The Extracellular Regulation of Bone Morphogenetic Proteins in *Drosophila* and Sawfly Wing

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Academic dissertation

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Contents

List of publications

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II. Matsuda S, Blanco J, and Shimmi O
A feed-forward loop coupling extracellular BMP transport and morphogenesis in *Drosophila* wing.
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III. Matsuda S, Yoshiyama N, Künnapuu-Vulli J, Hatakeyama M and Shimmi O.
Dpp/BMP transport mechanism is required for wing venation in the sawfly *Athalia rosae*.
Insect Biochem Mol Biol. Volume 43, Issue 5:466-473, 2013

Abbreviation

ActR	Activin receptor
ACV	Anterior crossvein
AE	Activation element
ALK	Activin receptor-like kinase
AMH	Anti-Mullerian hormone
AP	After pupariation
A-P	Anterior-posterior
Ar	Athalia Rosae
Babo	Baboon
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
Brk	Brinker
Bs	Blistered
CFP	Cyan fluorescent protein
CLP	Cleft lip/plate
Co-Smad	Common-Smad
CR	Cysteine rich
Cv	Crossveinless
Cv-2	Crossveinless-2
Cv-C	Crossveinless-C
Cv-D	Crossveinless-D
CV(s)	Crossvein(s)
Dad	Daughters against dpp
Dia	Diaphanous
Dl	Dorsal
Dpp	Decapentaplegic
DSRF	Drosophila Serum Response Factor
D-V	Dorsal-ventral
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
En	Engrailed
FRAP	Fluorescence recovery after photobleaching
GAPs	GTPase-activating proteins
Gbb	Glass bottom boat
GDF	Growth and differentiation factor
GEFs	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GSCs	Germline stem cells
Hh	Hedgehog
HSPGs	Heparan sulfate proteoglycans
I-Smads	Inhibitory Smads
Ltl	Larval translucida
LVs	Longitudinal veins

Mad	Mothers against dpp
MH1 and MH2	Mad Homology 1 and 2
MRLC	Myosin II regulatory light chain
NMJ	Neuromuscular junction
Omb	Optomotor blind
PCF	Post-Cocoon Formation
PCs	Proprotein Convertases
PCV	Posterior crossvein
PDP	Pyruvate dehydrogenase phosphatase
Pent	Pentagon
Put	Punt
Rok	Rho kinase
R-Smad	Receptor-regulated Smad
Sal	Spalt
Sax	Saxophone
Scw	Screw
sFRPs	Secreted frizzled-related proteins
shi	Shibire
Shn	Schnurri
Sog	Short gastrulation
Spz	Spätzle
TGFβ	Transforming growth factor β
Tkv	Thickveins
Tl	Toll
Tld	Tolloid
Tlr	Tolloid-related
Tsg	Twisted gastrulation
Ubx	Ultrabithorax
Wg	Wingless
Wit	Wishful thinking

Abstract

Evolutionarily conserved signaling pathways mediate cell-cell communications during development. While the extracellular signal is precisely regulated to achieve dynamic morphogenetic events at the species level, it must be also flexible to generate the diversified morphologies through evolution. However, little is known about the mechanisms behind the precision and flexibility. The insect wing vein pattern can provide an excellent model to address this fundamental question, since species-specific wing vein patterns have been diversified through evolution. In this thesis, I study how evolutionarily conserved bone morphogenetic protein (BMP)-type ligand specifies diversified insect wing vein patterns using Dipteran *Drosophila melanogaster* and Hymenopteran sawfly *Athalia rosae* as models.

In *Drosophila*, BMP-type ligand Decapentaplegic (Dpp) is expressed in the longitudinal veins (LVs) to maintain LVs and induce crossveins (CVs) fates during pupal stages. However, the distribution of Dpp remained largely unknown. Using GFP-tagged Dpp, I demonstrated that Dpp is directionally transported from LVs into the posterior crossvein (PCV) region by two extracellular BMP-binding proteins, Short gastrulation (Sog) and Crossveinless (Cv). In contrast, most of Dpp did not diffuse from LVs by Type I BMP receptor and a positive feedback mechanism. Thus the active transport and retention mechanisms allow diffusible Dpp to draw the complex wing vein patterns in *Drosophila*.

To investigate how BMP signal instructs wing vein morphogenesis that involves apposition and cell shape changes between two wing epithelial layers, I then focused on the function of RhoGAP Crossveinless-C (Cv-C) during the PCV morphogenesis. I found that *cv-c* mediates PCV morphogenesis downstream of BMP signal by inactivating various Rho-type small GTPases. Interestingly, I found that *cv-c* is also required for Dpp transport, while Sog/Cv mediated BMP signal is guided at the ectopic wing veins caused by loss of Rho-type small GTPases. These observations identified a feed-forward mechanism coupling Dpp transport and PCV morphogenesis.

To address how BMP signal specifies diversified insect wing vein patterns, I then introduced sawfly as a new model. I found that *dpp* is ubiquitously expressed but BMP signal reflects distinct fore- and hindwing vein patterns in sawfly. To address if Dpp transport mechanism is involved in wing vein formation, Cv/Tsg was identified from sawfly. Loss of *dpp* or *cv/tsg* by RNAi affected BMP signal and all of wing venations. These observations suggest that ubiquitously expressed Dpp is redistributed to specify distinct fore- and hindwing vein patterns in sawfly.

Taken together, I found that the extracellular distribution of Dpp/BMP is tightly regulated and coordinated to achieve precise patterning and morphogenesis of the insect wing veins. Furthermore, this study raises an interesting possibility that changes in the directionality of Dpp/BMP diffusion may underlie distinct insect wing vein patterns.

Review of the literatures

Introduction

Cell-cell communication plays a central role in development of multicellular organisms. BMP family ligands are evolutionarily conserved growth factors that mediate cell-cell communication in many developmental processes. Since ablation of BMP pathway causes a variety of developmental defects or diseases, investigating the regulatory mechanisms on BMP signaling is important in medicine as well as in developmental biology.

The fruit fly *Drosophila melanogaster* has the longest history as a model organism and has been widely used to study genetics and developmental biology. *Drosophila* is easily cultured, has a short generation time, has very sophisticated classical genetics, and mutant animals can be readily obtained. The fully sequenced genomes of 12 *Drosophila* species are also available. In particular, numerous studies in *Drosophila* have identified the components of evolutionarily conserved signaling pathways, including BMP signaling pathway.

In this thesis, I mainly focused on the extracellular regulation of BMP signaling in the *Drosophila* wing vein specification as a model to address its distribution and regulation during dynamic morphogenesis. To address how such regulation is used to specify the diversified insect wing vein patterns, I also introduced the sawfly *Athalia rosae* as a new model. In this review, I first review the overview of Dpp/BMP signaling pathway in vertebrates and *Drosophila*. I focus on the extracellular regulation of BMP signaling as a morphogen during patterning and growth of the wing imaginal disc and patterning of the early embryo in *Drosophila*. I then introduce a unique shuttling/transport mechanism to form morphogen gradient in the early embryo and to specify wing veins during pupal stages.

1. Overview of BMP signaling

1.1. Overview of BMP signaling in vertebrates

BMPs comprise the largest subgroup of the transforming growth factor β (TGFβ) superfamily. Around 20 BMP family members have been identified and intensively studied in vertebrates. BMP signal is transduced by type I and II serine/threonine kinase receptors. Type I receptors consist of BMPR-IA (ALK-3), BMPR-IB (ALK-6), ALK1, and ALK-2. Type II receptors consist of BMPR-II, ActR-IIA, and ActR-IIB (Table 1). Type I receptor binds to homo- or heterodimer ligands and then forms a heterotetrameric receptor complex consisting of two pairs of a type I and II receptor complex (Figure 1). Type II receptor is a constitutively active kinase and phosphorylates GS domain of Type I receptor upon ligand binding, which switches GS domain from a repressor element into a docking site for substrate Smads (Huse et al., 1999). Phosphorylated Type I receptor thus phosphorylates C-terminal of receptor-regulated Smads (R-Smads, Smad1, 5 and 8 for BMP signal). R-Smads have highly conserved Mad Homology 1 and 2 (MH1 and MH2) domains at the N-terminus and C-terminus respectively, and a less conserved linker region between them. MH1 domain is a DNA binding domain and inhibits MH2 function. Phosphorylation of MH2 domain at the two serine in the C-terminal SSXS residues by Type I receptor opens the MH2 domain to allow binding with common-mediator Smad (Co-Smad, Smad4), which has the similar structure but lacks the phosphorylation site at the C-terminus (Kretzschmar and Massague, 1998). The complex consists of two R-Smads and one Co-Smad and translocates into the

nucleus to act as a transcriptional factor to regulate downstream gene expression with other transcription factors (Chen et al., 2004). The overview of BMP signaling is summarized in Figure 1 (Kimelman, 2006). BMPs play critical roles in a variety of developmental processes in vertebrates, including development of germ cells, dorsal-ventral patterning of the body axis, induction of epidermis, and early patterning of the central nervous system, neural crest, and various placodes (Blitz and Cho, 2009).

1.2. Overview of BMP signaling in *Drosophila*

In *Drosophila*, three BMP-type ligands, Decapentaplegic (Dpp), Screw (Scw), and Glass bottom boat (Gbb) have been isolated (Arora et al., 1994; Doctor et al., 1992; Padgett et al., 1987). Phylogenic analyses showed that Dpp is BMP2/4 ortholog, Gbb is BMP5/6/7/8 ortholog, and Scw is a distant relative of the BMP5/6/7/8 subclass, with no clear vertebrate ortholog. As in vertebrates, ligands transduce the signal via type I receptors, ALK3/6 ortholog Thickveins (Tkv) and ALK1/2 ortholog Saxophone (Sax), and Type II receptors, Punt (Put) and Wishful thinking (Wit). Put and Wit also bind with the type I receptor Baboon (Babo) to mediate activin-like signals from dActivin and Daw (Moustakas and Heldin, 2009). Phosphorylated Mothers against *dpp* (pMad, R-Smad) translocates into the nucleus with Medea (Co-Smad) and regulates target gene expression (Figure 1 and Table 1). Thus the signal transduction is highly conserved. As *dpp* is named after a dramatic mutant phenotype in 15 imaginal discs (Spencer et al., 1982), Dpp plays critical roles in a variety of developmental processes, including maintaining germline stem cells (GSCs), patterning of the embryo, patterning and growth of the wing imaginal disc, and a variety of morphogenesis. *scw* expression is limited to the early embryo and required for the patterning of the early embryo (Arora et al., 1994). Gbb has been studied in maintaining GSCs (Kawase et al., 2004; Song et al., 2004), patterning and growth of the wing imaginal disc (Wharton et al., 1999), retrograde signaling regulating synaptic growth at the neuromuscular junction (NMJ) (McCabe et al., 2003), and maintaining energy homeostasis (Ballard et al., 2010).

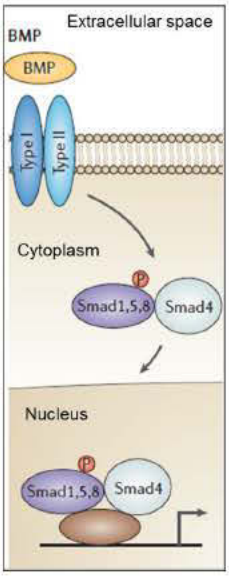

Figure 1. Overview of BMP signaling pathway. Upon BMP binding, type I receptor phosphorylates R-Smad. Phosphorylated R-smad, together with Co-Smad, is translocated into the nucleus to regulate target genes expression. (Modified from Kimelman

	Human	Drosophila
Ligands	BMP2/4	Dpp
	BMP5/6/7/8	Gbb
		Scw
Type I receptor	BMPRIA (ALK3)	Tkv
	BMPRIB (ALK6)	
	ALK1	Sax
	ALK2	
Type II receptor	BMPRII	Wit
	ActRIIA	Punt
	ActRIIB	
R-Smad	Smad 1,5,8	Mad
Co-Smad	Smad4	Medea
I-Smad	Smad6,7	Dad

Table 1.
Conserved components of BMP signaling pathway. BMP ligands, receptors, and Smads are conserved between Human and *Drosophila*. (Based on Moustakas and Heldin, 2009.)

2. Regulation of BMP signaling in the producing cells

BMP-type ligands are initially translated as an inactive proprotein consisting of a large N-terminal prodomain and a small C-terminal mature ligand domain. Mature ligand domains are dimerized as a heterodimer or homodimer in the endoplasmic reticulum, and are proteolytically cleaved in the Golgi compartment by Proprotein Convertases (PCs) prior to secretion of the mature dimers (Constam and Robertson, 1999; Cui et al., 1998; Ramel and Hill, 2012). The PCs constitute of a family of seven proteinases including Furin, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7 in vertebrates (Thomas, 2002). In *Drosophila*, three PCs (Dfurin1, Dfurin2, and Amontillado) have been identified. The optimal and the minimal furin recognition sequences are RX(R/K)R and RXXR, respectively. Recent studies showed that processing requirements of BMPs are diversified despite the conserved function of mature ligand domain, and propose that post-translational regulation at the level of processing provides diversity in both quantity and quality of BMP ligands.

In *Xenopus*, BMP4 has at least two furin recognition sites between prodomain and ligand domain; the upstream minimal site (S2) and the downstream optimal site (S1) located adjacent to the ligand domain (Figure 2). BMP4 is first cleaved at S1 to produce unstable non-covalently associated pro- and mature- domain. This allows subsequent cleavage at S2 to release stable mature BMP4 ligands (Cui et al., 2001; Degnin et al., 2004). Furin and PC6 can cleave the S1 and S2 redundantly, while PC7 selectively cleaves S1 (Nelsen and Christian, 2009). In *Drosophila*, Dpp has at least 3 furin sites; the upstream optimal site (FSII/S2), the downstream minimal site (FSIII/S1) and additional optimal site (FSI) (Kunnapuu et al., 2009) (Figure 2). Although FSI, FSII and FSIII sites can be cleaved independently, efficient cleavage of FSIII and FSI sites requires cleavage of FSII site. Thus, unlike BMP4, cleavage at FSII/S2 is likely to facilitate subsequent cleavage at FSIII/SI by Dfurin1/2 (Kunnapuu et al., 2009). Nevertheless, cleavage at S2 or FSII is critical for both BMP4 and Dpp signal (Cui et al., 2001; Kunnapuu et al., 2009; Sopory et al., 2010).

In the case of BMP5/6/7/8 ligands, Gbb and Scw also contain at least three furin recognition sites, one optimal site (Pro sites) within the prodomain, and two between prodomain and ligand domain (Main and Shadow sites) (Figure 2). Main site is optimal and Shadow site is not optimal sequence. In Gbb, all sites are cleaved independently, although efficient cleavage of Shadow site depends on the cleavage of Main site. Processing at either Pro or Main sites are important for the Gbb function. In Scw, Pro and Main sites are cleaved independently but cleavage at Shadow site requires the cleavage of Main site. Processing at both Pro and Main sites are critical for Scw function. In human, BMP7 has only Main site required for its activity (Fritsch et al., 2012) (Figure 2).

Thus, the processing in prodomain appears to evolve rapidly even within closely related ligands. These differential cleavages can produce distinct ligand forms with different properties. For example, BMP4 produces one, while Dpp produces two secreted molecular forms of ligands (Kunnapuu et al., 2009). The larger form of Dpp associates with heparin or Collagen IV stronger than the smaller form (Akiyama et al., 2008; Wang et al., 2008). Gbb produces even larger molecular form when processed within prodomain. The large form has stronger and longer-range signaling activity than the smaller molecular form. The cleavage site within Gbb prodomain is evolutionarily conserved in some BMP-type ligands and associated with human disease (Akiyama et al., 2012). For example, point mutations in the prodomain of human BMP4, BMP15, and Anti-Mullerian hormone (AMH) were

respectively found in the patients with cleft lip/plate (CLP) (Suzuki et al., 2009), premature ovarian failure (Dixit et al., 2006), and Mullerian duct syndrome (Imbeaud et al., 1994).

The cleavage can be also tissue specific to adjust amounts of BMP ligands. In mice, cleavage at S2 site is critical for testes and germ cells but dispensable for the limb, dorsal vertebrae, and kidney development, despite their sensitivity to BMP4 dosage (Goldman et al., 2006). In *Drosophila*, cleavage at FSII site was dispensable in the midgut epithelium, despite its critical role in the wing imaginal disc. Consistently, Dpp is less cleaved at FSII site in embryos than in the wing imaginal disc (Sopory et al., 2010).

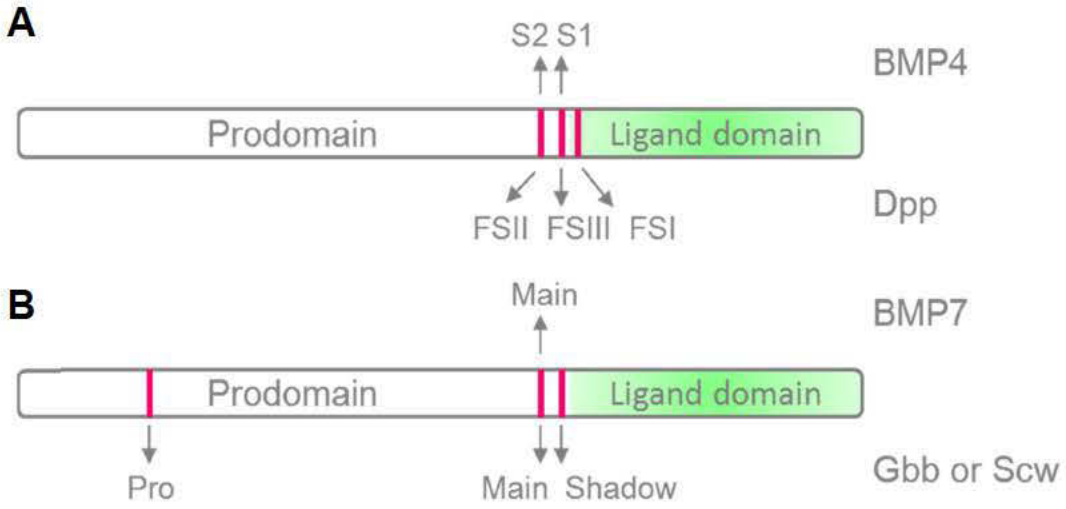

Figure 2. Overview of processing of BMPs.
(A) Overview of processing cites of BMP4 and Dpp. (B) Overview of processing cites of BMP7 and Gbb or Scw. BMP-type ligands are initially composed of N-terminal prodomain and C-terminal mature ligand domain (green). BMP-type ligands need to be cleaved in the producing cells for their function. The positions of processing sites by PCs (red) and their importance are diversified between BMP-type ligands. (Based on Cui et al., 2001, Kunnapuu *et al*., 2009, and Fritsch *et al*., 2012.)

3. Regulation of BMP signaling in the receiving cells

The intensity and duration of BMP signal can be also regulated in the ligand receiving cells, especially at the level of Smads. In addition to R-Smads and Co-Smad, there are inhibitory Smads (I-Smads) consisting of Smad6 and Smad7 or Dad (*daughters against dpp*) in *Drosophila* (Imamura et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997) (Figure 2). I-Smads contain MH2 domain, but lack MH1 domain and phosphorylation motif at the C-terminus. I-Smads inhibit the signal through degradation or dephosphorylation of Type I receptors (Hill, 2009). In addition, BMP signal induces the expression of the I-Smads, thus forming a negative-feedback loop to regulate signaling activity (Itoh and ten Dijke, 2007).

The linker region of R-Smads can be also phosphorylated at the Ser/Thr residues by various kinases such as MAPK or GSK3. The linker phosphorylation results in its polyubiquitination and degradation of Smads by the proteasome (Bruce and Sapkota, 2012). Smurf1 (Dsmurf in *Drosophila*) is an E3-ubiquitin protein ligase required for the degradation of Mad (Liang et al., 2003; Podos et al., 2001). Phosphorylation at the linker region allows cross talks of the signaling pathways. For example, FGF signal inhibits BMP signal through MAPK and Wnt signal prolongs BMP signal through inhibiting GSK3 (Eivers et al., 2008). The phosphorylation of Smads is reversible by phosphatases (Bruce and Sapkota, 2012). Recent studies identified a variety of phosphatases that dephosphorylate C-terminus and linker region of Smads. Among them, pyruvate dehydrogenase phosphatase (PDP) is the first identified phosphatase that dephosphorylates C-terminus of Mad in *Drosophila* (Chen et al., 2006).

The nucleocytoplasmic shuttling of Smad is also tightly regulated by karyopherins (Importins and Exportins). Signal-mediated nuclear import of Smad1 (Mad) requires Importin7/8 in *Drosophila* and mammals (Xu et al., 2007). Dephosphorylated monomeric Smad1 and Smad4 are exported by CRM1/Exportin1 (Pierreux et al., 2000; Watanabe et al., 2000). The nucleocytoplasmic shuttling of Smad is dynamic. Even if the signal is positive, Smads are constantly exported to the cytoplasm. This allows the immediate shut off of the signal (Hill, 2009).

4. Function of BMP signaling as a morphogen in *Drosophila*

In *Drosophila*, extracellular BMP regulation has been intensively studied as a morphogen in the wing imaginal disc and the early embryo. Therefore, I focus on the function and the extracellular regulation of BMP signal in these processes.

4.1. Morphogen

Morphogens are the substances that provide positional information in the developing tissues in a concentration-dependent manner. Cells acquire positional information by reading local morphogen concentration, and activate the transcriptional target genes depending on the different threshold (Rogers and Schier, 2011). Thus morphogens can subdivide the genetically identical uniform tissues into the distinct cell fates (French flag model) (Wolpert, 1969) (Figure 3). Several families of secreted proteins, including members of the Hedgehog (Hh), TGF-β, and Wingless (Wg) families, operate as morphogens during embryonic development. Among them, Dpp is the first validated secreted morphogen that functions directly at a distance to specify gene expression pattern in the *Drosophila* wing imaginal disc (Nellen et al., 1996).

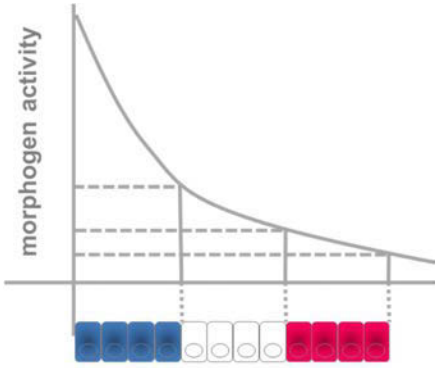

Figure 3. French flag model
French flag colors represent the effect of a morphogen on tissue patterning: high concentrations activate a blue gene, medium concentrations activate a white gene, and low concentrations activate a red gene based on distinct thresholds.

4.2. Patterning and growth in the wing imaginal disc

Imaginal discs are epithelial structures that give rise to adult body structures. The wing imaginal disc has served as an excellent model to study how morphogens regulate patterning and growth of the tissue. The wing imaginal disc grows from 20-30 cells in the embryo to about 50,000 cells during larval stages. The mature wing imaginal disc is a flattened sac that consists of two cell types, squamous peripodial cells and folded columnar epithelium (Figure 4). The peripodial cells undergo apoptosis in the early pupal stage but the columnar epithelium forms the wing blade, the hinge, and the notum of the adult fly. The wing pouch, which gives rise to the wing blade, is divided into the compartments by anterior-posterior (A-P) and dorsal-ventral (D-V) compartment boundaries (Figure 4). The compartment is defined as separated cell population with different lineage boundaries (Garcia-Bellido et al., 1973). Hh expression in the posterior compartment is regulated by the transcription factor *Engrailed* (*En*). Hh acts as a morphogen to induce a stripe of *dpp* expression at the A-P boundary. Dpp then acts as a morphogen to regulate A-P patterning and growth of the wing imaginal disc (Affolter and Basler, 2007; Tabata, 2001) (Figure 5). D-V patterning is mediated by Wg expressed in D-V boundary (Strigini and Cohen, 2000; Zecca et al., 1996). An important criteria showing that Dpp acts as a morphogen was the cell-autonomous action of constitutively active receptor and non-cell autonomous action of ligands. This excludes

the possibility that long-range action of Dpp is mediated by relay mechanisms that involve secondary signals downstream of BMP signal, and shows that Dpp acts directly at a distance (Nellen et al., 1996). BMP activity gradient visualized by phosphorylated Mad (pMad) antibody revealed a complex gradient pattern along the A-P axis. BMP signal is relatively weak in the A-P boundary region but high adjacent to the boundary region and then graded toward the peripheral region (Tanimoto et al., 2000) (Figure 5). This activity gradient reflects Hh signal-mediated transcriptional repression of *tkv* in the A-P boundary and induction of *tkv* in the posterior compartment. Low level of Tkv not only affects Dpp signal but also facilitates its dispersal (Lecuit and Cohen, 1998; Tabata, 2001).

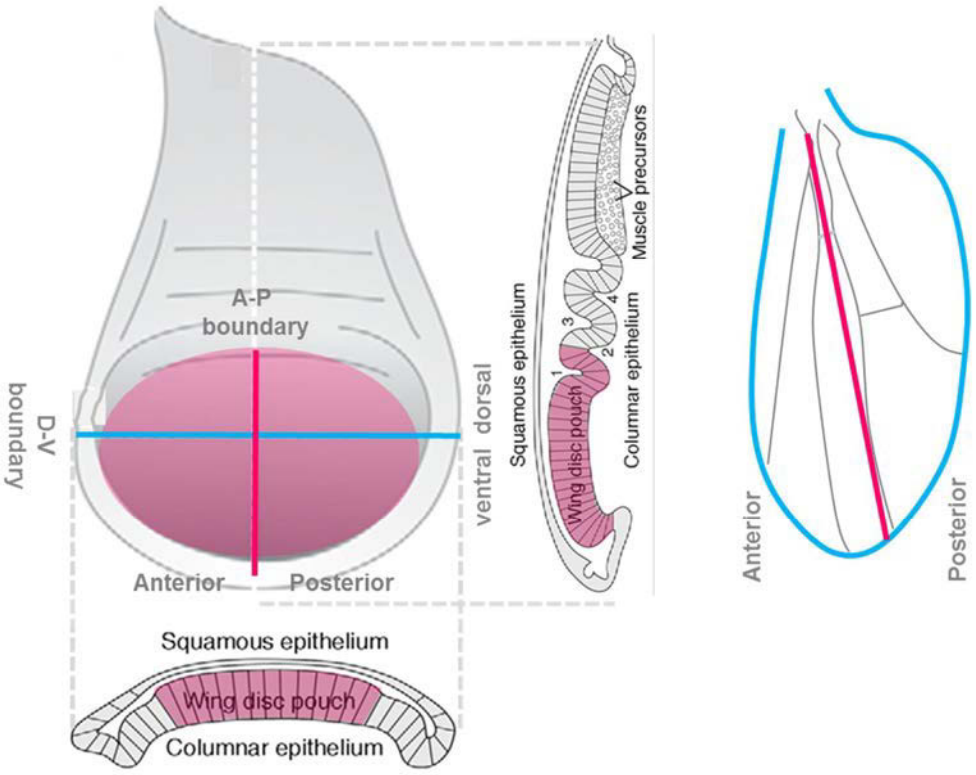

Figure 4. Structure of *Drosophila* wing imaginal disc (left) and adult wing (right). Wing pouch (pink) is the columnar epithelium and subdivided by A-P boundary (pink line) and D-V boundary (blue line). In the adult wing (right), A-P boundary corresponds to the line along L4 (pink) and D-V boundary corresponds to the wing margin (blue). (Based on Widmann and Dahmann, 2009.)

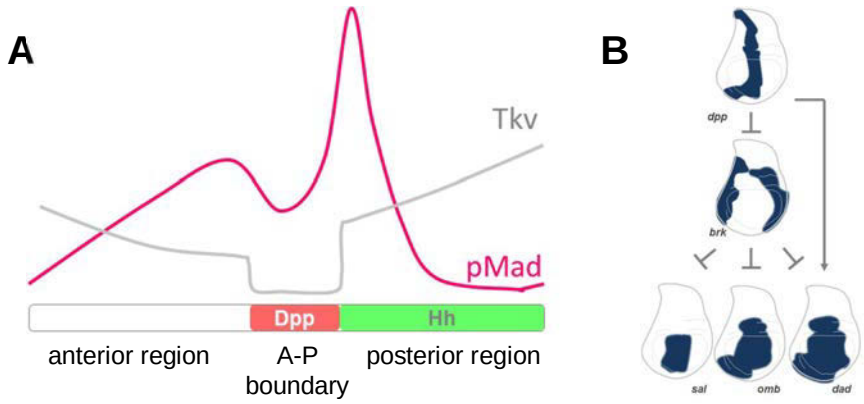

Figure 5. BMP morphogen activity gradient and expression patterns of downstream target genes. (A) BMP morphogen activity gradient (pink) and Tkv expression (gray). BMP morphogen activity gradient is low in the Dpp producing cells but rather high adjacent to them. This reflects *tkv* expression pattern. (B) Expression patterns of Dpp target genes. They are mainly regulated by derepression of repressor *brk* (Based on Tabata, 2001.)

The BMP activity gradient is translated into the nested expression patterns of target genes such as *dad*, *spalt* (*sal*), and *optomotor blind* (*omb*), and into adult wing vein positions along A-P axis (Nellen et al., 1996; Tsuneizumi et al., 1997) (Figure 5). It has been shown that BMP signal regulates these target gene expression mainly by repressing repressor *brinker* (*brk*) that suppresses them (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999) (Figure 5). The inverse gradient of *brk* expression is first formed by pMad/Medea/repressor Schnurri (Shn) complexes that bind to *brk* regulatory regions (silencer element (SE)) (Muller et al., 2003). The expression of target genes is then derepressed based on the threshold of Brk gradient. BMP signal can also directly activate target gene expression (*dad*, *sal*) through its regulatory element (activation element (AE)) (Weiss et al., 2010). The employment of two inverse gradients (BMP signaling activity gradient and Brk inverse gradient) may allow the cells to respond to the signaling more precisely. *gbb* is also required for A-P patterning of the wing (Khalsa et al., 1998). *gbb* is expressed ubiquitously, but clonal analyses showed that Gbb produced by the A-P boundary cells (*dpp* producing cells) is critical for patterning of more distal tissues compared with Dpp. Gbb appears to have a longer-range signaling than Dpp, in part, via Dpp-Gbb heterodimer formation (Bangi and Wharton, 2006).

BMP signal also regulates growth of the wing imaginal disc. Loss or severe reduction of *dpp* in the wing imaginal disc significantly reduces wing size (Spencer et al., 1982; Zecca et al., 1995), while activation of BMP signaling induces overgrowth (Burke and Basler, 1996; Capdevila and Guerrero, 1994; Martin-Castellanos and Edgar, 2002). Interestingly, growth of the wing imaginal disc is uniform despite the graded BMP activity gradient in the wing imaginal disc. In contrast to the genes that regulate patterning of the wing imaginal disc such as *sal*, *omb* and *dad*, little is known about the target genes that regulate the growth of the wing imaginal disc downstream of BMP signaling. It has been shown that Dpp regulates the growth also through Brk (Schwank et al., 2008). To date, micro RNA *bantam* and oncogene *myc* are reported as Brk targets (Doumpas et al., 2013; Martin et al., 2004). Here I review several models to address how BMP morphogen gradient is translated into the uniform growth, and how cells know when to stop growing (Schwank and Basler, 2010).

4.2.1. Gradient model

Gradient model postulates that cells recognize the differences in BMP signal that they are exposed to, and grow if the difference is sufficiently high. Thus the slope of morphogen gradient, rather than absolute intensity, is critical to promote growth (Day and Lawrence, 2000) (Figure 6). The model has been supported by the observation that either activation or inhibition of BMP signal in clones, which induced sharp gap in the BMP signal activation, stimulated transient non-autonomous cell proliferation (Rogulja and Irvine, 2005). Furthermore, uniform activation of Dpp signal in the entire wing disc, which disrupted the gradient, inhibited the growth in the medial region as expected. In contrast, this promoted the growth in the peripheral region (Rogulja and Irvine, 2005). This indicates that medial cells respond to the slope of BMP gradient but peripheral cells to the absolute BMP signal. However, contrast with the gradient model, it has been shown that the growth of the medial cells was actually promoted when Dpp signal was activated only in the medial cells and was inhibited non-cell autonomously by Dpp signal activation in the peripheral region (Schwank et al., 2008).

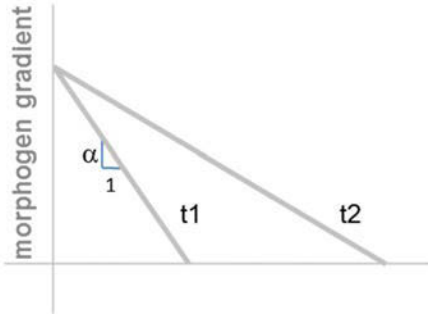

Figure 6. Gradient model.
In a simple model, in which morphogen activity is fixed at the morphogen producing cells and at the edge of the tissue, the slope of morphogen gradient (α) decreases as the wing grows (t1 to t2). Wing stops growing if the slope is below the threshold. (Based on Schwank and Basler, 2010.)

4.2.2. Mechanical compression model

Two independent models hypothesize morphogen-mediated growth promotion and mechanical compression-mediated growth termination. (Aegerter-Wilmsen et al., 2007; Aegerter-Wilmsen et al., 2010; Aegerter-Wilmsen et al., 2012; Hufnagel et al., 2007; Shraiman, 2005). Both postulate that differences in growth rates between cells produce mechanical stress that in turn affects growth. Shraiman proposed that cells continue to grow uniformly above a certain threshold of BMP signal. Peripheral cells stop growing when they are far away from the morphogen source and reach the threshold, but medial cells can still grow since they are still under growth control by morphogen. Thus differences in growth rates between medial and peripheral cells are generated during the growth of the wing imaginal disc and this induces mechanical compression towards the medial region to suppress the growth. This model predicts that BMP signal received by peripheral cells decreases during growth so that BMP morphogen gradient does not scale with wing disc size. However, this is not consistent with the recent reports that BMP morphogen gradient scales with the wing disc size (Hamaratoglu et al., 2011; Wartlick et al., 2011). In contrast, Aegerter-Wilmsen et al. proposed that intrinsic differences in the growth rates between the medial and peripheral cells are imposed by BMP morphogen gradient from the early phase of the growth. BMP morphogen activity induces growth in the medial cells. This then leads to stretching peripheral cells to trigger growth and the wing imaginal disc grows uniformly. According to the model, cells can grow above a certain threshold of mechanical property of

stretch. During the growth, remaining mechanical stress increases and compresses the medial cells to suppress their growth (Figure 7). The compression increases as wing cells grow and growth stops when the morphogen-induced growth cannot exceed the effect of compression (Aegerter-Wilmsen et al., 2007; Aegerter-Wilmsen et al., 2010; Aegerter-Wilmsen et al., 2012). The model can explain many experimental results including the data supporting gradient model, but mechanical property of the cells remains to be experimentally demonstrated.

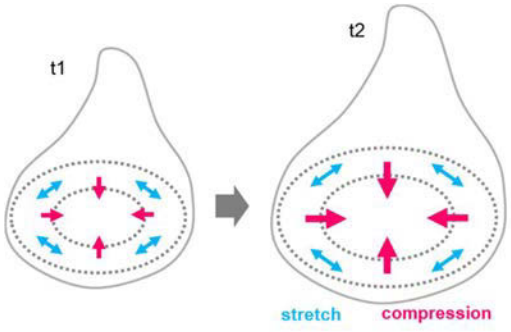

Figure 7. Mechanical compression model.
In the early stages (t1), the medial cells grow upon BMP morphogen activity and the peripheral cells grow upon mechanical stretching of the cells (blue) caused by growth of the medial cells. In the later stages (t2), the remaining mechanical stress in the peripheral cells compresses the medial cells to stop growth. This model can explain the uniform growth and termination of the growth. (Based on Aegerter-Wilmsen *et al.* 2007.)

4.2.3. Temporal growth model

Temporal increase of Dpp levels has been proposed to control growth. The model is based on the observation that the morphogen gradient (both ligand concentration and activity gradient) scales with the tissue size, and that the relative position of a given cell is constant in the wing imaginal disc due to uniform proliferation. Therefore all cells sense the same relative increase of Dpp or Dpp signal during wing disc growth (Figure 8), which was estimated to be about 40% for ligand concentration and about 50% for the signal during a cell cycle. Thus morphogen gradient can be converted into the uniform temporal increase of Dpp signaling, which accounts for the uniform growth (Wartlick et al., 2011). However, contradicting the model, it has been argued that cells grow normally even when the increase of Dpp signaling is genetically suppressed, and therefore argued that BMP signal has only the permissive role for wing growth (Schwank et al., 2012).

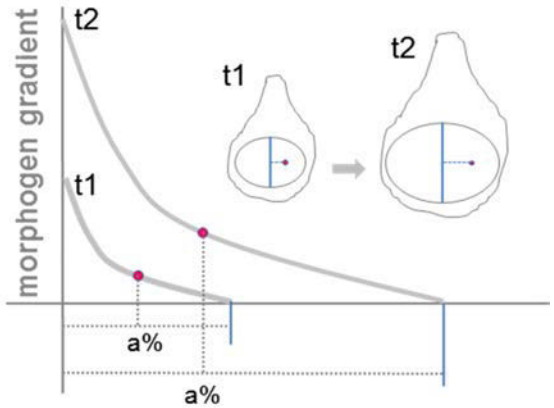

Figure 8. Temporal growth model.
BMP morphogen gradient expands as the wing imaginal disc grows. The gradient scales with the tissue size. Since the wing grows uniformly, relative position of a given cell is maintained during the growth. (Based on Le Goff and Lecuit, 2011.)

4.2.4. Coordination of patterning and growth

Given that the adult wing vein patterns are proportional to the adult wing size, growth and patterning of the wing must be coordinated during development. Consistently, recent studies showed the scaling of BMP gradient with wing disc size during growth (Hamaratoglu et al., 2011; Wartlick et al., 2011). Similar scaling was also observed when the growth rate was changed in the posterior compartment of the wing by modifying insulin pathway (Teleman and Cohen, 2000). According to a recent theoretical model, the scaling emerges naturally if a simple feedback circuit that involves two diffusible molecules, a morphogen and an expander, were taken into account (Ben-Zvi and Barkai, 2010). In the expansion-repression model, the morphogen represses the expander and restricts its expression far from the morphogen source. The expander is diffusible and stable, and increases morphogen gradient. Consequently, the expander can adjust the gradient until morphogen activity reaches the threshold to suppress the expander. A secreted protein Pentagon (Pent), which is suppressed by BMP signal and required for establishing BMP morphogen gradient (Vuilleumier et al., 2010), appears to be such an expander that realizes the scaling (Ben-Zvi et al., 2011; Hamaratoglu et al., 2011).

4.3. Dorsal-ventral patterning in the early embryo

In *Drosophila*, D-V axis of the early embryo is maternally defined during oogenesis and transmitted to the fertilized embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The initial D-V polarity involves the transfer of spatial information between the germline and the follicle cells surrounding the oocyte (Schupbach, 1987). Gurken, a TGF-α protein, derived from the dorsal source in the oocyte activates Torpedo/DER, a EGFR homolog, in the dorsal somatic follicle cells (Neuman-Silberberg and Schupbach, 1993; Price et al., 1989). The EGFR signal represses the expression of *pipe* encoding glycosaminoglycan-modifying enzyme heparan sulfate 2-O-sulfotransferase, and restricts its expression at the ventral side (~40%) of the follicle cells (Sen J et al., Cell 1998). After fertilization, the ventral vitelline membrane modified by Pipe facilitates processing and activation of Spätzle (Spz), a ligand for Toll (Tl) receptor, in the perivitelline space (DeLotto and DeLotto, 1998; Morisato and Anderson, 1994; Schneider et al., 1994). Since the initial *Drosophila* embryogenesis is a rapid process with synchronized mitotic divisions without cell divisions, Spz-Tl signaling generates a nuclear gradient of Dl along D-V axis of the embryo (Rusch and Levine, 1996). Dl acts as a morphogen to specify mesoderm and neuroectoderm by inducing the expression of *snail*, *twist*, and *sog* (Moussian and Roth, 2005) (Figure 9).

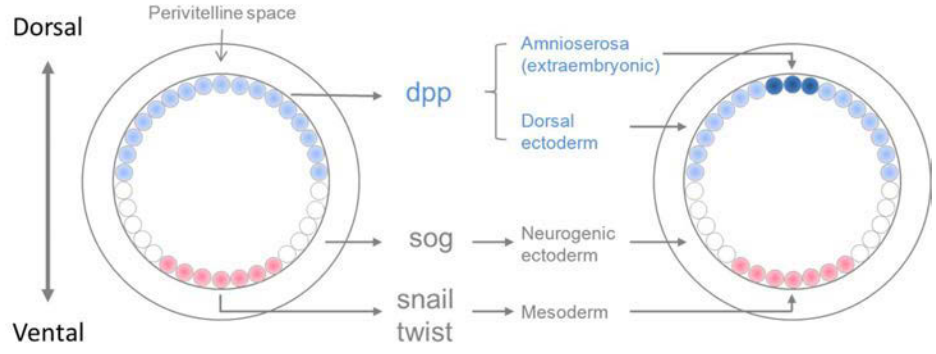

Figure 9. The expression pattern of Dl target genes. A nuclear gradient of Dl forms from ventral to dorsal side of the embryo. Dl induces *snail* and *twist* at a high threshold, *sog* at intermediate threshold, and repress *dpp*. *Snail* and *twist* are required for the mesoderm formation, *sog* for neurogenic ectoderm.

dpp is repressed by Dl and is zygotically induced at the dorsal half of the embryo (Figure 9, 10), where Dpp acts as a morphogen to pattern the dorsal surface of the blastoderm embryo into two fates, the extraembryonic amnioserosa from the dorsal midline and the dorsal ectoderm from the dorsal lateral side (O'Connor et al., 2006). In *dpp* null mutants, all dorsal cells acquire a ventral neurogenic fate (Sutherland et al., 2003). Moreover, injection of high levels of *dpp* mRNA converts all dorsal cells to an amnioserosa fate, whereas moderate levels specify dorsal ectoderm (Ferguson and Anderson, 1992). Dpp thus acts as a concentration-dependent morphogen for the specification of both tissues. BMP signal accumulates in the nucleus of dorsal cells during cellularization. Initially, anti-pMad staining is low and encompasses the dorsal region broadly. It then rapidly accumulates at the dorsal midline within 30-40min, and sharp BMP signaling gradient is established by the onset of gastrulation (Figure 10). pMad levels are high in the dorsal-most 5-10 cells, but rapidly drop off to undetectable levels in more lateral regions over several cell diameters (O'Connor et al., 2006).

In the typical morphogen concept, morphogen is expressed locally and establish a morphogen gradient outside the morphogen source. For example, BMP morphogen gradient in the wing imaginal disc is such a case despite the complex activity gradient (Figure 5). In contrast, sharp morphogen gradient can be established within the morphogen source (Figure 10). BMP morphogen gradient in the patterning of the embryo appears to be such a case (Figure 11). This appears to be unusual, since the passive diffusion tends to decrease the concentration at the source (Shilo et al., 2013). This observation predicts the qualitatively different mechanism for establishing the BMP morphogen gradient formation in the embryo and the wing imaginal disc.

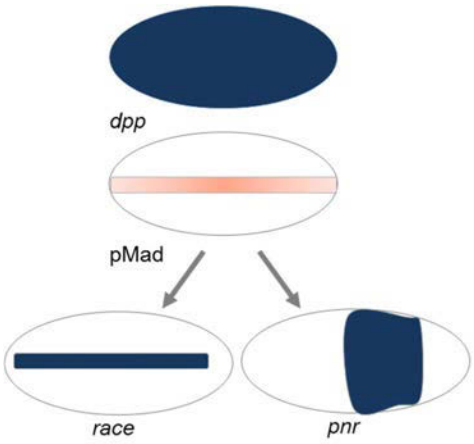

Figure 10. Sharp Dpp morphogen gradient in the dorsal surface of the embryo. *dpp* expression is uniform in the dorsal half of the embryo but pMad signal accumulates at the dorsal midline. Expression of *dpp* target genes, *race* and *pnr*. Dorsal view of the *Dorsophila* embryo.

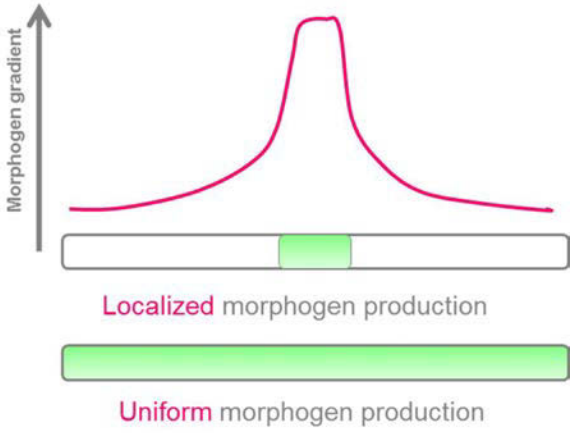

Figure 11. Morphogen gradient formation from localized or broad morphogen expression domain. In the wing imaginal disc, *dpp* expression is localized and Dpp morphogen gradient is established outside the *dpp* expression domain. In contrast, in the embryo, *dpp* expression is uniform in the dorsal surface of the embryo and Dpp morphogen gradient is established within the *dpp* expression domain.

4.4. Maintaining the wing imaginal disc architecture

In addition to non-cell autonomous function of Dpp as a morphogen, Dpp signal cell-autonomously plays critical roles in a variety of morphogenetic events, including embryonic dorsal closure (Ricos et al., 1999), pupal thorax closure (Martin-Blanco et al., 2000), and wing epithelial morphogenesis (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). Here I focus on the function of BMP signal in the wing epithelial morphogenesis.

Cell shape change is mediated by evolutionarily conserved members of the Rho GTPase family, including Rho, Rac, and Cdc42, that regulate actin dynamics. The activities of the Rho-type small GTPases are tightly regulated by the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The GEFs activate the GTPases by replacing GDP with GTP, while the GAPs inactivate the GTPases by enhancing their GTP-hydrolyzing activity (Moon and Zheng, 2003; Rossman et al., 2005). Rho GTPases promote polymerization of F-actin via actin nucleators, including ARP2/3 and Diaphanous (Dia). Rho GTPases also stabilize F-actin via Rho kinase (Rock or Rok in *Drosophila*). Phosphorylation of LIMK by Rho kinase inhibits actin depolymerizing factor cofilin through phosphorylation. Rho kinase also phosphorylates non-muscle Myosin II regulatory light chain (MRLC) to promote actomyosin contraction to regulate cell shape change (Jaffe and Hall, 2005).

BMP signal is an essential survival factor for wing disc cells since cells mutant for BMP signal components (*mad* and *tkv*) die due to JNK mediated apoptosis (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002). However, two studies challenged this view by showing that these mutant cells could actually survive but were extruded from the basal side due to disruption of cytoskeletal organization, when the activation of JNK signal was inhibited (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). These studies indicate that, in addition to its role for cell fate specification, Dpp signal is cell autonomously required to maintain cytoskeletal architecture of wing disc epithelia. A follow up study found that the onset of Dpp signal activation correlates with the cuboidal-to-columnar cell shape transition between the late second and the early third instar larval stage. Furthermore, during this stage, Dpp signal is associated with and required for increased activities of Rho1 and MRLC at the apicolateral region of the cells and apical-basal cell length. Localized actomyosin contraction at the apical side probably leads to narrowing of the apical domain and decreased cortical tension at the lateral side allows elongation of the cells (Widmann and Dahmann, 2009). Similar compartmentalization of Rho1 activity at the apical side has been also observed during posterior spiracle cell invagination in *Drosophila* embryo (Simoes et al., 2006). Interestingly, maintaining cell shape by Dpp signal is also mediated by Brk as in patterning and growth of the wing imaginal disc. The target genes of Brk remain to be identified.

5. Regulation of BMP signaling at the extracellular level

How does Dpp actually diffuse in the tissue to regulate patterning, growth, and morphogenesis? Here I review models on how Dpp diffuses in the wing imaginal disc and the early embryo.

5.1. BMP morphogen gradient formation in the *Drosophila* wing disc

Dpp morphogen gradient can be visualized by a GFP-tagged Dpp (Entchev et al., 2000; Teleman and Cohen, 2000). GFP has been inserted between the processing site and the mature ligand domain, so that the secreted mature ligand is tagged with GFP. GFP-Dpp is functional since it could rescue growth and patterning defects of *dpp* mutant in the wing. GFP-Dpp spreads into the wing pouch and forms a shallow gradient along A-P axis when expressed at the A-P boundary. Using this functional GFP-Dpp, a variety of mechanisms has been proposed on how the morphogen gradient is established (Figure 12).

5.1.1. Free extracellular diffusion model

The free extracellular diffusion and receptor-mediated degradation (Figure 12A) would be the simplest mechanism but had not been supported by too low diffusion coefficient of Dpp measured by the fluorescence recovery after photobleaching (FRAP) assay (Kicheva et al., 2007). However, the recovery of GFP-Dpp is not necessarily dependent on the slow diffusion process. If binding to immobile sites or degradation of ligands occurs with fast diffusion during the assay, the recovery of GFP-Dpp can be governed by these parameters rather than diffusion itself. Using fluorescence correlation spectroscopy (FCS) to visualize single molecule of Dpp, the free extracellular diffusion has been recently supported (Zhou et al., 2012). Formation of morphogen gradient by free diffusion has been also shown in FGF morphogen formation in zebrafish (Yu et al., 2009).

5.1.2. Restricted extracellular diffusion model

In the restricted extracellular diffusion model, Dpp diffuses along plasma membrane through repeated interaction with receptors and ECM proteins (Figure 12B). In this model, BMP receptors impede Dpp dispersal through receptor-mediated uptake and degradation (Lecuit and Cohen, 1998; Tanimoto et al., 2000). Heparan sulfate proteoglycans (HSPGs), *dally* and *dally-like*, and proteins required for biosynthesis of HSPGs promote Dpp dispersal or stabilize Dpp (Akiyama et al., 2008; Belenkaya et al., 2004; Bornemann et al., 2004; Fujise et al., 2003; Han et al., 2004; Takei et al., 2004). The model is consistent with the low diffusion coefficient of Dpp measured by the FRAP assay (Kicheva et al., 2007). A recent study estimated that 60-80 % of ligands are Tkv-unbound, since overexpressing Tkv (but not Punt) increases Dpp accumulation within clones, while *tkv* mutant clones did not affect the Dpp morphogen gradient (Schwank et al., 2011).

5.1.3. Receptor-mediated transcytosis model

According to the receptor-mediated transcytosis model, Dpp does not diffuse extracellularly but rather intracellularly via repeated receptor-mediated endocytosis and exocytosis through cells (Entchev et al., 2000; Gonzalez-Gaitan and Jackle, 1999; Kicheva et al., 2007; Kruse et al., 2004) (Figure 12C). The model was based on the observation that Dpp could not diffuse across the mutant clones of *shibire* (*shi*) encoding Dynamin GTPase required for endocytosis

(Chen et al., 1991; van der Bliek and Meyerowitz, 1991). However, the model has been refuted theoretically and experimentally (Lander et al., 2002; Schwank et al., 2011; Zhou et al., 2012). Theoretically, Lander *et al.* showed that accumulation of receptor in endocytosis defective cells can inhibit extracellular Dpp diffusion and cause the observed *shi* mutant results (Lander et al., 2002). Experimentally, majority of Dpp ligands has been shown to exist extracellularly by staining extracellular pool of Dpp (Belenkaya et al., 2004; Teleman and Cohen, 2000), and Dpp could actually move across *shi* mutant clones and activate BMP signal across *shi* mutant clones. Instead, *shi* was shown to be required for BMP signaling cell autonomously (Belenkaya et al., 2004). Furthermore, Dpp distribution was also unaffected behind *tkv* deficient cells (*tkv, brk* double mutant) (Schwank et al., 2011).

5.1.4. Cytoneme model

Dpp is proposed to be delivered to the target cells through the cytonemes, actin-based filopodia that extend from Dpp receiving cells towards Dpp producing cells (Ramirez-Weber and Kornberg, 1999) (Figure 12D). Tkv has been shown to move along them (Hsiung et al., 2005). Cytonemes are also produced in the eye imaginal discs and in the air sac primordia (Sato and Kornberg, 2002) and appear to respond to the different chemo-attractants (Roy et al., 2011). It is not known yet whether Dpp indeed moves along cytonemes, and how Dpp morphogen gradient can be established using cytonemes. Cytonemes have been also associated with Hh delivery from cap cells in the ovary (Hh producing cells) to the escort cells to induce BMPs to maintain GSCs (Rojas-Rios et al., 2012).

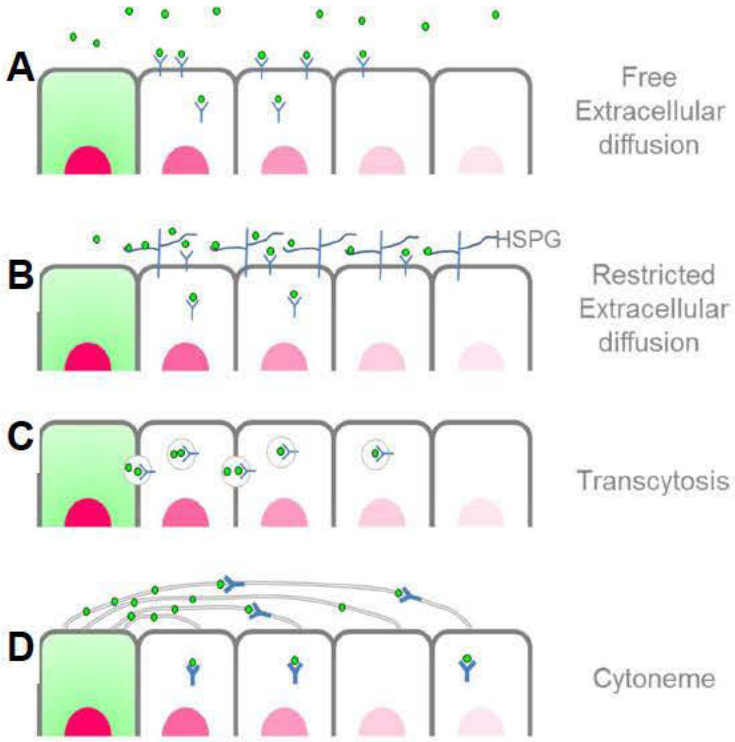

Figure 12. Models for Dpp morphogen gradient formation in the *Drosophila* wing imaginal disc. (A) Free extracellular diffusion model. Dpp diffuses into the lumen freely and receptor mediated degradation establishes the gradient. (B) Restricted extracellular diffusion model. Dpp diffuses through interaction with receptors and HSPGs. (C) Transcytosis model. Dpp diffuses intracellulary through repeated endocytosis and exocytosis. (D) Cytoneme model. Dpp is delivered through cytonemes connecting Dpp producing cells with receiving cells. (Based on Tabata, 2001.)

5.2. BMP morphogen gradient formation in the *Drosophila* embryo

In the early *Drosophila* embryo, the sharp BMP morphogen acitivity gradient is established within ubiquitous ligand expression domain. How is such a sharp morphogen gradient established?

5.2.1. Shuttling/transport mechanism

BMP morphogen gradient in the embryo requires evolutionarily conserved extracellular proteins, Short gastrulation (Sog), Twisted gastrulation (Tsg), Tolloid (Tld), and Scw (Arora et al., 1994; Francois et al., 1994; Ross et al., 2001; Shimell et al., 1991). Biochemical studies revealed their molecular function; Sog and Tsg are BMP inhibitors that prevent Dpp binding to the receptor (Ross et al., 2001). Tld is a metalloprotease that processes Sog to release the active ligands (Marques et al., 1997). Sog, Tsg, and Tld are evolutionarily conserved (Blader et al., 1997; Chang et al., 2001; Piccolo et al., 1997; Sasai et al., 1994; Scott et al., 2001).

How do they regulate BMP morphogen gradient? Since *sog* is expressed in the ventral-lateral regions and Sog protein diffuses into the dorsal side of the embryo (Srinivasan et al., 2002), the inhibitory ventral-to-dorsal gradient of Sog may establish opposing dorsal-to-ventral BMP morphogen gradient. This model predicts the uniform maximum pMad signal in dorsal region in *sog* mutant. However, pMad signal fails to refine and intensify, and amnioserosa markers are lost in *sog* mutant (Ross et al., 2001). Thus a paradox is that Sog and Tsg are BMP inhibitors but required for the dorsal midline development that requires high BMP signal. A model to solve this paradox is that Sog and Tsg can transfer BMP-type ligands towards the dorsal midline (Holley et al., 1996) (Figure 13). Consistently, long-range enhancement of BMP signaling by Sog has been reported (Ashe and Levine, 1999; Decotto and Ferguson, 2001; Eldar et al., 2002). The transport mechanism can also explain the observed robustness of BMP morphogen gradient (Eldar et al., 2002).

Two studies successfully visualized Dpp distribution in the embryo (Shimmi et al., 2005b; Wang and Ferguson, 2005). Shimmi *et al*. utilized genomic HA-tagged Dpp. Wang and Ferguson developed a method to inject anti-GFP antibody into perivitelline space to detect the receptor bound GFP-Dpp ligands (since the embryo develop fast, GFP signal was not directly detected). In both cases, Dpp was redistributed at the dorsal midline and this redistribution was inhibited in *sog* or *tsg* mutant. A current model is that Dpp/Scw heterodimer is redistributed by Sog/Tsg from dorsal-lateral side towards the dorsal midline, where Tld cleaves Sog to release ligands for the signal (Figure 13). Dpp or Scw homodimer contributes to the short-range signal in the dorsal-lateral region of the embryo. Sog cleavage by Tld in a complex with BMPs is critical to produce sharp and robust morphogen gradient (Peluso et al., 2011). The direction of Dpp diffusion is dictated by ventral-to-dorsal Sog gradient (Srinivasan et al., 2002). In addition, positive feedback mechanism appears to be involved in the gradient formation (Wang and Ferguson, 2005).

Biochemically, Dpp/Scw heterodimer has higher signaling activity than Dpp or Scw homodimer. Furthermore Dpp/Scw heterodimer interacts with Sog/Tsg and induces Tld-mediated Sog processing more effectively than each homodimer. Mathematical simulation indicates that morphogen gradient formation mediated by Dpp/Scw heterodimer is more robust against changes in gene dosage (Shimmi et al., 2005b). Usage of heterodimer may be a general mechanism to establish BMP morphogen gradient in the vertebrate embryo as well.

In zebrafish, BMP2b and BMP7 are required non-redundantly for D-V patterning of the embryo, and they have synergistic activity in ventralization of the embryo, suggesting involvement of BMP2b/BMP7 heterodimers (Schmid et al., 2000). Further study showed that only BMP2b/BMP7 heterodimers possess the sufficient signalling activity through distinct classes of type I receptor Alk3/6 and Alk8 (Little and Mullins, 2009). However, since pMad signal could be rescued in the embryo even when two ligands are expressed in non-overlapping regions, Dpp and Scw homodimer may form a complex *in vivo* (Neul and Ferguson, 1998; Nguyen et al., 1998; Wang and Ferguson, 2005). However, such a complex has not been identified yet.

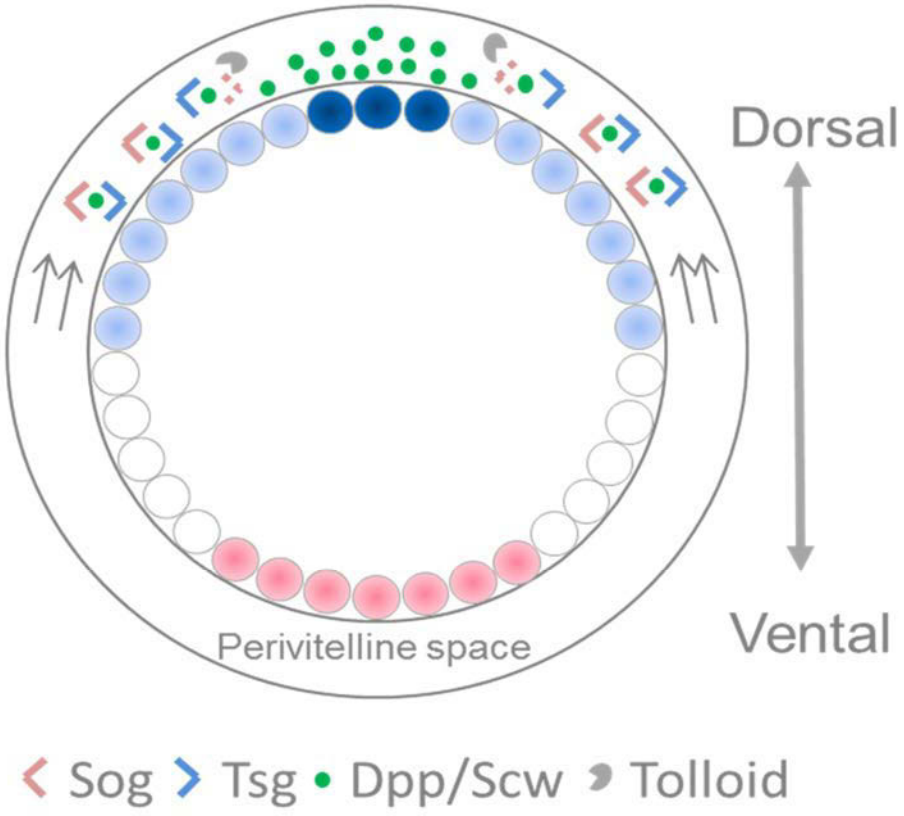

Figure 13. Facilitated Dpp/BMP transport model in the early *Drosophila* embryo. Sog and Tsg make a complex with Dpp/Scw heterodimer in the perivitelline space. This Dpp/Scw/Sog/Tsg complex is moved towards the dorsal midline based on the Sog gradient. Tolloid processes Sog in the complex to release the heterodimer in the dorsal side of the embryo, thus making a sharp morphogen gradient within the dorsal surface of the embryo, where *dpp* is uniformly expressed.

5.2.2. Involvement of ECM components

HSPGs play an important role in BMP morphogen gradient formation in the wing imaginal discs (Bornemann et al., 2004; Fujise et al., 2003; Han et al., 2004; Takei et al., 2004). In contrast, HSPGs are absent in the early embryo due to delayed translation of maternally supplied mRNA of enzymes essential for HSPG synthesis. Since extracellular proteins required for BMP morphogen gradient in the embryo have been shown to interact with HSPGs, the absence of HSPGs may be important to facilitate BMP transport in the embryo. Indeed, exogenous heparin inhibits BMP signaling and D-V patterning (Bornemann et al., 2008). Similarly, BMP diffusion is restricted by HSPGs in *Xenopus* (Ohkawara et al., 2002).

Collagen IV, an ECM component, has been recently shown to be in involved in BMP morphogen gradient formation. C-terminus of Collagen IV binds to Dpp homodimer or Dpp—Scw heterodimer through a basic region at N-terminus of Dpp mature domain, but not to Gbb or Scw, which lacks a basic motif. BMP binding motif in Collagen IV is conserved among species (Wang et al., 2008). Collagen IV also binds to Sog through cysteine rich 1 (CR1) and CR4 domains, but not to Tsg. Biochemical data suggested that Collagen IV acts as a scaffold to assemble Dpp/Scw/Sog/Tsg complex through multiple steps. (1) Dpp—Scw heterodimer and Sog independently bind to Collagen IV, (2) Scw-mediated release of CR4 domain of Sog from Collagen IV, and (3) Tsg releases CR1 domain of Sog from Collagen IV. Collagen IV also promotes ligand-receptor binding (Sawala et al., 2012).

5.3. BMP morphogen gradient in the early *Xenopus* embryo

The shuttling/transport mechanism has been shown to be conserved in the D-V patterning of the *Xenopus* (Ben-Zvi et al., 2008) and *Tribolium* embryo (van der Zee et al., 2006). Here I review BMP morphogen gradient formation in the early *Xenopus* embryo.

When cells in the dorsal blastopore lip (Spemann´s organizer) are transplanted into the ventral side of the host embryo, these cells transform ventral tissues to dorsal tissues to induce a well-proportioned secondary axis (Figure 15B). This classical experiment operated by Hans Spemann showed that the the organizer is critical for the D-V patterning of the early *Xenopus* embryo and introduced “induction” concept in developmental biology (Sander and Faessler, 2001). The search for molecules required for induction by Spemann organizer have identified BMP inhibitors, including Sog ortholog Chordin (Cho et al., 1991; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Smith and Harland, 1992). Many genes required for D-V patterning are conserved between *Drosophila* and *Xenopus*, but their expression patterns are inverted with respect to each other (De Robertis and Sasai, 1996; De Robertis and Kuroda, 2004) (Figure 14); In *Drosophila*, *dpp* is expressed at the dorsal side and *sog* is expressed at the ventral side of the embryo to form dorsal-to-ventral BMP morphogen gradient. In *Xenopus*, *bmp4* and *bmp7* are expressed in the ventral side, and *chordin* is expressed in the dorsal side of the embryo to establish ventral-to-dorsal BMP morphogen gradient.

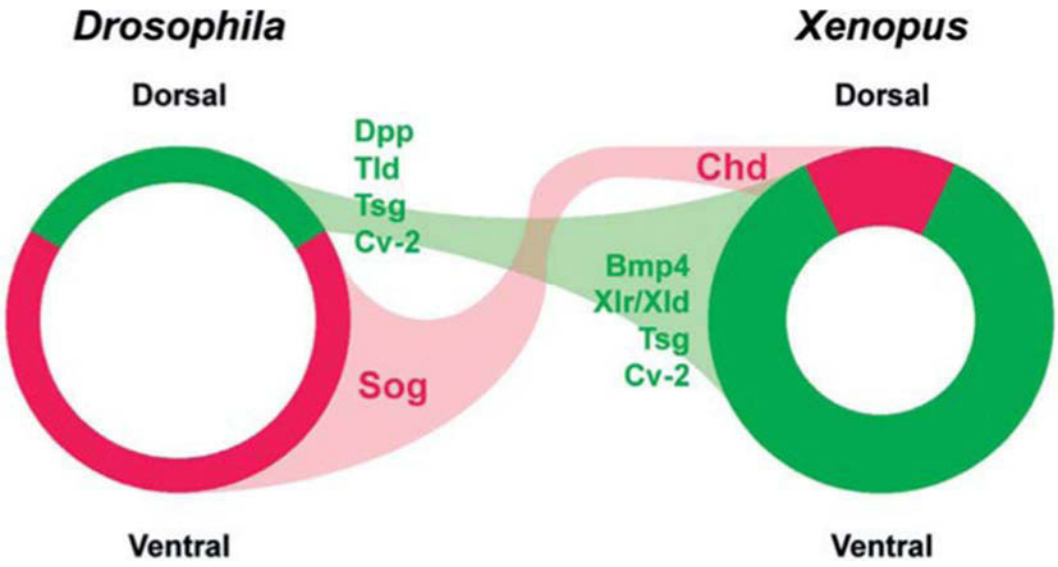

Figure 14. Inverted D-V patterning in *Drosophila* and *Xenopus* embryo. In *Drosophila* embryo (left), *dpp* is expressed in the dorsal side of the embryo and *sog* is expressed in the ventral side of the embryo. In contrast, BMP ligands are expressed in the ventral side of the embryo and Chordin, Sog homologue, is expressed in the dorsal side of the *Xenopus* embryo (right). (Based on De Robertis and Kuroda 2004.)

Recent studies showed that robust BMP morphogen gradient is also established by Chordin mediated transport mechanism in *Xenopus* embryo (Ben-Zvi et al., 2008). Tsg and Tolloid are also conserved in *Xenopus* (Chang et al., 2001; Piccolo et al., 1997). The mechanism also accounts for the remarkable plasticity of embryonic pattern against experimental disturbance (Ben-Zvi et al., 2008). For example, bisected dorsal halves of the embryos can still develop into well-proportioned tadpoles, and a well-proportioned secondary axis is induced by Spemann organizer (Figure 15). To achieve this remarkable scaling, BMPs secreted from ventral side are not sufficient but another BMP-type ligand Anti Dorsalizing Morphogenetic Protein (ADMP) is also required. Unlike other BMPs, ADMP expression is repressed by BMP signal and restricted in the dorsal side of the embryo (Moos et al., 1995) (Figure 16). ADMP can activate BMP signal through ALK2 (Reversade and De Robertis, 2005). Thus ADMP behaves as an expander in "expansion-repression" model (Ben-Zvi and Barkai, 2010). In the dorsal half of the dissected embryo (Figure 15A), ADMP expression increases and is transported towards the new ventral side by Chordin to compensate the reduction of BMPs (Ben-Zvi et al., 2008; Reversade and De Robertis, 2005). In addition, BMP2 is also expressed in the dorsal side of the embryo by low level of BMP signal and compensates the reduction of BMP signal (Inomata et al., 2008) (Figure 16).

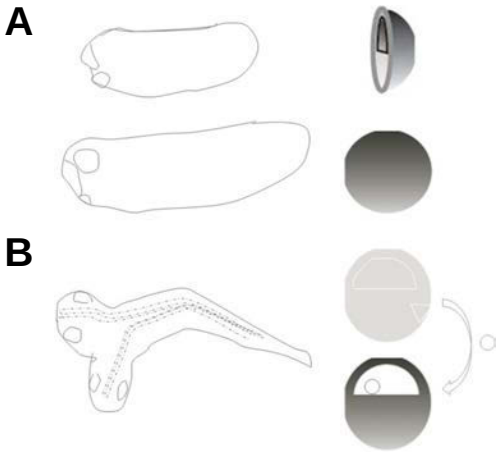

Figure 15. Spemann's experiments.
(A) The dorsal half of the embryo can still develop into well-proportioned tadpole despite its small side. (B) The secondary axis is induced when the Spemann's organizer is transplanted. (Based on Ben-Zv *et al*., 2008.)
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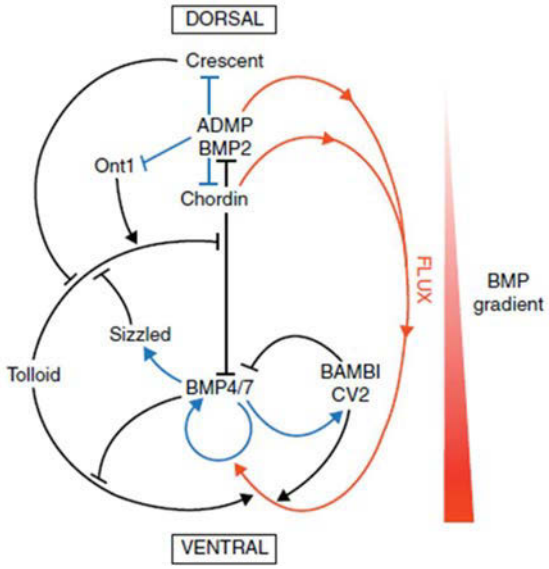

Figure 16. A complex network for BMP morphogen gradient in *Xenopus* embryo.
In contrast to *Drosophila* embryo, various extracellular factors help establish the BMP morphogen gradient in the *Xenopus* embryo. (Based on Plouhinec *et al*., 2011.)

There are some differences in the regulation of Tolloid activity between *Xenopus* and *Drosophila*. Process of Sog by Tolloid is dependent on BMP ligands in *Drosophila* but not in *Xenopus in vitro* (Marques et al., 1997; Peluso et al., 2011; Piccolo et al., 1997). In *Xenopus*, Tolloid-mediated Chordin degradation is enhanced by Ont1, a secreted scaffold protein of Olfactomedin family (Inomata et al., 2008). Tolloid activity is also inhibited by secreted frizzled-related proteins (sFRPs), Sizzled and Crescent (Cres) (Lee et al., 2006; Muraoka et al., 2006; Ploper et al., 2011) or by BMPs through non-competitive inhibition mechanism (Lee et al., 2009) (Figure 16).

In addition to core shuttling/transport components, additional extracellular regulators are expressed. For example, Goosecoid (Cho et al., 1991), Noggin (Smith and Harland, 1992), Follistatin (Hemmati-Brivanlou et al., 1994) are BMP inhibitors produced in Spemann organizer that physically prevent BMPs from binding to receptors but lack the activity of transporting ligands. Crossveinless-2 (Cv-2) is a secreted BMP binding protein expressed in the ventral side with five CR domains and structurally related to Sog/Chordin protein. Cv-2 is unique in that it shows both pro- and anti- BMP function. While Cv-2 generally acts as a negative feedback inhibitor, Cv-2 antagonizes Chordin when Chordin levels are increased, thus displaying pro-BMP function. Cv-2 is proposed to facilitate the flux of Sog by acting as a sink at the ventral side of the embryo (Ambrosio et al., 2008; Plouhinec et al., 2011). Cv-2 was originally identified in *Drosophila* as a mutant showing crossveinless phenotype. As discussed later, Cv-2 has also pro-BMP function in posterior crossvein (PCV) formation (Conley et al., 2000). In conclusion, the extracellular network to support the robust morphogen gradient appears more complex in *Xenopus* than in *Drosophila*.

5.4. Shuttling/Transport mechanism in other signaling pathways

Recent studies indicated that the transport/shuttling mechanism is more generally utilized in other signaling pathways.

5.4.1. Spz-Tl pathway

A recent study has shown that sharp and robust Spz/Tl activity gradient is also established by inhibitor-mediated shuttling/transport mechanism (Haskel-Ittah et al., 2012). As discussed, Spz/Tl signal is required for the graded nuclear localization of Dl that specifies D-V axis in the early *Drosophila* oocyte and embryo (Moussian and Roth, 2005). Interestingly, whereas Spz is activated by Pipe in the ventral 40 % of the embryo, the sharp Dl gradient is generated within the pipe domain.

Numerical screening identified the shuttling/transport mechanism as a mechanism that can produce the robust and sharp Spz/Tl activity gradient (Haskel-Ittah et al., 2012). According to the model, Spz is first processed into N-Spz and C-Spz by protease Easter (Ea), which remain associated as active ligands. Upon binding to Tl, the active ligand can transduce the signal and is also dissociated into N-Spz and C-Spz. N-Spz is a diffusible inhibitor and C-Spz is a less-diffusible active ligand. They rebind easily to form inactive diffusible complexes (Figure 17).

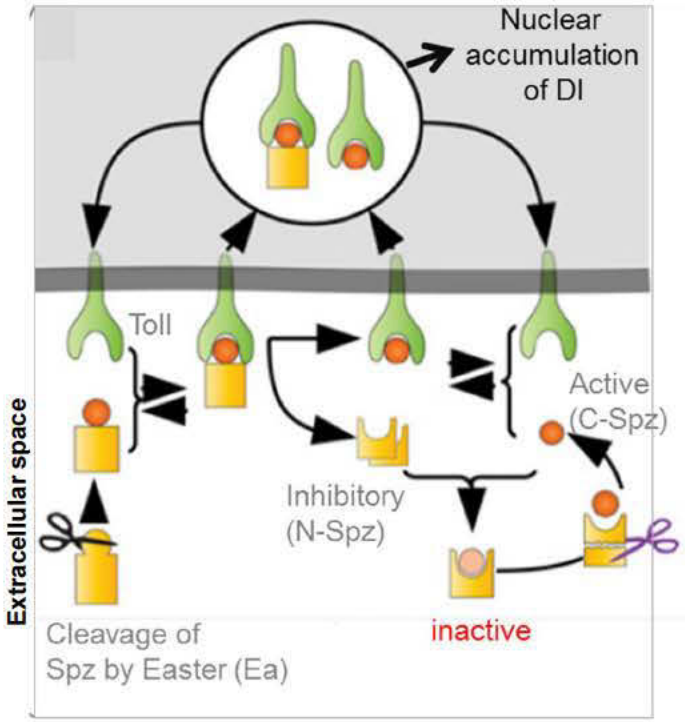

Figure 17. Biochemical reactions to produce active Spz ligands. Cleavage of Spz by Ea produces active ligands consisting of N- and C-Spz. The ligand is dissociated into N-Spz and C-Spz by binding to Tl. N-Spz inhibits the active C-Spz by forming inactive complex. Putative protease releases C-Spz by cleaving N-Spz. (Based on Haskel-Ittah *et al.*, 2012.)

The inactive complex is redistributed towards the ventral side, and released via inactivation of N-Spz by putative proteases (Figure 17, 18). The molecular characteristics are thus reminiscent of BMP transport mechanism, where the inhibitor Sog mobilizes BMPs, and Tld processes Sog to release BMPs. In BMP transport, *sog* expression outside the Dpp expression domain predicts the position of highlighted BMP signaling. However, such prepatterned cues do not exist in Spz transport. The driving force that redistributes C-Spz towards the ventral midline is the gradient of N-Spz generated by uniform diffusion of N-Spz and inactivation of N-Spz in the ventral 40% of the embryo. Thus the directionality is established in a "self-organized" manner. The putative protease predicted in the model remains to be identified.

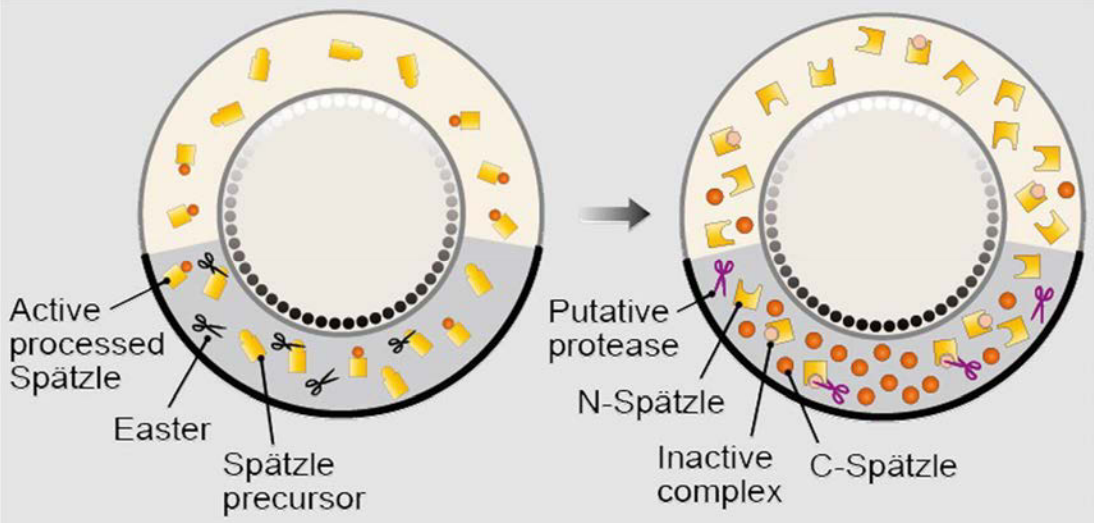

Figure 18. Self-organized shuttling mechanism for Spz morphogen gradient formation.
Easter processes Spz in the ventral 40% of the embryo (left). Upon binding to Tl, N-Spz and C-Spz are separated. N-Spz (inhibitory ligand) is diffusible and spread into the perivitelline space (right). C-Spz (active ligand) is not diffusible but mobile when bound to N-Spz. N-Spz in the inactive complex is cleaved by putative protease in the ventral side so that N-Spz gradient from dorsal to ventral side of the embryo is generated. The self-organized N-Spz gradient accumulates C-Spz ligands towards the ventral midline.
(Based on Haskel-Ittah *et al*., 2012.)

5.4.2. Wnt pathway

Similar transport mechanism is also proposed in the diffusion of Wnts in the early *Xenopus* embryo. It has been recently shown that Wnt8 and Wnt11 do not diffuse as previously thought. Instead, two sFRPs (Frzb and Cres) diffuse effectively and facilitate the diffusion of Wnts, although sFRPs have been mainly characterized as Wnt inhibitors. Accordingly, sFRPs are required for activity gradient of Wnt signaling. It has been thus proposed that sFRPs act as "conveyers" of Wnts to expand Wnt signaling area (Mii and Taira, 2009; Mii and Taira, 2011). Since sFRPs are not processed via binding to Wnts, Wnts appear to be released through reversible interaction of sFRPs and Wnts. The similar positive role of sFRPs for Wnt signaling was also reported in specification of optic cup in mice (Esteve et al., 2011). These data suggest that the shuttling/transport mechanism is more generally utilized in the early embryo. This is probably because a complex of transcriptional network consisting of zygotic genes cannot be used to refine the sharp gene expression boundaries in the early embryo (Haskel-Ittah et al., 2012).

5.5. BMP transport in the *Drosophila* wing vein development

Is the shuttling mechanism a specific mechanism in the early embryo? Recent studies proposed that BMP transport mechanism is also utilized in the wing vein specification during pupal stages in *Drosophila*.

5.5.1. Induction of LVs in the larval stages

Wing veins in *Drosophila* consist of four main longitudinal veins (LVs, L2~L5) that run from proximal to distal side of the wing, and two crossveins (CVs, anterior CV [ACV] and posterior CV [PCV]) that connect two LVs (Figure 19). Adult wing veins are hollow, fluid-filled tubes between the two wing epithelial layers that carry nutrients to living cells, and acts as rigid support structures for flight in adult fly. LVs are initially specified by morphogens (Hh and BMPs) along A-P axis during the larval stages (Figure 19). Hh induces L3/L4 and regulates the space between them. Dpp acts as a morphogen to specify L2 and L5. These morphogen activities define the expression of transcription factors that specify LVs (for example, *kni* in L2, *iro-C* in L3/L5) and intervein marker *blistered* (*bs*), encoding Drosophila Serum Response Factor (DSRF). LVs are further refined into narrow stripes by the mutually regulated EGFR signal that promotes vein fates and Notch signal that restricts them. Consequently, EGFR is active in LVs and Notch in the cells adjacent to LVs by Delta expressed in LVs (Blair, 2007).

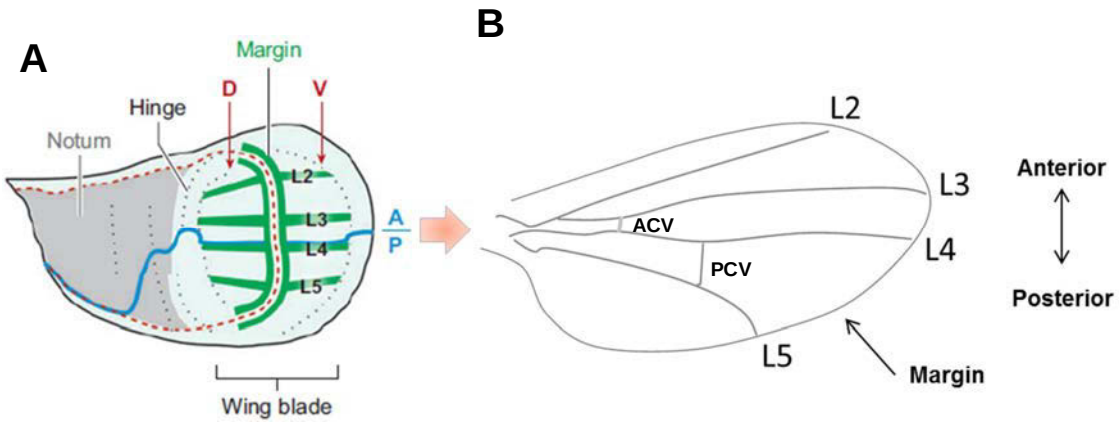

Figure 19. The positions of the future LVs in the Drosophila wing imaginal disc.
(A) The main LVs (L2-L4) are specified along A-P axis during the larval stages (B) The positions of LVs and CVs in the adult wing. (Based on Blair 2007.)

5.5.2. Maintenance of LVs in the pupal stages

During prepupal and initial pupal stages, the wing imaginal disc everts and folds to form apposed dorsal and ventral epithelia of the wing blade. Thus single wing epithelium becomes two layers during pupal stages. EGFR and Notch continue to maintain LVs fate in the pupal wing. In addition, *dpp* expression disappears from the A-P boundary region, and starts at the LVs and later also at the PCV region (27~28 hr after pupariation [AP]) (Ralston and Blair, 2005). BMP signal appears to maintain LVs fates downstream of EGFR and Notch signal. Molecularly defined wing veins undergo morphogenesis during pupal stages. The wing vein morphogenesis involves apposition of the two wing layers and cell shape change. EGFR/Ras

signaling regulates apical DE-cadherin localization to control wing vein morphogenesis in LVs, independently of BMP signaling that maintains wing vein cell fates (O'Keefe et al., 2007).

5.5.3. Induction of CVs: Long-range function of BMP signal in PCV

In contrast with LVs, CVs are defined during pupal stages. To date, the earliest molecular marker that positively recognizes the PCV region is BMP signaling. Interestingly, although *dpp* is expressed in part of ACV region, *dpp* is not expressed at the PCV region when BMP signal is detected during 18—28 hr AP (Ralston and Blair, 2005). This observation suggests a long-range function of Dpp to instruct PCV fate. Recent studies revealed that PCV development requires BMP-type ligands (Dpp and Gbb), BMP binding proteins (Sog and Tsg paralog Crossveinless [Cv]), and protease (Tld paralogue Tolloid-related [Tlr]), raising a possibility that the long-range BMP signaling at PCV region is mediated by BMP transport mechanism (O'Connor et al., 2006) (Figure 20). Although Dpp distribution has not been visualized in the pupal wing, biochemical data support the transport mechanism where Dpp—Gbb heterodimer is transported by Sog—Cv from LVs towards the PCV region and released by Tlr-mediated Sog processing (Serpe et al., 2005; Shimmi et al., 2005a). Despite the similarity, there are some differences in biochemical properties between two processes. First, Dpp—Gbb heterodimer signal requires only Tkv, and does not induce synergistic signal like Dpp—Scw heterodimer (Shimmi et al., 2005a). Second, Tlr processes Sog with slower kinetics than does Tld and Tld could not rescue the PCV-less phenotype of Tlr mutant (Serpe et al., 2005).

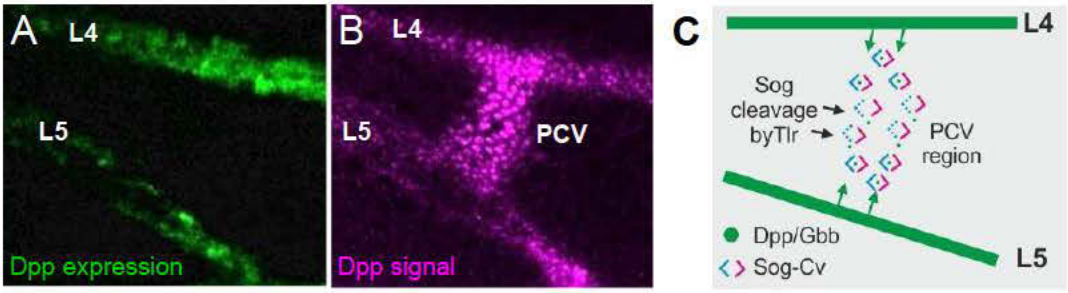

Figure 20. BMP transport model at the PCV region. (A) *dpp* expression pattern at 24 hr AP. (B) BMP signal at 24 hr AP. (C) A model of BMP transport from LVs towards the PCV region. (Based on I).

5.5.4. Identification of BMP signaling regulators using PCV as a model

The critical role of BMP signaling in the early PCV formation makes the PCV sensitive to loss of BMP signaling. This feature has been used to identify and characterize BMP signaling regulators. There are several naturally isolated *crossveinless* (*cv*) mutants including *cv*, *cv-2*, *cv-3*, *cv-like5*, *cv-like6*, *cv-b*, *cv-c*, and *cv-d*. To date, genes responsible for *cv*, *cv-2*, *cv-c* and *cv-d* have been identified.

Cv is an paralog of Tsg and an extracellular BMP binding proteins with CR domains that shares similar biochemical property as Tsg (Shimmi et al., 2005a). Cv-2 is an extracellular

BMP binding proteins with five CR domains (Conley et al., 2000). Unlike Sog, Cv-2 is not involved in transporting BMPs but is rather required for BMP signal in a short-range manner by promoting transfer of BMPs to the receptors via HSPG. Cv-2 expression is induced at the PCV region by BMP signaling, thus consisting of a positive feedback loop to refine the signal (Serpe et al., 2008). Crossveinless-D (Cv-D) has been recently identified as a lipoprotein similar to the vitellogenins that comprise the major constituents of yolk in animal embryo (Chen et al., 2012). Cv-D binds to Dpp and Gbb through its Vg domain, and to HSPGs to promote Dpp signaling at the PCV region. Interestingly, *cv-d* expression is absent from the wing but Cv-D protein is supplied by the hemocytes that circulate the lumen of the pupal wing. It has been shown that the function of lipoprotein is not only to transport lipid from the fat body to the tissue via the hemolymph but also to deliver the lipid-linked signaling molecules such as Hedgehog and Wnts (Panakova et al., 2005). BMP transport may require lipid-lipoprotein complex, although BMPs are not lipid-linked proteins (Chen et al., 2012). Unlike these extracellular BMP binding proteins, Crossveinless-C (Cv-C) has been shown to encode a RhoGAP. Cv-C is expressed in a variety of embryonic tissues undergoing morphogenesis and required for morphogenesis through regulating actin organization by inactivating Rho-type small GTPases (Brodu and Casanova, 2006; Denholm et al., 2005). However, how Cv-C is involved in the PCV formation and whether Cv-C is involved in BMP signaling remain unknown.

Although other mutant stocks were lost in past, *cv-3* is located in 67F-68A, *cv-like5* is in 3-48.1 (on chromosome III), *cv-like6* is in 1-59.1 (on chromosome X), *cv-b* is in 3-65 (chromosome III) (http://flybase.org/). This information may help identify novel BMP signaling regulators in the future.

5.5.5. Diversified wing vein patterns among insects

Insects wings are thought to have originated only once in the arthropod lineage. They are thought to have originated as two pairs of membranous wings and have undergone considerable variation in shape, size, color, and wing venation between species. These variations are also found between fore- and hind-wing within individuals (De Celis and Diaz-Benjumea, 2003).

In *Drosophila*, difference between the membranous forewing (wing) and the modified hindwing (haltere) (Figure 21) depends on the Hox gene *Ultrabithorax* (*Ubx*), which is expressed in the hindwing but not in the forewing. Ubx suppresses the forewing fate and promote haltere fate, while the forewing (wing) develops without Hox input. (Lewis, 1978; Weatherbee et al., 1998). The small haltere size is, in part, regulated by reducing transcription and mobility of Dpp in the haltere by Ubx (Crickmore and Mann, 2006; Crickmore and Mann, 2007). The forewing (wing) establishes stereotyped wing vein patterns via evolutionarily conserved signaling pathways as already described.

Tribolium castaneum (beetle) is one of the best studied model insects outside *Drosophila*. RNAi mediated gene knockdown system has been established in *Tribolium* (Philip and Tomoyasu, 2011) and its genome has been sequenced (Tribolium Genome Sequencing Consortium et al., 2008). In *Tribolium*, the forewing is the modified wing (elytra) and the hindwing is the ancient membranous wing (Figure 21). However, like in *Drosophila*, *ubx* is expressed in the hindwing to promote hindwing fate (Tomoyasu et al., 2005). Thus Hox-free forewing development has been evolved to form elytra, and Ubx inhibits these changes to promote membranous wing fate. Surprisingly, the core wing gene network was found to be

conserved between *Drosophila* and *Tribolium* (even in elytra). It has been proposed that the elytra developmental program has been co-opted downstream of the wing gene network multiple times (Tomoyasu et al., 2009).

Despite these studies, the wing vein development of other insects remains largely unknown. A potential model organism is the sawfly *Athalia rosae*, which belongs to the order Hymenoptera. The sawfly has two pairs of ancient membranous wings with diversified wing vein patterns (Figure 21). Since an RNAi system has been also established (Sumitani et al., 2005), sawfly serves as an excellent model to study how ancient insect wing vein patterns are established.

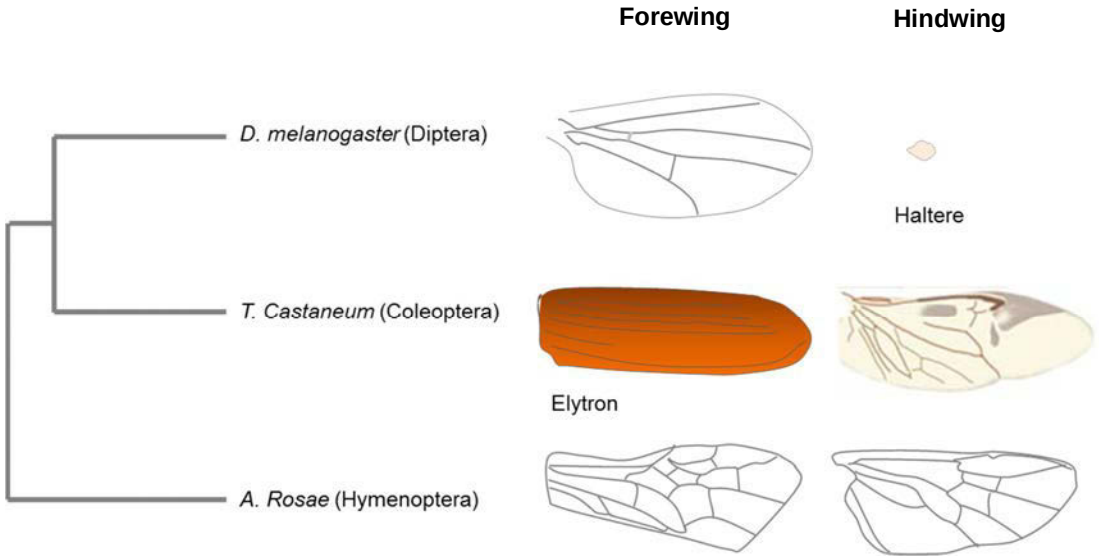

Figure 21. Diversified wing vein patterns among insects. In *Drosophila*, forewing is the membranous wing and hindwing (haltere) is the modified wing. In *Tribolium*, forewing is the modified wing (elytron) and hindwing is the membranous wing. In *Drosophila* and *Tribolium*, Ubx suppresses the forewing fate to promote the hindwing fate. In sawfly, both fore- and hind-wing have membranous wing with different wing vein patterns.

Aims of the study

In this thesis, I focus on how the distribution of BMP-type ligand is regulated at the extracellular level to specify diversified insect wing vein patterns using Dipteran *Drosophila melanogaster* and Hymenopteran sawfly *Athalia rosae* as models.

1. Visualization of Dpp/BMP distribution in the *Drosophila* pupal wing

Recent studies propose that Dpp/BMP transport mechanism utilized in the early embryo also functions during wing vein specification in *Drosophila*. However, Dpp distribution and its regulation have not been studied in the pupal wing. Using GFP-Dpp, I aim to visualize Dpp distribution in the *Drosophila* pupal wing and address the mechanism behind this.

2. The function of RhoGAP Cv-C during wing vein morphogenesis in *Drosophila*

Mature wing veins are formed through apposition and cell shape changes between two wing layers. To address how Dpp signal instructs wing vein morphogenesis, I focused on the function of RhoGAP Cv-C during the PCV formation. Cv-C viable mutant shows PCV-less phenotype but it remains unknown how *cv-c* is involved in the PCV formation.

3. The extracellular regulation of Dpp/BMP in the sawfly wing vein formation

To date, molecular mechanisms underlying wing vein specification are mostly derived from the studies in *Drosophila*. Little is known about how the wing vein patterns are generated in other insects. Since an RNAi system has been established (Sumitani et al., 2005), and Dpp has been isolated in sawfly (Yamamoto et al., 2004), I introduced sawfly as a model to test how Dpp/BMP signal is involved in establishing distinct fore- and hindwing vein patterns.

Materials and Methods

Materials and methods used in this study are listed as follows. For a detailed description of methods, see the original publications.

Methods and Materials	Article
Fly strains	I, II, III
Drosophila S2 cells and culture	III
Plasmid construction	I, III
Immunohistochemistry	I, II, III
in situ hybridization	I, II, III
sawfly	III
Luciferase assay	III
RT-PCR	III
Western blotting	III

Fly strains

Flies	Source
cv^{70}	Shimmi *et al*., 2005a
sog^{p129D}	Serpe *et al*., 2005
gbb^{5I}	K. Wharton
$cv\text{-}2^{KO1}$	M. O'Connor
cv-c1	H. Skaer
$cv\text{-}c^{c524}$	H. Skaer
mys^{nj42}	F. Schoeck
dpp^{s11}	Bloomington Drosophila Stock Center
dpp^{s4}	Bloomington Drosophila Stock Center
$cdc42^{2}$	Bloomington Drosophila Stock Center
$Rho1^{72O}$	Bloomington Drosophila Stock Center
$Rac2^{\Delta}$	Bloomington Drosophila Stock Center
$Rac1^{J11}$	Bloomington Drosophila Stock Center
UAS-GFP	Bloomington Drosophila Stock Center
sog^{P11885}	Bloomington Drosophila Stock Center
mys^{1}	Bloomington Drosophila Stock Center
UAS-GFP-Dpp	Teleman *et al*., 2000
UAS-sog-HA	Shimmi *et al*., 2005a, b
UAS-tsg-His	Shimmi *et al*., 2005a, b
UAS-scw-HA	Shimmi *et al*., 2005a, b
Ubi-CFP-Rab5	Marois *et al*., 2006

UAS-gbb	K. Wharton
UAS-tkv-HA	M. O'Connor
UAS-tkvQ199DHA	M. O'Connor
UAS-ΔE-*tkvQ199D*HA	M. O'Connor
UAS-PNKG58AeGFP	J.C. Hombria
UAS-Venus-gbb	This study (I)
UAS-Ar-dpp-HA	This study (III)
UAS-Ar-tsg/cv	This study (III)
UAS-tkv RNAi (#3059)	Vienna *Drosophila* RNAi Stock Center
UAS-Mad RNAi (#12635)	Vienna *Drosophila* RNAi Stock Center
UAS-Rho1RNAi	Bloomington Drosophila Stock Center
dpp^{shv}-lacZ	Bloomington Drosophila Stock Center
tkv-lacZ	T. Tabata
Vkg-GFP	Fly Trap stock collection
BS1348-Gal4	Bloomington Drosophila Stock Center
dpp^{shv}-Gal4	Ramel *et al*., 2007

Plasmid construction (I)

For generation of *Venus-gbb*, the *Venus*-coding sequence was inserted before the Flag tag of *gbb-Flag* (after amino acid E349 of the *gbb* cDNA). *Venus-gbb* was then cloned into pENTR (Invitrogen) and further subcloned into destination vectors available at the *Drosophila* Gateway Vector Collection (*Drosophila* Genomics Resource Center).

Immunostaining and *in situ* hybridization (I, II, III)

Drosophila pupal wings were fixed at 4 °C overnight and then dissected from the pupae. All immunohistochemistry and *in situ* hybridizations were performed using standard protocol. The primary antibodies were as follows: rabbit anti-pMad at 1:1000 (a gift from P. ten Dijke), mouse anti-LacZ at 1:1000 (Promega), mouse anti-*Drosophila* serum response factor (DSRF) at 1:2000 (Geneka), mouse anti-β-integrin (CF.6G11) at 1:100 (Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-aPKC (C-20) at 1:200 (Santa Cruz Biotechnology, Inc.), and mouse anti-Dlg (4F3) at 1:100 (DSHB). The secondary antibodies were as follows: anti-rabbit IgG-Alexa 568 or 647 and anti-mouse IgG-Alexa 488 were used at 1:1000, respectively (Invitrogen). Can Get Signal Solution B (TOYOBO) was used for staining with anti-pMad and anti-β-integrin. F-actin was visualized by Alexa Fluor 568 Phalloidin at 1:300 (Invitrogen). Fluorescent images were obtained with a Leica TCS SP5 confocal microscope.

Sawfly (III)

The general biology, staging, and maintenance of laboratory stocks of the sawfly *Athalia rosae* were described previously (Oishi et al., 1993). The last instar larvae were identified by their color and wandering behavior, and prepupae taken from the cocoons were staged as PCF 1–4 (each stage lasts 24 hr). Sawfly prepupal tissues were fixed in 4% formaldehyde at 4 ^{o}C overnight and subjected to immunohistochemistry or *in situ* hybridization. Fixed tissues were blocked with normal goat serum (Sigma) and 0.1% Tween-20 in PBS at 4 ^{o}C overnight and then incubated with the primary antibody rabbit anti-pMad at 1:1000 (a gift from P. ten Dijke) at 4 ^{o}C overnight. Anti-rabbit IgG-AP (alkaline phosphatase; Promega) was used at

1:1000 as the secondary antibody. *In situ* hybridization was done, using digoxigenin-labeled *Ar-dpp* or *Ar-tsg/cv* RNA probes, prepared as described previously (Yamamoto et al., 2004). RNAi applications to sawfly were described previously (Sumitani et al., 2005). Briefly, the last instar larvae and early or late PCF3 prepupae were anesthetized by chilling and dsRNA was injected into the dorsal hemocoel at the second and third abdominal segment, using a fine glass needle.

Isolation of sawfly-tsg/cv DNA (III)

The sequence of sawfly *tsg/cv* gene was isolated by PCR with degenerate primers corresponding to a conserved region among insect Tsg/Cv genes:
5'-TGYAAYGARGCNATHTGYGC-3' for CNEAICA
5'-CCDATRCAYTCRCARCANCC-3' for GCCECIG
Here, R = A+G, Y = C+T, N = A+C+G+T, H=A+T+C, and D = G+A+T. A cDNA library synthesized from 48 hr-old embryos was employed as a template. PCR reactions were carried out, using GoTaq polymerase (Promega) under conditions of 94 ^{o}C for 2 min, and 30 cycles of 94 ^{o}C for 2 min, 58 ^{o}C for 1 min and 72 ^{o}C for 2 min. The 5' and 3' adjacent sequences were obtained by RACE, as previously described (Yamamoto et al., 2004).

Preparation of dsRNA (III)

A cDNA fragment of *Ar-dpp* (252bp) was PCR-amplified from the fragment-carrying pSPT19 plasmid, using the T7 and SP6 sequences as primers. A cDNA fragment of *Ar-tsg/cv* was amplified from a partial cDNA in pSC-A (Stratagene) with the following primers flanked by T7 promoter sequences at each 5' end:
5'-TAATACGACTCACTATAGGGACGAGCTGCAAATGCGGTTTAGT-3'
5'-TAATACGACTCACTATAGGGACGAACCACCTGTAACTCGTCGC-3'
A 298-bp fragment of the *egfp* cDNA was amplified from the pPIGA3GFP/hspGFP-S65T transformation vector plasmid and cloned into pCRII-TOPO (Invitrogen). A fragment containing *egfp* cDNA, T7, and SP6 promoter sequences was amplified, using M13 forward and reverse primers. Sense and antisense strands of each fragment were transcribed, using a MEGAscript kit (Ambion) and annealed in distilled water to form dsRNAs.

Reverse transcription (RT)-PCR (III)

The head, abdomen, digestive tract, and fat body of sawfly prepupae were dissected in an RNAlater RNA stabilization reagent (Qiagen) and thoracic tissue was incubated at 4 ˚C overnight in the reagent. Pairs of wing buds (fore- and hindwing) with attached to larval cuticles were removed from the thorax, frozen in liquid nitrogen, and stored at -80 ˚C. Total RNA was extracted from frozen wing buds, using an RNeasy Micro kit (Qiagen). A OneStep RT-PCR kit (Qiagen) was utilized with 50 ng of total RNA as a template. The amplifications were conducted for 25 cycles for *Ar-dpp* and *Ar-tsg/cv* and 20 cycles for *Ar-elongation factor 1-alpha (Ar-ef-1α)*, with annealing temperatures of 65 ˚C for *Ar-dpp* and *Ar-ef-1α* and 60 ˚C for *Ar-tsg/cv*. The gene-specific primers were listed in the publication III:

Luciferase assay (III)

The reporter constructs were generous gifts from G. Pyrowolakis. *Drosophila* S2 cells were transfected with the Effectene reagent (Qiagen). We transfected 1×10^6 cells with 20 ng of the reporter Dad13 plasmid, 4 ng *Renilla* luciferase plasmid, and various combinations of *dpp*, *sog*, and *cv* expression plasmids. All experiments were done in duplicate. The cells were lysed 3 days after transfection and analyzed for firefly and *Renilla* luciferase activities, using a dual luciferase reporter assay (Promega).

Results and Discussion

1. Control of Dpp distribution in the *Drosophila* pupal wing (I)

1.1. Visualization of Dpp distribution in the *Drosophila* pupal wing

In the *Drosophila* pupal wing, BMP signal is activated at all the wing veins when Dpp expression is restricted at the future LVs at 24 hr AP. Dpp may diffuse ubiquitously and differential competence to the signal in the receiving cells may activate BMP signal only in the future wing veins. Alternatively, Dpp may diffuse directionally to the PCV region and act locally in LVs. To distinguish between these possibilities, GFP-tagged Dpp (Entchev et al., 2000; Teleman and Cohen, 2000) was used to visualize Dpp distribution in the *Drosophila* pupal wing. GFP-Dpp could rescue the loss of wing vein phenotype of *dpp* mutant when expressed in the LVs (Dpp expressing cells) by dpp^{shv}-Gal4, showing that GFP-Dpp is functional. Using this system, I found long-range Dpp diffusion from LVs to PCV region and short-range Dpp diffusion in LVs (I, Figs. 1 and 4) (Figure 22).

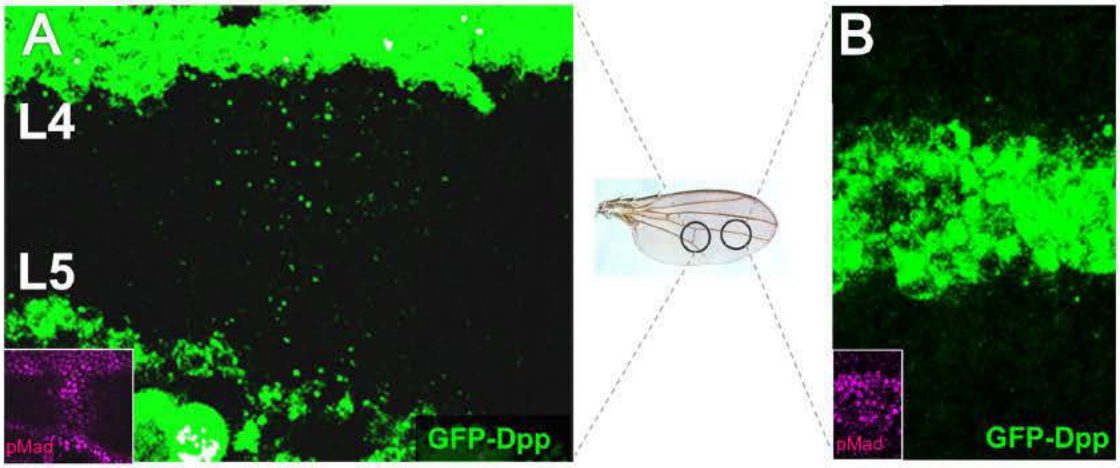

Figure 22. GFP-Dpp distribution in the *Drosophila* pupal wing. (A) Long-range GFP-Dpp diffusion from LVs towards the PCV region, where pMad signal is activated. (B) Short-range GFP-Dpp diffusion in LVs. (Based on I).

1.2. Long-range Dpp diffusion is mediated by BMP transport mechanism

Sog/Cv mediated BMP transport mechanism has been proposed for the long-range BMP signal at the PCV region (O'Connor et al., 2006; Serpe et al., 2005; Shimmi et al., 2005a). I found that GFP-Dpp diffusion was severely blocked in *sog* or *cv* mutant, and enhanced by overexpressing *sog* and *tsg*, a paralog of *cv* (I, Fig. 2). Furthermore, GFP-Dpp transport was also missing in *gbb* mutant (I, Fig. 3H). These data support the previously proposed BMP transport mechanism, in which Dpp-Gbb heterodimer is transported by Sog-Cv from LVs towards the PCV region.

1.3. Short-range Dpp diffusion is mediated by type I receptor Tkv

In contrast to the long-range Dpp transport towards the PCV region, Dpp did not diffuse from LVs (I, Fig. 4A) (Figure 22B). What restricts Dpp diffusion in LVs? In the larval wing imaginal disc, low level of Tkv in Dpp producing cells facilitates Dpp diffusion, whereas high level of Tkv in lateral region interferes with Dpp diffusion (Lecuit and Cohen, 1998; Tanimoto et al., 2000). To test this possibility, Tkv was knocked down in LVs by RNAi. Under these conditions, GFP-Dpp diffused from LVs to the adjacent intervein cells (I, Fig. 4B). This indicates that Tkv expressed in LVs restricts Dpp diffusion. However, *tkv* expression is actually low in LVs and high in the adjacent cells along LVs (I, Fig. 5A) and Dpp can potentially diffuse over the high Tkv expressing cells (I, Fig. 4B). Therefore additional mechanisms must be involved.

1.4. Short-range Dpp diffusion requires a positive feedback mechanism

It has been shown that mutant clones of *mad*, *shn*, or *tkv* generated on the wing veins induce split wing veins as in Tkv RNAi wing (Burke and Basler, 1996; Marquez et al., 2001; Torres-Vazquez et al., 2000). Therefore, I wondered if BMP signaling activity is also required to restrict Dpp diffusion in LVs. This hypothesis was demonstrated in three ways. First, when BMP signal was inhibited in LVs by Dad, GFP-Dpp diffused from LVs (I, Fig. 5B). Second, loss of *mad* in LVs by RNAi induced non-cell autonomous BMP signal activation along LVs (I, Fig. 5H, N). Third, when BMP signal was activated in LVs by constitutively active Tkv, BMP signal was blocked at the PCV region (I, Fig. 5E). In contrast, overexpression of wild-type Tkv in LVs did not inhibit BMP signal at the PCV region (I, Fig. 5C). These results suggest that BMP signal is required for Dpp retention in LVs. However, this raises a possibility that Tkv may not be directly involved in the retention of BMP, because BMP signaling is also reduced when Tkv is knocked down (I, Fig. 5B). To test this, constitutively active Tkv that lacks its extracellular domain (Δ-caTkv) was expressed in LVs. BMP signal at the PCV region was not blocked by Δ-caTkv (I, Fig. 5G). This suggests that extracellular domain of Tkv plays a critical role in Dpp retention through direct binding to Dpp and BMP signal is not sufficient to restrict Dpp diffusion. In summary, Dpp diffusion is restricted in LVs by a positive feedback mechanism as well as Tkv.

1.5. Is BMP transport directional towards the PCV region?

Visualization of GFP-Dpp distribution revealed that BMP transport is directed at the future PCV region (I, Fig. 1) (Figure 22A). Does this reflect directionality in the extracellular BMP transport? Since GFP-Dpp dots at the PCV region were highly co-localized with CFP-Rab5, an early endocytosis marker, GFP-Dpp dots do not actually reflect the extracellular Dpp distribution (I, Fig. S2) but reflect the ligands undergoing endocytosis. It is therefore possible that (1) Dpp diffuses directionally to the PCV region, or (2) Dpp diffuses uniformly but selectively binds to the receptor at the PCV region, or (3) Dpp diffuses and internalizes uniformly but differential stability or trafficking leads to Dpp accumulation at the PCV region. The latter two scenarios predict differential competence on Dpp binding or trafficking between the intervein regions and the PCV region. However, GFP-Dpp and pMad accumulated in the intervein regions via ectopic expression of *sog* and *tsg* in the LVs (I, Fig. 2E). Furthermore, loss of active retention in the LVs resulted in GFP-Dpp and pMad accumulation along the LVs (I, Fig. 4B). Thus the intervein regions retain the competence to receive Dpp signaling. These data indicate that Dpp diffuses directionally to the PCV region.

1.6. What directs Dpp transport towards the PCV region?

If Dpp is directionally transported towards the PCV region, what determines its direction? In other words, what specifies the PCV position? In D-V patterning of the embryo, Dpp is redistributed towards the dorsal midline by *sog* expression in the ventral-lateral side of the embryo (Srinivasan et al., 2002). Similarly, *sog* expression is high in the intervein regions but is missing from LVs and CVs (I, Fig. 6A, B). Importantly, the initial *sog* repression in the PCV region was BMP signal independent (I, Fig. 6C). Thus, Sog gradient appears to prepattern directionality in the Dpp transport towards the PCV region as in the embryo.

1.7. What are positive feedback factors to retain BMPs in LVs?

Active retention of Dpp in LVs requires not only Tkv but also unknown feedback factors. Potential feedback factors may facilitate the Tkv-ligand complex, sequester receptor-unbound ligands, or promote the turnover of the receptor-ligand complex. Two extracellular proteins, Cv-2 and larval translucida (Ltl), are known Dpp target genes in the pupal wings. *cv-2* is a BMP-binding protein required for enhancing BMP signaling at the PCV. However, *cv-2* is expressed at the PCV region but its expression is almost excluded from the LVs (Serpe et al., 2008). *ltl* is expressed at the pupal wing veins. Loss of *ltl* induces the ectopic Dpp signal in the intervein regions and overexpression of Ltl inhibits Dpp signaling at the PCV (Szuperak et al., 2011). Therefore Ltl may be involved in ligand retention. The molecular mechanism by which Ltl regulates Dpp signaling remains elusive. Alternatively, endocytosis may be involved in Dpp retention through promoting the Tkv-ligand complex turnover or degradation. Thickened wing vein phenotypes in *shi* mutant could be interpreted as loss of Dpp retention. Since endocytosis is also required for BMP signal (Belenkaya et al., 2004), further studies are required to allocate endocytosis function. Interestingly, similar positive feedback mechanism is also reported in D–V patterning of the early embryo, although in this case BMP signal promotes Dpp–Tkv binding to sustain the long-range Dpp diffusion (Wang and Ferguson, 2005).

2. The function of RhoGAP Cv-C during wing vein morphogenesis in *Drosophila* (II)

2.1. BMP signal is required for PCV morphogenesis

Optical cross-sections at the PCV region revealed that BMP signal coincides with wing vein morphogenesis recognized by apposition and cell shape changes between two wing layers (II, Fig. 1) (Figure 23). During 18—24 hr AP, F-actin and β-integrin preferentially accumulated at the basal side of the intervein region but less at the basal side of the PCV region, which can account for the lack of the apposition and cell shape change at the PCV region. To test if BMP signal is required for PCV morphogenesis, *cv* and dpp^{s4}/dpp^{s11} mutants were analyzed. In both mutants, in which BMP signal was severely affected at the PCV region, the initial PCV morphogenesis occurred but was subsequently disrupted with the accumulation of F-actin and β-integrin at the basal side of the PCV region (II, Figs. 1 and S2). These observations suggest that BMP signal is required for the maintenance of PCV morphogenesis by regulating F-actin and β-integrin localization at the basal side of the PCV region. In addition, BMP signal is not required for the initiation of PCV morphogenesis. I found that the initial PCV morphogenesis is also independent of *sog* transcriptional prepattern (II, Fig. 2).

2.2. RhoGAP Cv-C mediates PCV morphogenesis

A candidate for mediating PCV morphogenesis downstream of BMP signal is Cv-C (II, Fig. 3A-C). *cv-c* has been identified as a RhoGAP required for a variety of morphogenetic events in the embryo (Brodu and Casanova, 2006; Denholm et al., 2005). However, it remains unknown how *cv-c* is involved in the PCV formation. I found that *cv-c* is highly expressed at the PCV by BMP signaling during 20—24 hr AP (II, Fig. 3D-H). Consistent with the idea that Cv-C regulates PCV morphogenesis by inactivating the Rho-type small GTPases, adult PCV defects in *cv-c* mutant were efficiently restored by mutant alleles of various Rho-type small GTPases (II, Fig. 3I). Furthermore, defects in PCV morphogenesis in *cv* mutant were rescued in *cv, cdc42* double mutant independently of pMad signal (II, Fig. 3 J-L). In summary, these results suggest that Cv-C is induced by BMP signaling at the PCV region to regulate β-integrin and F-actin distribution at the basal side of the PCV region by inactivating various Rho-type small GTPases (II, Fig. 3M) (Figure 23).

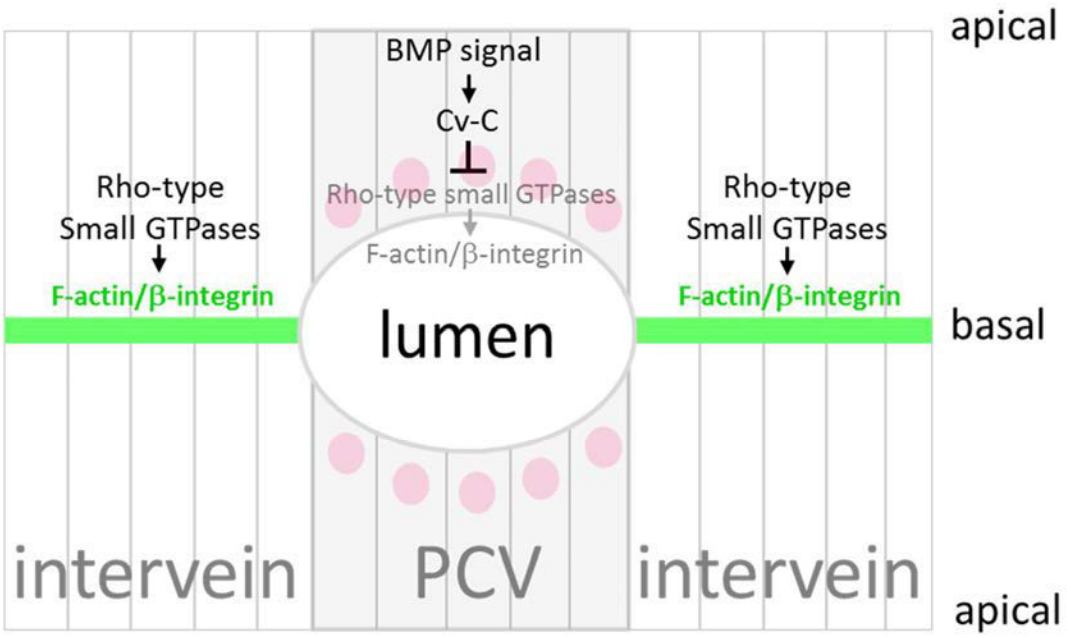

Figure 23. A model for PCV morphogenesis downstream of BMP signaling. Cross-section of the PCV region. BMP signal induces *cv-c* expression at the PCV region. Cv-C inactivates various Rho-type small GTPases including Rho1, Cdc42, Rac1, and Rac2 to inhibit β-integrin and F-actin localization from the basal side (Based on II).

2.3. Cv-C is required for BMP transport

Interestingly, BMP signal in the PCV region was also affected in *cv-c* mutant (II, Figs. 4A-D). To investigate how *cv-c* is involved in BMP signal, I generated *cv-c* null mutant clones using the MARCM system (Lee and Luo, 1999). BMP signal was normal in small mutant clones generated in the PCV region (II, Figs. 4E, F). Instead, the apical-basal cell length became longer with F-actin accumulation at the basal side in such clones (II, Fig. S4). This is consistent with the idea that *cv-c* is cell-autonomously required for PCV morphogenesis. Interestingly, BMP signal was affected non-cell autonomously when relatively large *cv-c* null mutant clones were generated in the PCV region (II, Figs. 4G-N). How could Cv-C affect BMP signal non-cell autonomously? The genetic interaction between *cv-c* and *sog* or *cv* (II, Figs. 5A-D) indicates that *cv-c* is involved in BMP transport. Indeed, GFP-Dpp diffusion was disrupted at the PCV region in *cv-c* mutant (II, Figs. 5E-H).

2.4. Cv-C inactivates β-integrin to promote BMP transport

How does RhoGAP Cv-C regulate extracellular BMP transport? Since *cv-c* is required for PCV morphogenesis, I hypothesized that PCV morphogenesis is required for BMP transport. To test this, a viable *cdc42* mutant displaying ectopic CVs was analyzed. In *cdc42* mutant, Sog/Cv dependent BMP signal was induced at the future ectopic CVs undergoing wing vein morphogenesis (II, Figs. 6A-I). This ectopic wing vein morphogenesis was independent of *sog* or *cv* (II, Figs. 6H-K), suggesting that BMP transport is guided towards ectopically induced wing veins. Since partial pMad defect in weak sog^{p11885} allele was rescued in sog^{p11885}, $cdc42^{2}$ double mutant (II, Figs. 6L-N), PCV morphogenesis can promote BMP transport towards the PCV region. What then links intracellular PCV morphogenesis and extracellular BMP transport? Ectopic BMP signal by loss of *cdc42* was associated with reduced β-integrin from the basal side (II, Figs. 6H-K and S5). Since integrins are the cell surface receptor critical for cell-ECM adhesion, they can potentially regulate BMP transport. In fact, integrins have been previously proposed to regulate Sog protein distribution from the intervein regions (high integrins) into the LVs (low integrin) during the pupal stages (Araujo et al., 2003). This raises the possibility that integrins link BMP transport and PCV morphogenesis. Indeed, Sog/Cv dependent ectopic adult wing veins and BMP signal were sufficiently induced in β-integrin mutant (II, Fig. 7). Finally, pMad and adult PCV defects in *cv-c* mutant were rescued in β-integrin, *cv-c* double mutant (II, Figs. 7L-N). This indicates that Cv-C inhibits β-integrin activity at the basal side of the PCV region to promote BMP transport as well as PCV morphogenesis.

2.5. A feed-forward loop coupling BMP transport and PCV morphogenesis

In summary, Cv-C is induced at the PCV region by BMP signal and inhibits β-integrin and F-actin localization at the basal side of the PCV region via inactivating Rho-type small GTPases. Low β-integrin activity at the basal side of the PCV region also facilitates BMP transport probably by regulating Sog gradient. These observations suggest a positive feedback mechanism coupling BMP transport and morphogenesis through Cv-C (II, Fig.8) (Figure 24). The initial BMP transport is thought to be guided by the *sog* prepattern information (I) (Ralston and Blair, 2005). However, *sog* mutant clones could not induce clear ectopic formation of CVs (I, Fig. 6) or PCV could be induced in some conditions by uniform *sog* expression (Serpe et al., 2008). Since the initial PCV morphogenesis prepatterns

the PCV position independently of *sog*, two prepattern factors (*sog* transcription and low β-integrin activity) probably collaborate to help establish Sog protein gradient.

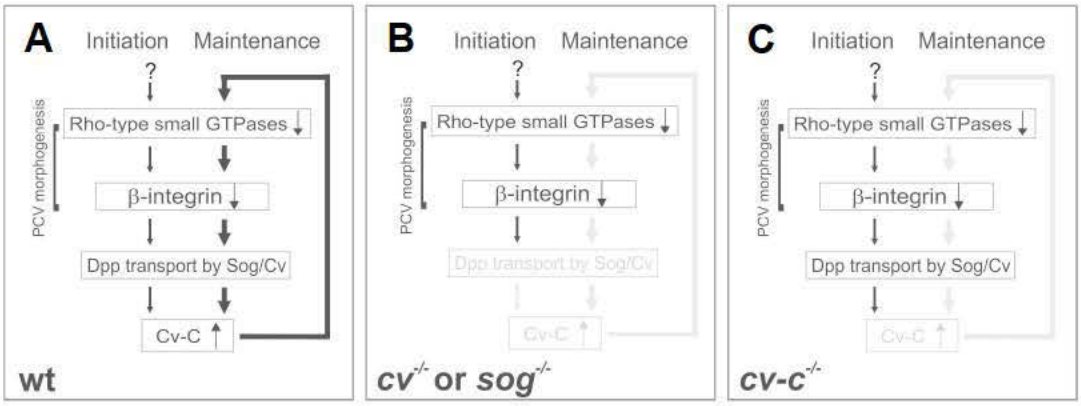

Figure 24. A feedback loop coupling BMP transport and PCV morphogenesis. (A) In wild-type pupal wing, BMP transport is first guided towards the PCV region through BMP signal independent low ß-integrin activity (~18 hr AP). BMP signaling induces RhoGAP Cv-C to maintain PCV morphogenesis and BMP transport through a feed-forward loop (20~26 hr AP). (B) In *cv* or *sog* mutant, the initial PCV morphogenesis occurs but is not maintained, due to the absence of BMP transport. (C) In *cv-c* mutant, BMP signaling and PCV morphogenesis are not maintained without a feed-forward loop. (Based on II).

2.6. In which route is BMP transported?

Consistent with the coupling of BMP transport and PCV morphogenesis at the basal side, GFP-Dpp accumulated along the basal side when expressed in the LVs (II, Fig. S6E). Ectopic Sog/Cv mediated BMP signal was induced when β-integrin activity was reduced from the basal side (II, Fig. 6). Furthermore, a recent study showed that Cv-D secreted from hemocytes floating between two wing layers regulates BMP signal at the PCV region (Chen et al., 2012). These observations indicate that BMP is transported along the basal side or in the lumen of the PCV region. In the initial PCV morphogenesis, BMP signal was detected prior to the lumen formation and rather associated with low level of β-integrin (II, Fig. 1). In addition, Sog directly binds to integrins (Araujo et al., 2003). Therefore, BMPs probably diffuse along the basal side. I speculate that BMPs released from Sog-Cv by Tlr can activate BMP signal in both wing layers (II, Fig. 8), based on the non-autonomous effects of *cv-c* mutant clones across the wing layer (II, Fig. 4) (Figure 25).

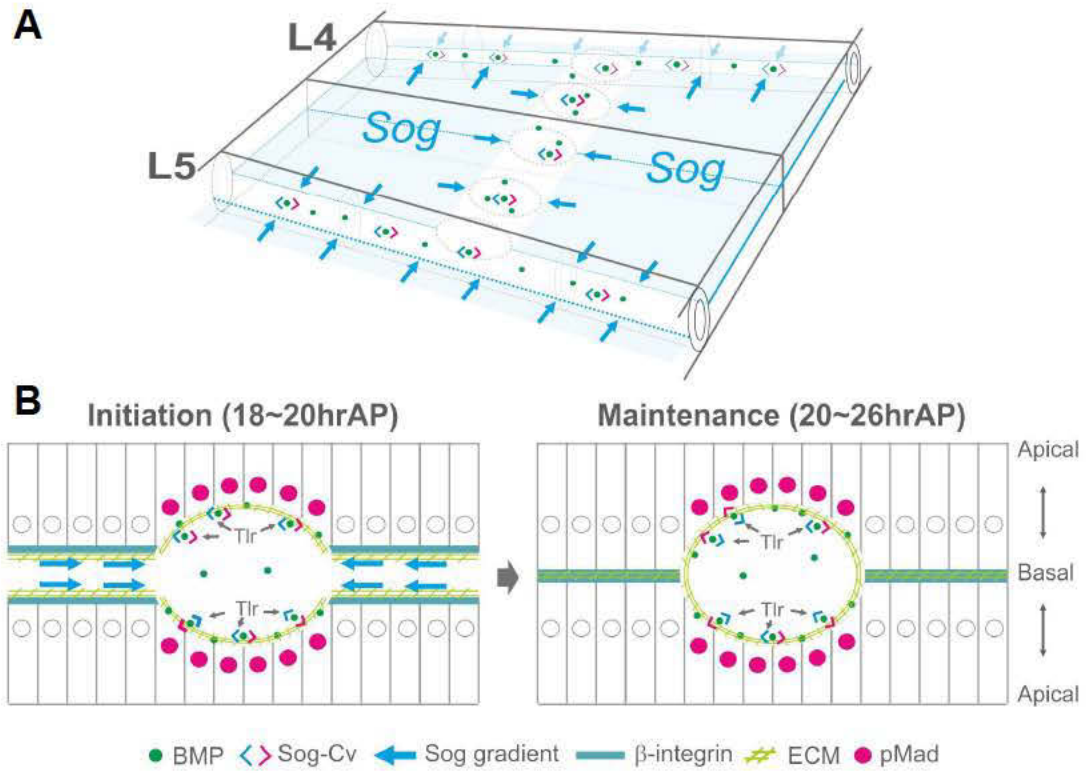

Figure 25. Schematic overview of BMP transport and cross-section in the PCV region. (A) Overview of BMP transport towards the PCV region. (B) Dpp-Gbb appears to be transported by Sog-Cv along the ECM of the PCV. Dpp-Gbb released by Tlr probably activates BMP signal in both side of the wing layers. (Based on II).

2.7. How is wing vein morphogenesis regulated?

Recent studies have revealed that cellular compartmentalization of the Rho-type small GTPases is critical to regulate epithelial morphogenesis (Bement et al., 2006; Simoes et al., 2006; Widmann and Dahmann, 2009). I found a similar biased distribution of Rho1 activity in the pupal wing using a GFP-based sensor that binds to the active form of Rho1 (Simoes et al., 2006). Rho1 activity was high at the basal side of the intervein regions and low in the PCV region (II, Fig. S3). Since overexpression of constitutively active form of Rho1 as well as Rho1 RNAi shortens apical-basal cell lengths in the intervein region (Yan et al., 2009), localized Rho1 activity rather than total activity is associated with the apical-basal cell length. Localized activities of Rho-type small GTPases then promote β-integrin and F-actin accumulation at the basal side (II, Figs. 6H-K) and maintain apical-basal cell length in the intervein region probably by mediating cell-ECM adhesion as in the larval wing epithelium (Dominguez-Gimenez et al., 2007). In the PCV region, BMP signal induces Cv-C to shorten the apical-basal lengths by inactivating various Rho-type small GTPases (II, Fig. 3). Interestingly, BMP signal has been shown to promote localized Rho1 activity that triggers apical-basal cell elongation in the larval wing imaginal disc (Widmann and Dahmann, 2009). Thus, BMP signaling may positively or negatively regulate the compartmentalization of Rho-type small GTPases in a tissue-dependent manner.

3. The extracellular regulation of BMP in the sawfly wing venation (III)

3.1. BMP signaling reflects the sawfly wing vein patterns

To address how BMP signal is regulated to generate diversified insect wing vein patterns, I then studied sawfly as a new model. I first addressed how sawfly wings generate mature fore- and hindwing vein patterns during development. At the early prepupal stage (Post-Cocoon Formation3, PCF3), both the fore- and hindwings display two primary LVs and additional short LVs are formed. The fore- and hindwing vein patterns are relatively similar until this stage. Subsequently, distinct numbers and positions of CVs establish the mature fore- and hindwing venation during PCF4. BMP signal reflected the fore- and hindwing vein patterns throughout prepupal wing vein development, as in *Drosophila* (III, Fig. 1).

3.2. *Ar-dpp* and *Ar-tsg/cv* are ubiquitously expressed in sawfly prepupal wings

I found that *Ar-dpp* was ubiquitously expressed in the fore- and hindwings during the prepupal stages of PCF3-5 (III, Fig. 2). This is contrast with the localized *dpp* expression in the future wing veins in *Drosophila*. One possibility is that Dpp is transported to the future wing veins. To test this, the homologue of *tsg/cv* (*Ar-tsg/cv*) was isolated in sawfly. *Ar-tsg/cv* is ubiquitously expressed in the prepupal fore- and hindwings (III, Fig. 3). The phylogenetic analyses suggest that Ar-Tsg/Cv is more closely related to Cv than Tsg (III, Fig. 3). Three *tsg*-related genes (*tsg*, *cv*, and *shrew*) are found in *Drosophila*, whereas a single *tsg*-related gene is found in *Apis mellifera*, *Anopheles gambiae* and *Tribolium castaneum*. They are more related to *cv* in *Drosophila* (Nunes da Fonseca et al., 2010). In fact, *cv* gene might have duplicated in the Dipteran lineage, which leads to subfunctionalization of *Drosophila tsg/cv*-related genes (van der Zee et al., 2006).

3.3. Functional analysis of *dpp* and *tsg/cv* in sawfly prepupal wings

To address the function of *Ar-dpp* and *Ar-tsg/cv* during wing development, dsRNA was injected into the dorsal hemocoel of the last instar larvae. The fore- and hindwing vein formation was disrupted by injection of *Ar-dpp* dsRNA in a dose-dependent manner (III, Fig. 4B, Table 1). Accordingly, loss of *Ar-dpp* strongly inhibited pMad accumulation in prepupal fore- and hindwings (III, Figs. 4D). Prepupal wing venation and pMad accumulation were also affected in the fore- and hindwing by *Ar-tsg/cv* dsRNA (III, Figs. 4E, Table 1). Since *Ar-tsg/cv* RNAi reduces *Ar-tsg/cv* transcripts, but not *Ar-dpp* transcripts (III, Fig. 4A), Ar-Tsg/Cv is required for BMP signal without changing *Ar-dpp* expression.

3.4. Ar-Tsg/Cv retains the ability to transport Dpp/BMP

To investigate whether Ar-Tsg/Cv retains the ability to transport Ar-Dpp, biochemical properties of Ar-Dpp and Ar-Tsg/Cv were addressed in *Drosophila* S2 cells using a luciferase assay (Weiss et al., 2010). In *Drosophila*, Sog and Cv efficiently bind with BMP ligands and block its binding to the receptors (Shimmi et al., 2005a). BMP signal activated by Ar-Dpp was inhibited by *Drosophila* Sog and Cv in S2 cells (III, Fig. 5A). Ar-Tsg/Cv was efficiently secreted into supernatants (III, Fig. 5B) and could inhibit Ar-Dpp signal (III, Fig. 5C). Moreover, *Ar-dpp* and *Ar-tsg/cv* could rescue PCV defects in *dpp* or *cv* mutant in *Drosophila* (III, Figs. 5D—G). These results indicate that Ar-Tsg/Cv retains the ability to transport Ar-Dpp for wing vein formation in sawfly.

3.5. Is BMP transport utilized to specify the ancient insect wing?

In summary, despite its ubiquitous expression, *Ar-dpp* is required for localized BMP signaling that reflects the sawfly fore- and hindwing wing vein patterns. Since Ar-Tsg/Cv retains the ability to transport BMP and is required for BMP signaling in all of the fore- and hindwing veins, BMP transport mechanism may be involved in redistributing ubiquitously expressed Dpp towards the future wing vein region. The severe phenotype caused by loss of *tsg/cv* in the sawfly is contrast with PCV-less phenotype of *cv* in *Drosophila*. BMP redistribution from ubiquitously expressed ligands to the narrow stripe is rather reminiscent of the D-V patterning of the early *Drosophila* embryo. What determines the direction for BMP transport? Ar-Dpp and Ar-Tsg/Cv are ubiquitously expressed (III, Fig. 2, 3). It would be interesting to address if *sog* expression also dictates the future sawfly wing vein positions (direction of BMP transport) as in *Drosophila*. In addition to the function of Tsg on BMP transport, Tsg appears to have BMP transport-independent pro-BMP function in *Drosophila* and *Tribolium castaneum* embryos (Nunes da Fonseca et al., 2010; Wang and Ferguson, 2005) and in vertebrates (Oelgeschlager et al., 2003; Xie and Fisher, 2005). Further studies will be required to elucidate how BMP transport mechanism contributes to formation of wing vein patterns in sawfly.

3.6. Diversity and variations in insect wing venation pattern

If Dpp redistribution plays a critical role in establishing fore- and hindwing vein patterns, this raises an interesting possibility that distinct wing vein patterns are generated, based on where Dpp is transported. In this case, differences in the direction of Dpp transport may be a mechanism underlying the different fore- and hindwing vein patterns in the sawfly. If *sog* expression provides the positional information for BMP transport, changes in the *Ar-sog* expression pattern may contribute to the distinct fore- and hindwing vein pattern. The difference in *dpp* transcription and Dpp mobility has been linked with the different sizes between the forewing (wing) and the hindwing (haltere) in *Drosophila* (Crickmore and Mann, 2006). In addition, the directionality of Dpp diffusion may be an important factor to generate variations between homologous structures within an individual and between species (Figure 26). In support of this notion, in some species of the Diptera, differences in wing vein patterns are based on the positions and numbers of CVs. For example, despite similar wing vein patterns to those of *Drosophila melanogaster*, the *planitibia* subgroup of the Hawaiian *Drosophila* has an extra CV, while species belonging to the family Asteiidae have no CVs (De Celis and Diaz-Benjumea, 2003; Edwards et al., 2007). It would be interesting to test if changes in the direction of Dpp transport give rise to distinct wing vein patterns in some winged insects.

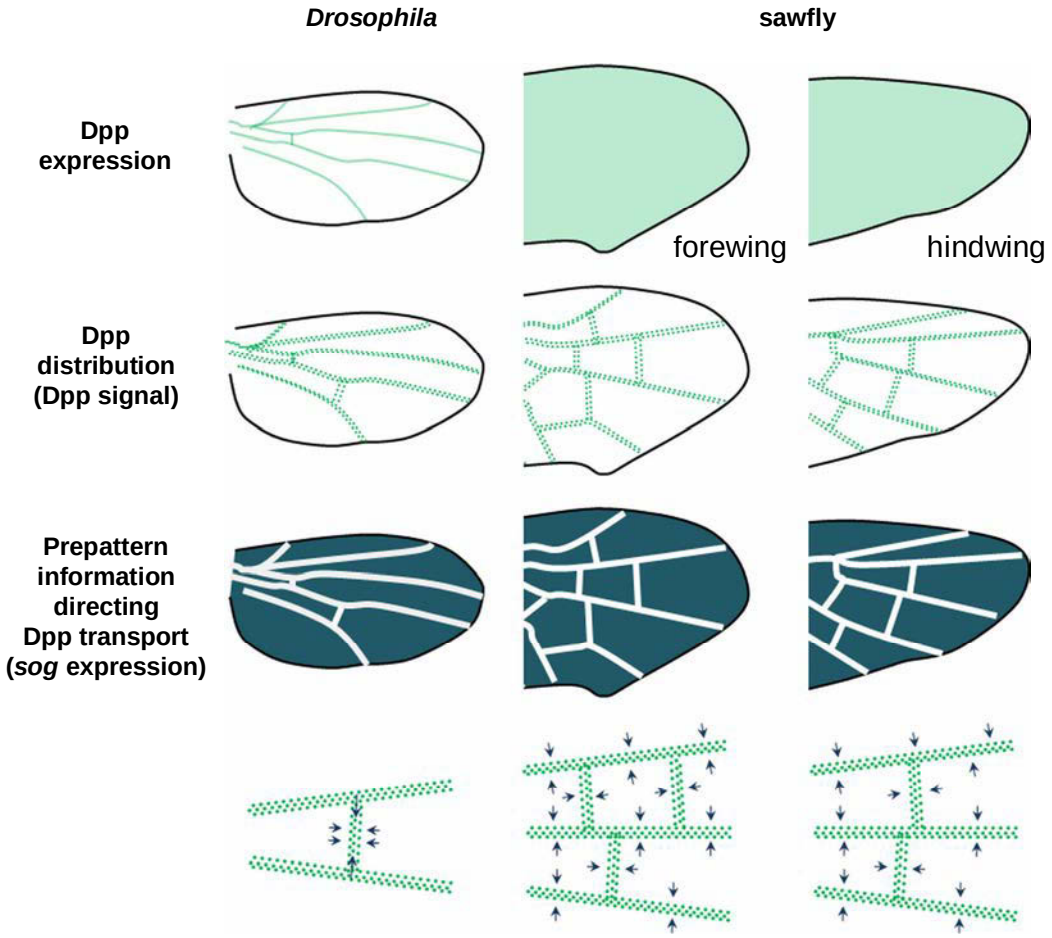

Figure 26. A model of how changes in directionality of Dpp/BMP transport may underlie variations in *Drosophila* and sawfly pupal wing vein patterns. In *Drosophila*, Dpp distribution (or Dpp signal) reflects wing vein patterns based on the prepattern information that regulates the Dpp directionality (ex, *sog* expression). In sawfly, Dpp signal mediated by Tsg/Cv reflects wing vein patterns despite ubiquitous *dpp* expression. Thus it is conceivable that Dpp distribution reflects wing vein patterns in sawfly through prepattern information that directs Dpp towards the distinct fore- and hindwing veins as in *Drosophila*.

Concluding remarks

To address how evolutionarily conserved Dpp/BMP signaling pathway mediates precise and flexible cell-cell communications during development and evolution, I focused on the extracellular regulation of BMP signal during the insect wing vein patterning and morphogenesis using Dipteran *Drosophila* and Hymenopteran sawfly *Athalia rosae* as models. The main findings are summarized as below.

1. Distribution of BMPs is tightly regulated at the extracellular level in *Drosophila* pupal wing by (1) Sog/Cv mediated directional BMP transport from LVs to PCV and (2) active retention of BMPs in LVs by Tkv and a positive feedback mechanism.

2. RhoGAP Cv-C mediates PCV morphogenesis downstream of BMP signal in *Drosophila*. Cv-C is also non-cell autonomously required for BMP transport through β-integrin affecting Sog flux. The feed-forward mechanism through Cv-C thus coordinates BMP transport and PCV morphogenesis in *Drosophila*.

3. The direction of BMP transport towards the PCV region is prefigured by BMP signal independent *sog* repression and wing vein morphogenesis in *Drosophila*.

4. Studies in sawfly suggest that BMP transport is required to redistribute ubiquitously expressed Dpp to reflect the distinct fore- and hind-wing vein patterns in sawfly.

Future directions

This thesis work raises a number of open questions to be addressed in the future studies.

1. What are the initial factors that specify the position of the PCV or direct BMP transport in *Drosophila*? BMP signal independent initial *sog* repression and low level of β-integrin provide clues to address this question.

2. What are the positive feedback factors that retain BMPs in the LVs in *Drosophila*?

3. What determines the direction of BMP transport in sawfly wing? It would be interesting to isolate *sog* in sawfly. More generally, isolating and characterizing core wing patterning genes in sawfly would shed light on how evolutionarily conserved systems generate diversified insect wing vein patterns through evolution.

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