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Directional transport and active retention of Dpp/BMP create wing vein patterns in *Drosophila*

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ABSTRACT

The bone morphogenetic protein (BMP) family ligand decapentaplegic (Dpp) plays critical roles in wing vein development during pupal stages in *Drosophila*. However, how the diffusible Dpp specifies elaborate wing vein patterns remains unknown. Here, we visualized Dpp distribution in the pupal wing and found that it tightly reflects the wing vein patterns. We show that Dpp is directionally transported from the longitudinal veins (LVs) into the posterior crossvein (PCV) primordial region by the extracellular BMP-binding proteins, short gastrulation (Sog) and crossveinless (Cv). Another BMP-type ligand, glass bottom boat (Gbb), also moves into the PCV region and is required for Dpp distribution, presumably as a Dpp–Gbb heterodimer. In contrast, we found that most of the Dpp is actively retained in the LVs by the BMP type I receptor thickveins (Tkv) and a positive feedback mechanism. We provide evidence that the directionality of Dpp transport is manifested by *sog* transcription that prepatterns the PCV position in a Dpp signal-independent manner. Taken together, our data suggest that spatial distribution of Dpp is tightly regulated at the extracellular level by combination of long-range facilitated transport toward the PCV and short-range signaling by active retention in the LVs, thereby allowing diffusible ligands to form elaborate wing vein patterns.

Introduction

The evolutionarily conserved bone morphogenetic protein (BMP) family ligands play critical roles in many developmental processes, including patterning, morphogenesis, proliferation, and apoptosis (Hogan, 1996). Since ablation of the signaling pathway causes a variety of developmental defects or diseases, it is important to address the mechanism of how the signaling activity is regulated. In *Drosophila*, decapentaplegic (Dpp) is the homolog of BMP2/4 in vertebrates, which binds to the BMP type I receptor thickveins (Tkv) and type II receptor Punt and phosphorylates the transcription factor Mad (referred to as pMad; an indication of active Dpp signal transduction). pMad is then, together with co-Smad Medea, translocated to the nucleus for regulating downstream target genes (Moustakas and Heldin, 2009). One interesting feature in the Dpp signaling pathway is that the signaling range is tightly regulated at the extracellular level. In dorsal–ventral (D–V) patterning of the embryo, *dpp* is ubiquitously transcribed in the dorsal half of the embryo and Dpp forms a morphogen gradient together with another BMP-type ligand, screw (scw), which is ubiquitously expressed. The BMP morphogen gradient is established by the facilitated transport of Dpp–Scw heterodimers to the dorsalmost region of the embryo by the extracellular BMP-binding proteins, short gastrulation (Sog) and twisted gastrulation (Tsg), and release of the ligands via enzymatic processing of Sog by tolloid (Tld) (O'Connor et al., 2006; Shimmi et al., 2005b; Wang and Ferguson, 2005). BMP transport toward the dorsal midline is dictated by a Sog gradient from the lateral regions toward the dorsalmost region of the embryo (Srinivasan et al., 2002). This facilitated transport mechanism is utilized among species (Ben-Zvi et al., 2008; De Robertis, 2008; van der Zee et al., 2006). During the larval stages, *dpp* is transcribed in a stripe at the anterior/posterior compartment boundary of the wing imaginal disk and the Dpp ligand forms a long-range morphogen gradient that organizes both patterning and growth of the wing imaginal disk (Affolter and Basler, 2007). Extracellular Dpp diffusion is regulated by interaction of Dpp with extracellular matrix (ECM) proteins (Belenkaya et al., 2004) and BMP type I receptor Tkv (Lecuit and Cohen, 1998; Tanimoto et al., 2000). It has also been proposed that long-range Dpp action occurs through receptor-mediated transcytosis (Entchev et al., 2000) or through cytonemes protruded from ligand-receiving cells (Hsiung et al., 2005), although the transcytosis model has been recently challenged (Belenkaya et al., 2004; Schwank et al., 2011). In contrast to the long-range morphogen gradient formation, Dpp can also act in a short-range manner. For example, the Dpp signal from niche cells is restricted to the adjacent germline stem cells (GSCs) to maintain the

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GSCs (Fuller and Spradling, 2007). Thus, the range of the Dpp signal is tightly regulated in a long- or short-range manner depending on the contexts, and these developmental processes have served as excellent models for addressing the molecular mechanisms regulating the Dpp signaling range. However, little is known about how long- and short-range signals are coordinated when both function in the same tissue.

To address this, we focused on Dpp signaling in wing vein development during the pupal stages, in which Dpp appears to function in both a long- and short-range manner. Unlike the larval wing disk development, Dpp does not appear to function as a morphogen but rather as a wing vein determinant to maintain longitudinal vein (LVs, L2-L5) fate and induce anterior and posterior crossvein (ACV and PCV) fate during the pupal stages (Fig. 1(A) and (B)) (Blair, 2007). Interestingly, *dpp* is only transcribed in the LVs, yet pMad is detected in all vein primordial, including the CVs, at 24 h after pupariation (AP) (Fig. 1(A′) and (A″)). Thus the function of Dpp is not simply attributed to *dpp* expression patterns. Recent studies have proposed that Dpp moves from the LVs into the prospective PCV region to initiate PCV formation (Ralston and Blair, 2005; Serpe et al., 2005; Shimmi et al., 2005a). Since Sog, Tsg paralog crossveinless (Cv), and Tld paralog tolloid-related (Tlr) are required for PCV development (O'Connor et al., 2006; Serpe et al., 2005; Shimmi et al., 2005a), the facilitated transport mechanism is thought to occur during PCV formation, as in the D–V patterning of the embryo. On the other hand, Dpp must function in a short-range manner in LVs, because pMad accumulation is highly localized at LVs in which *dpp* is expressed. Thus, the wing vein patterning would provide a model for addressing the mechanisms coordinating short- and long-range Dpp function in the same tissue. Dpp may diffuse ubiquitously from the LVs and then differences in the competence of the Dpp-receiving cells may define the wing vein patterns. Alternatively, extracellular Dpp distribution itself may reflect the wing vein patterns. In this case, the Dpp distribution must be tightly controlled. To date, however, Dpp distribution in the pupal wing has not been investigated.

In this study, we successfully visualized Dpp distribution in the pupal wings by applying GFP-Dpp and addressed the mechanisms by which diffusible Dpp draws complex wing vein patterns. We first showed that Dpp is directionally moved from the LVs to the future PCV by a Sog and Cv-mediated facilitated transport mechanism. The distribution is also dependent on another BMP-type ligand glass bottom boat (Gbb), indicating that the Dpp–Gbb heterodimer is the primary ligand for specifying PCV. In contrast, we found that Dpp functions in a short-range manner in LVs. We showed that Dpp mobility is tightly restricted in LVs by Tkv and a positive feedback mechanism through Dpp target gene(s). Finally, we showed that the direction of Dpp transport to the future PCV is prepatterned, in part, by Dpp signal-independent *sog* transcription. Taken together, our data suggest that Dpp spatial distribution reflects the wing vein patterns by combination of two distinct mechanisms, facilitated transport toward PCV and active retention in LVs, thus allowing diffusible Dpp to draw complex patterns in *Drosophila*.

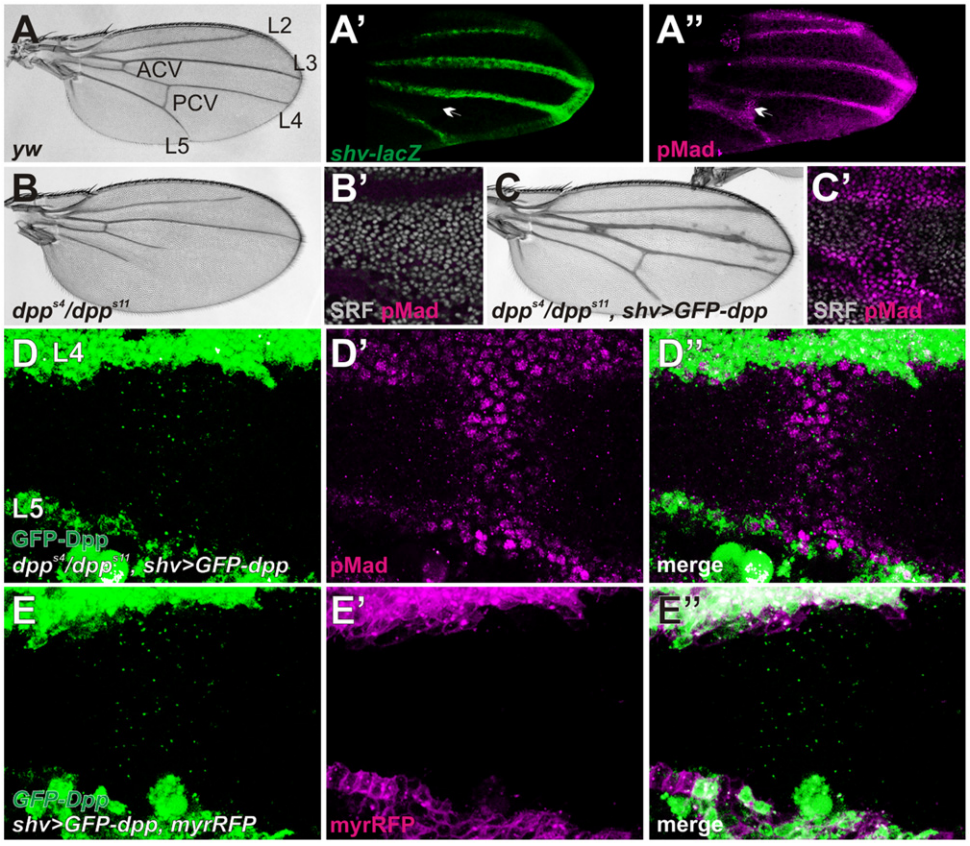

Fig. 1. *Visualization of GFP-Dpp distribution in Drosophila pupal wings.* (A)–(A″) wild-type adult (A) and pupal (A′), (A″) wings. *dpp* (*dpp^{shv}-lacZ*, green, A′) and pMad (purple, A″). The PCV is shown by an arrow. (B), (B′) *dpp^{s4}/dpp^{s11}* mutant adult wing (B) and pupal wing focused on the PCV region (B′). (B′) pMad (purple) and *Drosophila* serum response factor (DSRF) (white, intervein marker). (C), (C′) Rescued adult wing (C) and pupal wing focused on the PCV region (C′) (*dpp^{s4}/dpp^{s11}*, *dpp^{shv} > GFP-dpp*). (C′) pMad (purple) and DSRF (white). (D)–(D″) GFP-Dpp distribution in the PCV region in the rescued pupal wing (*dpp^{s4}/dpp^{s11}*, *dpp^{shv} > GFP-dpp*). GFP-Dpp (green, D), pMad (purple, D′) and merged image (D″). (E)–(E″) GFP-Dpp movement from the LVs into the PCV (*dpp^{shv} > GFP-dpp*, *myristoylated-RFP*). GFP-Dpp (green, E), membrane-bound RFP (purple, E′) and merged image (E″). All pupal wings were fixed at 24 h AP.

Materials and methods

Drosophila strains

The UAS-*Venus-gbb* flies were obtained by germline transformation. UAS-*sog-HA*, UAS-*tsg-His* and UAS-*scw-HA* flies were described previously (Shimmi et al., 2005a, b). The UAS-*tkv* RNAi (#3059) and UAS-*Mad* RNAi (#12635) flies were obtained from the Vienna *Drosophila* RNAi Stock Center. The *tkv-lacZ* flies were a gift from T. Tabata. The shv^{3Kpn}-Gal4 (dpp^{shv}-Gal4) was kindly provided by Ramel et al. (2007). The UAS-*tkv-HA*, UAS-tkv^{Q199D}*HA* (constitutively active Tkv), UAS-ΔE-tkv^{Q199D}HA, and $cv\text{-}2^{KO1}$ flies were gifts from M. O'Connor. The dpp^{s4} and dpp^{s11} are strong *shortvein* (*shv*) alleles (rearrangement of the *shv* regulatory region) (St Johnston et al., 1990).The gbb^{5l} and UAS-*gbb* flies were kindly provided by K. Wharton. The gbb^{4} and gbb^{5l} are strong hypomorphic alleles (Khalsa et al., 1998). The cv^{70} is a null allele (Shimmi et al., 2005a) and sog^{P129D} is a hypomorphic allele (Serpe et al., 2005). Ubiquitously expressed CFP-Rab5 flies were obtained from S. Eaton (Marois et al., 2006). To analyze GFP-Dpp distributions in *sog* or *cv* mutants on the X chromosome, homozygous mutant females of these genes were crossed with males of UAS-*GFP-dpp*: dpp^{shv}-Gal4/Cyo-Tm6BTb and male progeny carrying UAS-*GFP-dpp* and dpp^{shv}-Gal4 was analyzed as *sog* or *cv* mutant flies expressing GFP-Dpp.

Plasmid construction

For generation of *Venus-gbb*, the *Venus*-coding sequence was inserted just before the Flag tag of *gbb-Flag* (after amino acid E349 of the *gbb* cDNA) (Shimmi et al., 2005a). *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).

Immunohistochemistry and in situ hybridization

Drosophila pupal wings were fixed at 24 h AP at 4 °C overnight and then dissected from the pupae. All immunohistochemistry and *in situ* hybridizations were performed, as previously described (Shimmi et al., 2005a). 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) and mouse anti-*Drosophila* serum response factor (DSRF) at 1:2000 (Geneka). Secondary antibodies were as follows: anti-rabbit IgG-Alexa 568 and anti-mouse IgG-Alexa 488 or 647 were used at 1:1000, respectively (Invitrogen). F-actin was visualized by Alexa Fluor 568 Phalloidin at 1:300 (Invitrogen). Fluorescent images were obtained with a Leica TCS SP5 confocal microscope. To visualize ligand distribution in the pupal wings, approximately 10 confocal sections were taken at 1-μm intervals to cover one cell layer and images were processed by maximum intensity profile. All images were processed by Photoshop (Adobe).

Results

Dpp moves directionally from the LVs to the PCV region

To investigate Dpp distribution in the *Drosophila* pupal wing, GFP-tagged Dpp (Entchev et al., 2000; Teleman and Cohen, 2000) was expressed in the LVs under the control of the dpp^{shv} enhancer (a 5′ regulatory region of the *dpp* locus) (Sotillos and De Celis, 2005). We found that GFP-Dpp could restore pMad in the PCV and in the LVs at 24 h AP and also adult wing vein patterns in a dpp^{shv} mutant (dpp^{s4}/dpp^{s11}) (100% rescued, $n=136$ wings) (Fig. 1(B), (B′), (C), and (C′)). This shows that GFP-Dpp is functional for wing vein development during the pupal stages. We then examined GFP-Dpp distribution at 24 h AP in the rescued wing by maximal intensity profile. In contrast to the uniform distribution of GFP-Dpp in the LVs, we found that GFP-Dpp was detected as bright dots in the prospective PCV region, where pMad accumulation was also visible (Fig. 1(D) and (D″)). Single confocal plane analysis at different apico-basal level showed that GFP-Dpp dots are detected at or near the plasma membrane of the basal side of the PCV region but not in any focal planes covering the intervein regions (Fig. S1). We also found that the majority of GFP-Dpp dots highly co-localized with CFP-Rab5, an early endocytic marker. Thus, GFP-Dpp dots observed at the PCV region seem to be ligand-receptor complexes undergoing endocytosis rather than extracellular ligands (Fig. S2). In contrast, most of the GFP-Dpp in the LVs appears to reflect ligands being produced in the LV cells rather than ligands bound with receptors, since most of them were still detected when *tkv* was knocked down in the LVs (Fig. 4(B)). To test whether GFP-Dpp bright spots in the prospective PCV are ligands that are moved from the LVs, membrane-bound myrRFP was coexpressed with GFP-Dpp to visualize the GFP-Dpp producing cells. Indeed, GFP-Dpp spots clearly accumulated at the prospective PCV region beyond the LVs, where membrane-bound myrRFP was localized (Fig. 1(E) and (E″)). These ligand accumulations at the PCV region are consistent with the hypothesis that Dpp diffuses from the LVs and internalizes at the PCV region. Unlike the Dpp morphogen gradient observed in the third instar larval wing imaginal disk (Entchev et al., 2000; Teleman and Cohen, 2000), GFP-Dpp distribution in the pupal wings did not form a gradient from the ligand-producing cells (LVs) to the adjacent intervein region, but was rather restricted to the prospective PCV (Fig. 1(D) and (E)), suggesting that Dpp distribution is tightly regulated.

Dpp is directionally transported by Sog-Cv to PCV

How then is the long-range Dpp movement regulated? To test the facilitated transport hypothesis for PCV formation, we investigated the GFP-Dpp distribution at the PCV region in *sog* and *cv* mutants. GFP-Dpp dots and pMad were missing from the PCV region in both *sog* and *cv* mutants at 24 h AP and the crossveinless phenotype was not rescued in mutant flies (0% rescued, $n=116$ wings in *sog* mutants and 0% rescued, $n=117$ wings in *cv* mutants) (Fig. 2(A), (A″), (B), (C), (C″), and (D)). Furthermore, coexpression of *sog* and *tsg* (the functional homolog of *cv* (Shimmi et al., 2005a) in the LVs induced ectopic GFP-Dpp and pMad accumulation within the intervein and PCV regions (Fig. 2(E) and (E″)). Another BMP-binding protein, crossveinless-2 (Cv-2) is also required for Dpp signaling in the PCV (Serpe et al., 2008). To test whether Cv-2 is required for long-range Dpp transport, we investigated the GFP-Dpp distribution in *cv-2* mutant wings and found that GFP-Dpp dots and pMad accumulation were detected in the PCV at 24 h AP (Fig. S3). Furthermore the crossveinless phenotype of a *cv-2* null mutant was partially rescued by overexpression of GFP-Dpp, indicating that Cv-2 is not directly involved in Dpp long-range transport (57% partially rescued in *cv-2* mutants, $n=138$ wings) (data not shown). These results support a facilitated transport mechanism in which Sog and Cv assist Dpp long-range directional movement from the LVs into the PCV region.

Dpp-Gbb heterodimer is the primary ligand transported to the PCV

Another BMP ligand, Gbb (homolog of mammalian BMP5/6/7/8) is also required for PCV formation (Fig. 3(A) and (B)). Unlike *dpp*, *gbb* is ubiquitously expressed in the pupal wing (Conley et al., 2000).

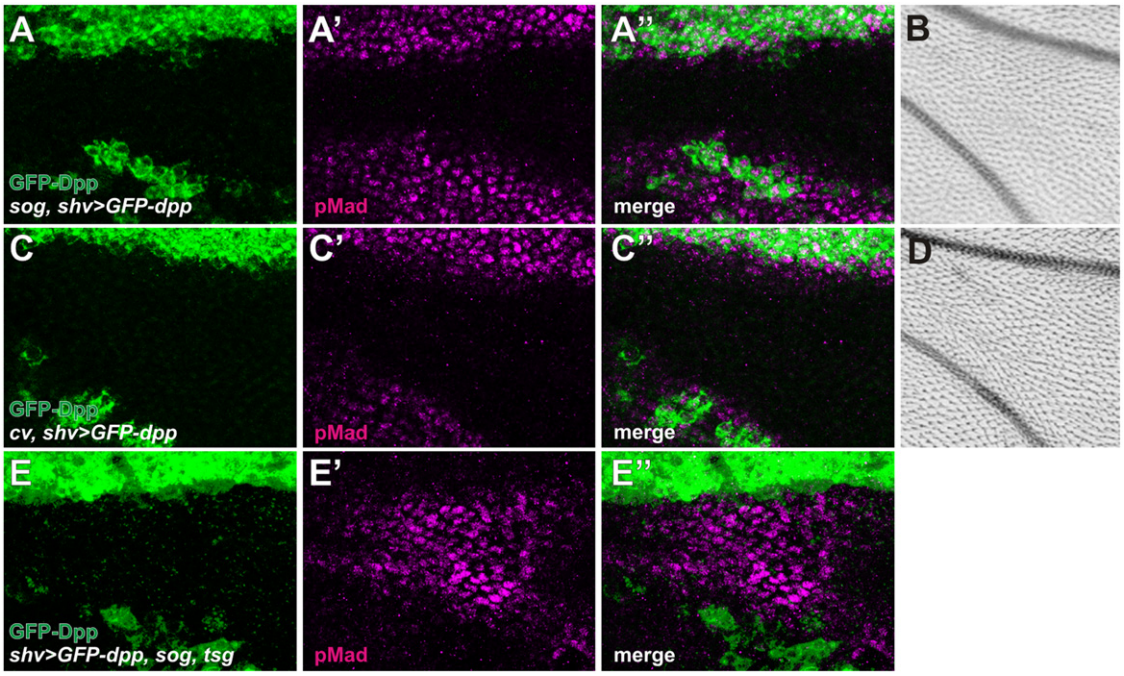

Fig. 2. *Directional transport of Dpp to the PCV in Drosophila pupal wings.* (A), (C), (E) GFP-Dpp distribution in pupal wings. (A)–(A″) *sog* mutant (sog^{P129D}, dpp^{shv} > *GFP-dpp*). (C)–(C″) *cv* mutant (cv^{70}, dpp^{shv} > *GFP-dpp*). (E)–(E″) pupal wing expressing Sog and Tsg (dpp^{shv} > *GFP-dpp, sog, tsg*). (B) Adult wing of sog^{P129D}, *shv* > *GFP-dpp*. (D) Adult wing of cv^{70}, dpp^{shv} > *GFP-dpp*. GFP-Dpp (green, (A), (C), (E)), pMad (purple, (A′), (C′), (E″)) and merged images (A″), (C″), (E″) focused on the PCV. All pupal wings were fixed at 24 h AP. Adult wings focused on the PCV (B) and (D).

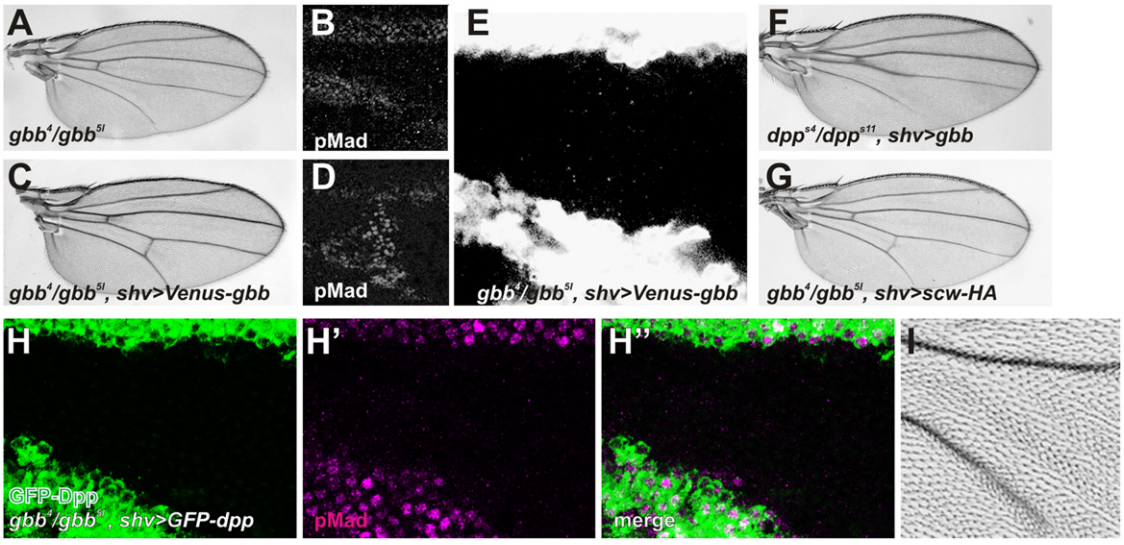

Fig. 3. *Gbb is required for Dpp diffusion toward the PCV.* (A) *gbb* mutant adult wing (gbb^4/gbb^{5l}). (B) pMad in *gbb* mutant pupal wing focused on the PCV (gbb^4/gbb^{5l}). (C) Wing rescued by *Venus-gbb* (gbb^4/gbb^{5l}, dpp^{shv} > *Venus-gbb*). (D) pMad in the rescued pupal wing focused on the PCV (gbb^4/gbb^{5l}, dpp^{shv} > *Venus-gbb*). (E) Venus-Gbb distribution in the PCV region in the rescued pupal wing (gbb^4/gbb^{5l}, dpp^{shv} > *Venus-gbb*). (F) Adult wing of dpp^{s4}/dpp^{s11}, dpp^{shv} > *gbb*. (G) Adult wing of gbb^4/gbb^{5l}, dpp^{shv} > *scw-HA*. (H)–(H″) GFP-Dpp distribution in *gbb* mutant pupal wing (gbb^4/gbb^{5l}, dpp^{shv} > *GFP-dpp*). GFP-Dpp (green, H), pMad (purple, H′), and merged images (H″) focused on the PCV. (I) Adult wing of gbb^4/gbb^{5l}, dpp^{shv} > *GFP-dpp* focused on the PCV. All pupal wings were fixed at 24 h AP.

Clonal analysis of *gbb* mutants indicated that *gbb* expressed in or near the LVs is required for PCV development (Ray and Wharton, 2001). We found that Gbb or Venus-tagged Gbb expressed in the LVs could rescue the *gbb* crossveinless phenotype in adults (100% rescued, $n=50$ wings) and restored pMad accumulation in the PCV region at 24 h AP (Fig. 3(C) and (D)). Moreover, Venus-Gbb punctate spots were detected in the prospective PCV in the rescued pupal wing (Fig. 3(E)). Thus, we conclude that Gbb produced in the LVs also moves to specify the PCV.

How do the two BMP-type ligands, Dpp and Gbb, specify the PCV? Since both ligands utilize the Tkv receptor for signal transduction, they may function redundantly. However, we found that Gbb could not rescue the crossveinless phenotype of the dpp^{shv} mutants (0% rescued, $n=88$ wings) (Fig. 3(F)), suggesting that the function of these two ligands is not redundant. We have previously shown that Dpp–Gbb heterodimers, but not Dpp or Gbb homodimers, selectively bind to the Sog–Cv complexes *in vitro*, suggesting that Dpp–Gbb heterodimers are the key regulators of the

PCV formation (Serpe et al., 2005; Shimmi et al., 2005a). To test this hypothesis, we examined the Dpp accumulation at the PCV region in *gbb* mutants. We observed that GFP-Dpp dots and pMad were absent from the PCV at 24 h AP and the PCV was not formed in adult wings (0% rescued, $n=34$ wings) (Fig. 3(H), (H″), and (I)). We also found that Scw, which forms a heterodimer with Dpp in the embryo, sufficiently rescued the *gbb* crossveinless phenotype (92% rescued, $n=132$ wings) (Fig. 3(G)), suggesting that heterodimer formation of BMP-type ligands is critical for PCV formation as well as embryonic patterning. Taken together, these results suggest that Dpp–Gbb heterodimers are the principle ligands moved by Sog and Cv to specify the PCV.

Dpp is actively retained in LVs by Tkv

In contrast to the long-range transport of Dpp into the PCV, we found that most of the Dpp protein appears to be immobilized within the LVs (Fig. 4(A) and (A″)). In many signaling pathways, receptors are capable of modulating ligand movement (Cadigan, 2002). For example, in the *Drosophila* third instar wing disk, a low level of Tkv in Dpp-producing cells facilitates Dpp diffusion and a high level of Tkv interferes with Dpp mobility (Lecuit and Cohen, 1998; Tanimoto et al., 2000). To test whether Tkv also modulates Dpp movement in the pupal wing, we utilized an RNAi approach. Knockdown of *tkv* in LVs mimicked the thick vein or split wing vein phenotype of hypomorphic *tkv* alleles, indicating that *tkv* expression in LVs is critical for maintaining vein width (Fig. 4(I) and (J)). We then examined the GFP-Dpp distribution in the *tkv* RNAi pupal wings. We found that GFP-Dpp spread ectopically at the intervein regions along LVs, where pMad also accumulated (Fig. 4(B) and (B″)). Consistent with a reduction in *tkv* expression in the LVs, we observed a concomitant reduction in pMad accumulation in the LVs. These data clearly show that Tkv is required to immobilize Dpp in the LVs. Our observation is in agreement with the previous observation that split wing veins were induced in a non-cell autonomous manner when *tkv* mutant clones were generated on the LVs (Burke and Basler, 1996). Interestingly, we found that the ectopic Dpp accumulation due to the loss of *tkv* is independent of *sog* or *cv* (Fig. 4(C), (C″), (D), and (D″)), and consistently, the loss of *tkv* in LVs in either *sog* or *cv* mutants shows the thickened vein phenotype (Fig. 4(K) and (L)). These data suggest that Dpp can move without interacting with Sog–Cv. Thus, Tkv is required to localize potentially diffusible Dpp within the LVs. In contrast, we found that *gbb* is required for ectopic pMad accumulation in the intervein regions by loss of *tkv* (Fig. 4(E), (G), and (M)). These observations suggest that Dpp–Gbb heterodimers are not only transported toward PCV primordial by Sog–Cv, but also actively retained in the LVs by Tkv. Sog–Cv-mediated transport probably liberates Dpp–Gbb heterodimers from active retention by Tkv in the LVs, thus enabling PCV formation.

Active retention of Dpp requires a positive feedback mechanism

In spite of the critical role of Tkv in retaining Dpp in the LVs, it has been shown that *tkv* expression is relatively low in the LVs (Dpp-producing cells) and high in intervein regions in the pupal wing (Fig. 5(A) and (A″)) (de Celis, 1997). Given that *tkv* transcription is suppressed by Dpp signal in the pupal wing (de Celis,

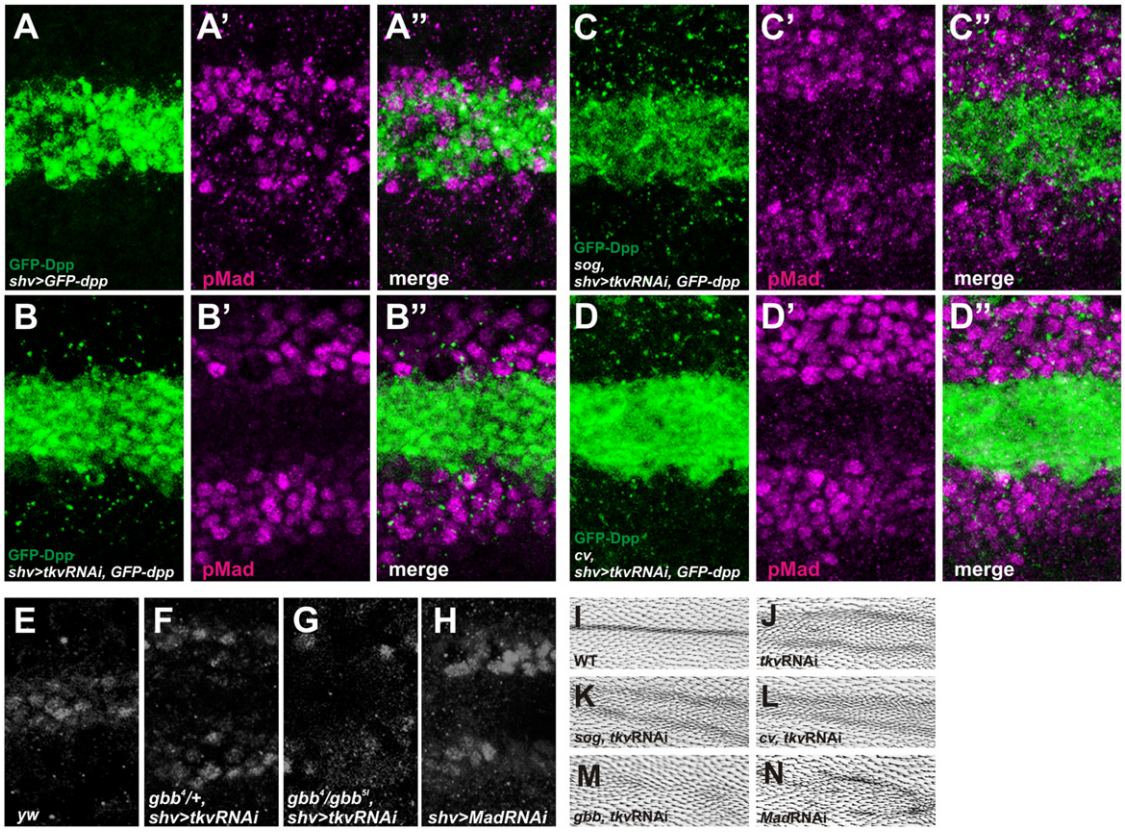

Fig. 4. *Active retention of Dpp in LVs in Drosophila pupal wings.* (A)–(D) GFP-Dpp distribution in pupal wings. (A)–(A″) Wild-type phenotype (dpp^{shv} > *GFP-dpp*). (B)–(B″) *tkv* RNAi (dpp^{shv} > *GFP-dpp*, *tkv RNAi*). (C)–(C″) *tkv* RNAi, *sog* mutant (sog^{P129D}, dpp^{shv} > *GFP-dpp*, *tkv RNAi*). (D)–(D″) *tkv* RNAi, *cv* mutant (cv^{70}, dpp^{shv} > *GFP-dpp*, *tkv RNAi*). (E)–(H) pMad focused on L4 in the pupal wing. (E) *yw* wild type phenotype. (F) *tkv* RNAi ($gbb^{4}/+$, dpp^{shv} > *tkv RNAi*). (G) *tkv* RNAi, *gbb* mutant (gbb^{4}/gbb^{51}, dpp^{shv} > *tkv RNAi*). (H) *Mad* RNAi (dpp^{shv} > *mad RNAi*). (I)–(N) Adult wings focused on L4. (I) *yw* wild-type phenotype. (J) *tkv* RNAi (dpp^{shv} > *tkv RNAi*). (K) *tkv* RNAi, *sog* mutant (sog^{P129D}, dpp^{shv} > *tkv RNAi*). (L) *tkv* RNAi, *cv* mutant (cv^{70}, dpp^{shv} > *tkv RNAi*). (M) *tkv* RNAi, *gbb* mutant (gbb^{4}/gbb^{51}, dpp^{shv} > *tkv RNAi*). (N) *Mad* RNAi (dpp^{shv} > *mad RNAi*). GFP-Dpp (green, (A)–(D)), pMad (purple, (A′)–(D′)) and merged images (A″)–(D″) focused on L4. All pupal wings were fixed at 24 h AP.

1997), how can low level of *tkv* retain Dpp in the LVs? Since Dpp could potentially spread 4–5 cell diameters into the intervein regions (Fig. 4(B)–(D)), where the *tkv* expression level is high, we wondered whether additional mechanisms are also involved in retaining Dpp within the LVs. In contrast to the widespread pMad gradient seen in the third instar wing disk, pMad was highly localized at or near the Dpp producing cells (LVs) in the pupal wing (Fig. 5(A) and (A′)). Therefore, we investigated whether Dpp signaling is required for Dpp retention. First, we hampered Dpp signal transduction in the LVs by overexpressing Dad (homolog of mammalian inhibitory Smad6/7) and examined the GFP-Dpp distribution. We found that GFP-Dpp dots accumulated into the intervein regions along the LVs, where pMad was enhanced at 24 h AP (Fig. 5(B) and (B″)). Second, we knocked down *Mad* (homolog of mammalian Smad1/5/8) in the LVs by RNAi. Like *tkv* RNAi wing (Fig. 4(B′)), pMad accumulation was reduced in the LVs and enhanced in the intervein regions along the LVs at 24 h AP (Fig. 4(H)). Accordingly, thickened or split veins were observed in the *mad* RNAi adult wings (Fig. 4(N)). Third, Dpp signaling was up-regulated in the LVs by a constitutively active form of Tkv. This resulted in the loss of pMad accumulation within the PCV region at 24 h AP and a crossveinless phenotype in the adult wing (Fig. 5(E), (E′) and (F)). In contrast, overexpression of wild-type Tkv in the LVs did not affect PCV formation (Fig. 5(C), (C′), and (D)). These results indicate that Dpp–Gbb heterodimer movement toward the PCV is sufficiently sequestered in the LVs by the activity of Dpp–Gbb target genes but not through the regulation of *tkv* transcription. To address whether extracellular domains of Tkv are involved in the active retention of Dpp in LVs, a constitutively active form of Tkv lacking its extracellular domains (ΔE-caTkv; (Haerry et al., 1998)) was expressed in the LVs. As shown in Fig. 5(G), (G′), and (H), over-expression of ΔE-caTkv did not affect pMad in the presumptive PCV or adult PCV formation, suggesting that the extracellular domain of Tkv is also required for sequestering Dpp. Thus, the extracellular ligand-bound domain of Tkv and the positive feedback mechanism are both critical for the active retention of Dpp in the LVs. Taken together, our data suggest that the spatial distribution of Dpp is tightly regulated to recapitulate the *Drosophila* wing vein pattern through two distinct mechanisms: directional transport of Dpp–Gbb heterodimers into the PCV primordia by a Sog–Cv complex and the active retention of these ligands in the LVs by Tkv and a positive feedback loop.

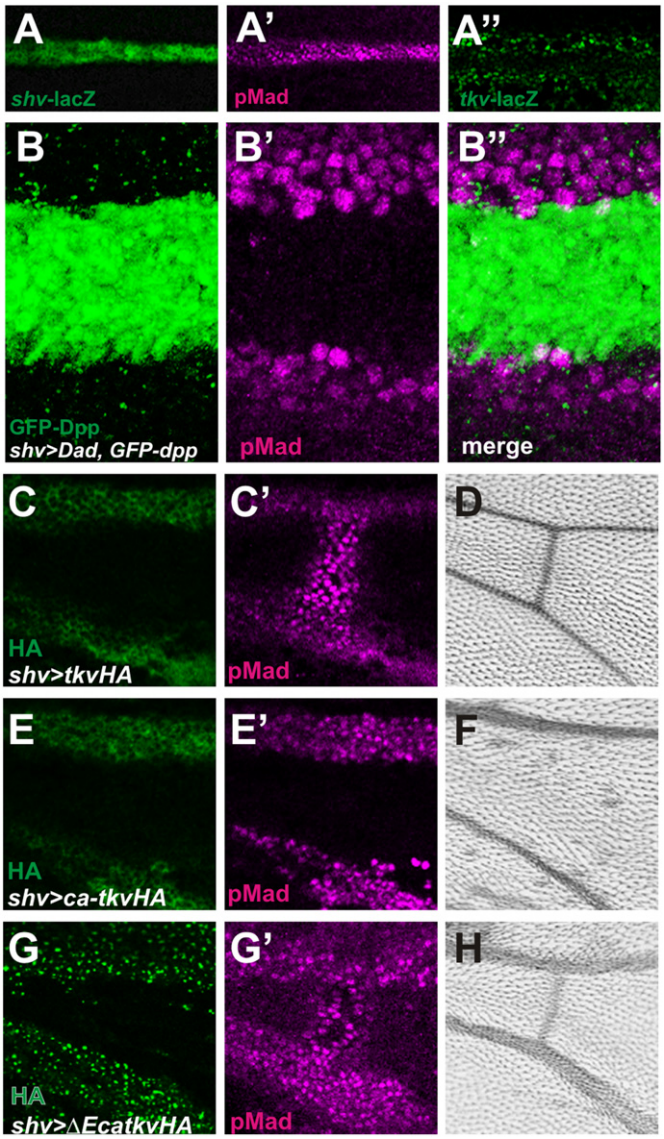

Fig. 5. *Active retention of Dpp requires a positive feedback mechanism.* (A)–(A″) Pupal wing focused on L4 at 24 h AP. (A) dpp^{shv}-*lacZ* (a reporter gene containing a portion of the *dpp* shortvein enhancer region located upstream of the *dpp* transcription unit). (A′) pMad (purple). (A″) *tkv-lacZ* (green). (B)–(B″) GFP-Dpp distribution in a pupal wing overexpressing Dad (dpp^{shv} > *GFP-dpp*, *Dad*). GFP-Dpp (green, B), pMad (purple, B′), and merged images (B″) focused on L4. (C)–(H) Pupal and adult wings focused on the PCV expressing wt-Tkv-HA (dpp^{shv} > *tkv-HA*) (C), (C′) and (D), *ca*-Tkv-HA (dpp^{shv} > *ca-tkv-HA*) (E), (E′), and (F) and *ca*-Tkv-HA lacking extracellular domain of Tkv (dpp^{shv} > ΔE-*ca-tkv-HA*) (G), (G′), (H). HA (C), (E), (G), pMad (C′), (E′), (G′) staining and adult wings (D), (F), (H). All pupal wings were fixed at 24 h AP.

Directionality of Dpp transport is prepatterned by sog expression

The accumulation of GFP-Dpp dots along the LVs was Sog–Cv-independent and was not directed to the PCV region when active retention of Dpp was disturbed (Figs. 4, 5). This indicates that directional Dpp movement to the PCV is not simply due to loss of active retention in the LVs but requires Dpp transporters. What then regulates the direction of facilitated Dpp transport? During the D–V patterning of the early embryo, Dpp movement to the dorsal side is regulated by a Sog inverse gradient from the lateral to the dorsal side of the embryo (Srinivasan et al., 2002). Similarly, *sog* transcription was restricted to the intervein regions but absent from the LVs (Dpp producing cells) and the CVs (Fig. 6(A)and (B)) and Sog proteins appear to move into the vein regions (Araujo et al., 2003). Thus, Dpp could be directed at the PCV, where *sog* expression is low. This view predicts that lowering *sog* transcription not only interferes with the signal at the PCV (by loss of Dpp transport) but also ectopically induces pMad accumulation (by modulating the direction of Dpp transport). Indeed, ectopic pMad signal was often observed in the intervein regions in viable hypomorphic *sog* mutants. (Fig. 6(E)–(H)) and ectopic vein fragments were formed in the adult wings (Fig. 6(K) and (L)), indicating that maintaining the *sog* expression pattern is critical for determining the direction of Dpp movement. If Dpp directional movement is based on a Sog inverse gradient, *sog* expression is predicted to be regulated independently of the Dpp signal. Consistently, it has been shown that early *sog* expression is excluded from the PCV at 19 h AP in *cv-2* mutant wings, where the Dpp signal is severely suppressed at the PCV (Ralston and Blair, 2005). However, since *cv-2* is induced by the Dpp signal and is not directly required for Dpp transport ((Serpe et al., 2008) and this study), it is possible that *sog* is sufficiently repressed by the initial Dpp signal at the earlier stages in *cv-2* mutants. To determine whether the initial *sog* transcription is regulated in a Dpp signal-independent manner, we investigated the *sog* expression in a *cv* null mutant, in which the facilitated Dpp transport is completely suppressed. We found that *sog* expression was excluded from the PCV region at 20 h AP, even in

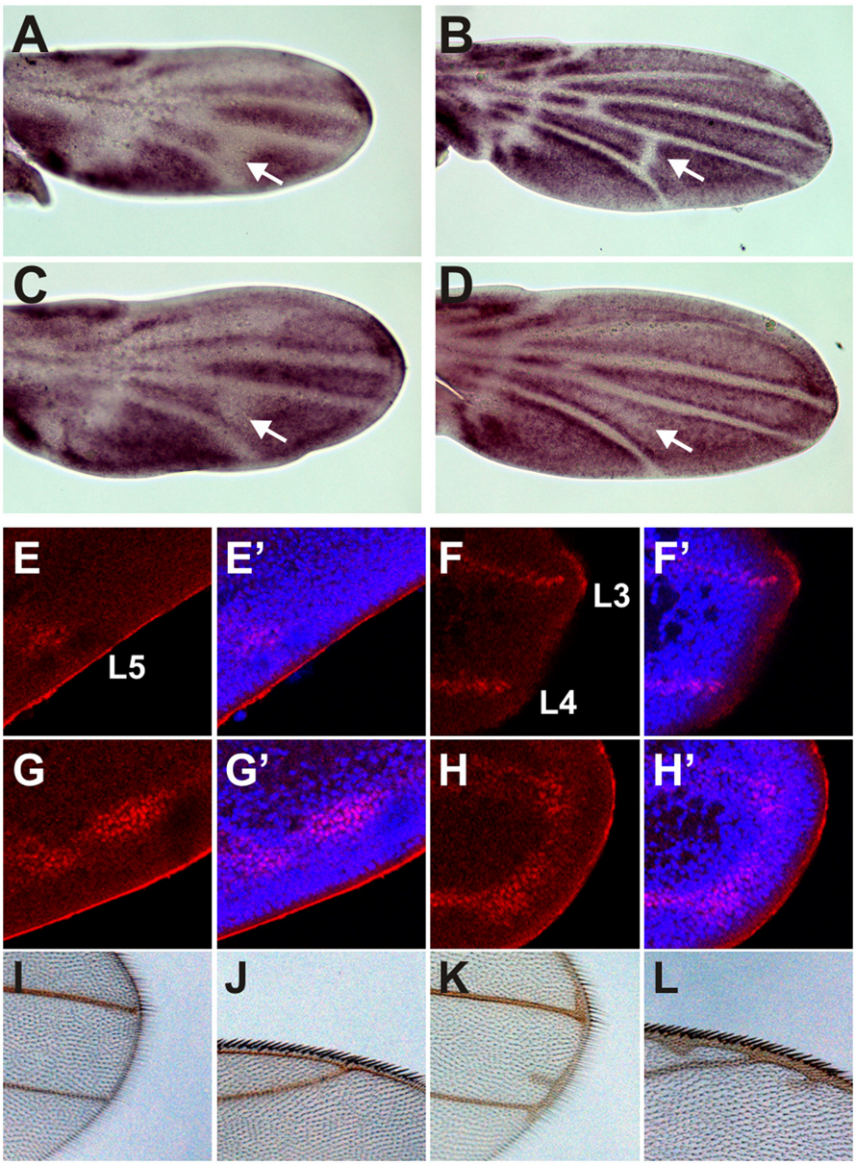

Fig. 6. *sog expression prepatterns PCV position in early pupal wings.* (A), (B) *sog* expression pattern in wild-type pupal wings. (A) 20 h AP. (B) 24 h AP. (C), (D) *sog* expression pattern in *cv* mutant pupal wings. (C) 20 h AP. (D) 24 h AP. The PCV regions are indicated by arrows. (E)–(H) Pupal wings at 24 h AP. (E)–(H) pMad (red). (E′)–(H′) Merged images of pMad and DAPI (nuclear marker, blue). (E), (G) Distal region of L5. (F), (H) Distal regions of L3 and L4. (E), (F) Wild-type phenotype ($sog^{P129D}/+$). (G), (H) *sog* mutant (sog^{P129D}). (I)–(L) Adult wings. (I), (K) Distal regions of L3 and L4. (J), (L) Distal region of L2. (I), (J) Wild type. (K), (L) *sog* mutant (sog^{P129D}).

cv mutants (Fig. 6(C)). Thus, our data suggest that absence of *sog* transcription prepatterns the future PCV position by regulating the direction of Dpp transport. Since *sog* repression was canceled at the future PCV at a later stage (24 h AP) without the Dpp signal (Fig. 6(D)), continuous Dpp signal must be required for maintaining *sog* repression at the PCV.

Discussion

Here we visualized the Dpp/BMP distribution in the *Drosophila* pupal wing and addressed the molecular mechanisms by which the spatial distribution of Dpp is regulated to form elaborate wing vein patterns. We showed that Dpp is directionally moved by Sog–Cv to the future PCV, while Dpp is actively retained in the LVs by Tkv and a positive feedback mechanism. Thus, our data suggest that spatial Dpp distribution is tightly regulated to reflect wing vein patterns by two distinct mechanisms: a facilitated transport mechanism and an active retention mechanism. Furthermore, our data suggest that the directionality of Dpp movement to the future PCV is prepatterned by Dpp signal-independent *sog* transcription at the PCV.

Directional Dpp diffusion toward the PCV region

Our data clearly showed that Dpp moves from the LVs to the future PCV region (Fig. 1). Although we could not visualize the extracellular-specific Dpp distribution due to technical difficulties, our data suggest that Dpp diffuses through the extracellular space for the following three reasons. First, the extracellular BMP-binding proteins, Sog and Cv, were required and sufficient for Dpp distribution in the PCV region (Fig. 2). A similar Dpp transport mechanism operating in embryo patterning also occurs in the extracellular space (Wang and Ferguson, 2005). Second, *cv* mutant clones affect adult PCV formation non-cell autonomously (Garcia-Bellido, 1977; Serpe et al., 2008). Third, since cytoneme-

like actin fiber directing to the LVs was not observed (Fig. S1), it is unlikely that the ligands are moved by cytonemes.

If Dpp diffuses at the extracellular level, how does Dpp specifically accumulate at the PCV region (Fig. 1 and S1)? Three possible mechanisms are considered: (1) Dpp diffuses directionally to the PCV region, (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 (Fig. 2(E)). Ablation of active retention in the LVs resulted in GFP-Dpp and pMad accumulation along the LVs (Fig. 4(B)). These data suggest that the invervein regions retain the competence to receive Dpp signaling. Therefore we favor the model in which Dpp diffuses directionally to the PCV region.

How does Dpp move into the PCV region? GFP-Dpp accumulation at the basal side of the PCV region (Fig. S1) raises a possibility that Dpp diffuses through the lumen in the veins and is internalized by cell surfaces near the lumen. Alternatively, Dpp may accumulate at the region where Dpp signaling is high, as proposed in the embryo (Wang and Ferguson, 2005). Since Dpp signaling appears to be required for wing vein formation and therefore the lumen formation, both possibilities may not be separated. These models may be addressed by asking whether Dpp accumulation is affected by modifying morphogenesis in a Dpp signaling-independent manner.

Although it remains to be investigated in which route Dpp diffuses, our data suggest that directionality of Dpp diffusion is prepatterned by Dpp signal-independent *sog* transcription (Fig. 6). Sog appears to form a gradient from the intervein region toward the future vein regions and its flux mobilizes the Dpp–Gbb–Sog–Cv complex to the PCV region. Tlr, a paralog of Tld, probably maintains Sog gradient by cleaving Sog at the PCV region (Serpe et al., 2005). Thus, the mechanism used for PCV formation is analogous to that of the embryo (O'Connor et al., 2006). The *sog* transcriptional regulation was mainly studied in the early embryo, where a nuclear gradient of the transcriptional factor Dorsal is a key regulator (Markstein et al., 2002), but little is known about the upstream factors required for *sog* transcription in the pupal wing. It would therefore be important to investigate how *sog* transcription is regulated in the pupal wings. Although the PCV position is prepatterned by Dpp signal independent *sog* transcription, we note that clear ectopic crossvein formation was not induced by large *sog* null clones or *sog* hypomorphic mutants ((Shimmi et al., 2005a), Fig. 6(K) and (L)). These results are probably due to severe loss of Dpp diffusion as a consequence of loss of the Sog function as a Dpp transporter. However, the PCV formation was recovered under some conditions even when *sog* is uniformly expressed in the pupal wing (Serpe et al., 2008). Thus, Dpp appears to move toward the PCV without *sog* transcriptional prepattern in this case. We assume that additional factors are required for creating the Sog protein gradient in addition to *sog* transcriptional regulation. For this, it would be interesting to investigate the mutants showing extra or aberrant CV phenotypes, where directionality of Dpp is predicted to be affected. For example, viable integrin mutants display extra or aberrant CVs (Wilcox et al., 1989). Interestingly, αPS1 integrin can bind to Sog and mutant clones of βPS integrin affect Sog distribution (Araujo et al., 2003). These suggest that the ECM may be critical for directional Dpp movement. Viable *Dcdc42* mutants also show extra CV phenotypes (Baron et al., 2000; Genova et al., 2000) and induce ectopic pMad accumulation in the intervein region without affecting *dpp* transcription (M.S and O.S., unpublished data). Although it remains unknown how Dcdc42 is involved in ectopic Dpp signaling, we note that RhoGAP88C *crossveinless-c* (*cv-c)* mutants show crossveinless phenotypes (Denholm et al., 2005). Thus the activity of small GTPases may be associated with the directional Dpp diffusion.

Active retention of Dpp in the LVs

In contrast to the facilitated Dpp transport to the future PCV, we found that Dpp mobility was tightly restricted by Tkv in the LVs (Fig. 4), while the Dpp signaling range is insensitive to overexpression of wild-type Tkv (Fig. 5(C) and (D)). This observation appears to be contradicted in the larval wing disk, where the Dpp-signaling range is manipulated by overexpression of wild-type Tkv (Lecuit and Cohen, 1998; Tanimoto et al., 2000). A recent paper suggests that most of the extracellular Dpp appears to be a receptor-unbound form and therefore gain-of-function of Tkv modulates Dpp distribution in the larval wing (Schwank et al., 2011). We presume that the insensitivity in the pupal wings reflects the limited number of free Dpp in the LVs, probably because most of Dpp is in a complex with receptors or Sog–Cv. Although Tkv plays a critical role for active retention of Dpp in LVs, *tkv* transcription is down-regulated by Dpp signaling in the pupal wings (Fig. 5(A″)) (de Celis, 1997). How could the low level of Tkv manage to restrict Dpp mobility? Our data showed that Dpp signaling is also important for retaining Dpp in the LVs (Fig. 5). This raises a possibility that active retention of Dpp is mainly achieved by Dpp signaling. However, comparisons of phenotypes between overexpression of caTkv and ΔE-caTkv suggest that both Dpp signaling and extracellular domain of Tkv are involved in the active retention of Dpp in the LVs (Fig. 5(E)–(H)). Despite this, loss of signaling alone caused an effect. A simple explanation for this is that ligand-receptor complex formation is not sufficient for the active retention of Dpp. Since the mutant clones for *schnurri*, a transcriptional cofactor for Mad, also caused forked or split veins when the clones occurred within the veins (Torres-Vazquez et al., 2000), the positive feedback mechanism is probably mediated by transcription of the Dpp target genes. Given that Dpp signal represses *sog* transcription at the PCV at 24 h AP, one possibility is that *sog* is also down-regulated by Dpp signaling in LVs, which helps generate Sog protein gradient from the intervein regions to the LVs to retain the ligands. However, since large *sog* null clone disrupts the PCV formation but not the LVs in adult wing (Shimmi et al., 2005a) and Dpp diffusion from the LVs by *tkv* RNAi is Sog independent (Fig. 4(C) and (K)), we think it unlikely that *sog* repression in the LVs plays a primary role for the active retention of Dpp. Potential mechanisms include (a) induction of factors facilitating the Tkv-ligand complex, (b) induction of factors trapping the extracellular receptor-unbound ligands, or (c) induction of factors modulating the turnover of the receptor-ligand complex. Previous studies indicated that two extracellular proteins, Cv-2 and larval translucida (Ltl), are the 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* expression is localized at the PCV and appears to be excluded from the LVs (Serpe et al., 2008). On the other hand, *ltl* is expressed at the pupal wing veins and its loss induces the ectopic Dpp signal in the intervein regions (Szuperak et al., 2011). Although overexpression of Ltl inhibits Dpp signaling at the PCV, the precise mechanism by which Ltl regulates Dpp signaling remains elusive. Alternatively, endocytosis may be involved in Dpp retention in the LVs. It has been shown that *Drosophila* Dynamin *shibire* (*shi*) temperature-sensitive mutants show thickened wing vein phenotypes during the pupal stages. Thus, the phenotype could be interpreted as loss of Dpp retention, for example, through decreasing the Tkv-ligand complex turnover, although it was interpreted as loss of Notch-Delta signaling (Parks et al., 2000). Since endocytosis is also required for the Dpp signal (Belenkaya et al., 2004), further studies are required to allocate endocytosis function. It is of note that the positive feedback mechanism also appears to be critical for D–V patterning of the embryo, although in this case the BMP signal promotes Dpp–Tkv

binding to sustain the long-range Dpp diffusion (Wang and Ferguson, 2005). Downstream factors remain to be identified in both the embryo and pupal wing. Unbiased approaches, such as gene expression profile analysis, would be needed to identify the factors responsible for active retention of Dpp.

Evolutionary aspects

Interestingly, variations in insect wing vein patterns are often based on the number or positions of the CVs. For example, within the Diptera, Astelids have no PCV and the *planitibia* subgroup of the Hawaiian *Drosophila* have an extra crossvein between L3 and L4, although in both cases their LV patterns are similar to that of *Drosophila melanogaster* (De Celis and Diaz-Benjumea, 2003; Edwards et al., 2007). Changes in the prepatterned directionality of Dpp diffusion may be responsible for such wing vein diversity. Further understanding of the mechanisms behind the direction of Dpp diffusion in *Drosophila* and other insects may explain such diversity in insect wing vein patterns.

Summary

In summary, our data suggest that Dpp distribution in the pupal wing is regulated at the extracellular level by the combination of two distinct mechanisms, long-range facilitated diffusion by Sog–Cv and short-range signaling by Tkv and a feedback mechanism, to form the elaborate wing vein patterns in *Drosophila*. Our study in the pupal wing also provides a new model for addressing the mechanisms underlying Dpp diffusion.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.04.009.

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