus* embryos involves the association of these molecules with secreted antagonists (Ben-Zvi et al. 2008, Eldar et al. 2002, Holley et al. 1996, Lewis 2008, Marqués et al. 1997, Reversade & De Robertis 2005, Shimmi & O'Connor 2003, Shimmi et al. 2005, van der Zee et al. 2006, Wang & Ferguson 2005, Zakin & De Robertis 2010). BMPs are initially found both ventrally and dorsally in *Xenopus* blastulae. They are mobilized when complexed with Chordin, a BMP antagonist that is secreted on the dorsal side. As Chordin diffuses away from its dorsal source, it binds to, inhibits, and mobilizes BMPs until the extracellular protease tolloid degrades the Chordin portion of this complex (**Figure 2**; Lewis 2008, Zakin & De Robertis 2010). This shuttling results in the deposition of BMPs near the site of Chordin degradation and reshapes the distribution and activity of BMPs into a gradient that peaks in the ventral region. Several additional extracellular molecules act as anti- or pro-BMPs and regulate the movement of BMPs within the embryo (Zakin & De Robertis 2010). A homologous mechanism is used during dorsal-ventral patterning in *Drosophila* (Umulis et al. 2009).

Lipidation modulates the movement of some morphogens (Eaton 2008, Steinhauer & Treisman 2009). For example, the mature, secreted Hh peptide is covalently modified by both cholesterol and palmitic acid (Gallet 2011). Modified Hh remains associated with the outer leaflet of the plasma membrane, possibly in lipid rafts, until its release, which is dependent on the transmembrane protein Dispatched (Gallet 2011). The release of Hh from the plasma membrane allows long-range spread and signaling.

Some morphogens appear to form higher-order structures (Eaton 2008, Gallet 2011). At the smallest scale, electrostatic interactions mediate the formation of Hh oligomers that cluster at the cell surface (Vyas et al. 2008). Mutant Hh proteins that retain signaling competence but lack the ability to aggregate lose long-range signaling capabilities. Other

Supplemental Material

studies suggest that Hh and Wg may be packaged through their lipid modifications into larger-scale lipoprotein particles (**Supplemental Figure 4**) (Callejo et al. 2008, Eaton 2008, Eugster et al. 2007, Gallet 2011, Panáková et al. 2005). As discussed below, lipidation might enhance the interactions with extracellular proteoglycans and act to concentrate morphogens at cell surfaces. Moreover, packaging of Hh and Wg into lipoprotein particles might account for the observations that lipoprotein receptor-related proteins can act as coreceptors for Hh and Wg (Fisher & Howie 2006, He et al. 2004) and that the Hh receptor Patched is itself a lipoprotein receptor (Callejo et al. 2008). Although these studies suggest important roles for Hh-containing particles, the composition, arrangement, and role of higher-order morphogen assemblies are still poorly understood.

Extra- and Intracellular Morphogen Routes

Low-affinity interactions with extracellular matrix components such as heparan sulfate proteoglycans (HSPGs) are thought to modulate several aspects of morphogen biology. HSPGs are composed of protein cores to which sulfated glycosaminoglycan chains are attached. They confine the movement of secreted morphogens to cell surfaces, thereby effectively increasing the concentration of molecules at the surface, promoting productive morphogen-receptor interactions, and preventing the release of morphogens into the lumen that overlies epithelia (**Supplemental Figure 5**) (Baeg et al. 2001, Belenkaya et al. 2004, Callejo et al. 2006, Gallet et al. 2006, Han et al. 2005, Takei et al. 2004, Vincent & Dubois 2002, Yan & Lin 2009, Yan et al. 2009). For example, extracellular Dpp is lost from the surface of cells lacking functional HSPGs, and HSPG mutant cells exhibit attenuated Dpp responses compared with their HSPG-expressing neighbors (Belenkaya et al. 2004, Takei et al. 2004). Thus, extracellular HSPG-mediated tethering of Dpp is required for morphogen signaling and spreading along the cell surface.

Interactions with HSPGs can have multiple additional effects. For example, HSPGs may act as coreceptors for morphogens (Fujise et al. 2003, Strigini & Cohen 2000, Tsuda et al. 1999). Morphogen binding to HSPGs can restrict movement not only to the surface of a cell but also to areas near the source, which shortens the signal's range. For example, cleavage of heparin sulfate chains from HSPGs in zebrafish embryos causes an expansion of the FGF8 activity range, presumably by allowing FGF8 to move farther from its source (Yu et al. 2009). In contrast, HSPGs are required for the long-range spreading of some morphogens (Baeg et al. 2001, Belenkaya et al. 2004, Marjoram & Wright 2011, Oki et al. 2007, Takei et al. 2004, The et al. 1999). For example, Vyas et al. (2008) proposed that the aggregation of Hh molecules discussed above allows Hh to interact with HSPGs, thereby facilitating its ability to interact with receptors as it moves through the wing disc. Lipoprotein particles can also interact with HSPGs and contribute to morphogen spreading (Eugster et al. 2007). HSPGs might even be involved in the release and packaging of morphogens at the source. For example, HSPGs might facilitate the assembly of lipidated Hh into Hh oligomers or lipoprotein particles (Eaton 2008, Eugster et al. 2007, Gallet 2011, Panáková et al. 2005). Finally, HSPGs might promote the stabilization and spreading of morphogens by preventing internalization and clearance (Akiyama et al. 2008, Takei et al. 2004). For example, Dpp mutant proteins that are unable to interact with the HSPG Dally are less stable in the extracellular space than wild-type Dpp and form shallower extracellular gradients of lower amplitude and decreased range (Akiyama et al. 2008).

The complex roles of HSPGs are also exemplified by their functions in Wg morphogen signaling. Strikingly, low levels of Dally-like protein (Dlp), a protein core of *Drosophila* HSPG, enhance Wg signaling, whereas higher levels inhibit signaling (Gallet et al. 2008, Yan et al. 2009). One model explaining this biphasic behavior of Dlp suggests that low (agonistic) levels of Dlp enhance signaling by

concentrating Wg on the cell surface, which prevents its loss into the extracellular space and makes it available to its receptor Frizzled2 (Fz2) (**Figure 3**). Conversely, high (antagonistic) levels of Dlp compete with Fz2 for Wg binding (Yan et al. 2009). Other biphasic modulators of morphogen signaling include *Drosophila* Crossveinless2 as well as *Xenopus* Syndecan-1 (Olivares et al. 2009, Serpe et al. 2008) and Ont1 (Inomata et al. 2008), which modulate BMP signaling, and *Drosophila* Ihog, which modulates Hh signaling (Dessaud et al. 2008, Yan et al. 2010). Biphasic modulation of receptor-morphogen interaction may help generate sharp borders of gene expression by creating bistable states in which morphogen binds either many or few receptors (Olivares et al. 2009, Serpe et al. 2008).

The multiple potential roles of HSPGs have made it difficult to interpret the results of mutant analyses. For example, although the loss of an HSPG might lead to reduced signaling, it is unclear if this is due to changes in diffusion, increased clearance, release from the cell surface, reduced presentation to receptors, abnormal packaging at the source, or other aspects of morphogen signaling. Quantitative imaging and biophysical studies will be needed to address the exact roles of HSPGs and their modulators in different systems (Kleinschmit et al. 2010, Szuperák et al. 2011, Vuilleumier et al. 2010).

Importantly, extracellular environments vary widely between different tissues and even within a tissue. For example, Dpp is much more mobile in early *Drosophila* embryos than in the wing discs. This increased mobility is thought to be caused, at least in part, by the absence of HSPGs in early embryos (Bornemann et al. 2008). Even within in the same tissue, a single morphogen can have different ranges. For example, Hh is secreted both apically and basolaterally from the wing disc epithelium (**Supplemental Figure 6**). Strikingly, basolateral Hh acts only at a short range, whereas apically released Hh acts at a long range (Ayers et al. 2010). Apical Hh is dispersed more widely due to its interaction with the HSPG Dally. Dally binds Hh and is released from the cell surface by the hydrolase Notum, which increases the mobility and range of Hh. In contrast, basolateral Hh signals move through cell-cell contact or through limited diffusion. This example highlights that overall morphogen distribution is a composite of different gradients that form by distinct mechanisms. Finally, even highly related morphogens can have different ranges within the same tissue. For example, the Nodal signal Squint has a longer range in zebrafish embryos than the related Cyclops signal (Chen & Schier 2001, Schier 2009).

In addition to the composition of the extracellular matrix, endocytosis has been implicated in modulating morphogen gradient formation. Blockage of endocytosis can prevent morphogen movement (Bejsovec & Wieschaus 1995, Entchev et al. 2000, Gallet et al. 2008, Kicheva et al. 2007, Moline et al. 1999). One interpretation of this result is that morphogens may be transported intracellularly by a process called transcytosis, in which cellular uptake and subsequent release mediate the spread of morphogen molecules through a tissue (**Supplemental Figure 6**; Entchev et al. 2000, González et al. 1991, Kruse et al. 2004). An alternative interpretation is that preventing endocytosis may lead to a buildup of extracellular molecules (e.g., receptors), thereby sequestering and inhibiting the spreading of morphogens (Belenkaya et al. 2004, Lander et al. 2002). Interestingly, however, transient blockage of endocytosis using temperature-sensitive mutants almost immediately precludes movement or accumulation of Dpp, which would be inconsistent with the latter interpretation, because receptor buildup should take time (Kicheva et al. 2007). In some cases, blocking endocytosis does not preclude gradient formation and can even increase gradient range, potentially by decreasing ligand clearance rates or altering trafficking (Dubois et al. 2001, Nowak et al. 2011, Scholpp & Brand 2004, Strigini & Cohen 2000, Yu et al. 2009).

Although extracellular diffusion appears to be a major mode of morphogen movement,

Supplemental Material

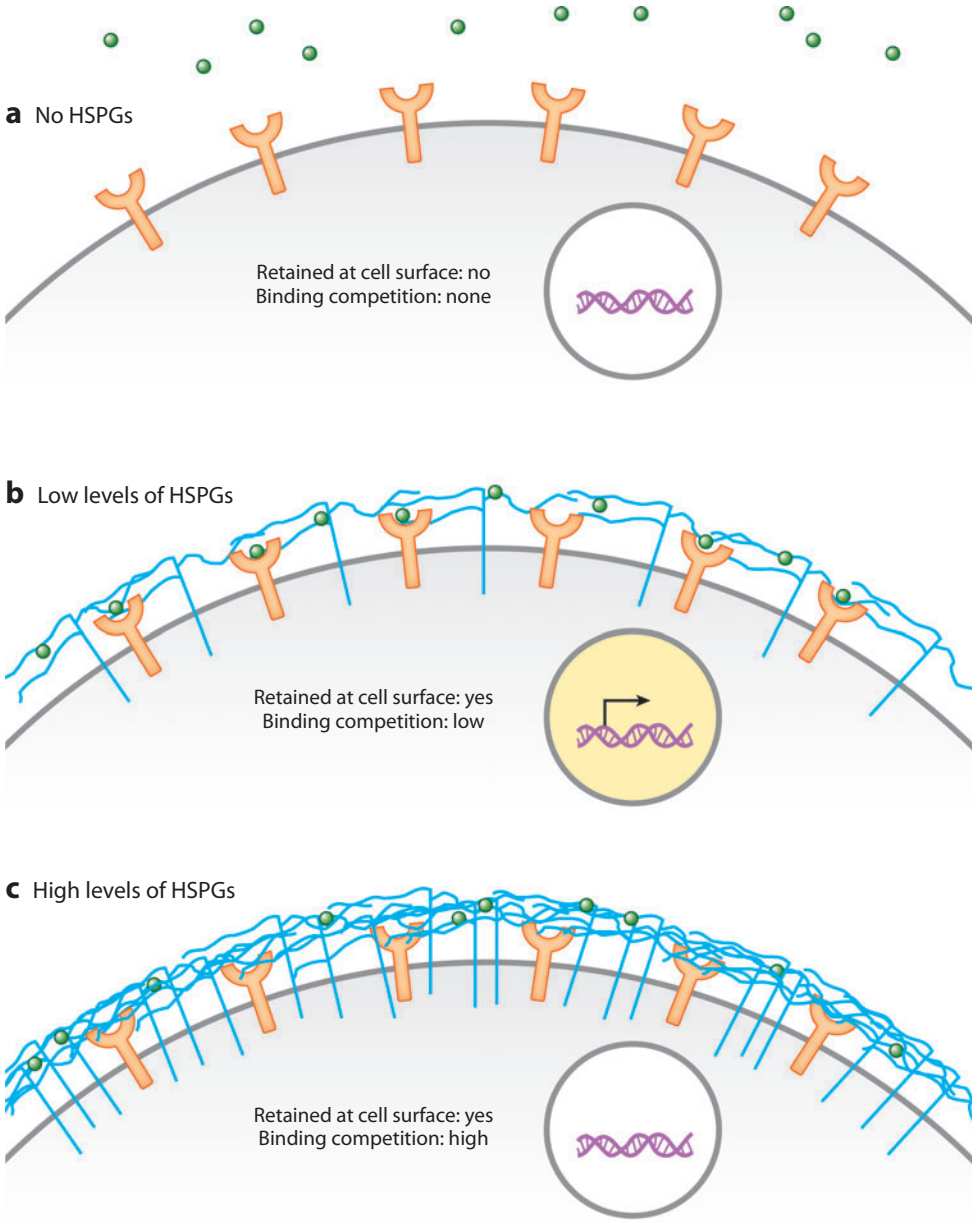

Figure 3

Biphasic activity of heparan sulfate proteoglycans (HSPGs). (*a*) In the absence of HSPGs (*blue*), morphogen molecules (*green*) are not concentrated at the cell surface; therefore, receptor-morphogen interactions are reduced and target genes are not induced. (*b*) When HSPGs are present in low abundance, they bind morphogen molecules, concentrating them at the cell surface and promoting receptor-morphogen interactions, resulting in target gene expression. (*c*) When HSPGs are present in high abundance, morphogen molecules are still retained at cell surfaces. However, because HSPG molecules bind morphogen, they outcompete receptors for morphogen binding. Thus, environments with high levels of HSPGs contain many additional binding sites that compete with receptors for morphogen binding, whereas environments with low levels of HSPGs contain fewer competing binding sites but still concentrate morphogen molecules at cell surfaces.

long filopodia-like extensions also have been implicated in morphogen signaling (Hsiung et al. 2005, Ramirez-Weber & Kornberg 1999, Roy et al. 2011). Although such structures are used during cell communication in processes such as synapse formation and Notch signaling (Cohen et al. 2010, De Joussineau et al. 2003), their importance in morphogen signaling has not been established.

Modulation of Morphogen Distribution by Target Tissue Feedback

Although the target tissues of morphogens have classically been portrayed as simple responders or readers of gradients, it is now clear that the dialogue between morphogens and target cells contributes to gradient shape and interpretation. The feedback regulation of morphogen receptor expression exemplifies this (Cadigan et al. 1998, Chen & Struhl 1996, Dessaud et al. 2007, Lecuit & Cohen 1998). Expression of the Hh receptor Patched (Ptc), for example, is upregulated by Hh signaling (Chen & Struhl 1996). Ptc is an unusual receptor in that it actively represses activity of the Hh signaling pathway in the absence of Hh. Binding of Sonic hedgehog (Shh) to Ptc sequesters Shh and prevents it from spreading farther from its source. Moreover, Shh-induced Ptc expression promotes the endocytosis and degradation of Shh (Incardona et al. 2000). The extracellular vertebrate protein Hip1 also binds and sequesters Shh, antagonizing Shh signaling (Dessaud et al. 2008). In contrast, members of the Ihog/Boi/Cdo/Boc family and Gas1 (in vertebrates) bind Shh to promote pathway activation (Dessaud et al. 2008). Before Shh exposure, target tissues such as the developing spinal cord express low levels of Ptc and higher levels of Gas1, Cdo, and Boc (**Figure 4*a*,*b***). Therefore, upon secretion Shh moves into a target field that is highly responsive to Shh signaling. Binding of Shh to Ptc activates the Shh signaling pathway, induces Ptc and Hip1 expression, and represses Gas1, Cdo, and Boc expression. As a result, the target field

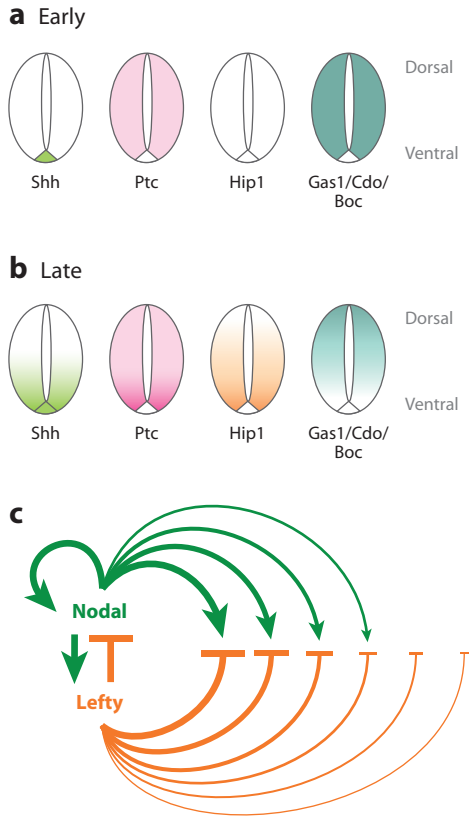

Figure 4

Target tissue feedback inhibition. (*a*,*b*) Model for tissue feedback during Sonic hedgehog (Shh) signaling in the developing spinal cord. (*a*) Before Shh secretion, levels of Gas1, Cdo, and Boc (*teal*) are high. Gas1, Cdo, and Boc are coreceptors for Hedgehog (Hh) and enhancers of Hh signaling. Patched (Ptc, *pink*) is expressed at low levels. Ptc represses Hh signaling in the absence of Hh but is inactive upon binding to Hh and sequesters Hh. Hip1 (*orange*), which binds and sequesters Hh, is not expressed in the absence of Shh. (*b*) Shh (*green*) is secreted into the ventral neural tube from the notochord (not shown) and floor plate (the ventral-most region in the neural tube). Upon Shh secretion, Shh signaling downregulates expression of Gas1, Cdo, and Boc and upregulates expression of Ptc and Hip1 near the source, which results in the sequestration of Shh and the dampening of pathway activation. (*c*) Model for feedback inhibition of Nodal signaling by Lefty. Nodal activity induces expression of the secreted Nodal inhibitor Lefty (*orange*), which is coexpressed with Nodal but has a longer activity range than Nodal (*green*). Model based on Chen & Schier (2002) and Shen (2007).

becomes less responsive and sequesters Hh, preventing its spread to regions more distant from the source. Moreover, Shh-induced Ptc expression promotes the endocytosis and degradation of Shh (Incardona et al. 2000). Thus, negative feedback loops can modulate the activity and spread of morphogen signals and are thought to contribute to the robustness of morphogen signaling (Barkai & Shilo 2009, Eldar et al. 2003, Irons et al. 2010, Lander et al. 2009).

In another example of morphogen-tissue dialogue, high levels of Dpp signaling in the wing disc repress the expression of the Dpp receptor Thickveins (Tkv), leading to an increase in the range of Dpp (Lecuit & Cohen 1998). This increase in range could be the result of an effective increase in Dpp diffusivity because fewer receptors are present to hinder Dpp mobility. In addition, a decrease in receptor expression could reduce the frequency of receptor-mediated endocytosis, thus decreasing clearance rates and increasing range. Strikingly, the repression of Tkv expression by Dpp is blocked in the developing haltere disc by the Hox transcriptional regulator Ultrabithorax (Ubx) (Crickmore & Mann 2006). Moreover, Ubx represses Dally (de Navas et al. 2006). Tkv expression and Dally repression restrict the range of the Dpp gradient in the haltere disk (Crickmore & Mann 2006, de Navas et al. 2006). Indeed, the Dpp diffusion coefficient in the haltere disc is smaller than in the wing disc (Wartlick et al. 2011). Thus, the limited diffusion and range of Dpp are thought to contribute to the smaller size and different patterning of the haltere compared to the wing.

In addition to morphogen receptors, secreted feedback regulators also modify the formation and interpretation of morphogen gradients (Piddini & Vincent 2009, Schier 2009, Szuperák et al. 2011, Vuilleumier et al. 2010). For example, Nodal ligands induce expression of Lefty proteins, which are secreted Nodal signaling inhibitors (Schier 2009). Lefty expression dampens Nodal activity and ensures that it does not extend beyond the appropriate range (**Figure 4*c***). Interestingly, the Nodal morphogen system shares several similarities with classical reaction-diffusion systems (Gierer & Meinhardt 1972, Kondo & Miura 2010, Meinhardt 2009) (see above section on Embryological and Theoretical Studies). In particular, Nodal and Lefty share the activator/inhibitor and self-enhancement features of this system. For example, in the zebrafish blastula, Nodal activates Nodal and Lefty transcription, and Lefty is required to restrict the range of Nodal signaling by blocking both the generation of Nodal locally and the response to Nodal at a distance (Chen & Schier 2002). The balance of Nodal and Lefty levels is further regulated by the microRNA miR-430 (Choi et al. 2007). The Nodal/Lefty activator/inhibitor pair also plays a role during left-right specification (Nakamura et al. 2006). In this system, Nodal/Lefty interactions appear to amplify small differences between the left and right sides. Analogous activator-feedback inhibitor pairs also have been implicated in head regeneration in *Hydra* (Meinhardt 2009), pigment stripe formation in zebrafish (Kondo & Miura 2010), and hair follicle spacing in mouse (Sick et al. 2006). However, in none of these systems have the tenets of reaction-diffusion models been tested by quantitative analyses.

TRANSDUCTION OF MORPHOGEN SIGNALING

As morphogens move through tissues, they bind receptors displayed on cell surfaces and initiate intracellular signaling cascades that result in the activation of transcriptional effectors. Different levels of effector activate and repress different sets of genes, which results in the execution of distinct developmental programs. In the following section, we discuss how binding of morphogens to receptors is translated into differential gene expression.

Reading Different Morphogen Concentrations

To detect differences in morphogen concentration, cells could measure either the absolute

number of occupied receptors or the ratio of bound to unbound receptors. With the possible exception of Hh signaling (Casali & Struhl 2004), the absolute number of activated, ligand-bound receptors is thought to determine morphogen signal transduction. For example, regardless of the total number of Activin receptors on a cell's surface, expression of a low-threshold target gene requires at least 100 occupied Activin receptors per cell, whereas expression of a high-threshold target gene requires at least 300 occupied receptors (Dyson & Gurdon 1998). This argues against models in which Activin signaling levels are measured by the ratio of bound to unbound receptors. Instead, Activin concentrations are transmitted by absolute receptor occupancy. Importantly, at the low morphogen concentrations found in developing tissues (i.e., concentrations lower than the dissociation constant of ligand-receptor complexes), receptor activation increases almost linearly with ligand level. Thus, total receptor activity is roughly proportional to morphogen concentration.

Linear Signal Transduction

Some biological signaling pathways can generate "all-or-none" or "switch-like" behavior of signal effectors or transducers. For example, exposure to increasing levels of progesterone, a signal that does not act as a morphogen, induces a switch-like activation of mitogen-activated protein kinase (MAPK) in immature *Xenopus* oocytes that leads to oocyte maturation (Ferrell & Machleder 1998). This all-or-none behavior generates a single response (oocyte maturation) from a graded input (different progesterone concentrations). Morphogen signaling, in contrast, generates multiple responses from a graded input (**Supplemental Figure 7**). In many cases, graded morphogen signaling is translated into graded transcriptional regulator activity, which leads to the expression of different sets of genes (Ashe & Briscoe 2006, Harvey & Smith 2009, Moussian & Roth 2005, Reeves & Stathopoulos 2009, Shimizu & Gurdon 1999, Stamataki et al. 2005, Wilson et al. 1997). For example, a 3× higher Activin receptor occupancy is transduced into a 3× higher nuclear concentration of the transcriptional regulator Smad2 (Dyson & Gurdon 1998, Shimizu & Gurdon 1999). Accordingly, the gradient of nuclear Smad2 along the vegetal-animal axis of the zebrafish blastula is thought to reflect the concentration gradient of both Nodal and Nodal-receptor complexes (Harvey & Smith 2009). Thus, extracellular morphogen concentration gradients can be maintained intracellularly as concentration gradients of activated transcriptional regulators. In this model, regulators have either an active or inactive form, and the concentration of active transcriptional regulators determines target gene expression.

Some morphogens may also utilize a mechanism in which individual signal transduction molecules have graded activities. For example, transduction of Hh signaling is mediated by the phosphorylation of the Smoothened (Smo) C terminus. Phosphorylation at individual sites leads to incremental changes in Smo activity (Jia et al. 2004, Zhao et al. 2007). This suggests a mechanism in which graded Hh signaling leads to graded Smo activity by inducing progressive phosphorylation of Smo. Increasing levels of Hh lead to a more extensive phosphorylation of individual Smo molecules as well as to a higher number of phosphorylated Smo molecules. Graded Smo activity results in the graded activation of Ci/Gli transcriptional regulators. In the absence of Hh, Ci is processed into a repressor form. Increasing levels of Hh block processing and convert Ci into an activator form. Thus, the extracellular gradient of Hh morphogen is translated into opposing nuclear gradients of Ci activator and Ci repressor.

INTERPRETATION OF MORPHOGEN SIGNALING

Graded morphogen distribution results in distinct domains of target gene expression within a tissue. In the following section, we discuss how DNA-binding affinity, combinatorial

interactions, and duration of signaling contribute to differential target gene induction.

Differential Affinities of Transcriptional Regulators for DNA Elements in Target Genes

A relatively simple mechanism by which graded activities of transcriptional regulators induce differential gene expression involves the DNA-binding affinity for *cis*-regulatory elements (Ashe & Briscoe 2006, Driever et al. 1989). In this model, regulatory elements with high affinity for the transcriptional effector control genes activated at low levels of morphogen signaling, whereas elements with low affinity for the transcriptional regulator control genes induced only at high levels of morphogen signaling (**Figure 5**). In support of this model, the expression domains of morphogen-responsive reporter genes can be broadened when enhancer affinity for transcriptional regulators is increased (Ashe & Briscoe 2006, Driever et al. 1989, Jiang & Levine 1993, Struhl et al. 1989, Wharton et al. 2004).

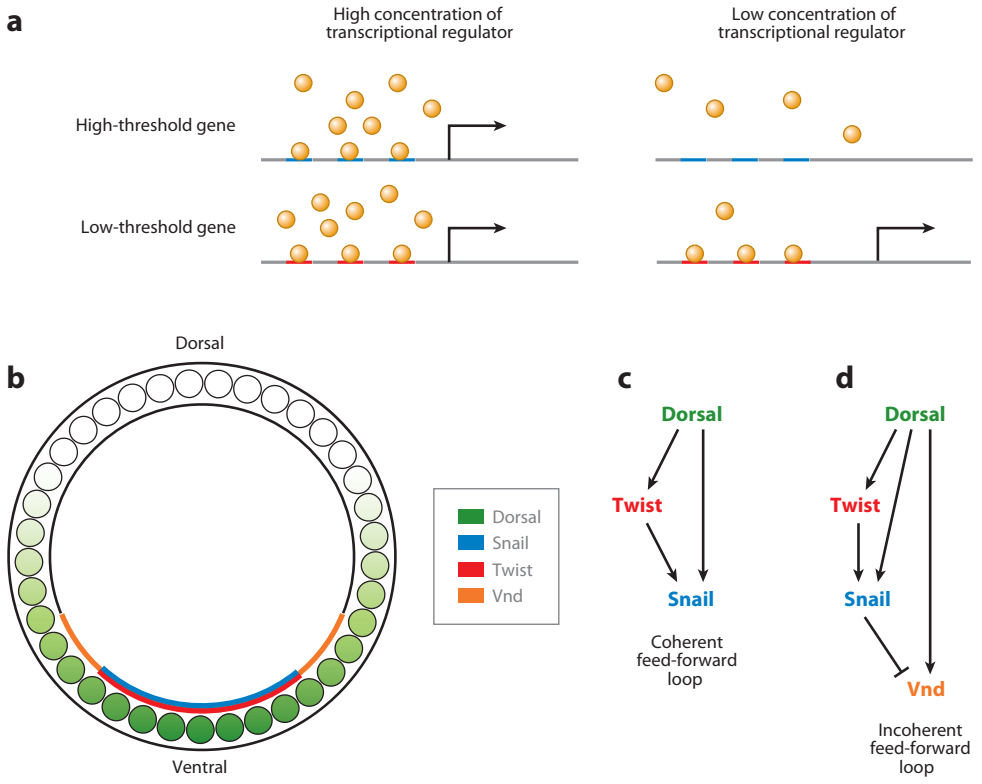

Figure 5

Gradient interpretation. (*a*) Interpretation by DNA-binding sites with varying affinity for transcriptional regulator (*gold*). The promoter of the top gene contains three low-affinity binding sites (*blue*; high-threshold gene); the promoter of the bottom gene contains three high-affinity binding sites (*red*; low-threshold gene). At high regulator concentrations, all sites in both promoters are bound, and both genes are expressed. At low concentrations, only the high-affinity sites are occupied, and only the gene with high-affinity sites is expressed. Based on Ashe & Briscoe (2006). (*b*) The ventral-to-dorsal nuclear Dorsal gradient (*green*) in *Drosophila* embryos is illustrated in a cross section. The expression domains of the Dorsal target genes Snail (*blue*), Twist (*red*), and Vnd (*orange*) are indicated. Based on Reeves & Stathopoulos (2009). (*c*) A coherent feed-forward loop initiated by Dorsal. (*d*) An incoherent feed-forward loop initiated by Dorsal. This loop restricts the expression of Vnd to the lateral regions of the embryo.

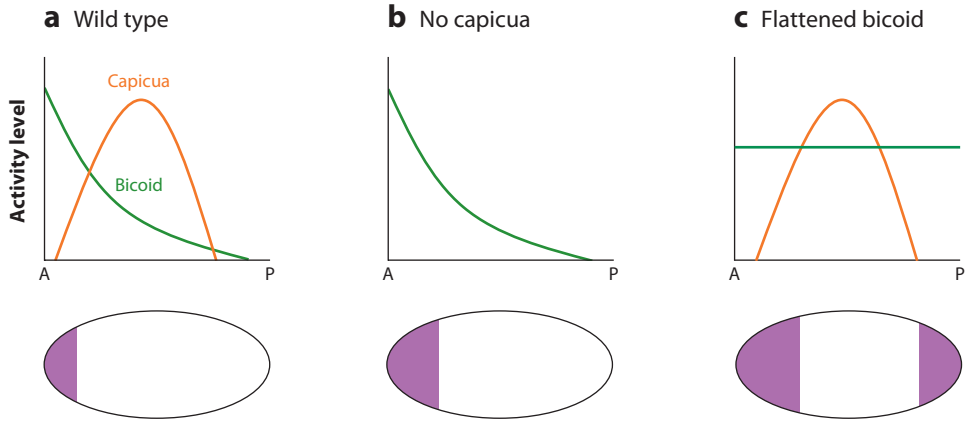

Figure 6

Capicua helps define the expression domains of Bicoid target genes. Capicua represses the expression of a subset of Bicoid target genes. (*a*) In wild-type embryos, Bicoid is distributed in an anterior-to-posterior gradient (*green*), whereas Capicua activity is repressed at the poles (*orange*). The expression domain of the Bicoid target gene *otd* is depicted in purple below. (*b*) In the absence of Capicua, the expression domain of *otd* is expanded posteriorly. (*c*) If the Bicoid gradient is experimentally flattened, mirror duplications of target gene expression are observed at the poles. For details see Löhr et al. (2009) and Ochoa-Espinosa et al. (2009).

Morphogen Interpretation by Combinatorial Interactions

Despite the importance of distinct DNA-binding affinities in responses to morphogen signaling, this parameter alone cannot predict the expression boundaries of most morphogen targets. For example, some genes controlled by high-affinity Bicoid binding sites have more restricted expression domains than do genes controlled by lower-affinity sites (Burz et al. 1998, Ochoa-Espinosa et al. 2005, Segal et al. 2008). In addition, when the Bicoid gradient is experimentally flattened, the expression boundaries of some target genes remain correctly positioned with respect to each other (Löhr et al. 2009, Ochoa-Espinosa et al. 2009). This suggests that additional inputs influence the positioning of target gene expression boundaries. One of these inputs is the transcriptional repressor Capicua (Löhr et al. 2009), which is repressed at the poles of the *Drosophila* embryo and binds to DNA regulatory elements that control expression of some Bicoid target genes. The balance between activation by Bicoid and repression by Capicua determines the expression boundaries of some Bicoid target genes (**Figure 6**). The modulation of Bicoid activity by the presence of Capicua is an example of prepatterning, in which preexisting factors modify the response to morphogen signaling. Another example of prepatterning is the cooperation of TGFβ signaling with asymmetrically localized, maternally deposited factors (Schier 2009). For example, the vegetally localized transcriptional regulator Eomesodermin cooperates with Nodal morphogen signaling to induce endoderm in zebrafish, whereas animally localized Ectodermin restricts Nodal signaling and promotes ectoderm formation in *Xenopus* (Bjornson et al. 2005, Dupont et al. 2005).

Modulation of morphogen interpretation by multiple inputs is also highlighted by the combinatorial regulation of Dorsal target genes (Reeves & Stathopoulos 2009). The transcriptional regulator Dorsal forms a ventral-to-dorsal nuclear gradient in *Drosophila* embryos and is required for patterning of mesoderm and ectoderm (**Figure 5**). Dorsal regulates the expression of at least 50 target genes and determines three activation thresholds. Although different Dorsal DNA-binding affinities contribute to target gene induction, the expression of Dorsal target genes is also controlled by additional transcriptional regulators. For example,

Dorsal induces expression of the transcriptional regulator Twist, which, together with Dorsal, promotes expression of several Dorsal target genes (Ip et al. 1992a, Kosman et al. 1991; **Figure 5**). Expression of reporter genes containing only Dorsal binding sites is patchy and stochastic, whereas reporters containing only Twist sites are not expressed. In contrast, reporters containing both Dorsal and Twist binding sites are expressed uniformly in domains with sharp borders (Szymanski & Levine 1995). Thus, the Dorsal-Twist coherent feed-forward loop leads to synergistic and robust activation of downstream genes. Robust gene expression may also be achieved by the preloading of RNA polymerase on the promoters of Dorsal target genes as well as by the presence of two or more enhancers that are both responsive to Dorsal (Boettiger & Levine 2009, Hong et al. 2008).

Dorsal-mediated patterning also employs incoherent feed-forward loops (**Figure 5**). Dorsal and Twist activate Snail expression, which in turn represses the expression of the Dorsal target gene Vnd (Ip et al. 1992b, Kosman et al. 1991, Reeves & Stathopoulos 2009). Thus, some Dorsal targets are repressed at high levels of Dorsal, at which Snail is induced, but activated at lower levels of Dorsal, at which Snail is not expressed. In this way, interactions between target genes can play an important role in defining target gene expression domains.

Although most current studies focus on DNA-binding affinity and transcriptional regulator networks in morphogen interpretation, additional factors such as nucleosome positioning (Kim & O'Shea 2008) or chromatin modifications might also affect target gene induction (Dahle et al. 2010, Vastenhouw et al. 2010). For example, studies in yeast suggest that changes in nucleosome positioning in promoter regions can modulate gene expression in response to graded signals (Kim & O'Shea 2008, Lam et al. 2008).

Temporal Effects of Morphogen Signaling

Formation of a morphogen gradient is dynamic and occurs over time. Therefore, not only are cells exposed to different concentrations of morphogen, they are also exposed to morphogen for different durations (**Supplemental Figure 8**). In some fast-developing early embryos, morphogen-mediated patterning is so rapid (2–5 h) that it is often assumed that increases in morphogen concentration are directly translated into expression of target genes (Bourillot et al. 2002, Harvey & Smith 2009, Schier 2009). However, even in these cases, the duration of morphogen signaling is important for proper cell fate specification. For example, premature inactivation of Nodal signaling blocks the induction of genes whose activation depends on high levels of Nodal (Gritsman et al. 2000, Hagos & Dougan 2007).

In more slowly developing tissues, the duration of exposure to morphogen can have dramatic effects on the response of cells (Ahn & Joyner 2004; Dessaud et al. 2007, 2010; Harfe et al. 2004; Kutejova et al. 2009; Nahmad & Stathopoulos 2009; Ribes & Briscoe 2009; Scherz et al. 2007; Yang et al. 1997). The dynamic nature of morphogen gradient interpretation is illustrated in the patterning of the spinal cord by Shh (Ribes & Briscoe 2009). A ventrally localized Shh source generates a ventral-to-dorsal gradient of Shh protein that patterns the nervous system. Shh is first detected close to its ventral source and then extends dorsally over several hours (Chamberlain et al. 2008). As the gradient emerges, target genes are activated, but importantly, their expression domains are induced and refined progressively. For example, Olig2 is first expressed close to the Shh source and then expands dorsally, whereas Nkx2.2 is expressed ventrally only later, when higher levels of Shh have accumulated (Chamberlain et al. 2008, Stamataki et al. 2005). Nkx2.2 expression then extends dorsally, repressing Olig2 in its wake. Thus, Shh, Olig2, and Nkx2.2 are engaged in an incoherent feed-forward loop (similar to that generated by Dorsal activity, as discussed above) that generates distinct gene expression domains in the developing spinal cord. In this scenario, the role of time could be to

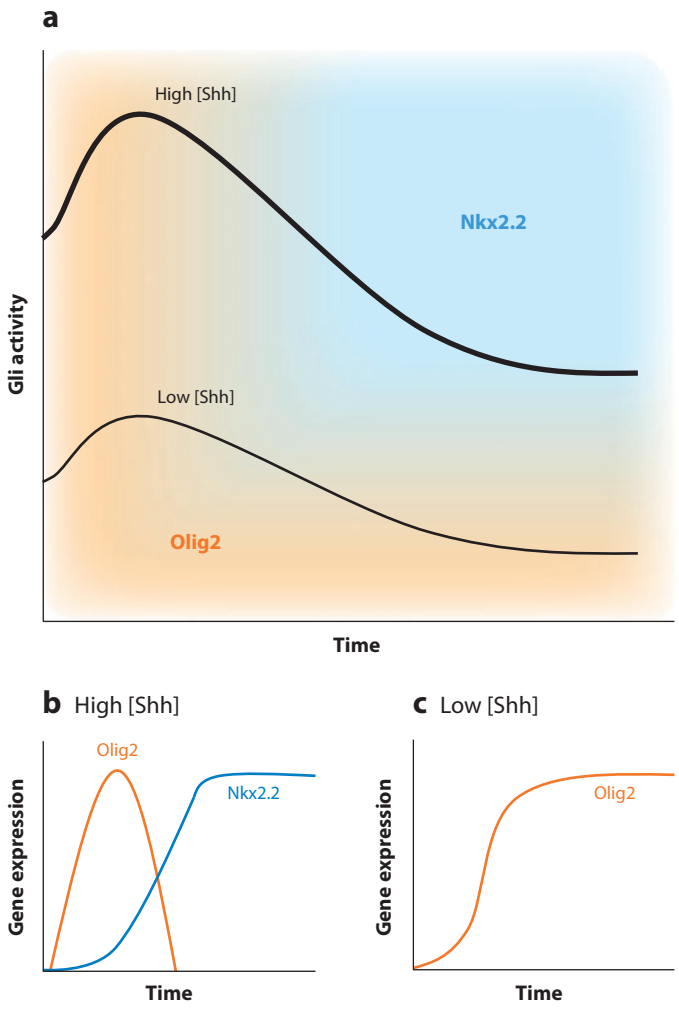

Figure 7

Temporal integration of Sonic hedgehog (Shh) signaling. (*a*) Hypothetical phase diagram describing the relationships between Gli activity, time, and gene expression. Nkx2.2 expression (*blue*) requires higher concentrations of Shh signaling for longer periods of time than Olig2 expression (*orange*). Nkx2.2 represses Olig2 expression. The thick line represents the temporal Gli activity in cells exposed to a constant, high concentration of Shh. The thin line represents Gli activity in cells exposed to a constant, low concentration of Shh. (*b*) Gene expression in cells exposed to a constant, high Shh concentration. Cells first express Olig2. Olig2 expression decays as Nkx2.2 expression initiates and represses Olig2. (*c*) Gene expression in cells exposed to a constant, low Shh concentration. Cells express Olig2 but never reach the activity level required for Nkx2.2 expression. Figure based on data from Dessaud et al. (2010).

establish the Shh gradient and allow regulatory interactions to occur between target genes.

Consistent with a requirement for Shh accumulation over time, higher levels of Shh are required to induce Nkx2.2 than Olig2 in neural plate explants. However, exposing neural tube explants to identical Shh concentrations for progressively longer times initially activates Olig2 and subsequently induces Nkx2.2 expression (**Figure 7**; Dessaud et al. 2007, 2010). Although it remains to be determined why Nkx2.2 induction requires longer durations of Shh exposure than Olig2, differential timing alone is sufficient to induce different target genes in this situation. These findings suggest that the adoption of distinct cell fates depends not only on the correct Shh concentration but also on the proper duration of Shh exposure.

The duration of Shh exposure alters cellular responses in several ways. First, extended exposure to Shh leads to an upregulation of Ptc expression (Dessaud et al. 2008). This negative feedback mechanism leads to the progressive desensitization of cells to Shh (Chen & Struhl 1996, Dessaud et al. 2007, Ribes & Briscoe 2009, Ribes et al. 2010). Cells that continue to be exposed to high levels of Shh maintain transduction of Shh signaling and can induce Nkx2.2 expression. In contrast, cells that are exposed to lower Shh concentrations and hence maintain transduction of signaling for shorter durations are unable to activate Nkx2.2 expression (**Figure 7**). Second, the transcriptional regulators that are activated or repressed by Shh signaling can modulate transduction of Shh signaling (Lek et al. 2010). For example, Nkx2.2 alters the transduction of Shh signaling, at least in part, by inhibiting the generation of repressive forms of Gli transcriptional regulators. This positive feedback loop could amplify Shh signaling and augment Nkx2.2 expression. These temporal adaptation mechanisms change the response of target cells even if extracellular Shh levels remain constant. Third, withdrawal of Shh can reverse patterning and result in the acquisition of a more dorsal fate. For example, cells that express Nkx2.2 can reexpress Olig2 after Shh withdrawal (Dessaud

et al. 2010). Thus, Shh-mediated patterning of the spinal cord depends on at least four factors: (*a*) the concentration of Shh, (*b*) the length of exposure to Shh, (*c*) feedback loops between the pathway and target genes, and (*d*) complex cross-regulatory interactions between target genes (Chamberlain et al. 2008; Dessaud et al. 2007, 2008, 2010; Lek et al. 2010; Ribes & Briscoe 2009).

Time appears to play important roles in the interpretation of other morphogens as well. For example, the ability of embryonic zebrafish cells to respond to BMP signaling changes over time, resulting in temporal patterning of the anterior-posterior axis (Tucker et al. 2008). In this case, it is thought that target tissue sensitivity changes so that BMP signaling has different effects during different periods. In addition, the interpretation of Wnt signaling may depend on the fold change in the level of the Wnt effector β-catenin in the nucleus over time (Goentoro & Kirschner 2009). Surprisingly, target genes appear to respond not to absolute levels of β-catenin but to the ratio of β-catenin before and after an increase in Wnt signaling. An analogous mechanism might be at play in the wing imaginal disc, where the timing of cell division correlates with 50% increases in Dpp signaling (Wartlick et al. 2011). Thus, temporal changes in morphogen signaling levels dictate target responses in several systems, but in none of these cases are the molecular mechanisms that mediate temporal integration understood. Interestingly, several other systems use temporal gradients to control the timing of target gene expression. For example, increasing concentrations of the FoxA transcriptional regulator Pha-4 contribute to the activation of different gene batteries at different times during *C. elegans* pharynx development (Gaudet & Mango 2002). Analogously, "just-in-time" transcription programs ensure the ordered activation of genes encoding proteins involved in the SOS response (Ronen et al. 2002) and flagellum assembly (Kalir et al. 2001). The different temporal onsets of target gene expression may be caused by differences in the affinity of a common transcriptional regulator for the promoters of the various genes in the pathway. It is conceivable that a similar mechanism underlies the interpretation of morphogen duration.

Although the duration of morphogen signaling may strongly affect responses in some contexts, it has relatively negligible effects in others. For example, cells exposed to Activin for as little as 10 min continue to express target genes for several hours after culture in Activin-free medium (Gurdon et al. 1995, Jullien & Gurdon 2005). The target genes that continue to be expressed are appropriate for the concentration of Activin previously experienced. When these cells are subsequently exposed to higher concentrations of Activin, they respond by expressing genes appropriate for the higher concentrations. Thus, a cell can increase its response, but it does not revert to a response appropriate for lower concentrations (**Supplemental Figure 9**). This "ratchet" mechanism affords a memory of the original positional information communicated to a cell in dynamic environments, such as when cells are changing their positions during gastrulation. The molecular basis of the Activin ratchet mechanism may involve long-lived Activin/receptor complexes that are endocytosed and elicit persistent cellular responses by maintaining a constant flow of phosphorylated Smad2 into the nucleus (Bourillot et al. 2002, Dyson & Gurdon 1998, Jullien & Gurdon 2005). However, the ratchet mechanism is not utilized by all morphogens. For example, as discussed previously, premature removal of Shh results in a loss of target gene expression, arguing against a long-term memory of previous exposure (Dessaud et al. 2010).

Supplemental Material

PRECISION, ROBUSTNESS, AND SCALING OF MORPHOGEN-MEDIATED PATTERNING

Despite its complexity, development is a reproducible, robust process. Embryos develop faithfully even in the face of environmental perturbations (such as low temperatures or

oxygen availability), genetic perturbations (such as heterozygosity for developmental control genes), and noise introduced by the stochasticity of gene expression and biochemical reactions. Even differently sized embryos of the same species give rise to mature organisms with proportionally patterned bodies. Here we discuss three important aspects of morphogen-mediated development that may influence reproducibility: precision, robustness, and scaling.

Precision

How do gradients give rise to precise gene expression domains that are at almost identical positions in different individuals? For example, the Bicoid-dependent posterior boundary of *hunchback* (*hb*) expression in the early *Drosophila* embryo varies by about 2% of embryo length in different individuals (Gregor et al. 2007a, He et al. 2008, Houchmandzadeh et al. 2002, Manu et al. 2009, Porcher & Dostatni 2010, Porcher et al. 2010). Strikingly, Bicoid levels in adjacent nuclei at this boundary differ only by 10%. Several studies suggest that the Bicoid gradient is very reproducible from embryo to embryo (~2–10% variability of embryo length) (Gregor et al. 2007a, He et al. 2008, Manu et al. 2009), and artificial *cis*-regulatory elements that contain only Bicoid binding sites drive expression in remarkably sharp and reproducible domains (Crauk & Dostatni 2005). How small differences in Bicoid levels are translated into sharp target gene thresholds is unclear (Porcher & Dostatni 2010). One model proposes that cooperative binding of Bicoid to regulatory elements can generate sharp on/off responses (Burz et al. 1998, Lebrecht et al. 2005, Ma et al. 1996, Struhl et al. 1989).

Although the Bicoid gradient appears to be remarkably precise, whether it provides positional information that is sufficiently precise to generate the observed low variability in the *hb* gene expression domain is controversial (Gregor et al. 2007a, He et al. 2008, Houchmandzadeh et al. 2002, Manu et al. 2009, Porcher & Dostatni 2010). The expression of many Bicoid target genes is controlled by multiple regulatory elements that bind transcriptional regulators other than Bicoid. It has therefore been proposed that *hb* expression is refined by the input of these transcriptional regulators [e.g., maternal Hb protein and Capicua (**Figure 6**)] and by cross-repressive interactions among transcriptional regulators that are coexpressed with *hb* (e.g., Kruppel and Knirps) (Clyde et al. 2003; Jaeger et al. 2004; Löhr et al. 2009; Manu et al. 2009; Ochoa-Espinosa et al. 2005, 2009). In this view, Bicoid is an essential component of a gene regulatory network that positions gene expression domains. Moreover, *hb* expression is dynamic during early development. Initially, nascent *hb* transcripts are found in a gradient that reflects the Bicoid gradient (Porcher et al. 2010). A sharp posterior boundary becomes apparent only at later stages. The molecular basis of this transition is not understood, further highlighting the complexities in deciphering when, where, and how morphogen gradients regulate precise expression boundaries (Bergmann et al. 2007, Bollenbach et al. 2008, de Lachapelle & Bergmann 2010, Gregor et al. 2007a, He et al. 2008, Houchmandzadeh et al. 2002, Manu et al. 2009, Porcher & Dostatni 2010).

Robustness

Modeling has demonstrated that feedback mechanisms can enhance the robustness (i.e., the resistance to perturbations) of morphogen systems (Barkai & Shilo 2009; Eldar et al. 2002, 2003; Irons et al. 2010; Lander et al. 2009; Meinhardt 2009; von Dassow et al. 2000). For example, as discussed above, Dpp and Hh signaling controls the expression of numerous genes that affect morphogen movement or interpretation. In particular, self-enhanced clearance is thought to help buffer changes in morphogen production rates (Barkai & Shilo 2009, Eldar et al. 2003, Lander et al. 2009). In this model, the higher the morphogen concentration, the more likely it is that a morphogen molecule will be cleared, thereby reducing transient increases in morphogen concentration. In

the absence of feedback mechanisms, decoding the presteady state of morphogen profiles can also enhance robustness (Bergmann et al. 2007, Saunders & Howard 2009). Similar to self-enhanced clearance, this strategy can reduce patterning errors caused by fluctuations in morphogen production rates.

Scaling

Although organisms can vary dramatically in size, the variations in proportion are often much less substantial, similar to a flag whose pattern is size invariant (Wolpert 2011). For example, the length of different dipteran embryos can vary by up to fivefold, but the Bicoid gradient and the expression of segmentation genes scale with embryo length (Gregor et al. 2005, Lott et al. 2007, Sommer & Tautz 1991). Thus, on average, Bicoid molecules must travel significantly farther in the larger embryos of some species. Even in differently sized *Drosophila* embryos, Bicoid gradients have congruent shapes when distribution is analyzed with respect to percent of egg length (He et al. 2008). The molecular basis for this scaling is unknown (Gregor et al. 2008), but longer *Drosophila* embryos express slightly higher levels of Bicoid than do shorter embryos (He et al. 2008). This suggests that egg length might be coupled to Bicoid RNA production during oogenesis or to Bicoid translation or stability in the embryo (Cheung et al. 2011).

Morphogen gradient scaling is also observed during tissue growth. For example, the decay length of the Dpp gradient increases over time but remains proportional to the size of the wing imaginal disc even as it grows more than fivefold (Wartlick et al. 2011). The diffusion coefficient of Dpp appears to stay constant during tissue growth, which suggests that other parameters of gradient formation change to increase the range of signaling. For example, the Dpp source widens during growth, and the clearance of Dpp might decrease, resulting in an increase in gradient amplitude and range. Because growth is uniform during gradient scaling, all cells experience the same relative, but not absolute, change in Dpp signaling over time. Cell division exhibits a striking correlation with a relative Dpp signaling increase of ~50%. Thus, cells are thought to divide every time they experience a 50% increase in Dpp signaling, but how cells measure such signaling changes over time is unclear.

Another striking example of scaling is observed during the earliest stages of embryogenesis, when many embryos can be split in two and one or both halves develop into smaller but normal animals (Cooke 1981). Studies in *Xenopus* suggest that multiple feedback mechanisms contribute to the scaling of BMP signaling in the smaller, dorsal half of split embryos (Barkai & Shilo 2009, Ben-Zvi et al. 2008, Eldar et al. 2002, Reversade & De Robertis 2005). Several theoretical models have been proposed to account for scaling, but which of these models best reflects the in vivo response of organisms to size differences is unclear (Ben-Zvi & Barkai 2010, McHale et al. 2006, Othmer & Pate 1980, Umulis et al. 2010).

Not all embryos are capable of developing into normal adults after being split, and ligation of *Drosophila* eggs does not result in scaling of the Bicoid gradient (Boring et al. 1993). Moreover, scaling of morphogen gradients is only one potential mechanism to deal with size differences. For example, embryos that contain higher levels of Bicoid initially display a posterior shift of target gene expression (**Supplemental Figure 1**) but develop into normal flies (Berleth et al. 1988). Cell death in the expanded regions appears to correct for abnormal patterning, which reveals that initial variations in patterning can be corrected at later stages (Namba et al. 1997). In addition, scaling could in theory be achieved by a change of competence in the cells themselves such that tissues are patterned correctly even if the morphogen gradient itself does not scale.

Supplemental Material

CONCLUSIONS AND PROSPECTS

The study of morphogens has seen remarkable progress in the past 25 years. Morphogens are no longer a theoretical concept but have been

identified in many developmental contexts. A plethora of regulatory interactions have been demonstrated to regulate morphogen gradient formation and interpretation. Although there are many variations on the theme, the general picture that emerges for morphogen gradient formation and interpretation is as follows. Morphogen molecules are released from a local but dynamic source, assemble with themselves and/or other molecules, and move via restricted diffusion through the extracellular milieu. Gradient shape is determined by the flux from the source, the diffusivity of the morphogen, and its clearance kinetics in target tissues. These properties are modulated by interactions with HSPGs and other extracellular proteins that tether morphogens to the cell surface, prevent loss of morphogen to receptor-free domains, and affect morphogen trafficking and reception. Morphogen concentration can be transmitted linearly to intracellular transduction molecules, which results in the graded activity of transcriptional effectors. These transcriptional regulators are part of complex regulatory networks that modulate the target gene response to morphogen concentration and duration. Thus, target genes are regulated not only by the morphogen signaling pathway but also by preexisting factors, cross-regulatory interactions, and feed-forward loops. Feedback mechanisms buffer fluctuations in morphogen production, affect signaling interpretation, and confer scalability and robustness to morphogen-mediated patterning.

Despite this general understanding of morphogen signaling, many questions remain to be addressed. In particular, sophisticated mathematical models have been proposed to explain how morphogen gradients form and how this information is translated into target gene induction. Strikingly, however, few of the parameter values in these models have been determined experimentally. Thus, how well theoretical considerations of robustness, scaling, and precision hold up to experimental scrutiny is unclear. For example, for most morphogen systems we lack measurements of diffusion coefficients, clearance kinetics, binding constants, on and off rates, and temporal dynamics. The current controversies surrounding the simple Bicoid gradient in the extensively studied *Drosophila* embryo indicate that gaining a full quantitative understanding will not be a trivial undertaking. At the molecular and subcellular levels, it is largely unclear how morphogens assemble to move through the extracellular milieu, what routes they take, and how they interact dynamically with other molecules. Finally, it remains largely unclear how morphogen function is modified to contribute to the striking variation of forms and shapes found in nature.

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