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REVIEW ESSAY

Prospects & Overviews

Is Drosophila Dpp/BMP morphogen spreading required for wing patterning and growth?

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Abstract

Secreted signaling molecules act as morphogens to control patterning and growth in many developing tissues. Since locally produced morphogens spread to form a concentration gradient in the surrounding tissue, spreading is generally thought to be the key step in the non-autonomous actions. Here, we review recent advances in tool development to investigate morphogen function using the role of decapentaplegic (Dpp)/bone morphogenetic protein (BMP)-type ligand in the Drosophila wing disc as an example. By applying protein binder tools to distinguish between the roles of Dpp spreading and local Dpp signaling, we found that Dpp signaling in the source cells is important for wing patterning and growth but Dpp spreading from this source cells is not as strictly required as previously thought. Given recent studies showing unexpected requirements of long-range action of different morphogens, manipulating endogenous morphogen gradients by synthetic protein binder tools could shed more light on how morphogens act in developing tissues.

KEYWORDS
Dpp/BMP, Drosophila wing disc, growth, morphogen, patterning, protein binder

INTRODUCTION

Morphogens are secreted molecules thought to control patterning in a concentration-dependent manner[1,2] (Figure 1A). A small number of secreted molecules, such as members of the bone morphogenetic protein (Bmp), Hh, Wg, and Fgf families of ligands, have been shown to act as morphogens. For over 20 years, the wing imaginal disc of *Drosophila melanogaster* has served as a leading model to study mechanisms underlying tissue development through morphogens.[3,4] Decapentaplegic (Dpp), a Bmp-type ligand in flies, represents the first validated secreted morphogen to control patterning of wing imaginal discs (wing precursor tissue).[5–10]

Dpp is produced and secreted from a stripe of anterior cells straddling the anterior–posterior (A-P) compartment boundary of the rosophila wing imaginal disc, and spreads into both compartments to form a concentration gradient (Figure 1B). Dpp (the fly homologue of vertebrate Bmp2/4) binds to type I receptor Thickveins (Tkv) and type II receptor Punt, which results in the phosphorylation of the R-Smad Mothers against Dpp (Mad), pMad. pMad and the Co-Smad Medea form a complex and translocate to the nucleus, where they enhance or suppress target gene transcription (Figure 1C). An interesting feature of Dpp signaling is that a majority of the Dpp target genes are indirectly induced by suppressing transcription of *brinker* (*brk*), which itself acts as a repressor for Dpp target genes.[12–14] In order to repress *brk*, the pMad/Medea tertiary complex recruits of the co-repressor Schnurri (Shn) to bind to well-characterized silencer elements (SEs) in the regulatory sequences of *brk* to block its transcription[15–19] (Figure 1C). Thus, *brk* is repressed in the medial region of the wing

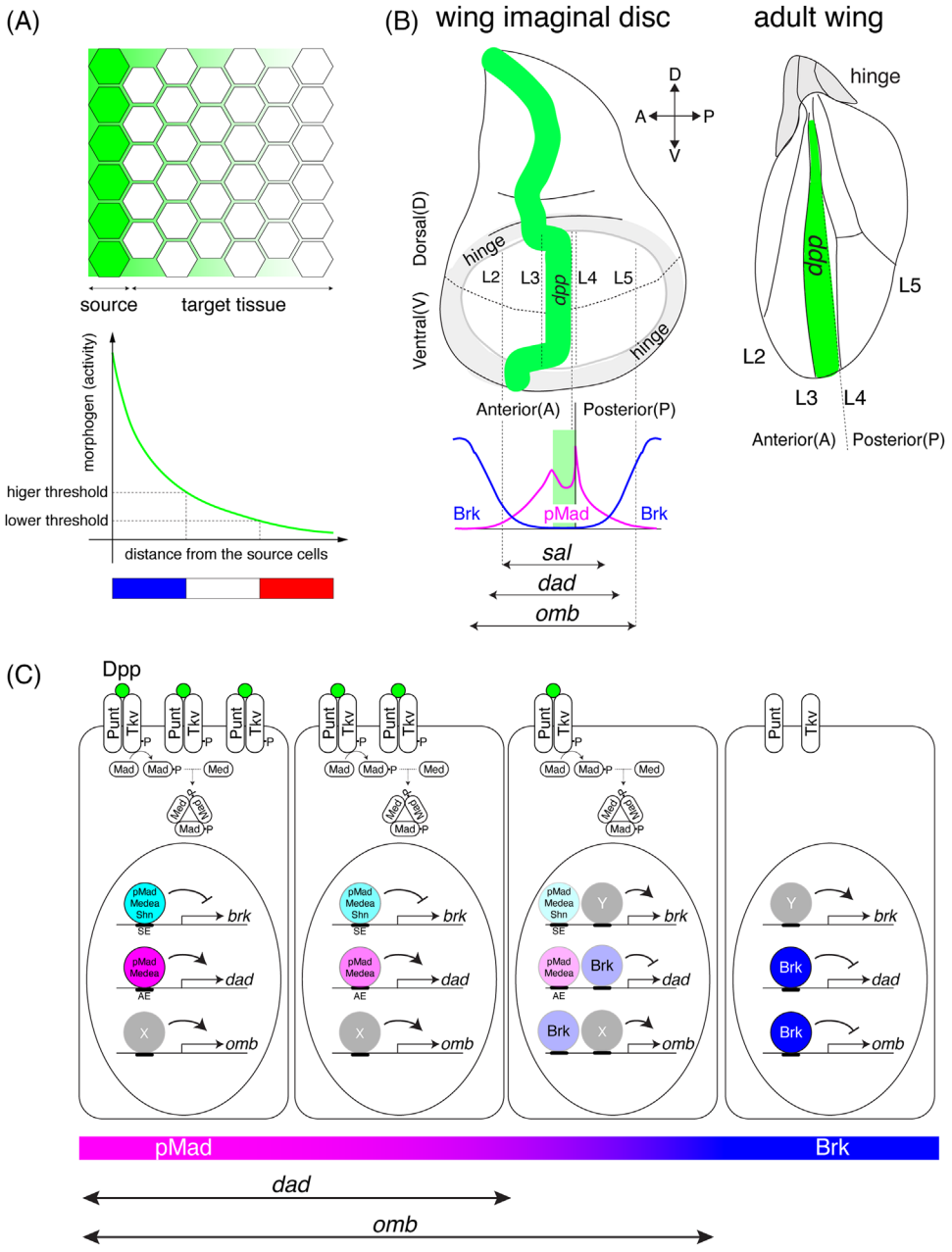

FIGURE 1 Overview of decapentaplegic (Dpp)/bone morphogenetic protein (BMP) morphogen gradient readout in the wing pouch. (A) Schematic view of morphogen gradient formation. In a text book view, morphogens produced from the localized source cells spread to form a concentration gradient in a developing tissue. Different cell types are generated based on distinct threshold of morphogen level (French flag model). (B) Schematic view of Dpp/BMP morphogen gradient formation in the wing imaginal disc. Dpp is produced from an anterior stripe of cells in the wing disc during late second and third instar larval stages. From the source cells, Dpp spreads into both compartments to form a concentration gradient. The Dpp gradient then generates nested target gene expression to specify adult wing vein positions L2 and L5. (C) Schematic view of the Dpp/BMP signaling pathway. Dpp binds to the type I and type II receptors Tkv and Punt, respectively, which leads to the phosphorylation of Mad (pMad). pMad then forms a complex with Medea, which enters the nucleus to regulate target gene expression. In the nucleus, a pMad/Medea/Shn complex blocks transcription of *brk* by binding to distinct silencer elements (SE). Brk acts as a repressor for the majority of Dpp target genes, including *sal*, *dad*, and *omb*. The pMad/Medea complex also directly enhances transcription of *sal* and dad by binding to activating elements (AEs). While Dpp signaling levels decrease away from source cells, Brk levels increase and repress *sal*, *dad*, and *omb* at distinct thresholds. Transcription factors that activate expression of *omb* or *brk* (X and Y) remain to be identified. Thus, the pMad gradient generates an inverse Brk gradient and the combined activity of both gradients generates nested target gene expression. The schematic wing images were taken and modified from ref. [11] under a CC-BY-NC-ND 4.0 International license.

pouch, where pMad levels are high, and de-repressed in the lateral region of the wing pouch, where pMad levels are low; thereby, the Dpp signaling gradient generates an inverse Brk gradient.[20] Brk, in turn, represses the expression of target genes such as *sal*, *dad*, and *omb* at different thresholds by binding to distinct GC-rich sequences of these genes in the lateral region. In *brk* mutant clones, these target genes are de-repressed but the expression levels of de-repressed *dad* and *sal* expression do not reach their maximum expression level, indicating that *dad* and *sal* are also directly (or indirectly) activated by Dpp signaling. Indeed, the pMad/Medea complex binds to activating elements (AEs) in the *dad* enhancer to activate *dad* transcription[21] (Figure 1C). In contrast to SEs, the AEs do not result in Shn recruitment and repression. Thus, the combined activities of the pMad gradient and an inverse gradient of Brk regulate the nested expression of target genes, which then specify adult wing veins L2 and L5 along the anterior border of Sal[22] and along the posterior border of Omb,[23] respectively (Figure 1B). In this way, the graded Dpp signaling levels are translated into precisely positioned patterning elements along the A-P axis.

In addition to the above mentioned and well-characterized role of *dpp* for gene expression patterning, *dpp* is also required for growth of the wing imaginal disc. This role has been inferred from by the complete lack of the wing tissue in *dpp* disc mutants[24] (Figure 3B see below), and from wing duplications or overgrowth arising upon ectopic expression of Dpp in small clones in the wing imaginal discs.[25–27] As is the case for patterning, growth is also controlled to a large extent by suppressing *brk*.[28] Indeed, while completely lost in *dpp* mutants, the wing pouch overgrows in *dpp*, *brk* double mutants, as it does in *brk* mutants.[12–14,28] Brk target genes controlling growth are less well understood when compared to Brk target genes controlling patterning.

Given that Dpp spreads from a stripe of cells in the center of the wing disc to form a concentration gradient in the surrounding tissue, it has been proposed that *dpp* is a bona fide morphogen.[31,32] How Dpp disperses to form a gradient and coordinates overall wing growth and patterning has served as an excellent model to investigate morphogen functions.[5–10] However, it remains controversial how Dpp disperses from its source,[31–37] how the resulting Dpp gradient controls uniform growth within the wing pouch,[28,29,38–47] how the Dpp gradient scales with tissue size,[32,46,48–51] and how wing discs stop growing when they reach the right size.[46,52–57] Most of the models proposed to account for these functions of Dpp are based on the assumption that dispersal from the anterior stripe of cells over the entire wing field is key to control wing patterning and growth.

Here, we first briefly outline the current models on how Dpp controls growth of the Drosophila wing disc and review recently generated genetic and synthetic protein binder tools to investigate the role of the Dpp morphogen for wing growth and patterning. Using these tools, we then argue that Dpp dispersal from the mains source cells is not as important as previously thought and discuss important open questions in the field. Given recent studies revealing unexpected requirements and roles of spreading of morphogens, designing and applying protein binder tools targeting distinct aspects of a morphogen protein would help to further dissect their functions in vivo.

GROWTH MECHANISM

In contrast to the patterning mechanisms described above, growth control by the Dpp gradient remains highly controversial. It has been shown that cell proliferation is uniform despite graded Dpp signaling activity in the wing pouch.[41,58–60] How can the Dpp gradient be translated into a uniform proliferation pattern? Interestingly, uniform activation of Dpp signaling does not lead to uniform proliferation. A variety of models have been proposed to solve this conundrum[28,29,38–43,46,61,11] (Figure 2) and discussed in previous excellent reviews.[9,55,62–64] Here, we will briefly summarize these models (see also Information Box) by focusing on how they explain the non-uniform proliferation pattern arises upon uniform Dpp activation. We will then ask how novel findings are either in line or not with each of them. Organ growth is also under hormonal control,[65] but we will not further discuss this aspect here.

The *gradient model* proposes that the slope of the Dpp gradient drives cell proliferation if the slope is steeper than a certain threshold[52] (Figure 2A, Information Box). The model predicts that uniform activation or blocking Dpp signaling blocks cell proliferation by flattening the gradient.

The *threshold model* proposes that cells proliferate if Dpp signaling is above a certain threshold level. The model predicts that uniform activation of Dpp signaling above the threshold does not affect cell proliferation, and that cell proliferation is blocked when Dpp signaling is below the threshold (Figure 2B, Information Box).

The *growth equalization model* proposes that the Dpp-Brk system is a growth modulator to equalize a default, non-uniform growth potential in the wing pouch, with higher inherent proliferation rates in the lateral region (Figure 2C).[28,43] Dpp signaling inhibits *brk* transcription in the medial region to allow its growth (thus the role of Dpp for medial growth is of utmost importance but appears to be permissive) and limits *brk* expression to the lateral region where it counteracts its higher proliferation rates. The model predicts that uniform activation of Dpp signaling does not affect cell proliferation in the medial region since *brk* expression is already repressed but increases cell proliferation in the lateral region by inhibiting *brk* expression. In contrast, loss of Dpp signaling should block cell proliferation in the medial region by de-repression *brk* but should affect cell proliferation less in the lateral region due to the default, Dpp signaling-independent growth potential of these cells (Figure 2C, Information Box).

The *temporal growth model* proposes that Dpp signaling is instructive for cell proliferation and that cells divide upon a relative increase of Dpp signaling by roughly 50%.[46] The model predicts that uniform activation of Dpp signaling induces higher proliferation in the lateral region since the relative increase of Dpp signaling is higher there, and that cell proliferation is blocked without Dpp signaling due to the lack of temporal increase of signaling (Figure 2D, Information Box).

The *mechanical growth model* proposes that growth is controlled not only by morphogens such as Dpp but also by mechanical forces. Morphogen-mediated growth in the medial regions stretches peripheral tissues to induce cell proliferation, and, as the wing disc grows, the

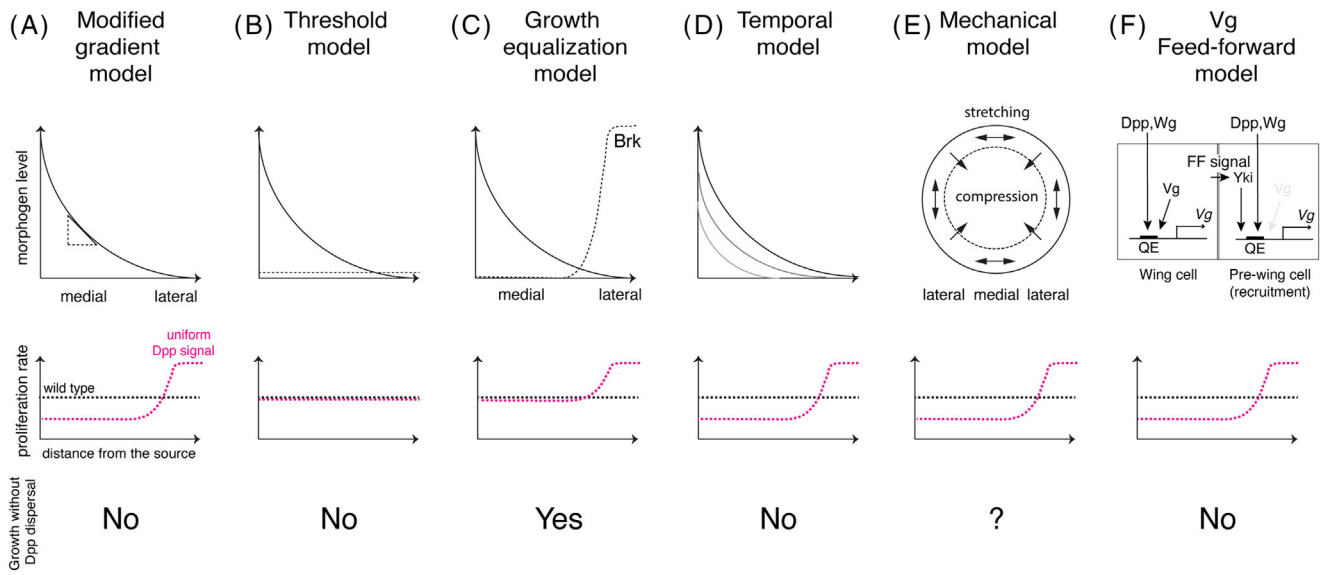

FIGURE 2 Different growth models for the posterior compartment of the wing imaginal disc. Since *dpp* is not expressed in the posterior compartment, the decapentaplegic (Dpp) signaling gradient in the posterior compartment is generated by Dpp derived from the anterior compartment. Thus, Dpp dispersal-dependent growth can be discussed in the posterior compartment. A variety of models have been proposed to explain the uniform proliferation pattern in the wing disc and higher proliferation in the peripheral regions upon uniform Dpp signal activation. Among these models, the *growth equalization model* predicts posterior growth in the absence of Dpp dispersal. The *mechanical model* might also be compatible with posterior growth under conditions in which anterior cells proliferate. The other models would not be in line with posterior growth in the absence of Dpp spreading in their simplest formulation.

peripheral tissues in turn compresses the medial regions to slow down and eventually inhibit cell proliferation[53,54,66,67] (Figure 2E, Information Box). Thus, the model does not explain how Dpp controls growth but can explain uniform growth and growth termination. The model predicts that the overgrowth of the wing pouch by uniform activation of Dpp signaling stretches the lateral regions, which in turn compress the wing pouch. The compression is stronger in the medial region than in the peripheral region, thereby generating non-uniform proliferation pattern. Interestingly, uniform activation of Dpp signaling causes growth of the wing pouch only toward the A-P axis. There must be additional signaling, such as Wg signaling, to control growth toward the D-V axis.[61,56]

The *Vg feed-forward model* proposes that Dpp controls wing growth through the autoregulation and feed-forward regulation of *vestigial* (*vg*), the selector gene that specifies wing fate and controls wing growth[47] (Figure 2F, Information Box). It has been shown that *vg* expression is initially induced in the center of the wing pouch and is gradually expanded into the entire wing pouch area during the third instar larval stages.[68] According to the model, Dpp and Wg spread to induce auto-regulation of *vg* expression to maintain wing fate and induce *vg* expression in the neighboring pre-wing cells via a feed-forward signal to recruit surrounding pre-wing cells to adopt wing fate. The maintenance and recruitment of *vg* expression is dependent on the spreading of the two morphogens and gated by Ecdysone.[56] The model predicts that uniform activation of Dpp signaling has little effects on the medial growth above the minimum thresholds to maintain *vg* expression but induces excess proliferation in the surrounding pre-wing cells to recruit them as wing cells since the proliferation of the surrounding cells is sensitive to the range of Dpp signaling, and that cell proliferation is blocked without Dpp signaling critical for maintenance and recruitment of *vg* expression.

NOVEL TOOLS TO MANIPULATE THE DPP MORPHOGEN GRADIENT

As described above, how the Dpp gradient controls growth remains highly controversial. To investigate in more detail how Dpp acts in this process, new tools have recently been developed.

Genetic tools

Given that *dpp* is an essential gene during embryogenesis, the critical requirement of *dpp* in wing development has initially been investigated using a series of hypomorphic *dpp* alleles (so-called *disc* alleles) that lack *cis*-regulatory sequences required for *dpp* expression in the imaginal discs (Figure 3A,B). Because flies cannot survive with only one copy of a functional *dpp* allele due to haploinsufficiency, it was not possible to generate *dpp* null clones using the available genetic tools. To address the precise spatial-temporal requirement of *dpp*, a flippase recognition target (FRT) cassette was inserted into the *dpp* locus using CRISPR/Cas9.[38,39] Excision of the FRT cassette using tissue-specific expression of Flippase (FLP) can induce tissue-specific conditional knockout of *dpp*.

INFORMATION BOX

1. The gradient model

By developing a system for temporal regulation of gene expression in genetic mosaics, it was found that generating gaps of Dpp signaling either by activating or inactivating Dpp signaling was sufficient to induce cell proliferation. In contrast, flattening the gradient by uniform activation of Dpp signaling in the entire wing pouch blocked cell proliferation in the medial region but increased cell proliferation in the lateral regions.[41] This led the authors to propose a refined gradient model, in which the slope of the Dpp activity gradient controls cell proliferation in the medial region and absolute Dpp signaling levels controls cell proliferation in the lateral region (Figure 2A).[41,42] However, a later study found that Dpp signaling activation in the medial region only did not block cell proliferation and Dpp signaling activation in the lateral region non-autonomously blocked cell proliferation in the medial region, indicating that cell proliferation in the medial region was not blocked by flattening the gradient.[28] Furthermore, the induced cell proliferation by generating gaps of Dpp signaling was transient,[41] raising a question whether transiently induced proliferation can explain the wing growth under physiological conditions.

2. Threshold model

The model predicts that growth is not induced above a certain threshold but uniform activation of Dpp signaling can induce overgrowth of wing disc.[28] However, it has recently been shown that weak uniform activation of Dpp signaling in the wing pouch can rescue growth but not patterning (nested target gene expression) in *dpp* mutants, supporting the threshold model, with distinct threshold for patterning and for growth.[39]

3. Growth equalization model

It has been shown that, similar to patterning, Dpp signaling controls wing growth via the suppression of *brk*.[28] Graded Dpp signaling generates an inverse Brk gradient, with highest Brk level in the lateral region (Figure 1B); wing discs lacking either *brk* or both *dpp* and *brk* overgrow with higher cell proliferation rates in the lateral region. These results suggest that the role of the Dpp-Brk system is to equalize default regional differences in proliferation rates, with higher proliferation rates in the lateral region, by setting higher Brk level there. The model predicts that the lateral wing growth is Dpp signaling-independent. Blocking GFP-Dpp spreading by morphotrap (under *dpp* mutant rescue condition) indeed unraveled such lateral growth in the posterior compartment.[78] However, since Dpp signaling was activated at least one cell row in the posterior compartment, it remained unclear whether the observed lateral growth is completely Dpp signaling-independent, and if so, whether the Dpp signaling-independent growth occurs within or outside the wing pouch.[78]

INFORMATION BOX

4. Temporal growth model

The model was based on the discovery of scaling of both the Dpp gradient (upon overexpression of GFP-Dpp) and Dpp signaling levels with tissue size during development. Both modeling and experimental findings suggest that on average cells divide when Dpp signaling level increase by 50% (references). The model explains why proliferative growth is homogeneous in space despite graded distribution of the Dpp morphogen. However, cells that fail to increase Dpp signaling (*mad*, *brk* double mutant cells) proliferate at normal rates, arguing against this model.[44,45] The model was also questioned since blocking GFP-Dpp spreading by morphotrap (under mutant rescue condition) still resulted in posterior growth. However, under this experimental setup, Dpp signaling was not completely lost in the posterior compartment.[78] Minor wing growth defects upon genetic removal of *dpp* via *dpp*-Gal4 using the $dpp^{FRT\text{-}TA}$ allele[38] argued again the model, but later studies showed that genetic removal of *dpp* when using $dpp^{FRT\text{-}TA}$ allele was not efficient inactivating *dpp*.[39,40]

5. The mechanical growth model

It has been postulated that differences in growth rates in the developing wing tissue induce mechanical stress.[102] Fast proliferating cells stretch the surrounding cells, and eventually get compressed to slow down cell proliferation at a similar rate to that of the surrounding tissue. The mechanical feedback mechanism can thus explain the uniform growth. Two subsequent growth models incorporated a feedback between mechanical forces and morphogen mediated proliferation in the wing disc.[53,54] Hufnagel et al. initially found that GFP-Dpp gradient (upon overexpression) and Dpp signaling remain constant and do not scale with the wing disc size,[54] although later studies showed that both GFP-Dpp distribution and signaling (upon overexpression) scale with the wing disc size.[46,50] According to the model, growth is induced above a certain threshold level of Dpp signaling and once the disc size reaches this threshold level, peripheral cells below the threshold stop cell proliferation and increase mechanical compression to the medial cells to stop morphogen-mediated cell proliferation. In contrast, Aegerter-Wilmsen et al., suggested that the high level of Dpp and Wg in the center of the disc initially promotes growth.[53,67] As the wing disc grows, peripheral cells are stretched to induce cell proliferation, and in turn compress the medial region to block cell proliferation. Tissue growth stops when the compression becomes stronger than morphogen-mediated growth. These models can explain the uniform growth as well as growth termination. However, it is difficult to experimentally challenge the model, since measuring and manipulating mechanical forces in tissues remains challenging.

INFORMATION BOX

6. Vg feed-forward model

The model was originally proposed regarding the growth control of the wing pouch by Wg. In *apterous* (*ap*) null mutant background, in which *wg* and *vg* expression are completely eliminated, Wg spreading and a feed-forward signal were sufficient and required to recapitulate the dynamics of *vg* expression seen during normal wing disc development.[93,94] The same model also applies to the growth control by Dpp, as experimentally shown using the same experimental paradigm in *ap*, *dpp* mutant background, in which *dpp*, *wg*, and *vg* expression are eliminated.[47] Thus, the authors proposed that both morphogens act together, through a common Vg feed-forward mechanism, to recruit additional wing pouch cells and thus control wing growth. However, although sufficient, these mechanisms might not necessarily account for how *vg* expression is controlled under physiological conditions. Indeed, Dpp signaling-independent *vg* expression was reported in various conditions.[11]

Protein binder tools

Genetic removal of *dpp* leads to the loss of the Dpp gradient and to a complete loss of Dpp signaling in the entire wing pouch (Figure 3B). Apart from its genetic requirement shown by using different mutant alleles, it is difficult to further address how the graded distribution of the Dpp ligand controls patterning and growth. To directly manipulate protein functions, protein binders have recently emerged as an important tool to address fundamental questions in developmental biology. Protein binders such as nanobodies, designed ankyrin repeat proteins (DARPins), and single chain variable fragments (scFvs) have binding affinities similar to antibodies toward their targets and can be easily expressed in cells, although scFvs need to be modified to fold properly in the intracellular milieu. Furthermore, protein binders can be functionalized by fusing them with domains of known function from other proteins, in order to manipulate their target proteins in various ways upon binding.[69–77] To study the importance of Dpp spreading in vivo, protein binders can be fused to the transmembrane domain of CD8 in order to trap Dpp on the cell surface and block its dispersal.

Morphotrap

Morphotrap is a membrane-anchored GFP nanobody that can trap GFP-tagged secreted proteins or transmembrane proteins on the cell surface.[78] Morphotrap was first applied to trap GFP-Dpp on the cell surface and thereby directly block GFP-Dpp dispersal, while allowing Dpp signal activation in the source cells. Since an endogenous *GFP-dpp* allele was not available at that time, morphotrap was expressed in the *dpp* source cells in *dpp* disc mutants rescued by a *GFP-dpp* transgene.

HA trap and Dpp trap

Morphotrap could not be used to manipulate spreading of endogenous Dpp since a recently generated endogenous *GFP-dpp* allele was not fully functional.[11] In contrast, an endogenous *HA-dpp* allele was homozygous viable and fertile without obvious phenotypes in the adult wing,[11] similar to previously reported endogenous *HA-dpp* alleles.[39] To manipulate endogenous HA-Dpp spreading, two novel trap systems analogous to morphotrap were generated; "HA trap," based on an anti-HA scFv to trap endogenous HA-Dpp (Figure 3C), and "Dpp trap," based on an Dpp-binding DARPin to directly trap endogenous Dpp (Figure 3D).[11]

ROLE OF DPP ON GROWTH REVEALED BY NOVEL TOOLS

Role of Dpp on growth revealed by genetic tools

Interestingly, inactivation of *dpp* using alleles allowing genetic removal showed different phenotypes. When the $dpp^{FRT\text{-}TA}$ allele was excised in cells of the anterior source stripe using *dpp*-Gal4, patterning was lost, but, quite surprisingly, growth was largely unaffected.[38] When *dpp* was removed from the entire anterior compartment using the same allele, both patterning and growth were severely affected. Based on these observations, the authors of this study proposed that *dpp* derived from the anterior stripe of cells controls patterning but is not relevant to control growth.[38] The model was intriguing, since it has been thought that *dpp* derived from the anterior stripe cells controls both patterning and growth. In sharp contrast, when the $dpp^{FRT\text{-}PSB}$ and $dpp^{FRT\text{-}CA}$ alleles were used to remove *dpp* using the same *dpp*-Gal4 line, both patterning and growth were severely affected.[39] Since the same Gal4 line was used, these authors speculated that genetic removal of an FRT cassette in $dpp^{FRT\text{-}TA}$ is somehow less efficient than the removal of the cassette in $dpp^{FRT\text{-}PSB}$ or $dpp^{FRT\text{-}CA}$. Indeed, Dpp signaling was not completely abolished during the wing growth phase upon removal of an FRT cassette in $dpp^{FRT\text{-}TA}$ using *dpp*-Gal4.[39,40] The critical requirement of *dpp* derived from the anterior stripe of cells was also seen when *dpp* was removed using the $dpp^{FRT\text{-}TA}$ allele with another driver line.[40] The authors found that *dpp*-Gal4 does not precisely recapitulate the expression pattern of *dpp*, especially in the early stages, but that *ptc*-Gal4 recapitulates *dpp* expression more precisely throughout development. When $dpp^{FRT\text{-}TA}$ was excised using *ptc*-Gal4 (instead of *dpp*-Gal4), both patterning and growth were severely affected.[40] Furthermore, knocking down of *dpp* using RNAi also showed the requirement of *dpp* from the anterior stripe of cells for wing growth.[29] In summary, new *dpp* alleles allowing genetic removal of *dpp* in a spatio-temporal manner confirmed that Dpp produced in the anterior stripe of cells is critical for both wing patterning and growth.

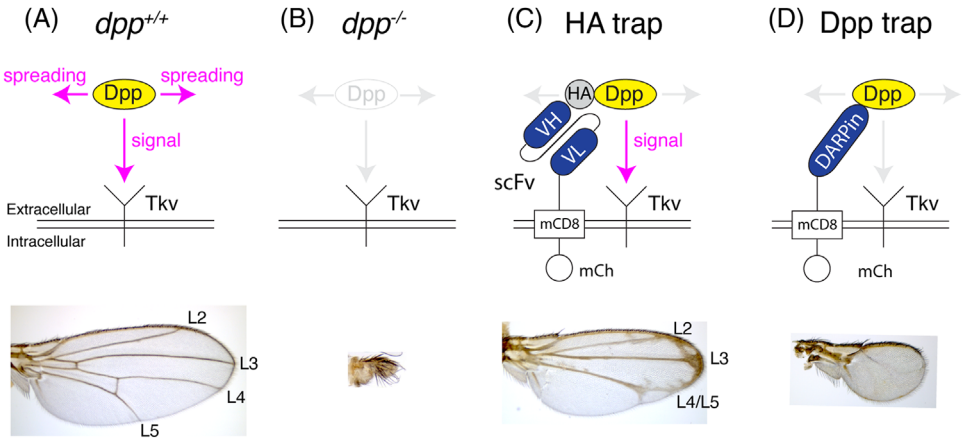

FIGURE 3 Summary of distinct phenotypes observed upon manipulating the decapentaplegic (Dpp) morphogen dispersal and signaling in the wing disc. (A) Upon secretion, Dpp disperses and activates Dpp signaling by binding to the receptors both in source cells and in neighboring cells. (B) In *dpp* mutants, both Dpp signaling in the source cells and in neighboring cells are lost. The resulting adult wing shows severe phenotypes. (C) When HA trap was used to trap endogenous HA-Dpp on the surface of source cells, Dpp signaling in the source cells was still activated (at roughly physiological level) but HA-Dpp dispersal was blocked. The resulting adult wing showed a much milder phenotypes when compared to the phenotypes seen in *dpp* mutants (B). (D) When Dpp trap was used to trap endogenous Dpp, both Dpp dispersal and signaling were efficiently blocked. The resulting adult wing phenotypes were more similar to the severe *dpp* mutant phenotype (B) and the phenotypes resulting from eliminating *dpp* function in the wing pouch area via RNAi.[29] The wing images (A–D) were taken from ref. [11] and the wing image (B) were taken from ref. [30] under a CC-BY-NC-ND 4.0 International license.

Role of Dpp on growth revealed by protein binder tools

While genetic removal of *dpp* via excision of an FRT cassette completely removes Dpp functions, two protein binder tools, HA trap and Dpp trap, were found to affect different aspects of Dpp function.[11] While the HA trap traps endogenous HA-Dpp to mainly block HA-Dpp spreading (Figure 3C), the Dpp trap traps Dpp to block both Dpp spreading and signaling (Figure 3D), probably because HA trap binds to HA-Dpp via the added HA epitope, thereby not strongly interfering with HA-Dpp-Tkv interaction, while Dpp trap directly binds to endogenous Dpp, thereby masking it and blocking interaction with Tkv. Thus, these two tools allow to distinguish the requirement of Dpp spreading and signaling in the control of growth and patterning (Figure 3C,D). Surprisingly, blocking Dpp spreading using HA trap revealed that Dpp dispersal is critical for posterior patterning and growth, but is largely dispensable for anterior patterning and growth.[11] The requirement of Dpp spreading is thus minor and asymmetric (Figure 3C), in sharp contrast to what has been thought or inferred from the severe phenotypes of *dpp* mutants (Figure 3B), and from the removal of *dpp* from cells of the anterior source stripe (see above). The authors found that the minor phenotypes were not due to weak Dpp signaling caused by leaked Dpp since *tkv* mutant clones survive well in the posterior compartment. In contrast, the minor phenotypes were most likely due to persistent Dpp signal in the source cells,[11] since blocking Dpp spreading and signaling in the source by Dpp trap caused severe phenotypes more similar to those seen in *dpp* mutants (Figure 3D), and cell-autonomous Dpp signaling activation in the source cells by a constitutively active Tkv receptor (caTkv) rescued *dpp* mutants to a large extent, generating a phenotype similar to the one seen when using HA trap.[11] These results suggest that Dpp signaling in the source cells can control important aspects of wing patterning and growth without Dpp spreading.[11] Here, we discuss how patterning and growth can be controlled without Dpp spreading in each compartment

Dpp spreading-insensitive anterior patterning and growth

How can relatively robust patterning and growth be achieved in the anterior compartment without Dpp spreading? It should be noted that a previous study also observed an asymmetric rescue of *dpp* mutants via the expression of caTkv.[79] The authors proposed that Dpp signaling in the stripe of cells induces both Sal and Omb, but only Omb expression persists in cells that move out from the stripe of cells upon proliferation.[79] However, an endogenous *dpp* transcription reporter and smFISH against *dpp* revealed that *dpp* expression is initially uniform in the anterior compartment before it is later refined to the narrow stripe of cells.[11] This raises the possibility that the transient *dpp* source in the lateral anterior compartment explains the relatively normal anterior patterning and growth in the absence of Dpp dispersal. Indeed, while weak pMad signal and Omb expression remained active in the anterior compartment when *dpp* was removed from the anterior stripe of cells using the $dpp^{FRT\text{-}TA}$ allele, the weak anterior activation of pMad and Omb was completely lost and the size of the anterior compartment became smaller when *dpp* was removed from all anterior cells (Matsuda et al., 2021). These results indicate that transcriptional refinement and persistent Dpp signaling by transient *dpp* expression (signaling memory) could account for the robust anterior patterning and growth when Dpp spreading is blocked.[11]

Dpp spreading-insensitive posterior growth

Interestingly, although critical for posterior patterning and growth, a significant part of the posterior wing cells appears to proliferate without Dpp dispersal and local Dpp signaling[11] (Figure 3C). Consistent with this, when *tkv* was removed from the entire posterior compartment using a *tkv* allele, in which an FRT cassette was inserted, part of the posterior wing pouch grew without local Dpp signaling.[11] How can these results be reconciled with the complete loss of wing tissue in *dpp* mutants? The observed severe growth defects upon blocking Dpp spreading and signaling by Dpp trap and the partial rescue of posterior growth in *dpp* mutants via anterior caTkv expression indicated that anterior Dpp signaling and/or the resulting anterior growth contribute non-cell autonomously to the posterior growth.[11] Loss of *dpp* by RNAi has been shown to cause growth defects equally in medial and lateral regions[29] but this is probably due to loss of Dpp signaling in the source cells. The observed posterior growth in the absence of local Dpp signaling is most consistent with the *growth equalization model* since this is the only model to clearly predict partial posterior growth without local Dpp signaling (Figure 2C). However, it is important to note that these results do not demonstrate how Dpp signaling-dependent growth is controlled, nor do they strictly exclude other growth models for Dpp signaling-dependent growth.

BIOLOGICAL AND EXPERIMENTAL RELEVANCE FOR OTHER MORPHOGEN SYSTEMS

These new observations using protein binder tools distinguish the requirement of Dpp spreading and local Dpp signaling, and unexpectedly, challenge the long-standing dogma that Dpp morphogen spreading is essential for the patterning and growth of the entire wing pouch area.[11] Interestingly, minor contributions of morphogen spreading have also been observed for other morphogens. Although Wingless (Wg), the main Wnt in *Drosophila*, is thought to be a morphogen required for wing growth, a membrane-tethered, non-diffusible form of Wg was able to replace endogenous Wg without strongly affecting appendage development.[80] Sonic hedgehog (Shh) is thought to be a morphogen controlling limb patterning and growth, but by bypassing its cell survival role during limb outgrowth, it was shown that Shh does not act as a long-range morphogen but rather as a short-range trigger to specify all the digits.[81]

Manipulating morphogen spreading also revealed previously unappreciated roles of morphogen dispersal. By applying HA trap to study Gbb (Glass bottom boat), another BMP type ligand, it has recently been shown that the active BMP-type ligands in the wing imaginal disc are heterodimers of Dpp and Gbb[82] as previously shown in the case of the formation of the posterior crossvein in the pupal wing.[83] While niche-derived Dpp was thought to act in a short-range manner to maintain male germline stem cells (GSCs) in Drosophila, blocking endogenous Dpp spreading revealed an unexpected role of a diffusive fraction of Dpp in differentiating cells further away from the source.[84] Replacing endogenous Wnt3a with a receptor-fused form that activates signaling only in producing cells in mice increased heterogeneity in Wnt signaling and sensitivity to retinoic acid, an endogenous antagonist of neuromesodermal progenitor (NMP) maintenance, indicating that intercellular exchange of Wnt ligands reduces cell population heterogeneity and achieves robustness to environmental stress in NMP cells.[85]

It would be therefore interesting to reinvestigate how morphogens act using protein binder tools. It has been difficult to address how morphogen gradients control tissue patterning and growth due to the lack of tools to manipulate different parameters, such as secretion, dispersal, and degradation of morphogens. Protein binder tools provide new approaches to directly manipulate morphogen gradients in a predictable manner. Using such tools, the importance of distinct morphogen parameters can be assessed, for example, by trapping morphogens as shown in the case of Dpp.[11] Trapping morphogens exclusively in the target tissue can also allow to artificially manipulate the gradient in shape and time, which is not easily possible by analyzing genetic mutants. More recently, the implementation of methods to manipulate protein secretion in time and space has further increased the tool box to study morphogens in vivo.[86,87] Protein binder tools can also be used to re-construct a morphogen system in order to address the minimum requirement of morphogen gradient formation.[88,89]

However, it is important to note that protein binders may cause leakage or artifacts that need to be taken into consideration. For example, the mild phenotypes observed upon expression of HA trap could be due to leakage of Dpp from the HA trap. However, *tkv* mutant clones survived well in the posterior compartment of the wing disc expressing HA trap, showing that posterior cells do survive well even if they are unable to respond to Dpp due to the lack of the receptor. Thus, Dpp signaling initiated via leaked Dpp is not a valuable explanation for the mild phenotypes.[11] The phenotype caused by HA trap was also mimicked by the rescue of *dpp* mutants via caTkv expression, again indicating that the mild phenotypes are unlikely due to leakage of Dpp. Similarly, the much more severe phenotypes cause by Dpp trap could be due to higher binding affinity for Dpp than HA trap; however, it was found that Dpp trap binds Dpp less efficiently than HA trap binds HA-Dpp.[11] Since trapping HA-Dpp by HA trap induced a sharp decrease in Dpp signaling along the anterior-posterior compartment boundary, if would formally also be possible that this may cause an artifact, ultimately resulting in the induction of posterior wing growth. Indeed, it has been shown that a large difference in Dpp signaling levels between neighboring cells can induce cell proliferation but only transiently.[41] Thus, we suspect that a steep decline of Dpp signaling levels cannot explain the sustained tissue growth. As discussed in the next section, we rather think that a gap of Dpp signaling levels could induce cell elimination.

It is important to apply protein binders to manipulate morphogens produced from the endogenous locus, since trapping overexpressed morphogens can lead to unphysiologically high signaling levels.[78] However, while a variety of long and short tags and the corresponding protein binders are available, genome engineering is time consuming, unless endogenously tagged alleles are already available. Isolating and characterizing protein binders against a given target protein is also a longer endeavor. One way to bypass this problem and to get a first

indication about the requirement of morphogen spreading is to test the extent to which cell autonomous activation of morphogen signaling can rescue mutant phenotypes.[11]

The distinct actions of HA trap and Dpp trap on Dpp signaling[11] raise the interesting possibility that it may be possible in the future to target one of the functions or properties of a protein of interest by designing a protein binder affecting exclusively a specific property. Indeed, a recent proof of principle study successfully designed such sequence-specific peptide-binding proteins.[90]

FUTURE DIRECTION FOR RESEARCH ON DPP

The new observations discussed here not only challenge the long-standing dogma that Dpp morphogen spreading is essential for patterning and growth of the entire wing tissue, but also raise a variety of questions about how Dpp spreads to control and coordinate wing patterning and growth.

First, it remains to be addressed how Dpp spreading-dependent growth is controlled. It would be interesting to artificially generate different Dpp gradient shapes by trapping Dpp with different affinity either in the source cells or in the posterior target tissue and ask how proliferation/growth rates respond to such changes. This was already done to some extent in mutant rescue condition,[78] but it would be important to repeat these experiments using endogenously tagged *dpp*. In addition, it would be interesting to manipulate the gradient at different time points during the development of the wing imaginal disc to ask how the duration of morphogen signaling controls wing patterning and growth; a recently established light-inducible Gal4/UAS system might be very useful to undertake such experiments.[91] Similar experiments have already been done using *dpp* RNAi,[29] but these experiments do not allow to distinguish between a requirement for Dpp signaling or for Dpp spreading.

Second, it remains unknown how Dpp spreading-independent patterning and growth is brought about. The robust anterior patterning and growth upon blocking Dpp spreading is proposed to be mediated by a "signaling memory" of a transient anterior *dpp* source outside the main *dpp* source. It remains to be tested how such a memory would work. Interestingly, a wing pouch marker (5xQE.DSRed), which contains five copies of a *vg* regulatory element (called Quadrate Enhancer [QE]), is thought to be activated by Dpp signaling[68,92,93] but was expressed in the Dpp spreading-independent posterior region upon HA trap expression.[11] Dpp signaling may be important for the initiation but not for the maintenance of the QE activity. Alternatively, given that Wg is also required for Vg expression, Dpp signal-independent QE expression could be mediated through Wg.[68,92–94] It is also possible that the *mechanical growth model* works in concert with the *growth equalization model* (Figure 2C,E), and Brk may repress the stretch-induced lateral wing growth. In this scenario, growth of the anterior compartment may stretch the posterior compartment, thereby inducing growth in the absence of local Dpp signaling.

Third, the substantial posterior growth in the absence of local Dpp signaling (upon blocking Dpp spreading) suggests that Dpp signaling is not cell-autonomously required for cell survival of wing pouch cells[25,95] or for tissue architecture[96] but that Dpp signaling-deficient cells are eliminated by surrounding Dpp-responsive cells.[97] This phenomenon is reminiscent of cell competition, in which less-fit but otherwise viable "loser" cells are eliminated when surrounded by "winner" cells.[98–101] It remains to be investigated how cells compare differences in Dpp signaling to eliminate Dpp signaling-deficient cells.

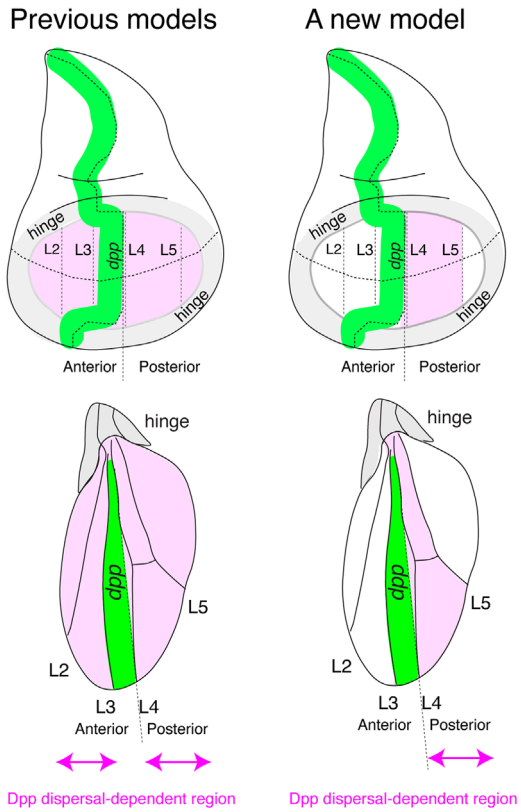

FIGURE 4 Modified model describing decapentaplegic (Dpp) dispersal-dependent wing patterning and growth. (A) Based on the severe *dpp* mutant phenotypes, previous models assumed that Dpp dispersal from the anterior source cells is essential to control patterning and growth of the entire wing pouch. (B) Using protein binder tools to manipulate endogenous Dpp spreading, a modified model proposes that requirement of Dpp dispersal from the anterior stripe of cells is required only for medial patterning and growth in the posterior compartment. The schematic wing images were taken and modified from ref. [11] under a CC-BY-NC-ND 4.0 International license.

SUMMARY

Dpp is the first validated secreted morphogen identified in Drosophila. It is thus surprising that we still do not reach much consensus about how Dpp controls and coordinates wing patterning, and in particular, wing growth. As we summarized in this review, understanding how Dpp acts as a morphogen has progressed with the generation and application of new tools and methods, including precise genome engineering and the use of protein binders. The results obtained by using these new

tools surprisingly challenge the long-standing view that Dpp spreading is strictly required for the patterning and growth of the entire wing pouch and proposed that Dpp spreading is essential for medial region of the posterior compartment (Fig. 4). While novel approaches will undoubtfully provide more insight, it is very likely that robust growth control of the size of the drosophila wing is the result of a combination of different modes of control and their cross interactions. In a wild type situation, Dpp clearly does disperse in a graded fashion in the developing wing imaginal disc, and could still finetune proliferation and growth to regulate wing size via mechanisms proposed by the different models outlined in the review. We believe it is now possible to experimentally address a variety of questions arising from these observations and resolve some of the controversies on how Dpp acts as a morphogen in the near future (Figure 4).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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