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Coordination of Patterning and Growth by the Morphogen DPP

Review

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The elegance of animal body plans derives from an intimate connection between function and form, which during organ formation is linked to patterning and growth. Yet, how patterning and growth are coordinated still remains largely a mystery. To study this question the *Drosophila* wing imaginal disc, an epithelial primordial organ that later forms the adult wing, has proven to be an invaluable and versatile model. Wing disc development is organized around a coordinate system provided by morphogens such as the TGF-β homolog Decapentaplegic (DPP). The function of DPP has been studied at multiple levels: ranging from the kinetics of gradient formation to the establishment and maintenance of target gene domains as well as DPP's role in growth control. Here, we focus on recent publications that both enrich our view of DPP signaling but also highlight outstanding questions of how DPP coordinates patterning and growth during development.

Introduction

Inspired by the contemplation of "all things organic" the architect Louis Sullivan coined a central axiom of modern architecture and design: "that form ever follows function" [1]. How are form and function established in animals? It is becoming increasingly clear that a small class of secreted molecules, called 'morphogens', serves as the architects that coordinate patterning and growth during development. Morphogens define organ function by generating a spatial pattern of differentiated cells and also modulate organ size and shape [2–4].

The 'French flag' model introduced by Lewis Wolpert [5] has proven to be a useful starting paradigm for describing the mechanisms of morphogen signaling. Briefly, the model posits that morphogens are locally secreted and diffuse from their source across a field of receptive cells while also being degraded to form a concentration gradient encoding positional information [3,6,7]. The local morphogen concentration read by receptive cells instructs them to adopt a specific cell fate dependent on their relative position within the morphogenetic field [3,8]. Boundaries of gene expression profiles correspond to specific concentration thresholds and often determine the future location of anatomical features in the fully developed adult.

How and whether morphogen gradients adapt to tissue growth (a process called 'morphogen scaling') is an important question that is not directly addressed in the original French flag model and is not completely understood. Further, the mechanism of growth regulation by morphogens is still hotly debated [1,2,4,9,10]. Many organs will not grow properly in the absence of key morphogens and ectopic morphogen expression can often trigger overgrowth. Yet, controversy still exists on whether morphogens are directly instructive or indirectly permissive for growth. Hence, several models have been developed to answer this question [2–4,10–13].

Much of what we know about morphogens has come from studies of *Drosophila melanogaster* wing imaginal discs — epithelial organs that grow during larval phases to later differentiate during metamorphosis into the adult wing (Figure 1A). In wing discs, the TGF-β homolog Decapentaplegic (DPP) functions as a morphogen that instructs cells to adopt position-specific fates that determine, for example, the location of wing veins along the anterior-posterior (A-P) axis (Figure 1A,B) [5,14–17]. DPP is secreted from a stripe of cells situated at the boundary between the anterior and posterior compartments of the wing disc from where it disperses and generates a concentration gradient (Figure 1A,B). A signal transduction cascade converts the local cellular concentration of DPP into a gradient of phosphorylated MAD (P-MAD) through the activation of the type-I receptor Thickveins (TKV) (Figure 2A) [3,6,7,18]. Phosphorylated MAD (P-MAD) acts as the sole known transmitter of DPP-receptor activity and regulates downstream gene expression. The target genes of DPP have different transcriptional activation thresholds [3,8,14–16] (Figure 2B). One of the most important patterning functions of DPP is to restrict the expression of the transcriptional repressor Brinker (BRK). The expression profile of *brk* is thus a mirror image of the DPP gradient [18–21] (Figure 2B).

Experimental evidence also links DPP expression to wing disc size: wing discs lacking DPP activity develop into little stumps, whereas discs with constitutively active DPP signaling grow significantly larger than wild-type discs [15,22–25]. Hence, DPP provides a link between relative position and cell fate, size and pattern, form and function, in the wing imaginal disc.

In this review, we discuss recent observations and models that attempt to explain the interactions between patterning and growth in the morphogenetic field of DPP (for a systems biology view on the interplay between patterning and growth, see [26]). Even though DPP is arguably one of the most thoroughly investigated morphogens, a consensus on the mechanisms of DPP gradient formation [12,24,27–34], scaling properties [31,35–39] and growth regulation — either indirect or direct — [12,24,28,31] has not been reached, partly due to differences in the experimental approaches between studies [2,4,9,12,13,27]. We will highlight key points of contention, propose solutions to reconcile discrepancies when possible, and discus future directions of research needed to clarify the multifaceted roles of DPP in patterning and growth.

Dynamics of DPP Signaling during Growth

Since the first studies of DPP gradient formation were published [35,40], the mechanism of long-range DPP dispersion has been ardently debated. The study of morphogen transport has proven technically challenging and a plethora of models has been proposed with varying degrees of experimental evidence (for a recent review on transport mechanism, see [41]). Broadly speaking, morphogen dispersal models can be divided into two categories: cell-based and

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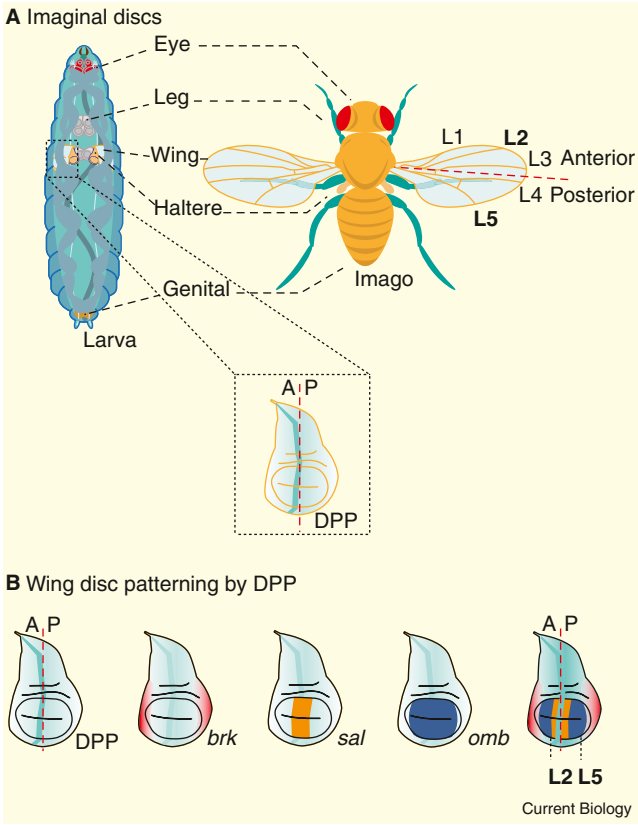

Figure 1. Imaginal discs and DPP-mediated patterning.

(A) Imaginal discs are primordial structures of adult insect appendages that are already present at the larval stage. During metamorphosis each imaginal disc develops into a specific adult appendage (eye, wing, leg, genital, etc.). *Drosophila* imaginal discs undergo patterning and growth during the larval stages. Imaginal discs are constituted of approximately 50 cells during the first larval instar and will grow up to 50,000 cells before the onset of pupariation. The larval stage depicted is late 3rd instar. (B) The DPP pathway patterns the wing disc along the A-P axis. DPP diffuses from a thin stripe of cells at the center of the disc and represses the expression of *brk*. The resultant activity of DPP and BRK leads to the nested expression domains of *sal* and *omb*. The domain boundaries of *sal* and *omb* will correspond to anatomical landmarks in the adult wing such as the position of the wing veins (L2 and L5).

diffusive [41]. Cell-based morphogen dispersal models invoke cellular mechanisms that actively transport morphogens, whereas diffusive dispersion models explain the spread of a morphogen due to 'random walks' through the extracellular space.

DPP Dispersal Models

The simplest mechanism of morphogen dispersal from its source to its target would be by diffusing through the extracellular space. A gradient is generated because the morphogen is not able to accumulate indefinitely but rather is cleared either by receptor-mediated uptake or by degradation [41]. However, questions regarding the capacity of morphogens to diffuse efficiently and reliably enough over long morphogenetic fields led to the proposition of cell-based models of morphogen transport (for a discussion, see [42]). Two active transport models have been proposed for DPP in the wing imaginal disc: transport by transcytosis or via cytonemes. As DPP cannot be directly visualized, DPP dispersion is usually studied with a transgene [31,35,40,43] that has an expression pattern closely approximating that of endogenous DPP (Figure 2C).

Transcytosis

Transcytosis is a mode of transport by which morphogens are shuttled across cells through repeated cycles of endo- and exocytosis (Figure 3A). A number of early observations linking receptor-mediated endocytosis to DPP signaling supported the idea that DPP disperses through transcytosis [29,31,40,44,45]. For example, the range of DPP signaling is restricted when endocytosis is hampered [44]. When DPP uptake is prevented exclusively in groups of sibling cells (referred to as 'clones') it tends to accumulate upstream in relation to the source of these clones while cells situated downstream, or farther away from the morphogen source, of the clone show reduced amounts [40]. Measurements of the diffusion kinetics of DPP with fluorescent recovery after photobleaching (FRAP) analysis [46] have been reported to be too slow for diffusive mechanisms [31,45]. Altogether, these results suggest that receptor-mediated endocytosis might be necessary for DPP movement. However, other groups have questioned whether endocytosis is really required for DPP transport [27,32,33,47].

Cytonemes

Morphogen dispersal by cytoneme-mediated transport proposes that long cytoplasmic processes that extend from target cells towards the source of morphogens (cytonemes) serve to shuttle the morphogen bound to its receptor back to target cell bodies (Figure 3B) [48–52]. The mechanism by which cytonemes could generate a morphogen gradient remains hypothetical. Two scenarios seem feasible: the morphogen could be degraded along the cytonemes relatively fast compared to its transport rate [48] or the quantity of cytoneme-based connections that cells manage to establish with the morphogen source could decrease as a function of the distance to the source [41]. Several observations link DPP to cytoneme formation: DPP is required for cytonemes to develop and can define the orientation of cytonemes [49]. In addition, cytonemes contain punctae of DPP and its receptor TKV [49,50]. However, the functional role of cytonemes in DPP signaling has not been elucidated yet, and it is unclear whether they play any role in generating the DPP signaling gradient. One of the challenges in addressing the role of cytonemes in gradient formation comes from a lack of genetic tools such as mutations that selectively abolish cytoneme function. A further challenge is the difficulty of observing cytonemes, which has limited the number of studies on this phenomenon. However, similar processes have recently been described in the chicken limb bud [53].

Diffusion-Based Models

Although there are several intriguing results linking DPP dispersal to cell-based mechanisms, there is a growing body of evidence supporting the alternative view that DPP gradient formation depends principally on diffusion. Arguably, the largest fraction of DPP is extracellular [35,47], and this fraction seems to correspond to the signaling pool [47]. The presence of a large and active fraction of DPP situated above the cell surface seems easier to reconcile with diffusion rather than with cell-based mechanisms (which should be associated with an intracellular signaling pool). Furthermore, several reports have suggested that the effects

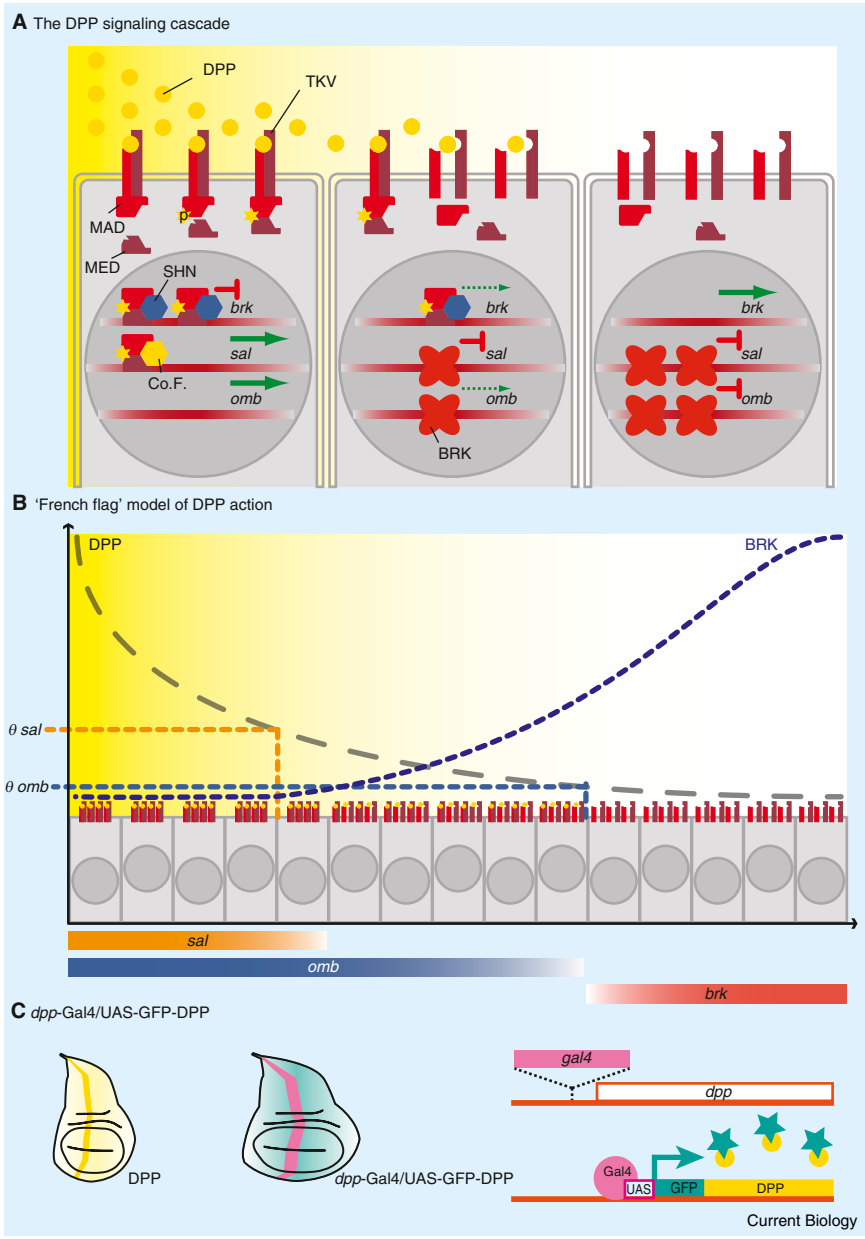

Figure 2. Wing disc patterning by the DPP pathway.

(A) DPP activates a signaling cascade by binding to its receptor TKV. Activated TKV phosphorylates the transcription factor MAD. P-MAD can bind to its interaction partners Medea (MED) and Schnurri (SHN). The P-MAD/MED/SHN complex can repress the expression of the transcriptional repressor Brinker (BRK). Low sensitivity target genes such as *omb* only require derepression of BRK to be expressed, whereas high sensitivity targets such as *sal* also require direct activation by a complex of P-MAD/MED and an unknown co-factor (Co.F.). (B) Threshold-dependent patterning by DPP in a "French flag" fashion. DPP limits BRK activity to the lateral edges of the A-P axis. *sal* is highly sensitive to BRK (hence *sal* has a high Dpp activation threshold, θ *sal*) and its expression is limited to the central regions of the A-P axis where BRK is totally absent. *omb*, with a low θ, can accommodate high levels of BRK and is expressed in a broader domain. (C) In order to observe DPP dispersion and the DPP gradient, researchers usually employ a GFP–DPP fusion protein. A GAL4-UAS binary transcription system [43] is used to drive GFP–DPP expression in a manner analogous to the endogenous *dpp* transcription. The system reproduces DPP expression sufficiently accurately such that it can rescue DPP loss-of-function alleles. However, there are some clear differences such as a larger expression domain and some degree of overgrowth.

on DPP transport caused by blocking endocytosis are not incompatible with diffusive processes [27,47]. From this perspective, the effect that blocking endocytosis has on DPP dispersion could be explained by an accumulation of DPP receptors at the cell surface (due a break in the balance between endo- and exocytosis) [27,47]. The accumulation of DPP receptors would form a barrier to the morphogen, significantly limiting DPP diffusion. For an endocytosis-deficient clone this would lead to accumulation of DPP upstream of the clone while generating a transient drop in DPP concentration on the side of the cell farther from the morphogen source [40].

Does TKV accumulate in endocytosis-deficient clones? Unfortunately, the evidence remains inconclusive [29,47]. In order to study the role of endocytosis in DPP dispersion a recent effort relied on a more targeted approach [32]. DPP endocytosis was selectively prevented by removing TKV in groups of cells. This was done together with removal of BRK to prevent the elimination of these clones from the epithelium. The result of this experiment demonstrated that DPP readily disperses over such clones in the absence of receptor-mediated endocytosis [32,41]. These points indicate that receptor-mediated endocytosis might be required for the transduction of the DPP pathway, but that the bulk of DPP dispersion occurs through diffusion. Two diffusion-based models have been proposed for DPP: free diffusion (Figure 3C) and hindered diffusion (Figure 3D). These models differ on the nature of interactions between extracellular factors and diffusing morphogens [41]. However, the differences between the models are relatively subtle and difficult to probe experimentally; thus, currently available data could arguably be used to support both [41].

Free Diffusion

Free diffusion implies that morphogen dispersion is mediated by a small pool of molecules that are not significantly affected either by the tortuosity, or 'crookedness' of the extracellular space, or by transient binding interactions with other proteins, such as receptors or components of the extracellular matrix [27,33,41] (Figure 3C). Recently, Lander and colleagues [33] noted that all reports that have supported morphogen transport by means other than free diffusion were based on FRAP. However, FRAP is ill-suited

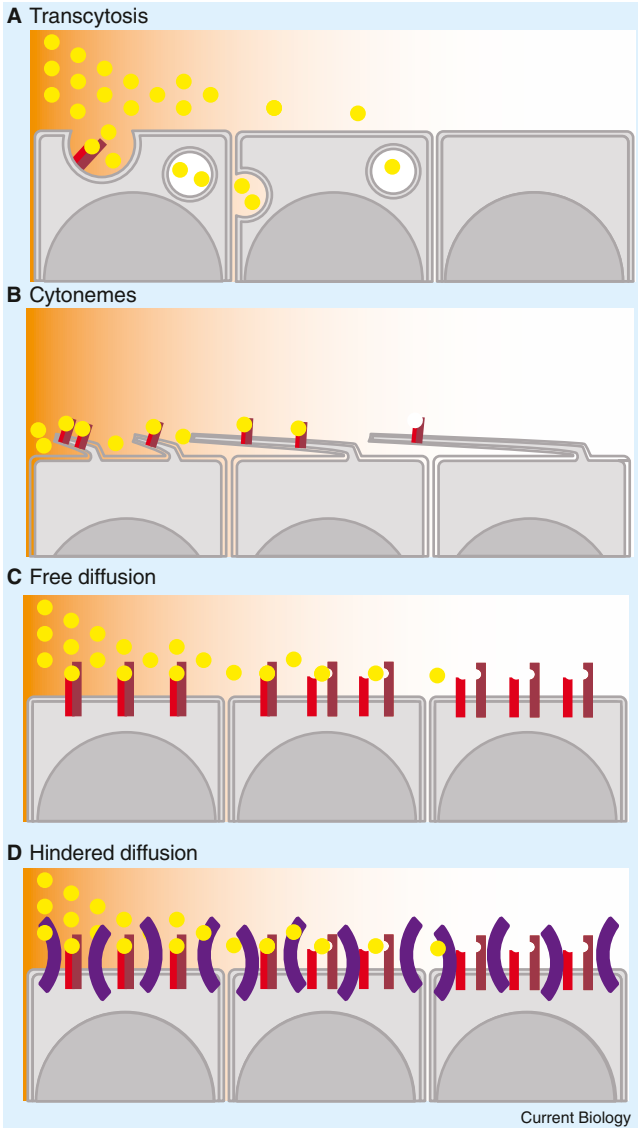

Figure 3. Models of DPP gradient formation.

(A) Transcytosis: following this hypothesis, DPP is shuttled from cell to cell along the A-P axis inside of endosomes. (B) Cytonemes: here DPP is transported on long cell extensions that contact the zone of DPP production. (C) Free diffusion: in this model, the DPP gradient is simply formed by the diffusion of DPP in the extracellular fluid. (D) Hindered diffusion: here, DPP diffuses through the extracellular milieu and interacts with local proteins such as DALLY.

to analyze DPP dispersion, because undistinguishable FRAP results would be obtained for DPP with diffusion coefficients spanning two orders of magnitude [33]. Consequently, more sensitive methods were employed to measure the diffusion rate of DPP [33]: fluorescence correlation spectroscopy [54] and pair correlation function spectroscopy [55]. These experiments revealed a highly mobile fraction of DPP locally moving at speeds that would correspond to those expected if DPP were dispersing by free diffusion [33].

Hindered Diffusion

Hindered diffusion considers that both tortuosity and binding interactions hinder the dispersion speed of a morphogen and might even be required for proper morphogen gradient formation (Figure 3D). In the case of DPP, interactions between DPP and the heparan sulfate proteoglycans (HPSGs) Dally and Dally-like are necessary for proper signaling activity [56–58]. Furthermore, clones mutant for these two proteins have both autonomous and non-autonomous phenotypes indicative of impeded DPP transport [47]. The data in support of free diffusion seem to conflict with the dependency of DPP gradient formation on HSPGs [47,56–58]. However, this discrepancy could be accommodated if one considers that HSPGs might be influencing gradient formation downstream of diffusion [33]. Nested FRAP experiments performed to distinguish between free and hindered diffusion models lend support to free diffusion [33]. However, these results contrast with previous nested FRAP reports [45] and it has been argued that both models cannot yet be untangled [41].

How DPP disperses from its source remains an unresolved issue. It is interesting to note how much the study of morphogen transport seems to depend on the development and combination of new genetic tools such as fluorescent reporters and quantitative assays with which to measure the kinetics of morphogen movement. Nonetheless, there is increasing evidence for diffusion-based models for DPP [32,33,41,59]. For the following discussion on scaling and growth control we will assume that DPP disperses through a diffusion-based mechanism.

Scaling of DPP Gradients during Growth

The scaling properties of morphogenetic fields are of intense interest in developmental biology [59,60]. The field is wrestling with several questions: to what extent do the boundaries of target gene expression domains expand proportionally with tissue growth to maintain constant relative positions? How does the morphogen gradient correspond to the downstream signaling profile? What are the mechanisms that direct spatial patterning in a uniformly expanding tissue? And how would different DPP dispersion models influence the means by which scaling might be achieved?

Scaling

The relative position of wing veins in the adult *Drosophila* wing is a natural benchmark for measuring the degree of anatomical scaling. Interestingly, starved larvae develop into diminutive adults with wings of almost perfect proportion with respect to the whole body [61]. The specific position of the different wing veins along the A-P axis depends on DPP activity thresholds. Thus, in starved animals the DPP system scales down to maintain correctly proportioned and fully functional wings.

The question of scaling is best approached from a mathematical perspective. The profile of the DPP gradient is often approximated as an exponential decay function (Figure 4A) [4,31,45], which is described by two parameters: the amplitude at the source, C_0, and the decay length, λ. Assuming a constant C_0, the DPP gradient scales if the decay length remains proportional to the magnitude of the length axis (L): λ/L = constant.

Several attempts have been made to quantitatively measure the gradients of DPP and P-MAD to determine the degree of scaling of DPP signaling activity. Initially, it was reported that the expression domain of the DPP target gene *spalt* scaled when the size of the posterior compartment was modified experimentally [35]. Two subsequent

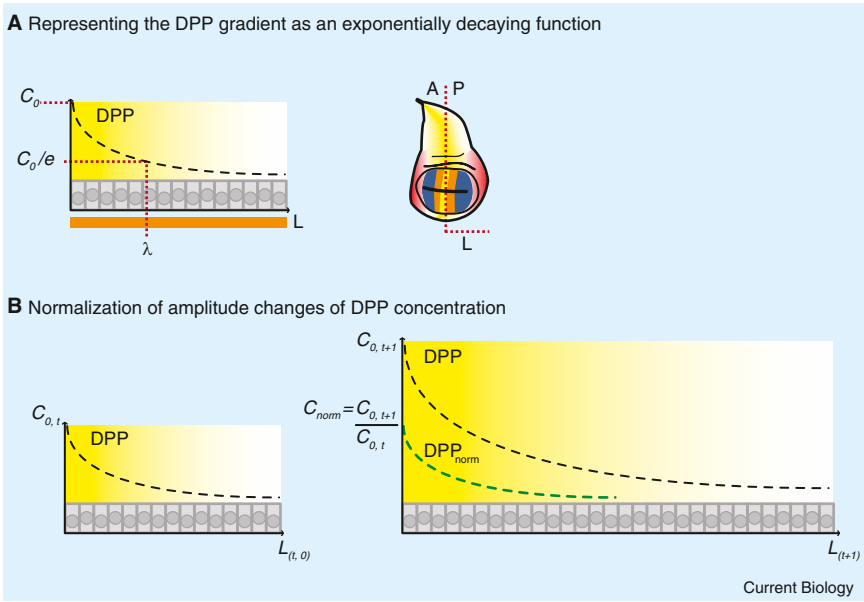

Figure 4. The DPP gradient as an exponentially decaying function.

(A) To a first approximation, the DPP gradient can be described as an exponentially decaying function whose properties are defined by the concentration at the source, C_0, and the decay length λ. (B) Visualization of the normalization of the amplitude of the DPP gradient.

studies focused on scaling during developmental growth and found no evidence of scaling [36,37]. One study reported that the normalized values of both the GFP–DPP and P-MAD gradients do not scale [36] while a second concluded that the DPP decay length (λ) does not scale towards the end of development [37]. However, more recent studies have reported scaling at different levels of the DPP signaling cascade and with different approaches to quantify scaling [31,39]. These two studies quantified scaling starting at earlier developmental time points whereas the previous reports were limited to the last ~48 hours of growth. Interestingly, if only later stages of third instar discs are considered [31], the data set is too noisy to support the hypothesis that the gradient scales. One possible resolution for the discrepancies between these studies could be that morphogen length scales become uncoupled to tissue growth during the final stages of development (our hypothesis is based on [31,36,37,39]).

In the study by Gonzalez-Gaitan and colleagues [31], the authors found that the amplitude of the DPP gradient increases during development. However, the authors note that the GFP–DPP intensity profiles of increasing developmental time points can be seamlessly overlaid if not only the length of the field but also the amplitude of GFP–DPP is normalized with respect to its concentration at the source, $C_{norm} = C_{abs}(x)/C_0(t)$ (Figure 4B). The authors propose that as the different profiles can be overlaid, they represent scaled versions of one another and that λ/L remains constant. The activity gradients of synthetic reporters of DPP signaling, like dad-nRFP (a nuclear RFP under the control of a synthetic enhancer of the DPP target gene *DAD*), also grow in amplitude, such that it is the normalized gradient that scales with tissue size [31]. Of note, normalizing procedures are not necessarily neutral and can influence the analysis [62].

An increase in the amplitude of DPP signaling over time would require a significant revision to the French flag model: the local thresholds of DPP signaling at target gene boundaries would need to increase over time to maintain proportional patterning. This finding creates the problem of identifying a new mechanism, other than fixed thresholds, for regulating target gene expression (Figure 5A). For example, a biological mechanism would be required in DPP receiving cells to normalize the DPP levels that they sense relative to the DPP concentration at the source.

A subsequent report by the Affolter group [39] provides an apparent conflicting result regarding increases in the amplitude of DPP pathway activity. In this study, the absolute amplitude of the P-MAD gradient was found to remain relatively constant while the length scale of the P-MAD gradient expands with tissue growth (λ/L is constant). This statement is qualified by an observed sharpening of the P-MAD gradient in the last hours of development [39]. Thus, while these two recent reports agree that some form of 'scaling' occurs, they disagree on whether the amplitude of the DPP activity gradient remains constant or increases during growth, and consequently on whether absolute values or normalized values scale.

On the surface, the finding that the P-MAD gradient expands while its amplitude remains relatively constant [39] fits better with the French flag model, as this would maintain the relative expression boundaries of downstream genes approximately constant as the disc grows (Figure 5B). This phenomenon would reveal a parsimonious mechanism for the scaling of the DPP activity gradient. The relative position of target gene thresholds would expand uniformly with an increasing A-P axis length while the concentration thresholds remain constant. However, several downstream target genes of DPP were found to follow different dynamics than P-MAD [39]. For example, *BRK* expression levels were found to increase during development even while P-MAD levels were found to remain constant through exponential growth. These results indicate that the French Flag model, while conceptually useful, requires future refinements [39].

One explanation that could reconcile differences in DPP and P-MAD dynamics may arise through a known negative feedback loop formed by the DPP target *DAD* [63]. Increases in DPP signaling lead to higher DAD levels and consequently stronger inhibition of P-MAD to buffer the signal [64]. The P-MAD gradient seems to be robust to upstream perturbations that increase the intensity of DPP signaling such that TKV receptor levels can be increased more than thirty-fold with only minor changes to the shape of the P-MAD gradient [64]. The increase of DAD expression levels with time (reported by both [31,39]) could reconcile increasing DPP amplitudes [31] with temporally invariant P-MAD values [39] (Figure 5C). DPP levels could be

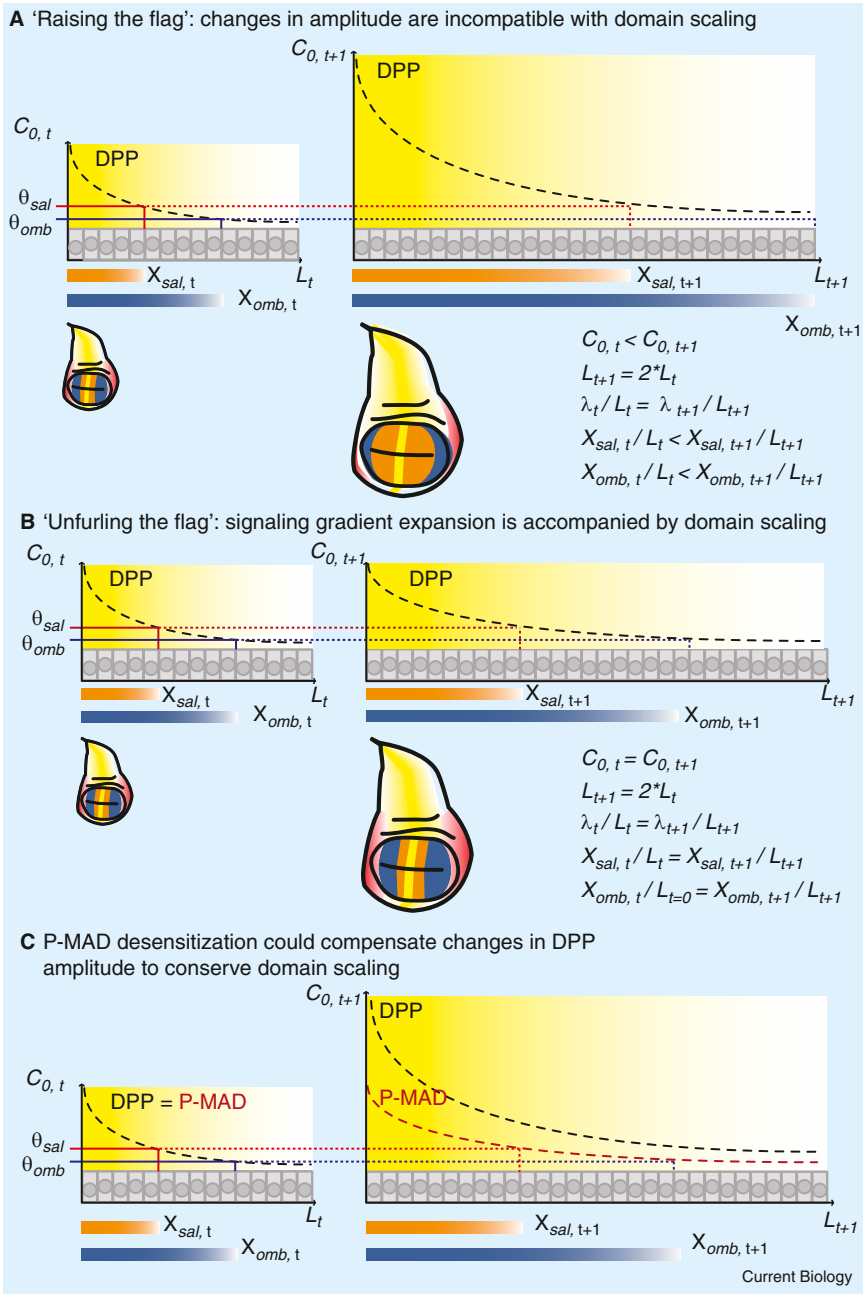

Figure 5. Gradient dynamics and signaling threshold activation.

(A) 'Raising the flag': if gene expression is controlled by a constant morphogen threshold of activation θ, changes in the amplitude of the DPP gradient with time render it impossible for the relative gene expression domains to scale with size. In this case, the amplitude of the DPP gradient increases with the length of the A-P axis. It is impossible to keep both the thresholds and the relative positions of target gene domains constant. (B) 'Unfurling the flag': The relative expression domains of DPP targets can scale with growth if the signaling gradient expands uniformly with tissue growth. Here, as the A-P axis length doubles, so does the length-scale of the DPP signaling gradient. The thresholds as well as the relative position of target gene expression domains remain fixed. (C) If the levels of P-MAD are buffered from changes in the amplitude of the DPP gradient such that the P-MAD gradient expands uniformly with tissue size, gene expression domain scaling can be achieved without adapting morphogen activation thresholds over time.

increasing to limit the signaling noise associated with low ligand levels. If absolute DPP levels would remain constant as the wing disc grows, an increasingly larger absolute number of cells would be exposed to low ligand levels. Could the wing disc rely on a two-tier system that compensates ligand noise by increasing ligand concentration in concert with size, while maintaining downstream pathway activity levels relatively constant such that scaling of target genes is parsimoniously obtained?

In conclusion, different approaches lead to different conclusions on the nature and degree of DPP scaling. These discrepancies could result from limitations of current experimental methods [62]. The study that reported invariant P-MAD amplitudes [39] might have been hampered by the poor comparability of immunofluorescence levels. Similarly, the GFP-DPP flies should be subject to caution as they tend to overgrow [31,45]. It will be important to further investigate how the DPP gradient scales with more accurate and faithful DPP activity reporters and assays.

Mechanisms of Morphogen Scaling

Biologically, scaling could be obtained either by decreasing DPP degradation or increasing DPP diffusion rates to accommodate tissue growth. In both cases, the result would be that the gradient would expand with growth [31,38,65].

Two scaling mechanisms have recently been proposed for the DPP pathway: expansion by dilution [31] and expansion–repression [38,39,65]. In the expansion-by-dilution model, a long-lived antagonist promotes DPP degradation, while tissue growth indirectly stabilizes DPP by diluting its antagonist (Figure 6A) [4,31]. In the expansion–repression model, an expander molecule diffuses from the edges of the disc while its expression is repressed by DPP, thereby linking DPP transport to the length of the A-P axis [65]. A recent publication [31] reported that, in mutants with anisotropic growth, the normalized DPP gradient scales better with area than length. This observation matches the predictions of an expansion-by-dilution model better than those of an expansion–repression model [4,31].

On the other hand, the molecular mechanisms behind an expansion–repression circuit might have started to be unraveled through the role of *pentagone* (PENT) [66], a likely DPP expander in the wing disc [38,39] (Figure 6B). PENT is a secreted molecule that is negatively regulated by

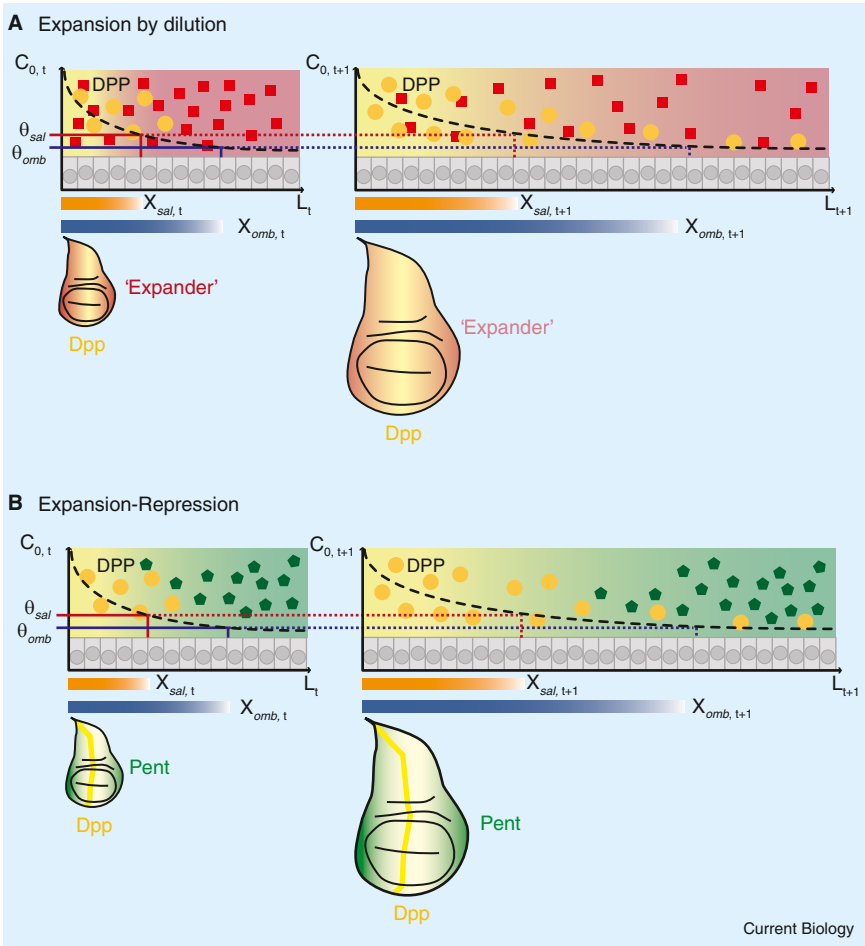

Figure 6. Scaling models.

(A) Expansion by dilution: a long-lived antagonist promotes DPP degradation and thus hinders its dispersion. However, growth dilutes the antagonist such that as the disc area increases, DPP movement is facilitated. In this way the DPP gradient can expand further as the disc grows. (B) Expansion-repression: An expander, PENT facilitates DPP diffusion but PENT expression is repressed by DPP. Initially DPP does not reach the expression domain of PENT, thus PENT is actively produced and diffuses through the wing disc. As PENT increases DPP diffusion, DPP starts to repress *pent* expression and the concentration of the expander decreases accordingly.

DPP, but does not bind directly to DPP [66]. PENT expression is limited to the edges of the wing disc and loss-of-function alleles of PENT perturb the scaling of the DPP gradient [38,39,66]. PENT seems to stabilize extracellular DPP [66] but the exact mechanism by which PENT helps to expand the DPP gradient by affecting the transport or the degradation of DPP remains to be elucidated.

Growth Regulation by DPP

The mechanism by which organs measure their size intrinsically is one of the great remaining mysteries of developmental biology [67]. The finding that morphogens control both pattern and size has attracted intensive scrutiny, because it links the two main phenomena underlying animal development: differentiation and proliferation. This observation provided developmental biologists with a prospective, morphogen-based, intrinsic growth control system for organs. Several models have been proposed to explain the rules of growth control by DPP in the wing disc [4,10,28,31,68,69]. Here, we focus on three of these models to illustrate the main challenges faced by the field in understanding the role of DPP in regulating wing disc growth.

Models of morphogenetic growth control address two fundamental questions: the control of final organ size and the conundrum of how a graded morphogen signal regulates uniform growth. Broadly, these models fall into two categories: instructive (direct) and permissive (indirect). Instructive models imply that DPP activity directly drives cell growth and/or division. Conversely, permissive models attribute the induction and control of cell proliferation to regulators other than DPP but propose that the competence of cells to respond to these mechanisms depends on position-specific DPP activity levels. Both instructive and permissive models strive to provide explanations for the observed uniformity of growth along the morphogenetic axis of DPP. However, only instructive models provide a morphogen-centric solution to the problem of growth termination. Below we will discuss two instructive models (morphogen slope and temporal dynamics) and our interpretation of a permissive model (growth equalization).

The Morphogen-Slope Model

Multiple models have been proposed to explain both the natural growth and regenerative response of the wing [10,11]. In particular, by extending the idea that differences in positional information could trigger growth to the values of the slope of a morphogen, Day and Lawrence [68] proposed that "local growth could depend on the local reading of the steepness of morphogen concentration gradients" (Figure 7A). In other words, cells read the local slope of the DPP gradient and this is translated into a decision to grow or not. The model further posits that the DPP morphogen gradient flattens as the disc grows. Hence, the slope of the gradient would be pinned to the dimension of the morphogenetic field. This provided an elegant solution to the question of final size control: cells would stop growing when the slope's steepness declined below a given value (Figure 7A).

In addition to providing a solution to the question of growth termination, this model also postulated an explanation for the uniform distribution of growth along the A-P axis. The original morphogen slope model posits that morphogens generate a linear gradient [68]. With a linear gradient, all cells along the morphogenetic field would measure the same slope and growth would be uniform (Figure 7A). In its original form this model was neither clearly instructive nor permissive, as it did not discuss whether the slope values directly

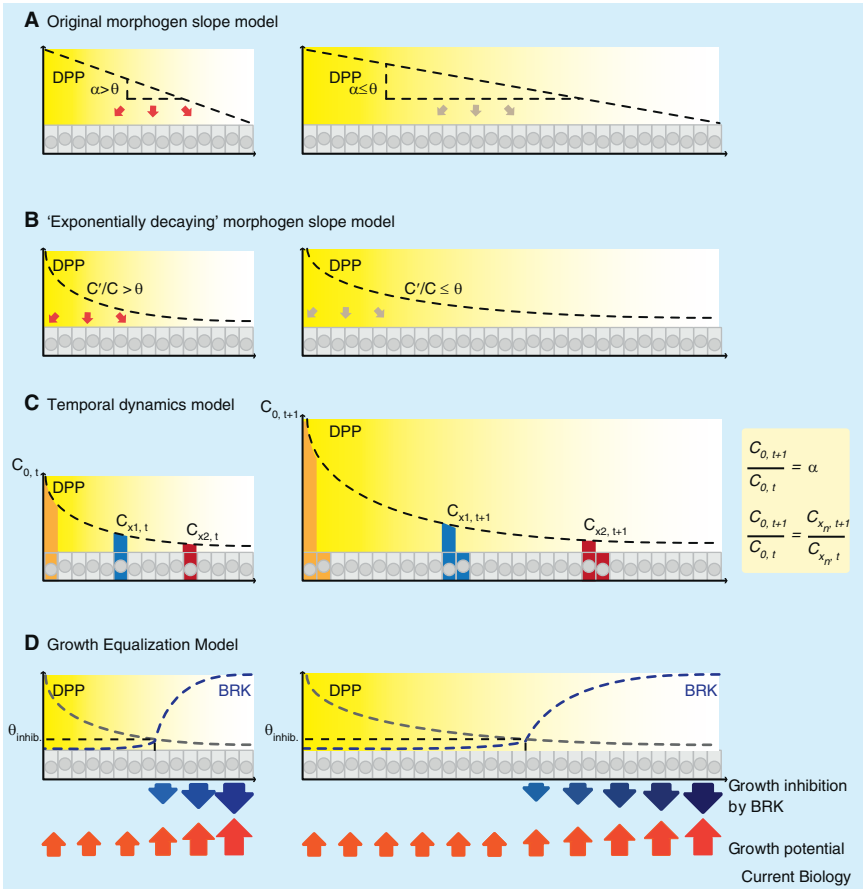

Figure 7. Growth models.

(A) In the original morphogen slope model, cells read the slope of a linear gradient. The slope is the same for all cells. If the value of the slope decreases below a threshold, θ, the cells stop dividing. (B) In the 'exponentially decaying' morphogen slope differences model, cells read the local spatial derivative divided by the local concentration C'/C. This value is constant for each cell along the A-P axis. If C'/C drops below a threshold, θ, proliferation stops. (C) The temporal dynamics model states that the amplitude of the DPP gradient increases with tissue size. The amplitude increases by the same relative value on all locations. The local amplitude has to increase by a factor α for cells to divide. (D) The growth equalization model proposes that the proliferation potential differs along the A-P axis. The exponentially decaying DPP ligand concentration gradient is transformed into a sigmoidal-shaped P-MAD signaling function (not shown) that delineates medial and lateral cells. BRK inhibition of growth is restricted by DPP to the lateral domains of the disc where the growth potential is higher. This results in a uniform distribution of growth.

drive the cell cycle. However, later interpretations of the gradient model assigned a more instructive role to the steepness of the gradient [4,10,31,69,70].

Can the morphogen slope model account for uniform growth along the A-P axis of the wing disc? In its simplest form, the original model was based on a linear gradient with a single, position-independent slope. However, it was later found that at both the ligand and signal transduction level, the DPP gradient is similar to an exponential decay function (with spatially-varying slopes) [31,45]. Thus, the slope of the DPP gradient is not position-independent: cells located close to the source of DPP see a steeper slope than more lateral cells. This rules out the possibility that a constant morphogen slope drives uniform growth. To circumvent this, it was recently suggested that the morphogen slope model could be adapted to an exponentially decaying DPP gradient by proposing that instead of reading a slope, the cells would read the relative spatial differences of DPP across their surface (the slope of the DPP ligand divided by the local concentration of DPP ligand) (Figure 7B) [4,31]. In an exponential gradient these values are position-independent and this would result, once again, in uniform growth.

Whether the slope of the DPP gradient regulates growth has also been difficult to ascertain. For example, ubiquitous DPP expression leads to overgrowth [15,28]. Two main types of experiment have been done to test the gradient model: clonal manipulations that create strong differences in DPP signaling levels between the cells at the interface of the clones, and ubiquitous manipulations that should render the gradient flat or, at least, very shallow.

An initial analysis of clones expressing a constitutively active allele of the DPP receptor *thickveins* (TKV^{QD}) reported a strictly cell-autonomous overgrowth effect, in contradiction to the morphogen slope model [24]. A later effort found a transient increase in cell proliferation around TKV^{QD} clones [69]. However, TKV^{QD} clones do not create a DPP gradient *per se*, but rather a stepwise function of non-physiologically varying levels of DPP signaling. By juxtaposing cells with very strong differences in DPP signaling levels (and thus according to the positional information model, very different positional values) these clones might be triggering intercalary growth [11,71,72] that could either reflect a normal feature of development and regenerative processes or an experimental artifact. From a mechanistic perspective, it is interesting to note that molecular mechanisms linking strong differences in DPP levels to growth may be connected to the Hippo signaling pathway [73].

The role of the DPP gradient in regulating growth was also studied in whole wing discs. DPP seems to modulate growth exclusively by restricting the expression of the transcriptional repressor BRK, which forms an inverse gradient to DPP [19–21,25,28]. Can discs homozygous for both *dpp* and *brk* loss-of-function alleles grow? These discs have no BRK or DPP gradient and no direct input from DPP. It can therefore be concluded that the slope of DPP activity is flat in these discs, although it has been argued that this might not be the case [31]. These discs could not only grow, but actually overgrew, just as $brk^{-/-}$ discs would, hence indicating that graded activity of the DPP signaling pathway might not be required to drive wing disc growth [28].

To conclude, it seems that, while strong differences in DPP signaling can trigger growth, wing discs can also grow in the absence of graded DPP signaling. How these findings relate to intercalary growth and whether intercalary growth is an

important feature of normal growth, if it is limited to regenerative processes, or whether it is equally important in both cases is unclear.

The Temporal Dynamics Model

The temporal dynamics model is based on the observation that wing disc growth rates correlate with increases in amplitude of the DPP activity gradient [31] (recall, however, that temporal increases in P-MAD amplitude were not found in [39]). Along the A-P axis, each cell measures the same relative increase of DPP pathway activity over the course of one size doubling (Figure 7C) [31]. Thus, the temporal dynamics model proposes that wing disc cells divide after reaching a constant (≈50%) relative increase in DPP signaling activity [4,31]. An attractive feature of the model is the simplicity of how growth termination is regulated: proportional increases in DPP activity become increasingly more difficult to obtain, and thus cell cycle rates progressively slow until a certain threshold is reached at which point growth stops. The observed uniformity of growth is a natural outcome of position-invariant temporal increase in normalized DPP concentration.

The strongest evidence in support of this model comes from manipulations of the rate at which DPP signaling activity increases. When the levels of TKV^{QD} expression were modulated with an inducible construct, it was found that: cell division again correlated with a local increase in DPP activity of 50%, and clones in which the local activity was increasing faster were also growing faster [31]. However, these results only indicate a strong correlation between the rate at which DPP increases and cell doubling times.

Could the model be tested in a different way? Because the temporal dynamics model requires increases in DPP signaling amplitude over time, groups of cells in which DPP signaling is kept constant should show impaired growth. This situation can be generated with clones that are homozygous double mutants for the key DPP pathway effectors *mad* and *brk* (Figure 2). Importantly, these clones are impervious to any input from DPP or other BMP ligands expressed in the discs (in this case GBB) and should be insensitive to any potential feedback loop modulating MAD activity (that such a feedback loop could exist is hypothesized in [31]). Thus, these clones should be composed of cells in which the levels of DPP activity remain fixed both temporally and spatially. Surprisingly, medially situated clones mutant for both *mad* and *brk* grow normally with respect to their twin-spot wild-type counterparts while laterally situated *mad, brk* double mutant clones overgrow [74]. These results suggest that wing disc cells do not require increasing DPP levels in order to divide (as long as BRK is not directly repressing growth). However, it was argued that DPP pathway activity, as indicated by dad-nRFP, still increases in clones missing both *mad* and *brk* [75]. It will be interesting to see if the molecular mechanism through which temporal increases in DPP signaling could throttle the cell cycle will be uncovered in the future. It will also be interesting to see if the increased activity of dad-nRFP observed in *mad, brk* double mutant clones [75] really reveals dynamic DPP signaling activity in the absence of MAD and BRK.

The Growth-Equalization Model

Here, we propose an alternative perspective in which DPP plays an indirect role in growth control primarily by equilibrating inherent non-homogenous growth potentials across the A-P axis of the wing disc. The model postulates that wing disc cells can grow and proliferate independently of a direct input from DPP as long as BRK, which functions as a growth repressor, is absent. This model highlights the role of other potentially stimulatory/instructive signals such as insulin/TOR signaling that could drive growth independently of the DPP signaling pathway. Importantly, we propose that this 'basal' growth is inherently non-uniform, as lateral cells appear to have a growth advantage compared to medial cells. This non-uniform growth potential is possibly due to other biochemical signals or micro-environmental factors deriving from tissue geometry (Figure 7D) [76,77], or through mechanical feedback [36,78–80]. DPP could balance the default state of non-uniform growth by restricting the expression domain of *brk* such that BRK limits the proliferation of lateral cells to rates that can be sustained by medial cells (Figure 7D). Interestingly, it has recently been shown that BRK represses MYC, an important growth regulator, in the lateral regions of the wing disc [81]. This finding indicates a broad mechanism through which BRK could limit the growth potential of lateral cells by controlling MYC levels and consequently decreasing ribosome biogenesis and the general transcription of active genes [81–83].

The uniform distribution of growth along morphogenetic fields has often been seen as a paradox in the face of graded morphogen levels [4,10,36,68,79]. However, the uniform growth paradox arises only if it is assumed that the growth potential is naturally uniform across the field. We propose that uniform growth is not a paradox but rather an objective of morphogenetic growth control.

This model is based on the ability of discs lacking *dpp* and *brk* to grow normally but non-uniformly [28]. A similar experiment with *mad, brk* double mutant clones led to the same conclusion. Medially located homozygous *mad*, *brk* clones can grow and do so at the same rate as their wild-type twin spots [74]. This highlights that the growth modulating function of DPP is to repress BRK and that medial cells do not require a direct DPP input to grow. It also suggests that the function of BRK could be to counter a higher proliferation potential of lateral cells.

Previous reports provide some challenges to this model. For example, the DPP activity reporter *dad-nRFP* shows spatial and temporal variations of activity in discs lacking *dpp* and *brk* [31] and in *mad, brk* double mutant clones [75]. However, the reliance on this synthetic reporter implies the assumption that any activity is exclusively due to the DPP pathway, which is difficult to prove unequivocally. Further, experiments showing that tkv^{QD} clones can induce a transient non-autonomous increase in cell proliferation [69] also argue against a strictly passive role for DPP in growth control. However, the transient nature of this phenomenon could indicate that whereas strong differences in DPP activity between neighboring cells can have an instructive effect on growth this might not be required for growth under normal conditions.

The growth equalization model raises several questions: if DPP patterns rather than drives growth, what are the signals that directly control growth and proliferation? It will be interesting to study whether the insulin and TOR signaling pathways are equally active along the A-P axis or if BRK is modulating the capacity of lateral cells to respond to insulin or TOR as it does with MYC [81]. Further, exactly how and through which signals (metabolites, ligands, mechanical

forces) could the microenvironment influence the growth potential of wing disc cells?

Conclusions

In spite of numerous efforts, many aspects of DPP signaling, such as the dispersion mechanism, scaling properties, and the role of DPP in wing disc growth control, remain open. To solve these conundrums new and more precise quantitative tools need to be devised to observe and analyze morphogen dynamics and pathway activity. As we continue to "contemplate all things organic," we still have much to learn about how morphogens such as DPP regulate form and function.

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