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Buffering Global Variability of Morphogen Gradients

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Morphogen gradients determine tissue pattern by triggering differential cell responses to distinct morphogen concentrations. The strict quantitative dependence of the emerging patterns on morphogen distribution raises the challenge of buffering variability in morphogen profile to ensure a reproducible outcome. We describe the underlying principles of two modules for buffering morphogen distribution: buffering morphogen amplitude by storing excess morphogen in a limited spatial region, and buffering morphogen spread by pinning morphogen levels at a distal position through global feedback that adjusts morphogen diffusion or degradation across the tissue. We also present concrete examples of patterning systems that implement these modules.

Overview

The original "French flag" morphogen model proposed by Lewis Wolpert was inspirational as a guiding concept for patterning a large number of cells by a single diffusible molecule (Green and Sharpe, 2015; Wolpert, 1969, 1971). According to this model, an initial asymmetry provides a defined source for morphogen production. Subsequent diffusion of this morphogen through a field of adjacent cells generates a concentration gradient. The receiving cells can sense different levels of the morphogen and induce distinct sets of target genes accordingly. The capacity of a morphogen to function as a global determinant of pattern is the basis for shaping embryos and organs during development. At the same time, this direct link between morphogen levels and the resulting cell fates entails a significant hazard, as any deviation from the desired distribution profile will directly lead to large-scale patterning abnormalities. The striking reproducibility of the body plan between different individuals that develop in variable environmental conditions and differ in size implies the existence of mechanisms that buffer morphogen gradients against unavoidable variability. This feature is further highlighted by the limited patterning effects of halving the dosage of genes encoding morphogens, their receptors or downstream signaling components (Barkai and Shilo, 2009; Lander et al., 2009; Umulis et al., 2008). Patterning systems are therefore geared toward reproducibility.

Approaches for studying the biological significance and molecular basis of this robustness are different from the conventional genetic approaches by which developmental signaling pathways have been uncovered. First, variability is measured at the population level rather than in individual embryos. Second, buffering may not be precise as a certain level of variability may be tolerated by the population, and some imprecision in the initial patterning may be corrected at subsequent developmental stages, for example by apoptosis of excess cells. Still, existing results support the notion that variability is buffered also at the level of the initial patterning events that are guided by morphogen gradients (Barkai and Shilo, 2009; Lander, 2013; Umulis and Othmer, 2013).

Most of what we know about variability in morphogen gradients relies on measurements of biological readouts of morphogen activity rather than on the morphogen distribution itself. Ideally one would like to measure the morphogen distribution directly. This is more feasible in cases where the morphogen is a transcription factor such as Bicoid, and its nuclear level can be reliably monitored (Morrison et al., 2012). When dealing with extracellular morphogens, which represent the more general scenario, the actual quantitation of the morphogen level is highly problematic. First, low levels of the morphogen that could have pronounced biological effects may not be detectable. Second, it is not possible to distinguish between the active morphogen fraction and a "dead" morphogen pool which may be trapped on the extracellular matrix or stored in endosomes (Zhou et al., 2012).

Variability in morphogen distribution may be generated either by local irregularities that extend over a limited spatial domain and affect individual cells within the same field, or by global alterations that modulate the entire profile. Local perturbations can be buffered by some spatial or temporal averaging (Lander, 2011, 2013; Lander et al., 2009). For example, in the case of the Bicoid gradient, averaging over time and around adjacent nuclei gives rise to a smoother response (Garcia et al., 2013). Indeed, effective local averaging can take place at any step along the signal transduction cascade. In contrast, global variations in morphogen distribution affect most of the responding cells. Such variations cannot be corrected by averaging but require a mechanism that can monitor the distribution profile and recruit feedback controls to adjust it in a broad manner. This review focuses on sensing and buffering global variations in the morphogen profile across the entire patterning field.

Here we consider morphogen profiles in scenarios where the tissue is not growing and steady state has already been reached. We note that there are also cases where morphogen gradient is established concomitantly with tissue growth, which further complicates the dynamics (Averbukh et al., 2014; Romanova-Michaelides et al., 2015; Wartlick et al., 2011a). In addition, cases where the duration and history of exposure play a role also introduce the element of variability in timing, but may also provide some buffering (Balaskas et al., 2012). These more complicated situations are not reviewed here.

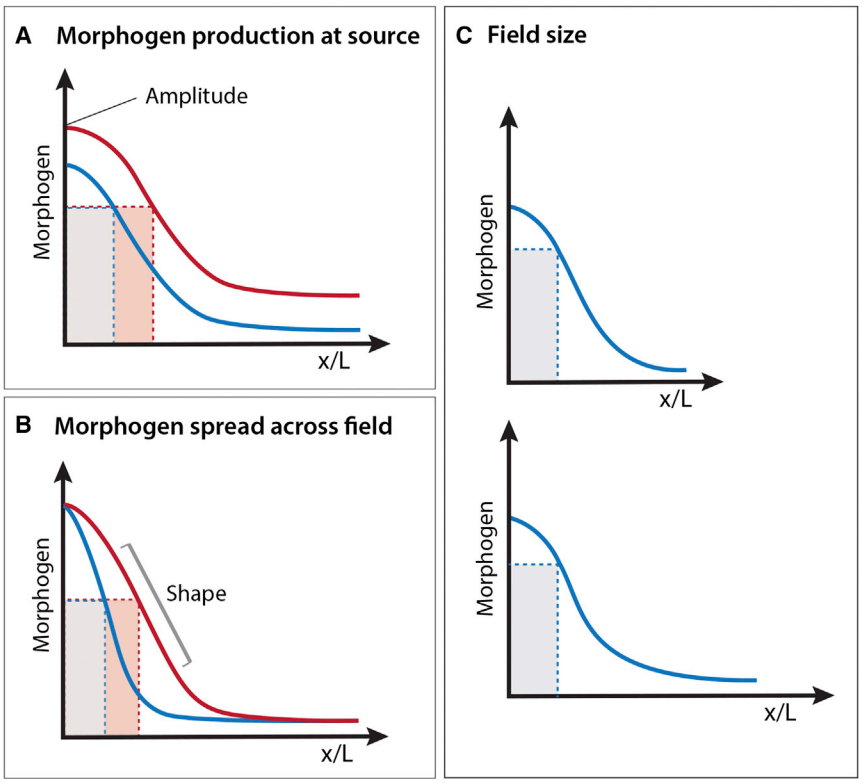

Figure 1. Types of Variabilities in Morphogen Distribution

(A) Variability in morphogen production rate will lead to differences in the distribution of the morphogen across the field. Note that different gene expression zone sizes (marked by rectangles) will be induced according to the global morphogen distribution profile.

(B) Variability in the properties of the receiving cells, e.g., in the level of receptor they express, will lead to different profiles of morphogen distribution, even when the amount of morphogen that is produced is similar. The red line represents a situation with reduced morphogen-receptor levels. Again, note the different sizes of gene expression zones that are induced.

(C) If embryos differ in size while the distribution of the morphogen is not altered accordingly, the resulting pattern will not be scaled. Here the induced gene expression zone size is similar, but its proportion relative to the total embryo size differs, as the lower panel represents a larger embryo.

Variability in Amplitude and Shape of Morphogen Gradients

When considering variability of morphogen distribution, it is useful to distinguish between the amplitude and the shape of the gradient (Figure 1). By amplitude we refer to the total level of morphogen at its source, while shape denotes the spread of the morphogen across the field. The amplitude of the morphogen profile depends largely on morphogen production rate at the source. On the other hand, the shape of the profile is determined by the parameters controlling morphogen spread, typically diffusion, degradation, or receptor binding, but is not linked to the rate at which morphogen is produced. Thus, gradients can be of the same amplitude but of different shape, or conversely, of the same shape but of different amplitude.

A reproducible pattern is achieved when both the amplitude and shape of morphogen distribution at steady state are invariable to biological fluctuations. Furthermore, the shape of morphogen distribution needs to be adjusted to the size of the field to ensure proportionate patterning in embryos of different sizes. The differential dependence of these two properties—morphogen amplitude and shape—on biological parameters suggests the involvement of distinct mechanisms that ensure reproducibility. Such modalities are now beginning to be unveiled in several morphogen patterning systems, using a combination of experimental and computational approaches. Although these systems are characterized by distinct features and molecular components, fundamental unifying concepts underlying buffering of variability are emerging. Next we discuss global buffering mechanisms and their implementation in different systems, with a particular focus on *Drosophila* development.

"Limiting Profile": Buffering Gradient Amplitude by Storing Excess Morphogen at a Restricted Spatial Region

Fluctuations in the amplitude of morphogen profiles can be buffered by storing excess morphogen within a small spatial region, without affecting morphogen distribution (and therefore cell-fate determination) in most of the field (Figure 2). Indeed, increasing morphogen levels in a region where the highest cell fates are determined will not alter cell fates in that region. Following this mechanism, more or less morphogen will be localized to this limited zone, depending on the total level of morphogen, leaving intact the rest of the profile and the resulting pattern. We denote such profiles, which approach an invariable shape distal to the source when morphogen level increases, limiting profiles.

Limiting Profile through Self-Enhanced Morphogen Decay

How can effective confinement of higher morphogen levels close to its source be achieved? For morphogens produced from a localized source, a limiting profile is obtained when the morphogen triggers its own degradation or sequestration ("self-enhanced degradation") (Eldar et al., 2003), or when it limits its own diffusion ("self-repressed diffusion") (Bollenbach et al., 2005). Under these circumstances, the morphogen levels will decline rapidly close to the source, and the rate of decline will taper off at more distal positions (Figure 2A). To see how this mechanism works, we note that the shift in cell-fate boundaries following some fluctuation in morphogen production rate is proportional to the rate by which morphogen decays close to the source. Fast decay in the region (through increased degradation or reduced diffusion) will therefore maintain excess morphogen in this zone. However, as morphogen levels are reduced its decay length will decrease as well, converging to the limiting profile distribution and allowing it to reach further distances. Self-enhanced degradation or self-repressed diffusion therefore enable rapid decline close to the source, without compromising

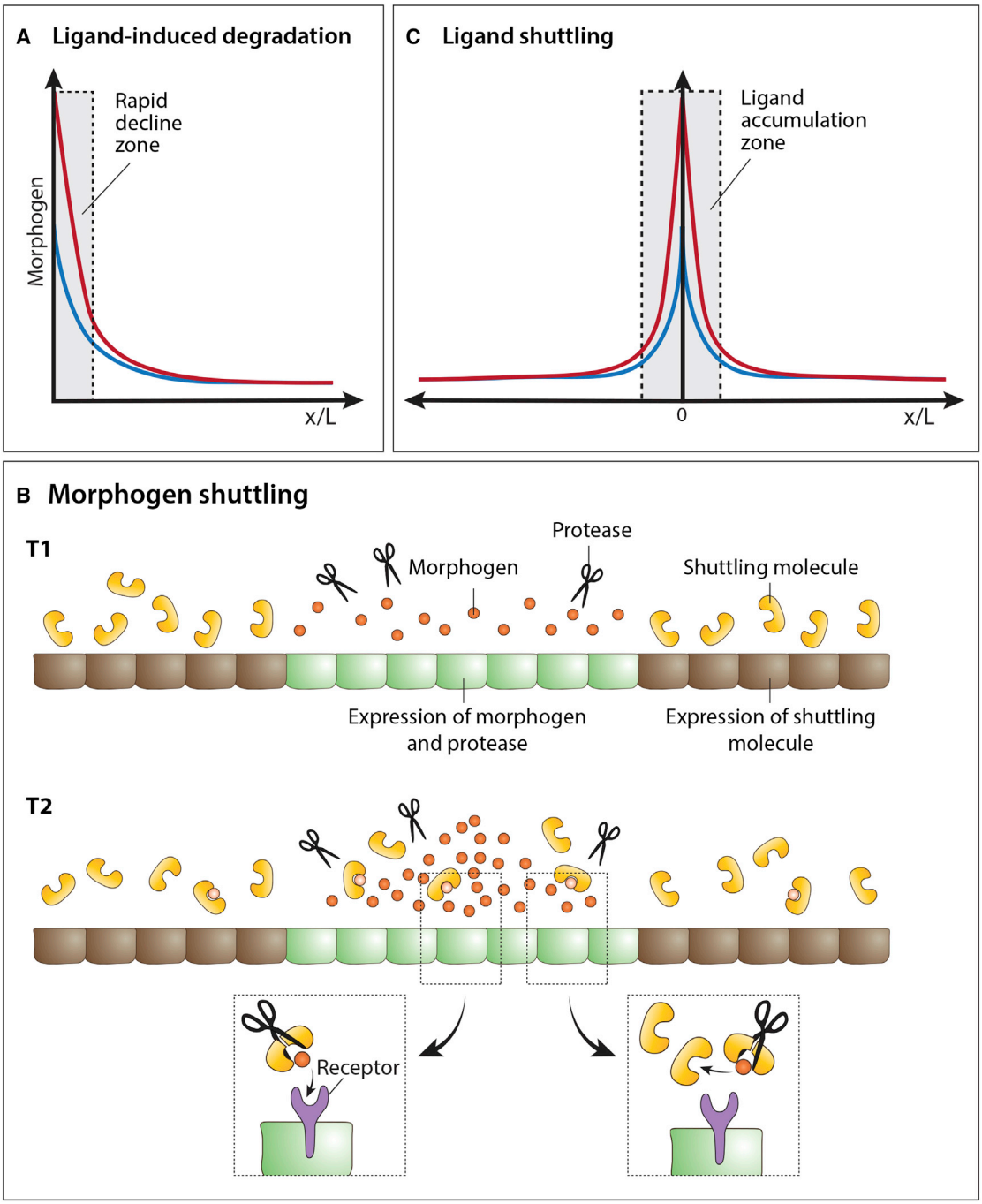

Figure 2. Limiting Profiles Buffer Variability in Morphogen Production

(A) When a morphogen induces its own degradation, higher levels of morphogen at the source (red line) will lead to a more rapid decline of the profile, and the distributions will converge distal to the source. Close to the source, where high levels of morphogen are sensed, reproducible cell fates will be induced despite the increase in morphogen levels.

(B) At T1, shuttling of morphogen is initiated by broad expression of the morphogen within the patterned region, flanked by domains expressing the shuttling molecule. The morphogen and shuttling molecule can associate, to generate a biologically inactive and highly diffusible complex. At T2, due to the activity of a protease within the patterned region, the shuttling molecule in the complex will be cleaved and will release the ligand. When cleavage takes place within the lateral region, the released ligand will bind another shuttling molecule. Conversely, cleavage at the center of the patterned region will preferentially lead to binding of the free ligand to the receptor, due to the graded distribution and declining abundance of the free shuttling molecule within the patterned region. This will give rise to physical concentration of the free ligand toward the center. A low diffusion rate or rapid endocytosis of the free ligand is essential to preserve this graded distribution.

(C) In systems where ligand is shuttled to the center of its broad expression domain, higher levels of ligand production will lead to concentration of more ligand at the center. Again, the resulting cell fates in this region will be maintained. The red line marks a higher level of morphogen production.

the ability of the morphogen to reach further distances (Eldar et al., 2003).

In terms of molecular mechanisms that can generate a limiting profile, the Hedgehog (Hh) pathway comes to mind. Binding of Hh to its receptor Patched (Ptc) sequesters Hh and triggers signaling by Smoothened (Smo). Since *ptc* is a prominent target gene of the pathway (Hidalgo and Ingham, 1990; Marigo et al., 1996), the higher the level of Hh, the higher its rate of sequestration by Ptc, which is functionally equivalent to degradation. Self-enhanced degradation was also implicated in the robustness of the retinoic acid (RA) gradient in zebrafish (White et al., 2007). RA provides long-range positional cues in the embryonic hindbrain. In collaboration with fibroblast growth factor, it induces expression of Cyp261a1, the major RA-degrading enzyme (Schilling et al., 2012). A similar mechanism was recently identified also in the formation of intracellular gradients in yeast (Hersch et al., 2015).

Limiting Profile through a Diffusion-Based Shuttling Mechanism

A critical aspect of "classical" morphogens is their spread from a restricted source of producing cells, as discussed above. However, early embryos may not have sufficient positional information or resolution to define a small group of cells that can serve as a localized source. When such circumstances prevail, the morphogen is therefore expressed broadly by a large number of cells. Despite this uniform expression, a sharp gradient can still be generated within the broad expression domain, through a mechanism termed ligand shuttling. A limiting profile is also realized in this class of morphogens, where instead of spreading, effective diffusion-based trafficking is used to concentrate the active extracellular signaling molecule.

In brief, the ligand shuttling mechanism relies on expression of a molecule that can associate with the ligand and generate an inactive diffusible complex, thereby providing a vehicle for ligand trafficking. A second critical component is an extracellular protease that cleaves the shuttling protein when it is in the complex, to liberate the trafficked ligand. The shuttling vector plays a dual role of facilitating high levels of signaling at the center of the ligand expression domain and inhibiting signaling at its edges (Ashe and Levine, 1999; Decotto and Ferguson, 2001; Eldar et al., 2002; Shilo et al., 2013) (Figure 2B). In this manner, the morphogen distribution converges to a sharp limiting profile that is well within the ligand expression domain. The overall level of morphogen impinges on the distribution very close to the center, in a region that is largely devoid of the shuttling molecules but has no effect on the profile outside this region (Barkai and Shilo, 2009; Mizutani et al., 2005; Umulis et al., 2006) (Figure 2C).

This ligand shuttling mechanism was shown to operate in the case of bone morphogenetic protein (BMP) signaling in the early *Drosophila*, *Tribolium*, and *Xenopus* embryos, where the Short gastrulation (Sog) or Chordin proteins, respectively, function as the shuttling vector (Ben-Zvi et al., 2008; Eldar et al., 2002; Reversade and De Robertis, 2005; Shimmi et al., 2005; Wang and Ferguson, 2005). The emerging profile of BMP activation was shown to depend on the Sog/Chordin expression domains and their biochemical properties (Peluso et al., 2011; van der Zee et al., 2006).

Shuttling was also identified in early *Drosophila* embryos as the basis for the graded activation profile of the Toll receptor, which is generated by a sharp distribution profile of the Toll ligand, Spätzle (Spz). In the case of Spz, a distinct shuttling molecule does not exist. Rather, the pro-domain of the ligand Spz fulfills this role. A defined production domain for this pro-domain is established in a self-organized manner: the pro-domain is released only upon binding of the cleaved, but still associated, ligand complex to free receptor (Weber et al., 2007). As the activated ligand is concentrated and saturates the receptors at the center of the field, effective production of the pro-domain is shifted laterally. The pro-domain is therefore produced away from the center, to drive ligand shuttling and the creation of a limiting profile (Haskel-Ittah et al., 2012).

Interestingly, in parallel to Spz ligand shuttling at the extracellular milieu, another shuttling mechanism may be operating within the cytoplasm of the embryo to concentrate the downstream elements of activated Toll to the ventral region. This may be achieved by ventral trafficking of the transcription factor Dorsal (Dl), the final target of Toll signaling. In the first three hours of development the *Drosophila* embryo is a syncytium, possibly allowing diffusion of components within the cytoplasm across the embryonic axes, which could lead to "flattening" of Dl nuclear distribution. A recent study provided evidence that this spreading is prevented, and that Dl is in fact further restricted ventrally through its effective shuttling by the Cactus (Cact) protein (Carrell et al., 2016). Cact forms an inactive complex with Dl, and phosphorylation of Cact by Toll at the ventral side leads to its degradation and release of Dl (Roth et al., 1991).

"Distal Pinning": Buffering Gradients by Fixing Morphogen Level at a Distal Position

In contrast to morphogen amplitude, which can be adjusted close to the source, the shape of the profile depends on the overall diffusion and degradation of the morphogen across the field. Buffering the morphogen profile is therefore more challenging since it requires some means of monitoring the spread of the morphogen across the field, and also entails global feedback that expands or narrows the morphogen distribution until reaching the desired spread. For this reason, local adjustment of morphogen levels, e.g., through boundary conditions, is not sufficient to buffer the morphogen spread.

Mechanisms that adjust morphogen levels with tissue size (scaling) were described in particular situations (Aegerter-Wilmsen et al., 2005; Houchmandzadeh et al., 2005; Howard and ten Wolde, 2005; McHale et al., 2006; Umulis, 2009). For example, integrating information from two opposing gradients was proposed as a way for scaling the anterior-posterior Bicoid gradient in the early *Drosophila* embryo. Most mechanisms, however, are specifically tuned for scaling pattern with size variations, and their applicability for the more general case of variability in parameters controlling morphogen distribution is not clear.

Recently a class of mechanisms that buffer morphogen spread was described, being capable of scaling morphogen distribution with system size as well as buffering variations in morphogen diffusion and degradation. These mechanisms function by the adjustment, or "pinning down," of morphogen levels at a particular localized position. Critically this local adjustment is achieved through a global feedback that regulates

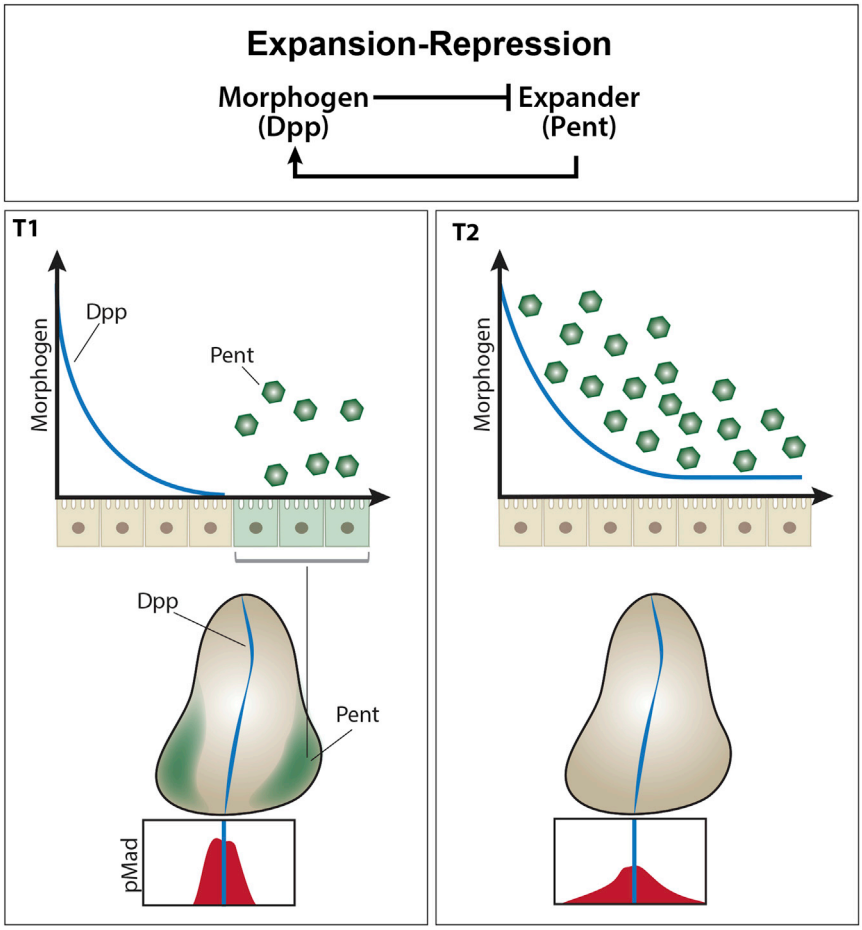

Figure 3. Expansion-Repression by Dpp and Pentagone in the Wing Disc

Dpp signaling represses the expression of *pent*. (T1) At the initial stages, Pent will be produced in the distal regions of the disc (marked by green cells), where morphogen levels are low. The secreted Pent protein will be distributed extracellularly and will facilitate further expansion of the Dpp gradient by reducing the affinity of secreted Dpp to its receptor complex. The expander protein is sufficiently stable to allow its accumulation. (T2) When the levels of Dpp at the disc edge are suitably high to reach the fixed "distal pinning" point where *pent* expression is blocked, steady state will be reached. In this case, the distal pinning point is defined by the levels of morphogen that will block expander expression. It does not correspond to a fixed "geographical" location, but is rather correlated with the time at which the distal morphogen levels will reach this fixed concentration. Note that the expander protein will be present and functional even when its actual production is reduced or terminated, due to its stability. In discs of different sizes, the overall time of Pent production will vary accordingly, and a scaled distribution of the morphogen will be obtained.

morphogen spread throughout the field (Ben-Zvi and Barkai, 2010). It is noteworthy that the eventual distribution of the morphogen gradient does not depend on morphogen diffusion or degradation, as these are continuously adjusted by the induced feedback. Rather, the final profile will be determined exclusively by the cellular parameters controlling the expression threshold of the feedback molecule, and these are expected to be less variable.

This concept defines a general class of circuits and can be implemented by a variety of molecular manifestations. As long as the extracellular feedback mechanism affects the *global* morphogen distribution, it may involve a variety of modalities such as ligand stabilization, or alteration in ligand-receptor and ligand-extracellular matrix affinities. To illustrate this, we next describe two general classes of molecular circuits, together with concrete examples whereby "distal pinning" was shown to operate.

Distal Pinning through Expansion-Repression: BMP in the Drosophila *Wing Imaginal Disc*

Distal pinning can be implemented by a general class of circuits termed "expansion-repression" (Ex-R). Here, morphogen represses the production of a secreted "expander" molecule, which functions to broaden the gradient. The pinning point is positioned at the edge of the field, where morphogen levels are the lowest. The expander stops accumulating when morphogen levels at this point rise above the threshold required for repression of expander expression (Ben-Zvi and Barkai, 2010). Rapid diffusion and stability of the expander protein are required for effective buffering.

Several studies implicated Ex-R in scaling the BMP gradient in the *Drosophila* wing imaginal disc. Here, the BMP protein Dpp is produced at the center of the disc along the anterior/posterior axis, and diffuses across the disc epithelium to generate a concentration gradient (Figure 3). Binding of Dpp to its hetero-tetrameric receptor leads to phosphorylation of a Smad protein (pMad) and induction or repression of different classes of target genes (Affolter and Basler, 2007). The *pentagone* (*pent*) gene was initially identified by virtue of its transcriptional repression by Dpp. In *pent* mutants the posterior wing vein is not patterned (Vuilleumier et al., 2010). Pent appeared to fulfill the criteria of an expander: first, its expression is repressed by Dpp, and hence is restricted to the edges of the disc where Dpp levels are lowest. Second, Pent is a secreted protein, and was shown to reduce accessibility of Dpp to its receptor. Presence of Pent effectively promotes Dpp diffusion and morphogen gradient expansion (Figure 3). Thus, the level of Pent within the extracellular milieu functions as a tunable "knob" that determines the propensity of Dpp to bind its receptor, thereby modulating its withdrawal from the extracellular pool.

Experimental analysis supports the notion that a Pent-dependent Ex-R mechanism scales the Dpp gradient with disc size. When discs of different sizes were compared in age-matched larvae their Dpp signaling profiles, as monitored by phosphorylated Mad, were scaled (Ben-Zvi et al., 2011; Wartlick et al., 2011a, 2011b). In the absence of Pent, the distribution of Dpp signaling is tighter and scaling is lost. Furthermore, scaling of Dpp signaling was also eliminated upon constitutive expression

of Pent, with pMad profile becoming independent of disc size (Ben-Zvi et al., 2011; Hamaratoglu et al., 2011; Restrepo and Basler, 2011). Mechanistically, Pent was shown to trigger internalization of Dpp glypican co-receptors, thus modulating the ability of cells to trap ligand and transduce the signal (Norman et al., 2016).

With the concept of Ex-R in mind, it will be interesting to look at other morphogen systems for secreted molecules that could function as expanders. For example, Ex-R was also suggested to provide scaling during regeneration (Werner et al., 2015). Another system that appears to display Ex-R properties is the zebrafish neural tube. Here, expression of the secreted matrix protein You/Scube2 is elevated on the dorsal side of the neural tube and somites, in the region furthest away from the source of Sonic Hedgehog (SHH) emanating from the notochord. In the absence of this protein, cell fates that are induced close to the source of SHH such as *isl2* are retained, while more distal fates such as *en1* are lost (Hollway et al., 2006; Kawakami et al., 2005). Since epistasis analysis placed You/Scube2 at the level of the SHH ligand, consistent with its extracellular localization, it is tempting to speculate that this protein indeed functions as an expander for SHH. Specifically, it would imply that the transcription of *you/scube2* is repressed by SHH signaling, and the protein extends the effective diffusion range of SHH. Molecular characterization of Scube2 function is indeed consistent with its role as an expander: Scube2 is recruited to the surface of SHH-producing cells, where it regulates proteolytic SHH processing, facilitating ligand release (Jakobs et al., 2014, 2016).

Distal Pinning through Induction-Contraction: Toll Signaling in the Early Drosophila *Embryo*

A second class of circuits implementing the distal pinning mechanism is termed induction-contraction (In-C). Here, the morphogen induces a secreted feedback molecule functioning as a contractor, to narrow down the morphogen spread. Cells expressing the feedback response require sufficiently high morphogen levels. In addition, they need to be positioned away from the source, at a zone that could be defined, for example, by intersection with another signaling pathway. Again, rapid diffusion and stability of the contractor protein are required for effective buffering.

We recently implicated the In-C mechanism in buffering Toll pathway signaling in the early *Drosophila* embryo, identifying WntD as the contractor (Figure 4). WntD is a secreted protein that functions to block the capacity of Toll to bind its ligand and dimerize (Ganguly et al., 2005; Gordon et al., 2005; Rahimi et al., 2016). Notably, by blocking this binding, WntD not only reduces signaling level but also promotes the shuttling of the ligand toward the ventral midline, thereby concentrating the gradient. Expression of *wntD* is confined to the posterior-most part of the embryo, since its promoter activity requires two orthogonal inputs: binding of the Toll-target Dl as well as removal of transcriptional inhibition by Capicua (Cic) achieved by the Torso/MAPK pathway, operating at the termini of the embryo (Helman et al., 2012). Importantly, due to the morphology of the embryo the maximal values of Toll signaling at the termini correspond to those sensed by cells positioned ~30% lateral to the ventral midline (at mid anteroposterior position). Thus, *wntD* expression is restricted to intermediate levels of the Toll gradient, as required by the In-C mechanism. Taken together, WntD complies with all requirements for a contractor in terms of its transcription regulation and the highly diffusible secreted protein product that inhibits the extracellular activity of Toll. Indeed, our recent studies have shown that WnD is essential for reducing variability among embryos in the Toll activation gradient; its deletion unmasks a high variability in the positioning of *snail* expression, an early target gene for Toll-dependent patterning (Rahimi et al., 2016).

Expansion-Repression versus Induction-Contraction

While both Ex-R and In-C circuits represent an integral feedback loop, they employ inverted directionality: repression versus induction of a widely diffusible molecule which globally expands or contracts the gradient, respectively. In both cases the effectiveness of the buffering mechanism relies on rapid spread and slow degradation of the expander or contractor. The rapid spread is essential to transmit accurately to the entire field the information that is processed at the edge of the gradient, and is reflected by the expression profile of the expander or contractor. The stability of the secreted buffering protein facilitates its accumulation in the extracellular milieu, and reduces overshoots and oscillations. Both of these mechanisms are also best suited for buffering variations that impact on the length scale of the gradients, including degradation rate or diffusion coefficient, but are less well suited for buffering changes in morphogen production, which require complementary mechanisms such as the limited profiles described above.

It is interesting to compare the two circuits. First, we note that In-C may be more difficult to implement than Ex-R. In the Ex-R topology the pinning point does not rely on a fixed "geographical" location, while the In-C requires some mechanism to prevent contractor expression by the cells positioned close to morphogen source. In Ex-R, morphogen represses expression of the expander and will therefore expand until morphogen levels at the edge of the field are sufficient to repress expression of the expander. At this point expander expression will stop and the system will reach a steady state. Conversely, in the In-C mechanism, at steady state the morphogen levels need to be reduced below a certain level to prevent contractor expression. The expression of the contractor will be excluded from high morphogen levels by defining its fixed distal pinning point through a cue that is independent of the morphogen. In the example we identified in the *Drosophila* embryo, localized contractor expression was enabled by interaction between the two orthogonal axes patterning the embryo, and by the embryo morphology. In would be interesting to examine other morphogen situations whereby such localized expression is enabled.

Another difference between the two topologies is that in addition to buffering the morphogen spread against variations in its parameters, the Ex-R circuit allows to scale the pattern with tissue size with no prior positional information at the edge of the field. In contrast, the In-C circuitry will provide scaling only if the pinning point, defining its allowed expression domain, is also scaled with embryo size. Since, as described above, this pinning point is defined by an additional cue independent of the morphogen system itself, this poses an additional constraint on the mechanism. Notably, both mechanisms can scale

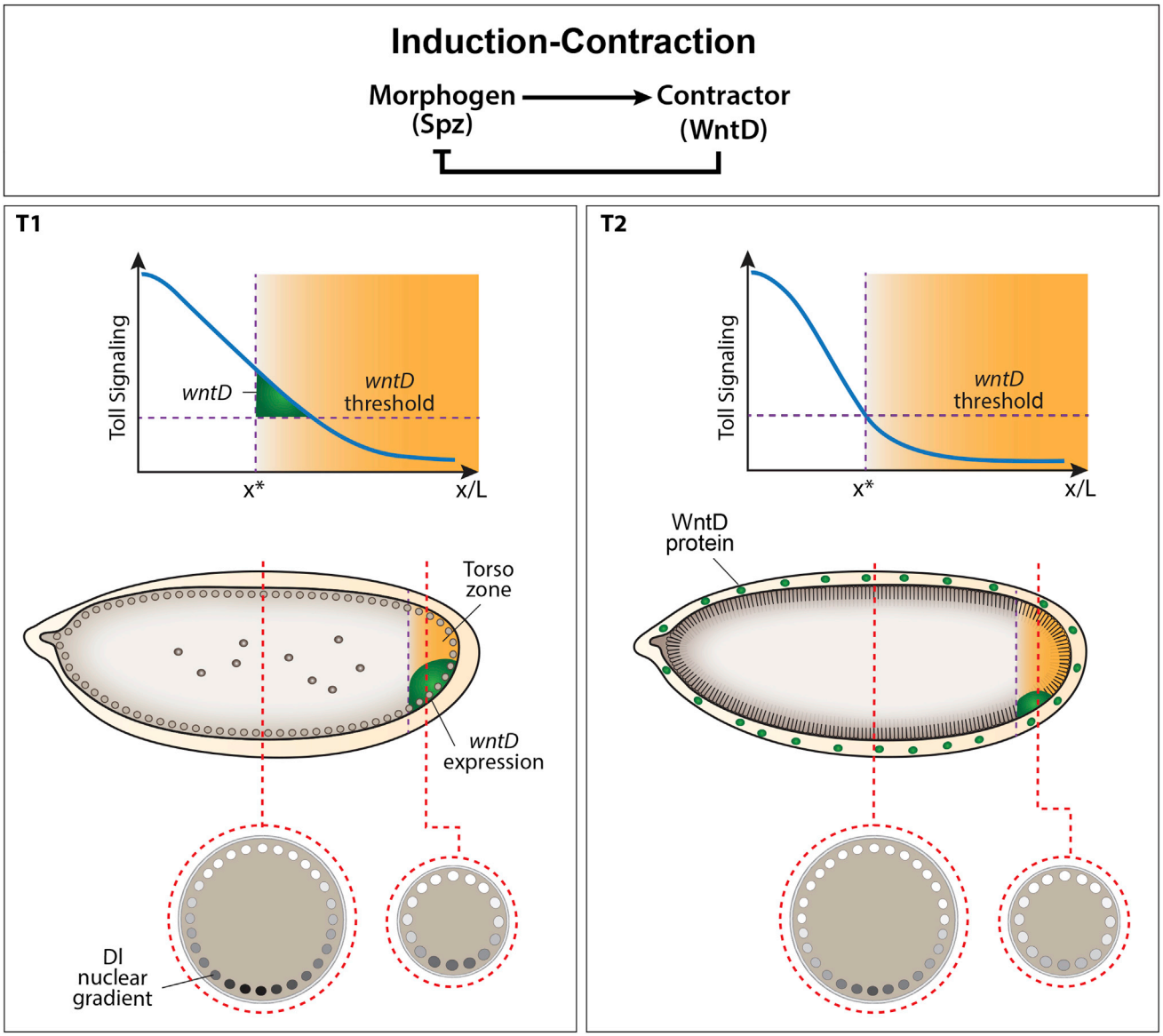

Figure 4. Induction-Contraction by Toll and WntD in the Early Embryo

Toll signaling induces the expression of *wntD* only at the termini of the embryo, where Torso/MAPK signaling also removes repression of *wntD* by Capicua. (T1) Initially, the level of *wntD* expression at the termini will be correlated with the level of Toll signaling. Note in the cross-sections that due to the morphology of the embryo the levels of Toll signaling at the terminus are lower. (T2) Secretion and uniform diffusion of WntD in the extracellular milieu will attenuate the global Toll activation profile, by interfering with binding of the Toll ligand Spätzle. Attenuation will prevail until the gradient reaches the fixed "distal pinning" point (x*) where signaling at the terminal domain will fall below the threshold for *wntD* induction. Note that in this case the distal pinning point corresponds to a fixed "geographical" location, defined by the activity zone of Torso. In the rest of the embryo, signaling will be reduced and reach the desired distribution profile. The same profile will be obtained in different embryos, regardless of variations in the initial level of Toll signaling among them. Rapid diffusion and stability of WntD protein are required for effective buffering.

constant-size or slow-growing tissue, but are less effective when patterning occurs concomitantly with rapid growth.

Finally, the opposite regulatory topology of Ex-R and In-C may be linked to the distinct challenges posed to the respective morphogen gradients by the two tissues. Ex-R expands the gradient and is therefore more appropriate for conditions in which diffusion is long-range, or slow. In the wing disc, for example, Dpp has to diffuse from its source over more than two dozen cells, such that the capacity to reach more distant cells becomes limiting, and is facilitated by the expander. In contrast, In-C functions to narrow down the gradient, and may therefore be more appropriate for conditions of rapid, or broad, diffusion. In the early embryo, for example, diffusion in the perivitelline fluid comprising the extracellular milieu is rapid, making it challenging to maintain a sharp and restricted extracellular gradient of the active ligand (Stein et al., 1991). Thus, expression of a contracting molecule across the entire circumference may help to keep the signaling profile tighter and more confined.

Concluding Remarks

Patterning by morphogen gradients is utilized in different developmental settings, and involves a variety of signaling pathways and distinct secreted morphogen molecules. In this review we highlighted several classes of mechanisms that can buffer variability in the morphogen profile. These mechanisms are generic, and can be realized by a variety of molecular pathways. In fact, the basic building blocks of these mechanisms, i.e., extracellular feedbacks that modulate morphogen diffusion or degradation, are widespread and have been described for a large number of morphogen systems. They impinge on the most basic features of the morphogen: its stability and capacity to interact with the respective cell-surface receptors. With the concepts of "limiting profile" and "distal pinning" in mind, future examination of morphogen systems may identify additional molecular manifestations of these modalities, and attribute generic buffering roles to these components.

The mechanisms of limiting profile and distal pinning distinctly buffer variability in morphogen production or distribution, respectively. Both mechanisms could operate in parallel, on the same morphogen system. For example, in the case of the Toll activation gradient that involves ligand shuttling (Haskel-Ittah et al., 2012), limiting profile may buffer variability in the amount of processed Spz ligand that is generated, by storage of excess ligand at the ventral midline. In parallel, distal pinning employing induction of *wntD* expression can adjust the global shape of the gradient in the lateral domains that lie beyond the central ventral zone (Rahimi et al., 2016).

This review focused on variability and adjustment of morphogen profiles in scenarios where the tissue is not growing and steady state is reached. Clearly, the situation becomes more complex when tissue growth takes place in parallel to patterning (Averbukh et al., 2014; Romanova-Michaelides et al., 2015; Wartlick et al., 2011a). In addition, it has been argued that in some morphogen systems the trafficking takes place along cytoplasmic extensions (termed cytonemes), rather than in the extracellular milieu (Kornberg and Roy, 2014). The concept of distal pinning described here may still operate in such a system, although implemented in different ways. To modulate propagation and final distribution of the morphogen within the field, an extracellular expander or contractor would need to impinge, for example, on morphogen stability or receptor association, while the morphogen is trafficked on the surface of cytonemes.

In the future it will be interesting to explore possible links between stabilization of morphogen gradients within the same species and evolutionary modifications of patterns. In a recent study, adjustment of the patterning response to the SHH gradient in avian species with differently sized neural tubes was examined. Modulation of the ratio between activating and inhibitory Gli proteins within the cells was shown to impinge directly on the slope of the response to the fixed SHH gradient (Uygur et al., 2016). Thus, a genetically programmed uniform change in all cells can adjust the shape of the response to the fixed morphogen distribution gradient. While this mechanism alters the global response, it is "hard-wired" and does not provide a dynamic feedback that is necessary to adjust fluctuations in morphogen profiles between members of the same species. Can the feedback responses that are used to reduce variability within the same species, discussed in this review, also be recruited during evolution to alter gradient properties? Do the morphogen gradients adjust to changes in embryo size as new species evolve, and which properties of the morphogen gradient are modified? The quantitative dependence of the threshold for expander or contractor production on morphogen levels, appears to be central for defining the ultimate shape of the morphogen gradient. Changes in the regulatory properties that define the distal pinning point of these genes could therefore tune the global profile of the gradient they control.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.12.012.

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