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Regulation of BMP activity and range in *Drosophila* wing development

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Abstract

Bone morphogenetic protein (BMP) signaling controls development and maintenance of many tissues. Genetic and quantitative approaches in *Drosophila* reveal that ligand isoforms show distinct function in wing development. Spatiotemporal control of BMP patterning depends on a network of extracellular proteins Pent, Ltl and Dally that regulate BMP signaling strength and morphogen range. BMP-mediated feedback regulation of Pent, Ltl, and Dally expression provides a system where cells actively respond to, and modify, the extracellular morphogen landscape to form a gradient that exhibits remarkable properties, including proportional scaling of BMP patterning with tissue size and the modulation of uniform tissue growth. This system provides valuable insights into mechanisms that mitigate the influence of variability to regulate cell-cell interactions and maintain organ function.

Introduction

Components of Bone Morphogenetic Protein (BMP) signaling pathways are encoded in the genomes of axially organized animals [1]. Within a single species, BMP ligands have a remarkable diversity of physiological roles [examples in 2,3–6]. This diversity of function is reflected in the number of molecular mechanisms that regulate the pathway both inside and outside the cell. Emerging data from the fruitfly *Drosophila melanogaster* indicate that different mechanisms for extracellular BMP transport are used to accommodate the different constraints in each tissue.

A defining feature of BMP signaling in each *Drosophila* tissue is the range of ligand action. Specific cells produce ligands, which may travel over many cell diameters (long-range signaling), or travel locally to nearby cells (short-range signaling). Fly blastoderm embryos and larval wing primordia are current paradigms for long-range signaling by BMPs [7,8]. Short range signaling occurs in other tissues, such as the embryonic midgut [9], and the germline stem cell (GSC) niche [4]. Extremely short-range BMP signaling occurs at

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neuromuscular junctions [2]. The range of action provides a valuable framework for understanding tissue-specific regulation of BMP activity. This review focuses on the roles of cell surface proteins in modulating the flux of BMP ligands across the developing primordium for the *Drosophila* wing, and how feedback regulation shapes this extracellular landscape.

Signaling by two BMP ligands converges on the gradient of P_{Mad} responses

BMPs are morphogens in wing development because they induce multiple cell types at distinct positions that presage the formation of adult wing veins [7,10] (Fig. 1A). Growth and early lineage restriction occur in larval primordia, [11] (Fig. 1B), when BMPs also regulate cell shape [12]. Two BMPs, Dpp and Gbb, regulate wing development [13] through association with the type I receptors Tkv and Sax, and type II receptor, Punt [7,14]. Studies focus on the core signal transduction pathway via the BMP R-Smad, Mad, and the co-Smad Medea (Fig. 2), although the co-Smad is dispensable for at least one transcriptional response, induction of the micro-RNA gene *bantam* [15].

The gradient, or distribution, of BMP activity is visualized by immunofluorescent detection of receptor-phosphorylated Mad (P_{Mad}, Fig. 1C shows example from the end of larval development). Ligand binding recruits a type I-type II receptor signaling complex that induces P_{Mad} accumulation in the nucleus. Levels of P_{Mad} are graded across the primordium in a reproducible pattern (Fig. 1C), with highest levels on either side of a medial strip of cells that we will call the A/P organizer [16]. The region of detectable P_{Mad} defines the range of BMP activity, and expands or contracts in accordance with primordium size [17,18], a phenomenon called scale-invariance.

The BMP activity gradient is the output of combined Gbb and Dpp activity. Gbb is essential to establish the most lateral veins (Fig. 1) [13]. Overall, Gbb is most important laterally, and endogenous Dpp is most important medially, although genetically increased Dpp levels can compensate for reduced Gbb. Mosaic studies suggest that Gbb is highly mobile [13]; in addition, a portion of long-range BMP activity could involve Gbb-Dpp heterodimers produced in medial cells. However, Dpp and Gbb differ in their sites of expression (Fig. 1C) and binding to cell surface proteins.

dpp gene expression is induced in a band of medial cells by Hh signaling [19]; the cells with high Hh responses form the A/P organizer [16]. Dpp pro-protein undergoes multi-step processing to generate two secreted isoforms [9,20]. Only the longer isoform is active in wing development [9,20]; both isoforms are active in short-range signaling [9]. A clue to their differential activity comes from heparan sulfate proteoglycan (HSPG)-mediated stabilization of extracellular Dpp, which involves a heparan sulfate binding site near the Dpp short isoform N-terminus [21]. Dpp strongly binds Tkv, but not Sax [22].

gbb is expressed throughout the wing primordium [23]. Gbb binds Sax more strongly than Tkv, but productive signaling only occurs through Sax-Tkv heteromeric complexes [14,22], perhaps because Sax-Punt interactions are ineffectual for P_{Mad} activation [22]. A potential mechanism for the essential Tkv function comes from GSCs, where Lis1 interacts with Tkv-Punt-Mad complexes to increase P_{Mad} accumulation [24]. It will be valuable to determine how this function relates to previously identified functions for Lis1 in membrane cytoskeletal organization and Dynein microtubule motor complexes [25].

Terminal differentiation of the wing veins requires local signaling from both BMPs during pupal development, with *dpp* expressed in developing veins [26]. Formation of the posterior

cross-vein (Fig. 1A) requires transport of latent BMPs from longitudinal vein cells in a Sog/Cv complex [8]. At the target site, latent complexes are trapped by Cv2; Tlr releases active ligand. A paralogous system is used in blastoderm embryos, where a latent BMP-Sog-Tsg complex diffuses freely in the surrounding fluid. Active ligand is released in dorsal regions by Tld [27], and trapped by Collagen IV [28].

Additional signals regulate growth of the wing primordium (this issue and [29,30]). Later in pupal development, vein-intervein pattern refinement can correct morphology [26,31]. Quantitative phenotype variations have been associated with genes in these pathways [32], but likely arise from multiple rounds of signaling during initial larval patterning and later pattern refinement in pupae.

Receptors and Glypicans balance local signaling with long-range BMP activity

After secretion, BMP ligands move through the extracellular matrix, where they may be captured by cell-surface receptors for signaling or continue moving to a neighboring cell. The mechanism for BMP movement across the wing primordium balances the tradeoff between signal strength in medial cells and the amount of signal received at lateral cells. While numerous factors modify BMP signaling range (Table 1), mechanisms are best understood for Tkv and Glypican HSPGs.

The amplitude of BMP activity is highest in medial cells on either side of the A/P organizer, and diminishes progressively over cells farther from the organizer, (amplitude is commonly measured as the P_{Mad} level detected in immunofluorescence, Fig. 1C). Diminishing amplitude is referred to as signal decay in quantitative analyses, which focus on the posterior activity peak [33]. The length-scale (or decay length) is a measure of BMP range defined by the distance required for the concentration to drop to $1/\exp$ (approximately 0.367) times its peak value. The shape, amplitude, and dynamics of BMP activity result from a fine-tuned balance between factors that modulate transport and destruction of ligand and thus the resultant length-scale for signaling range.

Type I receptors are critical for removal of ligand from the mobile extracellular pool. Tkv strongly affects all BMP activity [19], whereas Sax affects Gbb [14]. The type II receptor Punt does not influence ligand removal [34]; the BMP type II receptor Wit appears dispensable in wing development [35]. Ligand removal may be a direct result of signaling, since BMP responses in each cell require endocytosis [36,37]. Taken together, the gradient of BMP activity is maintained by continuous removal of ligands as a consequence of ligand-type I receptor endocytosis.

The critical role for Tkv in the pattern of BMP activity derives from the pattern of *tkv* expression, which is regulated by Hh and BMP signaling [19]. Low Tkv levels in the A/P organizer create the two peaks of BMP activity by increased ligand movement to adjacent cells. Depletion of extracellular Dpp by elevated Tkv is detected both for endogenous Dpp and for transgenic tagged-Dpp [38]. Tkv levels are high in all posterior cells, so that the posterior peak of BMP activity has high amplitude, but the length scale is short. In contrast, the anterior peak has lower amplitude and diminishes more gradually. Regulation of *tkv* expression is a key determinant for morphology in other fly tissues as well [39–41].

The Glypicans Dally and Dlp are required for BMP responses in this tissue, and are sometimes referred to as co-receptors [42]. Glypicans may promote signaling by increasing BMP concentration at the cell surface, or they may have a more direct role. Mammalian HSPGs enhance BMP type II receptor recruitment to BMP4-BMP type I receptor complexes

[43], although free heparan inhibits BMP signaling in *Drosophila* S2 cells [44]. Glypicans can activate signaling in neighboring cells that lack Glypicans [44–46]; this trans effect is evident in mosaic wing primordia [36].

Trans-signaling by the two *Drosophila* Glypicans, Dally and Dlp, on neighboring cells supports a model where BMPs pass from cell to cell via association-dissociation interactions with Glypican HSPGs. Dally, which can bind either Dpp or Gbb [44], is required to move BMP activity from medial to lateral cells, thus decreasing BMP activity medially while increasing it laterally [21]. Dlp, which binds Gbb and not Dpp [44], function in BMP ligand mobility is unclear; widespread overexpression of Dlp decreases the domain of P_{Mad} accumulation [21]. One interpretation is that Dlp influences the BMP activity gradient indirectly through Hh [42], with minimal direct contribution to BMP distribution. Altogether, the data indicate that Dally mediates BMP flux from medial to lateral regions.

Glypican gene expression is sculpted by multiple developmental signaling systems [42]. Combined BMP and Hh signaling depress *dally* expression medially near the A/P organizer [47], whereas Wg signaling at the wing margin strongly decreases expression of *dally* and *dlp* and also decreases their function by inducing post-translational modification via Notum [42] and Sulf1 [48]. The net effects are a Wg-dependent BMP flux away from wing margin cells, and BMP/Hh-dependent flux of BMP activity away from the A/P organizer. Additive effects from genetic manipulation of Dally and Tkv levels [21] suggest that the stoichiometric ratio of Tkv to Dally determines whether BMP is trapped locally to increase signal amplitude, or is handed-off to adjacent cells to increase length-scale.

Feedback regulation on the extracellular distribution of BMP activity

Quantitative studies of the BMP activity gradient in wing primordia have significantly revised our understanding of morphogen gradients. Cells do not passively receive information from extracellular BMP activity. Feedback mechanisms modify their production of extracellular BMP binding proteins, consequently altering the tissue-wide distribution of BMP activity. We mentioned BMP down-regulation of *dally* and *tkv* expression; this cell-autonomous negative feedback locally enhances ligand flux to neighboring cells. Additional modes of feedback regulation are emerging: modulating the levels of BMP ligands, their flux across the primordium, and the resultant amplitude of signaling within individual cells.

Intracellular feedback decreases *dpp* expression [38]. Furthermore, the inhibitory Smad Dad is induced by BMP activity [49], and feeds back on Mad interactions with Sax and Tkv to prevent accumulation of P_{Mad} [50]. This feedback loop prevents elevated Tkv levels from increasing steady-state intracellular responses [38]. Feedback mediated by Dad ensures reliable signal amplitude within each individual cell. However, the minimal effects on adult wing phenotype from manipulating Dad and Tkv levels [38] raises the question of how intracellular feedback compensates the effects of increased Tkv on the overall length-scale of extracellular BMP distribution. One possibility is that feedback repression of *pent* balances increased Tkv that would otherwise limit signaling range [51,52].

Pent is a secreted BMP regulator that is repressed by high BMP activity, creating a Pent gradient from lateral sites of high expression [52]. Loss of *pent* is associated with reduced wing size and shorter gradient length-scale, with increased amplitude at the A/P organizer. Remarkably, lateral *pent* expression is required for BMP flux outward from the A/P organizer. Thus, Pent acts non-autonomously to regulate ligand mobility, presumably by Pent movement from its lateral source to medial regions.

While *pent*, *dally*, and *tkv* are downregulated in response to increased BMP signaling, expression of another feedback regulator, the secreted leucine-rich repeat protein Larval

Translucida (Ltl), is up-regulated by BMP activity [53]. Ltl physically interacts with Dally and is necessary to restrict BMP activity to the pupal cross-vein. The effect of Ltl on the range of pupal BMP activity is reminiscent of Cv-2 function [54], but the mechanisms for Ltl action are unknown. To summarize, BMP signaling down-regulates genes that elicit opposite effects on BMP activity distribution, and some of the targets themselves have biphasic function. Mathematical modeling provides a tool to probe the concerted behavior of these feedback loops, and thus guide future experiments.

Modeling the regulation of tissue-wide regulation of BMP activity distribution

Computational analyses are increasingly used to understand how multilayered feedback loops work together to reliably control developmental patterning, and in combination with experimental manipulations is unraveling how feedback regulation improves the performance of BMP patterning in uncertain environments. In blastoderm embryos, intracellular feedback increases amplitude and decreases length-scale for BMP distribution, and modeling has explored how this feedback can stabilize boundary formation through reinforcement of low versus high signaling states [55].

Recent modeling of the feedback between Dpp, Tkv, Dally, and Pent (Fig. 2), demonstrated how this network could scale Dpp activity distribution with primordium size [51]. The critical elements for scale-invariance were 1) repression of Pent by Dpp signaling and 2) free diffusion of Pent, which either increased Dpp diffusion or decreased receptor binding and resultant Dpp degradation. This “expansion-repression” system of Dpp repression of Pent expression and Pent-mediated expansion of Dpp length-scale was supported by experimental tests, in which forced expression or loss of Pent abolished scale invariance by eliminating feedback. Additional mechanisms of scale-invariance have been proposed, including decreases in the Dpp removal rate by varying levels of receptors [18,56] in proportion to tissue size. Each feedback gene shows different precision in scaling with primordium growth [17]. Undoubtedly, multiple mechanisms work together to achieve related performance objectives such as scale-invariance between individuals in a population and dynamic scale-invariance during tissue growth within an individual.

Feedback regulation of the length-scale for BMP activity distribution, similar to the above “expansion-repression system”, is an attractive mechanism to link growth and patterning in systems like wing development. In the wing primordium, computational evaluations have focused only on variations in length-scale for the posterior peak of BMP activity. Numerous studies have reported that manipulating the amplitude of BMP activity is associated with a proportional change in the local rate of cell division; recently, this relationship has been quantified [18]. The pattern of *dpp* expression is important for the activity gradient to scale with size, as forced widespread expression of Dpp is associated with an activity gradient that is not scale-invariant [18]. While there is a correlation between the overall shape of the BMP activity gradient and uniform proliferation rates, other mechanisms have been proposed to contribute to uniform proliferation and the resultant final wing size, including parallel activity of other signals, slope sensing, and mechanical feedback [57–60].

Conclusions

We are just beginning to scratch the surface of how the properties of each BMP isoform and cell-autonomous feedback mechanism impact the tissue-wide distribution of BMP activity. The daunting complexity of potential regulatory interactions [e.g. 61] is simplified by focusing on a single physiological event such as wing development. Even then, complexity

impedes intuitive design and interpretation of experimental manipulations. Computational modeling is becoming essential to gain insight into network function.

The results summarized here also emphasize the continuing importance of studying signaling networks in the context of an entire tissue. New insights will come with studies of signal dynamics over time [62]. We have only mentioned a few examples of interconnectivity with other signaling networks, such as Hh, Hippo, and Wnt pathways. Additional mechanisms for pathway cross-talk have been identified [e.g. 63,64], but have not been investigated for effects on the distribution of BMP activity. Cross-regulation is important for uniform growth [15,30,60], and will undoubtedly contribute to the stability of developmental outcome [65,66]. We anticipate that *Drosophila* physiology will continue as an experimental paradigm where integration of mathematical modeling, image analysis, and experimentation yield critical insights into the rich mechanisms at work in BMP signaling.

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Figure 1. Drosophila wing development and the distribution of BMP activity

A. Adult wing Example of an adult wing blade, showing the longitudinal veins L2, L3, L4, L5, and the posterior crossvein (PCV). L2 and L5 are the most lateral veins; L3 and L4 flank cells from the A/P organizer. Veins are separated by flat intervein cells, and stiffen the wing blade. **B. Mature larval wing primordium** Top image shows a fully developed wing imaginal disk, with the wing primordium circled in yellow. Bottom image shows immunofluorescent staining of a similar wing primordium with anti-Delta (C594.9B, DSHB) to illustrate some of the cell types present at the end of larval development (corresponding to late-third instar larvae by the criteria of Maroni and Stamey [67]): cells competent to become veins L3, L4, and L5 (orange arrows), cells competent to become wing margin cell types (blue bracket), the A/P organizer region (gold bracket), and the wing primordium (gold oval). Cells on either side of the margin form the two surfaces of the adult wing. **C. Expression patterns in mature larval wing primordium** Center image shows immunofluorescent staining of a larval wing primordium (same developmental stage as 1B) with anti-phosphoSmad1/5 (Ser463/465, Catalog #9516 Cell Signaling, [68]), at sufficient magnification to distinguish nuclear localization. A/P organizer (gold bracket) and wing margin (blue bracket) regions are indicated. Gold rectangle indicates approximate location for PMad levels in profile. An example of a PMad gradient profile from image analysis is shown on top. To aid readers of this review, a composite of approximate gene expression patterns is shown at bottom. Each gene expression pattern is estimated from an independent published figure; for a quantitative evaluation, consult original data for each gene as indicated: *dally* (green [69]), *tkv* (blue [38]), *pent* (purple [52]), *dad* (orange [17]), *gbb* (gray) and *dpp* (brown), both ligand gene patterns from [Maryanna M. Aldrich, Ph.D. thesis, Brown University, 2011]. All images are anterior left, posterior right; primordia are dorsal up.

Figure 2. Schematic of feedback regulation in the developing wing primordium

BMP signaling regulates genes that modify signal activity both intracellularly (autonomously) and extracellularly (non-autonomously). The relative activity of each component of the signaling network is shown for a medial cell (left) and a lateral cell (right). Within each cell (blue with red nucleus), intracellular signaling and gene expression responses are shown by one network of arrows. Above each cell, extracellular regulation of ligand mobility (range) and activity are shown with a separate network of arrows. The strength of each regulatory effect is indicated by line thickness and shade. Relative levels for each protein are depicted by the size of its symbol in each region. For instance, signaling levels are high in medial cells and low in lateral cells, and this is depicted by the relative sizes of the ligand-receptor signaling complexes and phospho-Smad oligomers. **Medial cells** experience moderate to high BMP signal activity giving down-regulation of target genes *dally*, *pent*, and *tkv* and inducing expression of *dad* and *ltl*. As a result, the local extracellular network promotes BMP ligand movement to lateral cells at the expense of local BMP signaling. **Lateral cells** experience low BMP signal activity, so that *dally*, *pent* and *tkv* are strongly expressed and *dad* and *ltl* are not expressed. As a result, the local extracellular network reduces BMP ligand mobility and promotes local BMP signaling.

Table 1

Table of BMP Pathway Proteins/Genes Discussed

Protein	Gene Name	Molecular Function	FlyBase ID	Gene expression altered by BMP?	Autonomous effect on PMad levels	Tissue-wide effect on signal range
-	<i>bantam</i>	microRNA	FBgn0262451	Up	•	•
Cv	<i>crossveinless</i>	extracellular BMP binding protein	FBgn0000394	•	Down	CV Decrease
Cv2	<i>crossveinless 2</i>	secreted protein	FBgn0000395	CV Up	CV Up	CV Decrease
Dad	<i>daughters against dpp</i>	inhibitory Smad	FBgn0020493	Up	Down	Normalize
Dally	<i>division abnormally delayed</i>	glypican	FBgn0011577	Down	Up	Increase
Dlp	<i>dally-like</i>	glypican	FBgn0041604	•	Up	Decrease †
Dpp	<i>decapentaplegic</i>	BMP2/4 type ligand	FBgn0000490	Down	Up	Increase
Gbb	<i>glass bottom boat</i>	BMP 7 type ligand	FBgn0024234	•	Up	Increase
Hh	<i>hedgehog</i>	extracellular signaling protein	FBgn0004644	No	††	Increase
		protein associated with membrane		•	GSC Up	•
Lis1	<i>lissencephaly-1</i>	cytoskeleton and Dynein complex	FBgn0015754			
Ltl	<i>larval translocida</i>	Leucine-rich repeat, secreted protein	FBgn0052372	Up	Down †	CV Decrease
Mad	<i>Mothers against dpp</i>	BMP R-Smad	FBgn0011648	•	Up	•
Medea	<i>Medea</i>	co-Smad	FBgn0011655	•	Up	•
Notum	<i>notum</i>	α/β hydrolase	FBgn0044028	•	•	•
Pent	<i>pentagone or magu</i>	secreted protein	FBgn0262169	Down	ND	Increase
Put	<i>punt</i>	Activin type II receptor	FBgn0003169	•	Up	ND
Sax	<i>saxophone</i>	type I receptor (Alk1-like)	FBgn0003317	•	Up	CV Decrease
Sog	<i>short gastrulation</i>	extracellular BMP binding protein	FBgn0003463	•	EDM Down	EDM Decrease
Sulf1	<i>sulfated</i>	heparin sulfate endosulfatase	FBgn0040271	•	•	•
Tkv	<i>thickveins</i>	BMP type I receptor	FBgn0003716	Down	Up	Decrease
Tld	<i>tolloid</i>	metalloprotease	FBgn0003719	•	S2 ND, EDM Dual	EDM Dual
Tlr	<i>tolkin or tolloid-related</i>	metalloprotease	FBgn0014998	•	CV Up	CV Dual
Tsg	<i>twisted gastrulation</i>	extracellular BMP binding protein	FBgn0003865	ND	EDM Dual	EDM Decrease
Ttv	<i>Tout velout or DExtI</i>	heparin sulfate co-polymerase	FBgn0020245	•	Up	•
Wit	<i>wishful thinking</i>	BMP type II receptor	FBgn0024179	•	N Up	•

* Functions are reported for the wing primordium, unless indicated as follows: EDM = dorsal midline region of blastoderm embryos [8]; CV = pupal cross-vein cells [8]; GSC = germline stem cells [4]; N = neurons [2]; S2 = S2 cells. For detailed information on these tissues, consult the indicated reference. Other citations are in text.

** This column notes how the gene product regulates PMad levels within the same cell, usually tested by mosaic analysis. *Italics indicate a test in whole animal mutants.*
 *** This column notes whether there is a global change in BMP signal range when gene function is reduced, and does not imply a direct, extracellular regulation of signal range.
 † indicates that the gene's regulation of PMad levels in the wing primordium has only been examined using tissue-wide over-expression.
 ND = No effect Detected
 • not currently reported
 †† Although Hh signaling induces *dpp* gene expression, it also decreases PMad responses by down-regulating *kv* and *daily* expression.