Development 133, 183-193 doi:10.1242/dev.02214

Shaping BMP morphogen gradients in the *Drosophila* embryo and pupal wing

Michael B. O'Connor[1,2,*], David Umulis[3], Hans G. Othmer[4] and Seth S. Blair[5,*]

In the early *Drosophila* embryo, BMP-type ligands act as morphogens to suppress neural induction and to specify the formation of dorsal ectoderm and amnioserosa. Likewise, during pupal wing development, BMPs help to specify vein versus intervein cell fate. Here, we review recent data suggesting that these two processes use a related set of extracellular factors, positive feedback, and BMP heterodimer formation to achieve peak levels of signaling in spatially restricted patterns. Because these signaling pathway components are all conserved, these observations should shed light on how BMP signaling is modulated in vertebrate development.

Introduction

Key to many developmental processes is the ability of cells to reproducibly interpret information regarding their spatial position within a developing field so that patterns and, ultimately, tissues form with the proper dimensions and connectivity. Nowhere have these processes been more thoroughly studied than in the early *Drosophila* embryo and larval imaginal discs. In each case, morphogens, special classes of signaling molecules that specify cell fate in a concentration-dependent manner, have emerged as key components that guide patterning. In recent years, great efforts have been made to elucidate how cells interpret and respond to morphogen concentration gradients with specific gene expression outputs. Equally important, however, is to determine what mechanisms generate extracellular concentration gradients in the first place. Here, we review recent work on how the gradients formed by, and the signaling output of, a specific family of morphogens, the bone morphogenetic proteins (BMPs), are influenced by the formation of ligand heterodimers and by their binding to extracellular factors. We concentrate specifically on how these features enhance BMP signaling during early embryonic patterning and late wing development. We also review recent experimental data on the existence of positive feedback as an important additional component for proper BMP signaling in both the embryo and pupal wings. Furthermore, we discuss how computational modeling has offered insights into how extracellular gradients form and how the gradients function reliably in the face of genetic variation. Due to space limitations, we only briefly allude to related issues from vertebrates (for reviews, see Balemans and Van Hul, 2002; De Robertis and Kuroda, 2004; Kishigami and Mishina, 2005; Schier and Talbot, 2005).

[1]Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455, USA. [2]The Howard Hughes Medical Institute. [3]Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN 55455, USA. [4]School of Mathematics and Digital Technology Center, University of Minnesota, Minneapolis, MN 55455, USA. [5]Department of Zoology, 250 North Mills Street, University of Wisconsin, Madison, WI 53706, USA.

*Authors for correspondence (e-mail: moconnor@mail.med.umn.edu and ssblair@wisc.edu)

The basics of BMP signaling in *Drosophila*

BMPs belong to the TGFβ superfamily of growth and differentiation factors. Three BMP-type ligands are present in *Drosophila*: Decapentaplegic (Dpp), a functional ortholog of vertebrate BMPs 2 and 4, Glass bottom boat (Gbb), a member of the BMP 5,6,7 subgroup, and Screw (Scw), a distantly related family member (Newfeld et al., 1999). In the embryo and wing, ligand dimers signal through a common set of receptors that include the type II receptor Punt, and the two type I receptors Saxophone (Sax) and Thickveins (Tkv) (Fig. 1) (reviewed by Parker et al., 2004). Upon ligand binding, Sax and Tkv phosphorylate Mad, the sole *Drosophila* BMP Smad. Phosphorylated Mad (pMad) forms a complex with the co-Smad Medea, which then translocates into the nucleus. Smad proteins either activate or repress transcription, depending upon the particular complement of co-factors present.

Dpp as an embryonic morphogen

In the early *Drosophila* embryo, two major tissues, amnioserosa and dorsal ectoderm, form from the 40% dorsal-most cells. The amnioserosa derives from the eight to ten cells that lie adjacent to the dorsal midline, while dorsal ectoderm derives from more lateral cells. In *dpp* null mutants, all dorsal cells acquire a ventral neurogenic fate (reviewed by Sutherland, 2003). Moreover, injection experiments have shown that high levels of *dpp* mRNA convert all dorsal cells to an amnioserosa fate, whereas moderate levels specify dorsal ectoderm (Ferguson and Anderson, 1992). Dpp therefore acts as a concentration-dependent morphogen for the specification of both tissues.

The visualization of pMad levels using a phospho-specific antibody has shown that pMad accumulates in the nucleus of dorsal cells midway through cellularization. Initially, anti-pMad staining is low and encompasses the dorsal-most 18-20 cells, but then it rapidly contracts and strengthens, and by the onset of gastrulation a sharp, step gradient of pMad has formed (Fig. 2B), in which pMad levels are high in the dorsal-most five to nine cells, but rapidly drop off to undetectable levels in more lateral regions over two to three cell diameters (Dorfman and Shilo, 2001; Ray and Wharton, 2001; Ross et al., 2001). Similarly, by the end of cellularization, the co-Smad Medea accumulates in the nuclei of the dorsal-most cells, forming a sharp stripe (Sutherland et al., 2003).

The embryonic Dpp gradient requires extracellular modulators

Although in the early embryo Dpp activity is highest near the dorsal midline and lower at the lateral boundaries, *dpp* is transcribed uniformly throughout the entire dorsal domain (Fig. 2A). In other words, the sharp, step distribution forms within a domain of uniform *dpp* expression. Thus, additional extracellular factors must be involved in shaping the ligand activity gradient.

Mutations in *short gastrulation* (*sog*), *twisted gastrulation* (*tsg*) and *tolloid* (*tld*) produce phenotypes similar to, but less severe than, those exhibited by *dpp* mutants (Arora and Nusslein-Volhard, 1992).

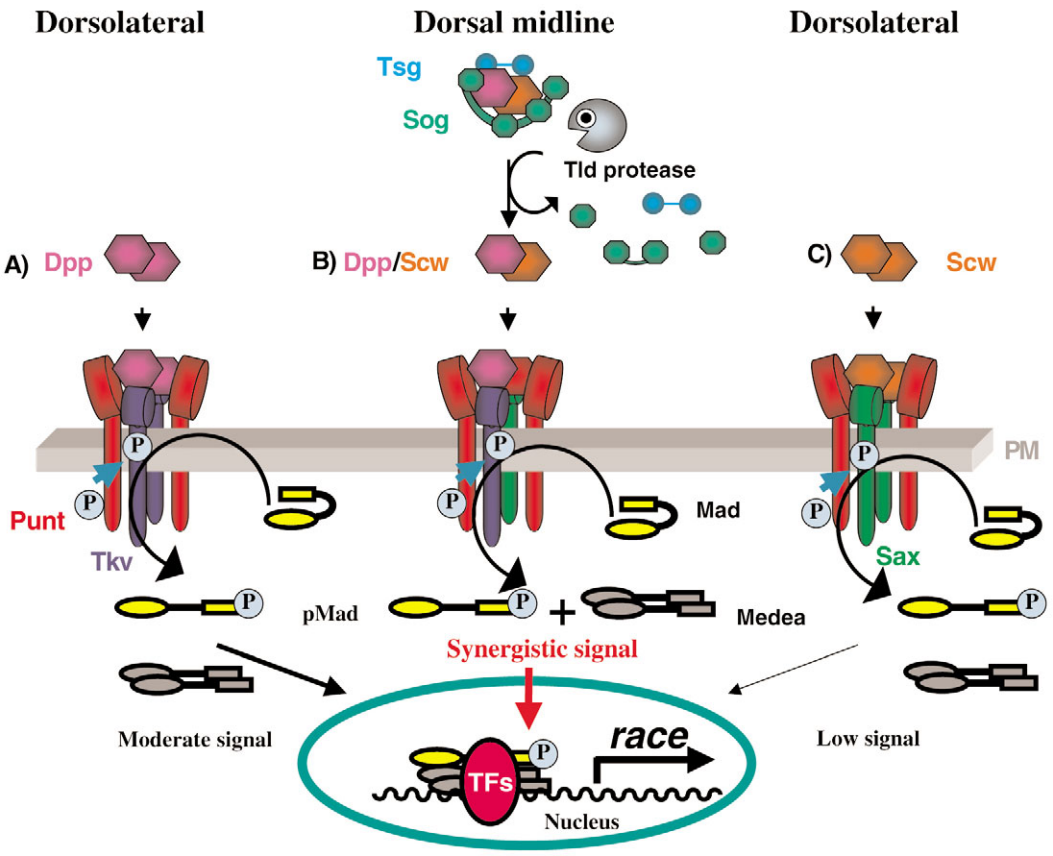

Fig. 1. BMP signaling in *Drosophila*. Three different BMP ligand species exist in the early embryo. (**A**) Dpp homodimers, (**B**) Dpp/Scw heterodimers, and (**C**) Scw homodimers. (B) Dpp/Scw heterodimers are preferentially transported to the midline through the action of Sog/Tsg and Tld. At the midline, the heterodimer accumulates and is free for signaling as Tld processes Sog. This heterodimer binds to a heteromeric receptor complex, probably a tetramer located in the plasma membrane (PM), composed of two type II receptors (Punt), and one subunit each of the type I receptors Tkv and Sax. Punt activates Tkv and Sax by phosphorylating residues within their GS boxes (a glycine-serine rich segment near the membrane). Once activated, the type I receptors phosphorylate Mad. Mad then associates with the co-Smad Medea (probably in a trimeric complex of uncharacterized subunit composition), and the complex translocates to the nucleus where it binds to and activates or represses target genes in conjunction with other transcription factors (TFs). At the midline, the Sax and Tkv receptors produce a synergistic signal that results in the activation of high-threshold target genes, such as *race*. In the lateral regions, homodimers of Scw and Dpp produce moderate and low levels signals, respectively, that can activate low-threshold response genes, such as *pannier* (*pnr*) (for details, see Shimmi et al., 2005b).

In fact, these mutations all disrupt the step gradient. In *tld* mutants, all signaling is reduced, but in *sog* and *tsg* mutants, signaling in dorsolateral regions increases, and signaling in the dorsal-most cells decreases, producing a broad dorsal region of pMad that much more closely resembles the pattern of *dpp* expression (Ross et al., 2001). These genes all encode secreted products (Arora et al., 1994; Francois et al., 1994; Mason et al., 1994; Shimell et al., 1991). Both Sog and Tsg contain cysteine-rich motifs (CRs) that facilitate the binding of these proteins to Dpp in a ternary complex that sequesters Dpp from its receptors (Ross et al., 2001). Tld is a metalloprotease that can cleave Sog (Marques et al., 1997; Shimmi and O'Connor, 2003).

Thus, Sog and Tsg inhibit signaling in dorsolateral cells, but promote it in the dorsal-most cells. The ventrolateral expression of Sog is thought to be the key to both of these events (Fig. 2). In lateral regions, the complex of Sog, Tsg and Dpp should inhibit the binding of Dpp to its receptors to locally inhibit signaling. However, because the complex prevents Dpp from interacting with its receptor or with other cell-bound ligand-binding molecules, it should also facilitate long-range Dpp diffusion. As the net flux of Sog is away from its site of synthesis in ventral lateral regions and towards the dorsal midline (Srinivasan et al., 2002), the flux will facilitate the transport of Dpp out of lateral regions towards the midline. This should promote Dpp signaling by increasing the Dpp concentration at the dorsal midline (Holley et al., 1996; Eldar et al., 2002; Mizutani et al., 2005).

In this model, a key component that helps create Sog flux is the processing of Sog by the metalloprotease Tld (Holley et al., 1996; Marques et al., 1997; Shimmi and O'Connor, 2003). This dorsally expressed protease acts locally (Wang and Ferguson, 2005) to cleave Sog when bound to Dpp. Released Dpp has two possible fates: it can bind to its receptor and signal, or it can be recaptured by another Sog/Tsg complex. When Sog levels are maximal, as in the lateral regions, the probability of recapture is high, whereas at the midline, where Sog levels are low, released Dpp is more likely to bind to its receptors and signal (Eldar et al., 2002; Mizutani et al., 2005).

Sog/Tsg/Tld-mediated Dpp transport

Although the basic Dpp transport mechanism was proposed over ten years ago, it is only recently that the distribution of the Dpp protein in the early embryo has been visualized. In one study, epitope-tagged Dpp was expressed in its normal dorsal-on ventral-off blastoderm pattern using a transgene construct driven by the endogenous *dpp*

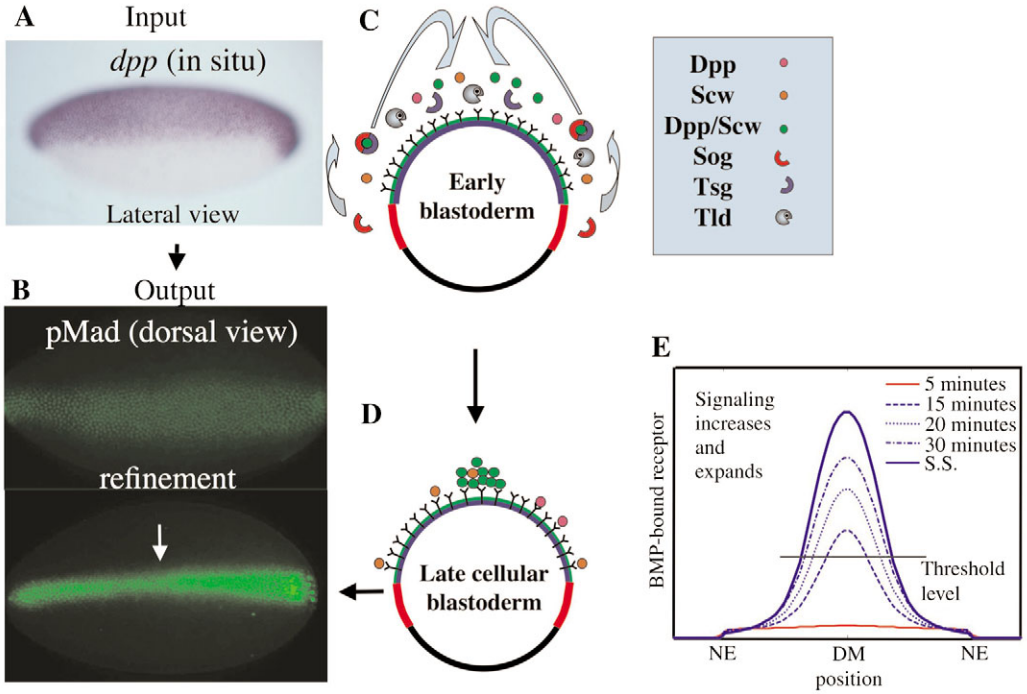

Fig. 2. Patterning the dorsal side of the *Drosophila* embryo by BMP transport. (**A**) Dpp is transcribed uniformly within the dorsal half of the embryo in the early blastoderm. (**B**) Initially, Mad phosphorylation is wide and of low intensity at the mid-cellular stage, but then refines during late blastoderm into a sharper and more intense stripe. The refinement requires an additional unknown factor that is induced by the early low-level Dpp signal (see Wang and Ferguson, 2005). (**C**) A schematic cross-sectional representation of an embryo showing the expression domains of the various extracellular components (red, *sog*; blue, *tsg* and *tld*; green, area of overlap in *dpp* and *scw* expression). Sog diffuses into the dorsal domain from its ventrolateral site of synthesis, and preferentially complexes with Dpp/Scw heterodimers and Tsg. (**D**) Net diffusion of this complex, driven in part by Tld processing of Sog, promotes accumulation of the Dpp/Scw heterodimer near the midline from mid- to late-cellular blastoderm stage. Homodimers of Dpp and Scw are not transported efficiently as they have a lower affinity for the Sog/Tsg complex (see Shimmi et al., 2005b). (**E**) The spatial distribution of BMP-bound receptor at various times obtained using modeling methods similar to those described by Mizutani et al. (Mizutani et al., 2005) in which there is a constant BMP production/degradation. Note that the model predicts that, at a given threshold, the intensity of the pMad stripe should both increase in time and widen. NE, neuroectoderm; DM, dorsal midline.

'hinR' promoter/enhancer (Shimmi et al., 2005b). The tagged Dpp accumulates in a profile that is very different from its mRNA pattern and, ultimately, high levels of Dpp protein amass near the dorsal midline. To determine whether Dpp was located inside or outside the cell, Wang and Ferguson (Wang and Ferguson, 2005) developed a novel staining protocol called perivitelline injection (PVI), in which an antibody is injected into the space between the cell membrane and the vitelline membrane of live embryos. Under these conditions, the antibody has no access to the cytoplasm and can only interact with secreted Dpp. Once again, a stripe of Dpp is seen at the dorsal midline, confirming that it represents the accumulation of extracellular Dpp, presumably bound to receptors.

The Dpp profile nicely matches the pMad and nuclear Medea profiles, suggesting that Dpp transport is responsible for achieving the peak signaling levels necessary for the activation of high-threshold target genes. Furthermore, as predicted by the transport model, Dpp accumulation at the midline requires the activity of the Sog, Tsg and Tld proteins (Shimmi et al., 2005b; Wang and Ferguson, 2005). However, mutations in these genes affect extracellular Dpp accumulation differently (Wang and Ferguson, 2005). In *sog* mutants, extracellular Dpp binds to all cells in the dorsal domain, but the total level of Dpp bound by any one cell at the midline is lower than normal, whereas in lateral regions it is higher than normal. Accordingly, pMad staining is broader but weaker at the midline, resulting in an expanded mid-level target gene expression and the loss of high-level target gene expression (Ross et al., 2001). By contrast, *tsg* mutant embryos accumulate little extracellular Dpp anywhere in the dorsal domain. In addition, *tsg* mutant embryos exhibit lower levels of residual pMad staining than do *sog* mutants and, unlike *sog* mutants, cannot be rescued by increasing Dpp levels (Wang and Ferguson, 2005). These observations suggest that Tsg, in addition to its role as a co-inhibitor and a component of the transport complex, is likely to have a Sog-independent role in promoting BMP signaling. Recent work suggests that the zebrafish Tsg homolog also has role in promoting BMP signaling that is independent of Chordin, the vertebrate Sog homolog (Xie and Fisher, 2005). Possible mechanisms include the enhanced binding of Dpp to either its signaling receptors or to some other cell surface-binding protein, or inhibition of some, as yet unidentified, extracellular antagonist of BMP signaling.

Hetero- and homodimers produce biphasic signaling

In addition to Dpp, amnioserosa specification also requires Scw, a second BMP-type ligand (Arora et al., 1994). Unlike *dpp*, *scw* is expressed uniformly, and only at the blastoderm stage. Mutations in *scw* result in less severe phenotypes than do mutations in *dpp*. Amnioserosa is lost in both cases, but in *scw* mutants some dorsal ectoderm is still formed. Dpp and Scw display an asymmetric relationship in their ability to compensate for one another in the early

embryo, i.e. injection of *dpp* mRNA can rescue *scw* mutants, but *scw* mRNA cannot rescue *dpp* mutants (Nguyen et al., 1998). Furthermore, injection of *scw* mRNA synergistically enhances Dpp signaling.

Intriguingly, Shimmi et al. (Shimmi et al., 2005b) recently showed that Dpp and Scw form heterodimers in cell culture and in the embryo. Furthermore, they found that, in cell culture, the heterodimer produces tenfold more signal (pMad phosphorylation) than an equimolar mixture of Dpp and Scw homodimers. Interestingly, Scw homodimers exhibit very little signally ability in cell culture, which might explain the asymmetric rescuing ability of the two ligands in mRNA injection experiments.

The increased signaling ability of the Dpp/Scw heterodimer may be due to synergy between the two type I receptors Tkv and Sax. In vitro, the Dpp/Scw synergistic output required both Tkv and Sax, whereas Dpp homodimers only required Tkv (Shimmi et al., 2005b). In the embryo, the injection of activated *tkv* mRNA, but not *sax*, rescues *dpp*-deficient embryos in a dose-dependent fashion (Neul and Ferguson, 1998). Furthermore, just as *scw* mRNA injection augments *dpp* signaling, the injection of activated *sax* mRNA stimulates activated *tkv* signaling. This suggests that Scw probably signals through Sax, whereas Dpp primarily signals through Tkv, and that the two ligands synergistically activate the two receptors.

In vitro, the Dpp/Scw heterodimer has a higher affinity for Sog and Tsg than do their homodimers, and, as a result, the heterodimers are more likely than the homodimers to diffuse toward the dorsal midline as part of a Dpp/Scw/Sog/Tsg complex. Consistent with this view, it has been shown that in the absence of Scw, extracellular Dpp homodimers do not localize to the midline but instead remain broadly distributed, producing a low-level signal (Shimmi et al., 2005b; Wang and Ferguson, 2005). Taken together, these results suggest that the subdivision of dorsal cells into two tissues results from a biphasic signal that exploits unique aspects of both homo- and heterodimers. In this scenario, Dpp/Scw heterodimers are preferentially transported to the dorsal midline in a complex with Sog and Tsg. There, they are released from the complex by Tld and produce optimal output through the synergistic activation of a Sax/Tkv heteromeric complex, resulting in the specification of amnioserosa. By contrast, Dpp and Scw homodimers are not as efficiently transported as they have a lower affinity for Sog/Tsg. For this reason, they remain broadly distributed in the dorsal domain and produce a low-level signal by binding to homomeric complexes of Tkv and Sax, respectively. The broad, low-level signal pre-patterns the dorsal ectoderm and suppresses neurogenic activity. However, final specification of the dorsal ectoderm probably requires a second round of signaling via Dpp, but not Scw, that occurs later during germ band extension stages and that is likely to account for the differences in cuticular phenotypes exhibited by the two mutants (Dorfman and Shilo, 2001).

Although the above model can explain many aspects of dorsal patterning, evidence against the role of heterodimers in signaling comes from experiments in which Dpp and Scw are expressed in non-overlapping regions of *scw* mutant or *dpp scw* double-mutant embryos (Neul and Ferguson, 1998; Nguyen et al., 1998; Wang and Ferguson, 2005). As heterodimer formation is thought to occur in the Golgi during secretion (Gray and Mason, 1990), expression of the two ligands in different regions of the embryo should only allow for homodimer formation. These results demonstrated that at least moderate levels of signal can be produced by homodimers and led to the suggestion that some novel higher order receptor complex might contribute to synergistic signaling. It is interesting to note in this regard that, when BMP ligands are added to vertebrate cells, the aggregate size of preformed receptor complexes, which presumably represent tetramers, has been shown to increase (Hassel et al., 2003). These observations indicate that additional work will be required to ascertain the relative contributions of homo- and heterodimers to the patterning process.

Modeling of BMP embryonic patterning

It has now been directly demonstrated that Dpp ligand accumulates on the surface of cells at the dorsal midline, in agreement with the transport model. However, because of the complexity of the network, it is difficult to predict how the system will behave in the face of genetic perturbations without a quantitative mathematical model that incorporates diffusion and the known kinetic interactions. A desirable characteristic of such models is ‘robustness’, meaning that the output (e.g. the level of signal response) is relatively insensitive to variations in parameters over a physiologically reasonable range. Robust systems have a distinct evolutionary advantage, as they can better cope with naturally occurring fluctuations in the levels of system components.

The first computational model of dorsal patterning demonstrated the plausibility of a Sog-mediated transport mechanism for BMPs (Eldar et al., 2002), and provided a framework within which to explore the issue of pattern robustness. A combination of large-scale computation and analytical manipulation demonstrated that robustness of the patterning response requires several conditions regarding parameter choices. The conditions for robustness are that: (1) the processing of Sog by Tld depends on BMPs; (2) free BMPs do not diffuse; (3) BMPs bind irreversibly to receptors; (4) Sog displaces BMPs from receptors; and (5) Dpp homodimers are transported by the Sog/Tsg complex, whereas Scw is transported by Sog. Condition 1 has been shown in vitro (Holley et al., 1996; Marques et al., 1997; Shimmi and O’Connor, 2003), demonstrating the utility of analyzing a network for robustness requirements. However, no evidence exists for conditions 3 and 4, and condition 5 is not met as Dpp and Scw are not independently targeted for transport by Sog and Sog/Tsg, but are instead likely to be preferentially transported as a heterodimer (Shimmi et al., 2005b). Condition 2, that diffusion of Dpp is limited when Dpp is not bound to a soluble inhibitor, is more controversial. Although it is true that accumulation of Dpp near the dorsal midline requires Sog/Tsg-mediated transport, it is not clear what limits the spread of Dpp once it is localized. Early experimental data supported Dpp immobilization (Eldar et al., 2002). However, recent studies of embryonic patterning suggest that, even in the absence of carrier proteins, Dpp can act over 15-20 cell diameters (Mizutani et al., 2005), and that Scw acts at even greater distances (Wang and Ferguson, 2005). The disparity between Dpp and Scw is not caused by differences in their intrinsic diffusion rates because the molecules are of similar size and shape; instead, it probably reflects differences in production, degradation, and/or binding to other components of the system.

If Dpp is widely diffusible, how does its distribution evolve into a very sharp and narrow gradient in the model? The answer is that, even with high diffusion coefficients, the diffusion length of a ligand can be very short if other kinetic processes, such as binding to immobile receptors and subsequent degradation, act upon it. Another model of embryonic patterning allows the primary ligand to diffuse but incorporates receptor-mediated BMP degradation as a means to limit its spread (Mizutani et al., 2005). This model can simulate many of the observed in vivo distributions of pMad in different genetic backgrounds and on the appropriate time scales (see Fig. 2E for an example). In essence, this achieves what was

DEVELOPMENT

accomplished in the Eldar model by restricting the spread of BMPs, but in a more realistic manner. Moreover, the model suggests that any soluble BMP-binding protein can potentially expand the range of BMP action simply by protecting the ligand from receptor-mediated degradation.

Another interesting experimental result that can be explained by modeling is that the robustness of the system to changes in gene dosage depends on the gene. For instance, the embryo is not robust to changes in the levels of *sog* gene dosage (Mizutani et al., 2005), but is to changes in the level of *tsg*. Similarly, the embryo is quite robust with respect to changes in *scw* gene dosage, but not to *dpp*. If the conditions 3 to 5 set forth by Eldar et al. (Eldar et al., 2002) are not met, then what leads to the robustness of the system to changes in the concentrations of these proteins? To some extent the answer lies in the formation of heterodimers. Mathematical analysis of heterodimer formation demonstrates that it can provide an effective buffer against changes in gene dosage, at least for one partner of the heterodimer (Shimmi et al., 2005b). For instance, robustness with respect to Scw can be explained provided that Scw is produced in slight excess of Dpp, and that the more effective signaling form is the heterodimer. Analysis of the local dynamics of Sog/Tsg complex formation also suggests that this contributes to robustness. Furthermore, a series of dimerization reactions, in which the output of one step becomes the input to the next step (e.g. Dpp/Scw binds to Sog/Tsg), has an additive effect that further reduces the effect of perturbations (Shimmi et al., 2005b). This shows that sequential dimerization steps increase robustness in a spatially homogenous system, but this idea needs to be further analyzed to see whether the conclusions hold true when diffusion is incorporated into the model.

Positive feedback sharpens Dpp localization

While the mathematical models of extracellular BMP transport can account for the final distribution of pMad, other regulatory mechanisms probably contribute to the temporal formation and step-like distribution of this pattern. The mathematical models with time-independent BMP production predict that peak signaling originates near the dorsal midline, and, as time progresses, that pMad signaling widens and increases in intensity (Fig. 2E). In reality, the pMad levels increase with time at the dorsal midline, but the width of the region of high pMad actually contracts towards the midline over the course of about 30 minutes (Fig. 2B), which suggests that a key component is missing. The enhanced intensity probably reflects the continued accumulation of Dpp near the midline, but what accounts for the rapid loss of pMad signal from nearby lateral cells? One possibility is that it results from an increased Sog concentration in the extracellular space; this requires, however, that prior signaling is rapidly lost through the degradation and/or recycling of ligand-activated receptor complexes, together with Mad degradation (Podos et al., 2001), and/or Mad de-phosphorylation coupled with nuclear export. However, recent results suggest an intriguing additional mechanism. Localized injection of activated *tkv,* but not of wild-type *tkv*, mRNA leads to the accumulation of extracellular Dpp, implying that the activation of BMP signaling enhances future ligand-receptor interactions (Wang and Ferguson, 2005). One explanation for this observation is that the initial BMP signal activates a target gene whose product either reduces the interaction of ligand with an inhibitory component, or aids in further ligand capture by receptors. Consistent with this is the finding that blocking signal transduction with *medea* mutants also blocks the sharpening of extracellular Dpp. At present, the identity of the induced factor remains unknown, but a signaling-induced, cell-surface BMP-binding protein (CSBBP) could produce the observed contraction of pMad signaling and lead to a step-like distribution of surface-localized ligand. To see how this works, however, we must first introduce a different system, wing vein development.

BMP signaling during vein development

Recent studies indicate that Sog and other extracellular regulators of BMP activity promote BMP signaling in a quite different developmental context, the specification of a subset of *Drosophila* wing veins. Although the constraints of this system have so far prevented the type of direct assessment of ligand movement performed in the early embryo, mutants affecting venation have been used to identify an additional extracellular component that provides a nice example of the type of positive feedback predicted to exist in the embryo.

Wing veins arise as stripes of cells in the wing imaginal disc just before and after pupa formation. Each vein is positioned by a slightly different mechanism (Bier, 2000; de Celis, 2003), but, for our purposes, we will divide the veins into two categories, the longitudinal veins (LVs) and the crossveins (CVs), on the basis of their orientation and timing of development (Fig. 3). The precursors of the LVs, those that run along the proximodistal axis of the wing, first appear in larval wing discs, whereas the anterior and posterior CVs (ACV and PCV), those that bridge the LVs, do not appear until the early stages of pupal development (Conley et al., 2000).

BMP signaling plays at least two different roles in vein development. The first is to position the LVs along the anteroposterior axis of the wing disc. During larval development, Dpp is expressed in a stripe down the midline of the wing disc, forming a long-range gradient of BMP signaling. A subset of the LVs are positioned in response to specific levels of BMP signaling, and reductions in BMP signaling can either shift the positions of these veins or lead to gaps. There is no evidence that Sog, Tsg-like or Tld-like proteins modulate BMP activity at this stage (Shimmi et al., 2005a; Yu et al., 1996).

During pupal stages, the expression of *dpp* changes: it is lost from the midline stripe, and now appears in all of the LVs (de Celis, 1997; Yu et al., 1996). This Dpp acts locally to maintain the previously specified LV fate; its loss leads to the '*shortvein*' *dpp* phenotype (de Celis, 1997; Posakony et al., 1990; Ray and Wharton, 2001). However, Dpp also acts as a long-range signal for the initial specification of the CVs. BMP signaling is activated in the prospective CV regions prior to the appearance of other known vein-promoting signals; manipulations that inhibit BMP signaling block the formation of the CVs, often with minimal effects on LV development, leading to a '*crossveinless*' phenotype (Conley et al., 2000; Ralston and Blair, 2005). CV development has thus provided a sensitive assay for studying BMP signaling.

Signaling in the ACV is prefigured by *dpp* expression in a stripe that intersects the ACV (Ralston and Blair, 2005). However, localized BMP signaling in the incipient PCV is not initially accompanied by a higher expression of ligand within the PCV itself (Fig. 3C). Rather, this signaling requires the expression of *dpp* in the adjacent LVs, and thus the movement of Dpp from the LVs into the PCV region (Ralston and Blair, 2005). In this respect, the discrepancy between the regions of ligand expression and signaling is even more extreme in the PCV than in the early embryo.

Sog, Tolloid-related and Crossveinless

As in the embryo, Sog is required for this long-range Dpp signaling in the PCV; removing endogenous Sog causes a loss of signaling in the PCV and a *crossveinless* phenotype (Serpe et al., 2005; Shimmi

et al., 2005a). This was surprising, as the initial studies of Sog in the wing suggested just the opposite; strong overexpression of Sog led to loss of the PCV (Yu et al., 1996), and co-expression of Sog and Tsg led to a loss of signaling during even the early stages of Dpp signaling in the larval imaginal disc (Ross et al., 2001). However, although high levels of Sog can inhibit signaling in the wing, low levels of overexpression actually stimulate signaling distant from the site of misexpression (Shimmi et al., 2005a), much as occurs after localized misexpression of *sog* in the embryo (Ashe and Levine, 1999). A truncated form of Sog that contains only the first two CRs can also stimulate BMP signaling in the developing wing (Yu et al., 2004).

Mosaic analysis indicates that Sog acts over a long range in the pupal wing, consistent with it having a role in transporting Dpp (Shimmi et al., 2005a). The parallel with the embryo also extends to Sog's partners. As in the embryo, signaling in the PCV requires a Tolloid family protease, in this case Tolloid-related (Tlr, also known as Tolkin) (Finelli et al., 1995; Nguyen et al., 1994; Serpe et al., 2005), and the presence of the Tsg family member Crossveinless (Cv or Tsg2) (Shimmi et al., 2005a; Vilmos et al., 2005).

Like Tld, Tlr can cleave Sog in vitro and its loss leads to loss of signaling in the PCV, probably through the accumulation of excess full-length Sog (Serpe et al., 2005). Although the excess Sog can presumably transport Dpp, it apparently sequesters Dpp from its receptor. Indeed, lowering endogenous Sog levels can rescue the *tlr* mutant phenotype. The embryonic protease Tld cannot substitute for Tlr in the wing. This may be explained by the slower kinetics of Tlr activity observed in vitro; such kinetics may be required for the movement of the intact Sog-Cv-ligand complex over the longer time scale of the developing wing.

Loss of the Tsg-like protein Cv also leads to loss of BMP signaling in the PCV and a *crossveinless* phenotype. Cv acts with Sog to bind ligand in vitro, and thus Cv might be required to form the transport complex; this is consistent with rescue experiments indicating that Cv acts over a long range in the wing (Shimmi et al., 2005a; Vilmos et al., 2005). In addition, Cv can substitute for Tsg in the early embryo and Tsg can substitute for Cv in the wing, although they differ in the strength of their genetic interactions with ligands (Shimmi et al., 2005a; Vilmos et al., 2005).

Heterodimers and crossveins

A final parallel with the embryo is that signaling in the PCV may be driven partly or wholly by ligand heterodimers. Although *scw* is not transcribed at pupal stages (Arora et al., 1994), another BMP-like ligand, Gbb, is expressed at this time (Khalsa et al., 1998; Wharton et al., 1999). Loss of either *dpp* or *gbb* blocks signaling in the PCV (Ralston and Blair, 2005). Gbb is expressed ubiquitously in the pupal wing (Conley et al., 2000); however, mosaic analysis indicates that the PCV is only disrupted when Gbb is removed from the adjacent LVs (Ray and Wharton, 2001), the same cells that express *dpp*.

Unlike Scw, Gbb can signal in the absence of Dpp, and in vitro Dpp/Gbb heterodimers do not produce a synergistic signal (Shimmi et al., 2005a). Why then would a Dpp/Gbb heterodimer be more effective at signaling within the PCV? As in the embryo, there may

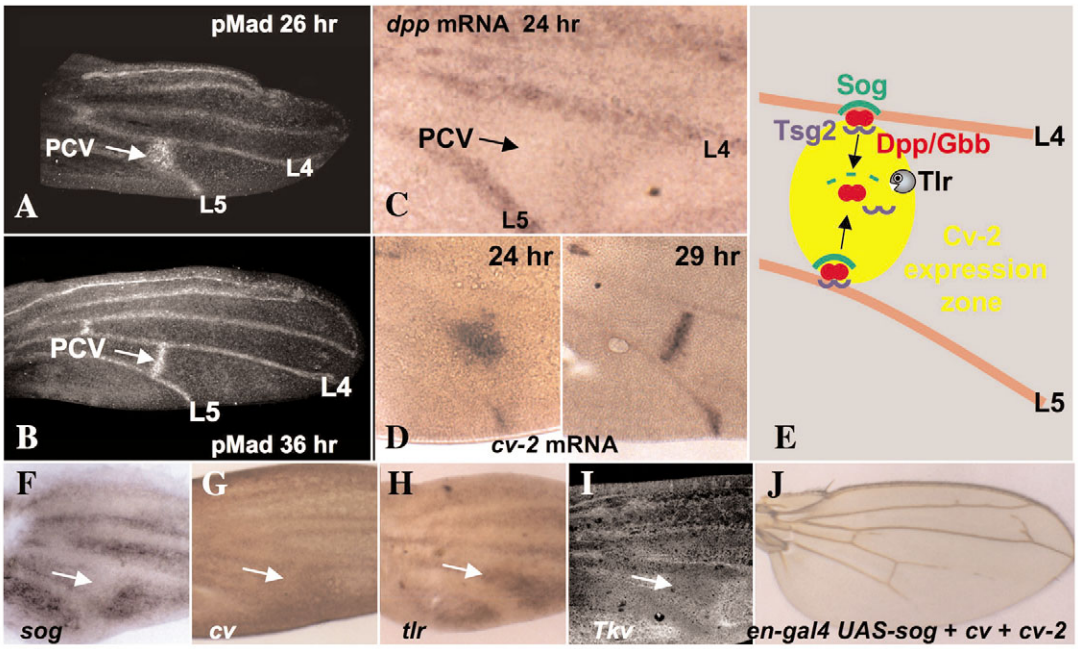

Fig. 3. Posterior crossvein formation requires BMPs and BMP transport components. (**A**,**B**) pMad accumulation in the longitudinal veins (LVs) and posterior crossvein (PCV) at 26 (A) and 36 (B) hours post-puparium formation (ppf). Note that at 26 hours ppf pMad accumulates at the PCV in a wide domain that then refines considerably by 36 hours ppf. (**C**) *dpp* mRNA expression in the LVs, but not in the PCV, at 24 hours ppf. (**D**) *cv-2* mRNA expression at 24 and 29 hours ppf. Note the sharpening in the *cv-2* mRNA profile as time progresses. (**E**) A schematic representation of one possible patterning mechanism. Dpp is only produced in the LVs, whereas Gbb is uniformly expressed. The Dpp/Gbb heterodimers formed in the LVs preferentially bind to a complex of Sog and Cv (also known as Tsg2). Tlr cleaves Sog to release the heterodimer for signaling. Initial low signal levels, together with other unknown positional cues, induce *cv-2* transcription (yellow) in a zone that will form the PCV. Cv-2 protein accumulates on the cell surface and creates a positive-feedback loop that presents BMP ligand to the signaling receptors. (**F-I**) Expression patterns of *sog* (F), *cv* (G) and *tlr* (H) mRNA, and Tkv protein (I) in 19-24 hour ppf wings. (**J**) Uniform overexpression of *sog* (*UAS-sog*), *cv* (*EP(X)1349*) and *cv-2* (*EP(2)1103*) in the posterior of the wing with *en-gal4* does not disrupt PCV formation. (C,F,I) Reproduced, with permission, from Ralston and Blair (Ralston and Blair, 2005); (D,H,J) reproduced, with permission, from Ralston (A. Ralston, PhD thesis, University of Wisconsin, 2004); (G) reproduced, with permission, from Shimmi et al. (Shimmi et al., 2005a).

DEVELOPMENT

be an effect on transport. Because the Dpp/Gbb heterodimer binds to the Sog-Cv complex with a higher affinity than homodimers, it may be preferentially transported over long distances (Shimmi et al., 2005a). Heterodimers may also decrease the levels of uncleaved Sog, as cleavage of Sog by Tld or Tlr requires the presence of ligand, and Dpp/Gbb heterodimers more effectively stimulate processing than does either homodimer (Serpe et al., 2005). Thus, the simplest model is that Sog and Cv form a complex with a Dpp/Gbb heterodimer and help to carry it into the PCV region where Sog is cleaved by Tlr, freeing the Dpp/Gbb heterodimers for signaling (Fig. 3E).

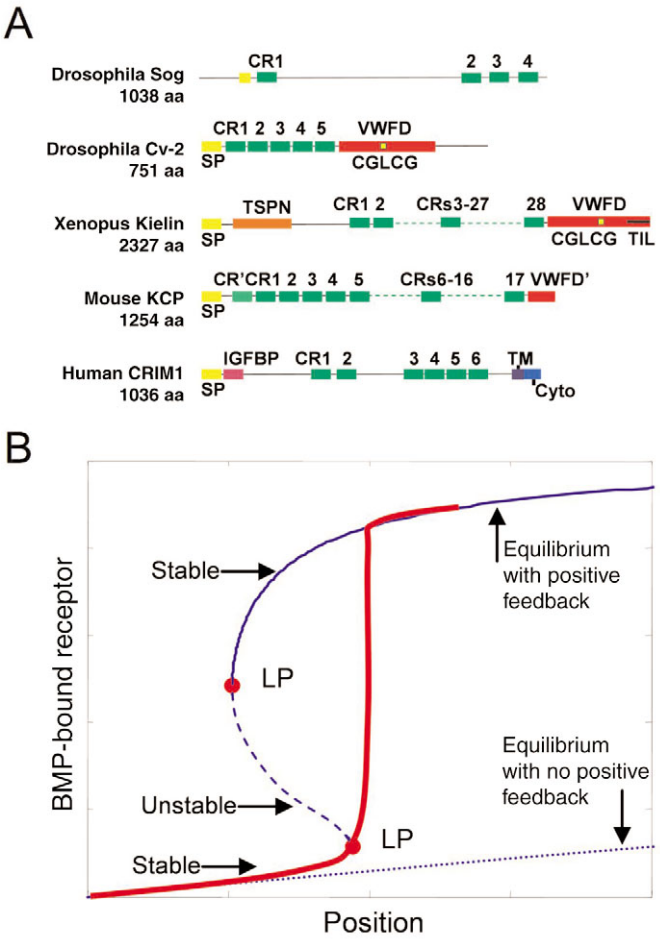

Fig. 4. CR-containing proteins and their possible roles in positive feedback and spatial bi-stability. (**A**) The domain structures of several BMP-binding proteins. Sog is a secreted (signal peptide, SP, yellow) protein that contains BMP-binding modules [cysteine-rich motifs (CRs), green], whereas Cv-2, and its vertebrate homologs (not shown), and other related vertebrate proteins, such as Kielin or KCP, contain both CR domains and a von Willebrand Factor D (VWFD) motif (red) that may promote cell surface localization. The CRIM1 protein also contains CR domains but instead of a VWFD, it contains an insulin-like growth factor binding protein domain (IGFBP), a transmembrane domain (TM) that is likely to anchor it to the cell surface, and a small cytoplasmic tail (Cyto). (**B**) Position (or BMP) versus BMP-bound receptor for on/off equilibrium (dotted line) and positive feedback induced bi-stability (solid line). In general the distribution of morphogen is non-linear in x [$BMP=f(x)$]; however, in this case, the extracellular gradient of BMP is linear in position (i.e. BMP~α*x). As the level of BMP increases, the level of BMP-bound receptor follows the on/off equilibrium solution until it reaches a limit point (LP) where the lower equilibrium solution ceases to exist. For levels of BMP above this point, the level of BMP-bound receptor approaches the upper stable branch.

Crossveinless 2 and positive feedback

One BMP-binding protein that is crucial for signaling in the CVs, but whose function in the early embryo has not been examined, is Crossveinless 2 (Cv-2). Loss of Cv-2 causes loss of BMP signaling in the developing CVs (Conley et al., 2000). Cv-2 contains five N-terminal CR domains (Fig. 4A), similar to the BMP-binding CRs of Sog, followed by a partial von Willebrand Factor D (VWFD) domain. Vertebrates have a Cv-2 homolog with a larger VWFD domain that includes a Trypsin inhibitor-like cysteine-rich (TIL) domain (Binnerts et al., 2004; Coffinier et al., 2002; Coles et al., 2004; Kamimura et al., 2004; Moser et al., 2003). Vertebrates also have large 'Kielin-like' proteins, which contain varying numbers of CRs and lengths of VWFD domains, and CRIM1, which has CR domains and a transmembrane-spanning segment (Fig. 4A) (Kolle et al., 2000; Lin et al., 2005; Matsui et al., 2000).

All Cv-2 and Kielin-like proteins so far tested are secreted and bind BMPs, presumably through their CR domains (Binnerts et al., 2004; Coffinier et al., 2002; Coles et al., 2004; Lin et al., 2005; Matsui et al., 2000; Moser et al., 2003), indicating that Cv-2 might promote signaling by aiding ligand transport, perhaps as part of the Sog-Cv complex. However, mosaic analyses indicate that *cv-2* expression, unlike that of *sog* and *cv*, is required locally within the crossvein itself. Thus, Cv-2 is not a long-range transporter but is likely to act as a co-factor to concentrate ligand near the receiving cells or to free it from the Sog-Cv complex. This is consistent with the behavior of chick Cv-2 and mouse Kielin/Chordin-like protein (KCP) in vitro, where conditioned medium containing either protein enhances BMP signaling and, in the case of KCP, the binding of BMP7 to its receptor (Kamimura et al., 2004; Lin et al., 2005). Likewise, studies in chick, *Xenopus* and mice suggest that Cv-2 and KCP have agonist roles (Coles et al., 2004; Lin et al., 2005), but these proteins have also been reported to antagonize BMP in various overexpression and in vitro assays (Binnerts et al., 2004; Coles et al., 2004; Matsui et al., 2000; Moser et al., 2003). There may be several reasons for this. As with Sog, high levels of Cv-2 overexpression may sequester ligand. It may also be that localized processing and/or co-factors are required for Cv-2 to promote BMP activity.

As in the embryo, pMad accumulation in the CVs refines from a broad to a narrow domain (Fig. 3A,B), and this is paralleled by a similar increase and refinement in *cv-2* expression in the CVs (Conley et al., 2000) (Fig. 3D). *cv-2* expression is responsive to BMP signaling, and thus likely provides positive feedback that aids in the refinement process (A. Ralston, PhD thesis, University of Wisconsin, 2004; Fig. 3D). However, other factors must be present that initially increase the movement or accumulation of ligand from the LVs into the PCV region, or that raise the sensitivity of those cells to signaling. Although in the embryo the ventrolateral expression pattern of *sog* is sufficient to provide directionality to gradient formation, this is not the case in the PCV. In the pupal wing, *sog* mRNA expression is reduced in the developing PCV (Fig. 3F), but clones lacking *sog* do not induce ectopic signaling (Ralston and Blair, 2005; Shimmi et al., 2005a; Yu et al., 1996). *cv* expression is slightly higher at vein boundaries and *tlr* is higher in the intervein (Fig. 3G,H), but uniform overexpression of either does not significantly alter PCV signaling (Serpe et al., 2005; Shimmi et al., 2005a; Vilmos et al., 2005). Uniform overexpression of Cv-2 does not expand signaling outside the PCV, either alone or in combination with Sog and/or Cv (Fig. 3J) (Conley et al., 2000; Ralston and Blair, 2005; Vilmos et al., 2005) (A. Ralston, PhD thesis, University of Wisconsin, 2004). Nor is the cue likely to be provided by changes in receptor

expression. Sax is not required for formation of the PCV (Ray and Wharton, 2001; Singer et al., 1997). Tkv expression is reduced in the PCV (Fig. 3I), which could in theory increase ligand diffusion, but this reduction is apparently the result, not the cause, of heightened signaling (Ralston and Blair, 2005).

One obvious place to look for additional factors that modulate BMP signaling is the other *crossveinless* mutations, several of which are uncharacterized. However, not all of these have provided an obvious link to BMP signaling. *crossveinless c* (*cv-c*) encodes a RhoGAP protein (Denholm et al., 2005), and signaling in the PCV is reduced in *cv-c* mutants (A. Ralston, PhD thesis, University of Wisconsin, 2004). This is intriguing, as reductions in Cdc42 activity can induce ectopic CVs (Baron et al., 2000; Genova et al., 2000), but the connection between small GTPase activity and PCV development is, as yet, poorly understood, and could be quite indirect.

Positive feedback and bi-stability

The positive feedback potentially provided by molecules like Cv-2 not only increases signaling globally, but in theory can create the increasingly sharp step-gradients observed in both the embryo and the PCV by producing spatial bi-stability. Here, spatial bi-stability means that the response to the extracellular BMP distribution divides a region into a stable high signaling zone and a stable low signaling zone separated by a sharp boundary (i.e. the spatial distribution of signaling is step-like). Bi-stability frequently arises in models of complex networks, particularly in those that include positive-feedback loops, in which the balance between competing processes can lead to multiple steady states for a given set of conditions. A typical response diagram that illustrates bi-stability is shown in Fig. 4B. Without positive feedback, the level of BMP-bound receptor is fixed by the binding equilibrium (on-off rate, dotted line Fig. 4B), but intracellular positive feedback can shift the equilibrium curve and lead to bi-stability (S-shaped curve, Fig. 4B). For low levels of BMP, the level of BMP bound to its receptor follows the binding curve equilibrium (dotted line, Fig. 4B) until a point (red dot, Fig. 4B) where the lower steady state ceases to exist and only the upper stable branch is accessible. Thus, regions with BMP levels higher than the limit of stability for the lower branch will adopt a high signaling fate, while cells below that point will adopt a low signaling fate (spatial bi-stability). Thus, cells can re-interpret the extracellular gradient at the level of BMP-bound receptor (red line, Fig. 4B) and produce a step-like response in space to a more gradual change in BMP levels.

Previous analysis of the Patched/Hedgehog patterning system suggests that positive feedback on receptor expression can lead to a spatial bi-stability (Eldar et al., 2003). However, it is unlikely that Tkv or Sax is the positively regulated target of BMP signaling in *Drosophila*. Misexpression of Tkv does not significantly affect the pMad output in the embryo (Mizutani et al., 2005; Wang and Ferguson, 2005), and, in the wing disc and pupal wing, BMP receptors are actually downregulated by BMP signaling (de Celis, 1997; Lecuit and Cohen, 1998; Ralston and Blair, 2005; Tanimoto et al., 2000). However, a positively regulated co-receptor or a CSBBP, like Cv-2, can play the same role (Fig. 5). Vertebrate Cv-2, Keilin-like and CRIM1 proteins could also act in a similar manner.

Conclusions and perspectives

The data reviewed here show that we now have a reasonably complete understanding of how the molecules and their interactions lead to the spatial distribution of BMPs in the early *Drosophila* embryo, and, to a lesser extent, in the PCV of the pupal wing. However, there are still unanswered questions that will continue to drive research in this area over the next few years. Not the least of these is the identification of new players, such as those responsible for positive feedback in the embryo and the spatial regulation of signaling in the PCV. The *Drosophila* genome encodes several uncharacterized proteins that contain CRs like those known to bind BMPs. We also need to factor in new findings about interactions between the known players and other extracellular elements. For instance, Tsg, Sog, and its vertebrate ortholog Chordin, have been shown to interact with cell-surface components such as integrins, proteoglycans and heparin, and these interactions may influence gradient formation (Araujo et al., 2003; Jasuja et al., 2004; Larrain et al., 2003).

Comparisons between *Drosophila* and vertebrate components are also of interest. For example, Tsg in vertebrates seems to have both pro- and anti-BMP effects, and some of these appear to be independent of the BMP antagonist Chordin (Chang et al., 2001; Little and Mullins, 2004; Oelgeschlager et al., 2000; Oelgeschlager et al., 2003; Ross et al., 2001; Scott et al., 2001; Xie and Fisher, 2005; Zakin and De Robertis, 2004). The precise biochemical mechanism responsible for the agonist activity has not been explained, but may be related to the promotion of BMP accumulation that has been noted in the *Drosophila* embryo (Wang and Ferguson, 2005). There are also some intriguing differences

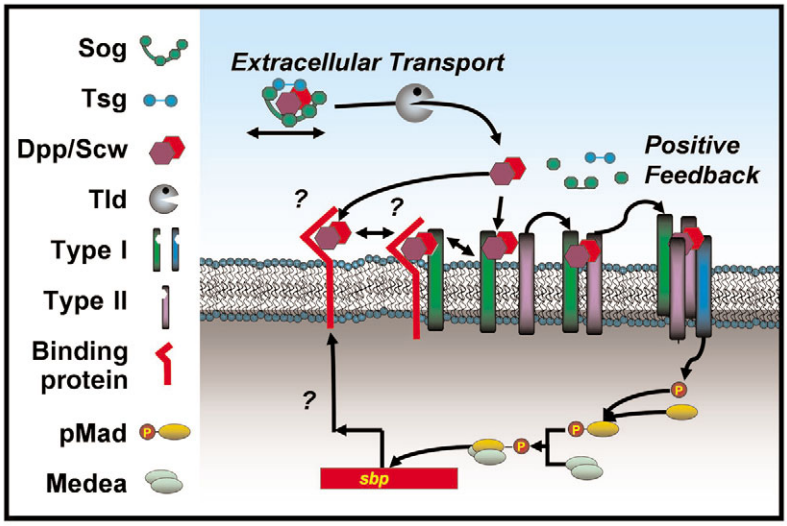

Fig. 5. A model for positive feedback. Initially BMP ligands, such as Dpp and Scw, bind to the type I and type II signaling receptors. This signal activates transcription of a cell surface BMP-binding protein (sbp), such as Cv-2, that helps to present ligand to the signaling receptors. This may account for the production of spatial bi-stability, as proposed by Ferguson and Wang (Ferguson and Wang, 2005). P, phosphorylation. The question mark indicates that the identity of this component is not yet established in the embryo.

DEVELOPMENT

between *Drosophila* and vertebrate proteins that might give them different properties. The Tsg protein of *Drosophila* can bind heparin, whereas the vertebrate proteins do not (Jasuja et al., 2004; Mason et al., 1997). Similarly, in *Drosophila*, Sog is processed in at least three positions by Tld in a ligand-dependent fashion, whereas, in vertebrates, Chordin is processed at only two major sites, and this processing does not depend on Chordin forming a complex with BMPs (Marques et al., 1997; Piccolo et al., 1997; Scott et al., 1999; Shimmi and O'Connor, 2003). Do these differences account for the inability of Chordin to have agonist activity when expressed in flies (Decotto and Ferguson, 2001)? Lastly, individual fragments of Sog have been found to have either antagonistic or agonistic function when overexpressed in the wing (Yu et al., 2004; Yu et al., 2000). Do these fragments play a role in the endogenous modulation of BMP activity? Presumably rescue experiments employing mutant versions of these sites, together with the production of Sog/Chd chimeric proteins, will provide definitive answers to each of these issues in the near future.

The biochemical mechanism of receptor synergism is also an important issue. Are Smads more efficiently recruited to the Tkv/Sax-containing complex than either homomeric complex? Is there a novel cross phosphorylation of the two receptors in a heteromeric complex that contributes to the synergism? Are there intracellular regulators of receptor activity that differentially bind to the different receptor complexes? Alternatively, the heteromeric receptor complex may be routed through a different signaling endosome that persists and signals longer than homomeric receptor complexes do. It is also important to determine whether the synergism is even a necessary component of the early developmental process, as overexpression of one isoform of Tkv has been shown to partially rescue *sax* mutations (Brummel et al., 1994). Finally, is receptor synergism a feature of vertebrate systems? Two type I BMP receptors exist in vertebrates, and BMP heterodimers have been implicated in regulating several developmental events (Butler and Dodd, 2003; Schmid et al., 2000). In addition, heterodimers can produce stronger signals in vertebrate cell culture systems than homodimers can (Aono et al., 1995).

For developmental processes, bi-stable behavior has several implications. What determines whether the cell will have a high or low signal-reception fate? With positive feedback it is entirely possible that cells adopt distinct fates based on the history of their exposure to a changing extracellular morphogen gradient instead of on an absolute concentration at a given point in time (Dillon and Othmer, 1999). A last issue, raised by cell culture signaling assays, is whether stochastic influences have to be considered when modeling the embryonic patterning mechanism. Previous studies indicate that BMP responsiveness in cell culture is saturated at the 10-nanomolar level (Shimmi and O'Connor, 2003). If this holds true in the embryo, then it extrapolates to only several thousand BMP molecules in the perivitelline space. Such a low number would make patterning susceptible to stochastic fluctuations (England and Cardy, 2005). Once again, a solution might be positive feedback, which should dampen stochastic influences on signaling output (Dillon and Othmer, 1999). Because computational analysis shows that step gradients in morphogen interpretation can form in the absence of feedback, might buffering against stochastic fluctuations be the primary reason that positive feedback is employed in this system? Measuring the actual levels of ligands in the perivitelline space is therefore crucial to obtaining a more complete understanding of the patterning mechanism.

We thank Guillermo Marques and MaryJane Shimell for critical comments on the manuscript. S.S.B. is supported by grants from the NSF and the NIH, H.O. by a grant from the NIH, and D.U. by a NIH Biotechnology Training Grant. M.B.O. is an Investigator with the Howard Hughes Medical Institute.

REFERENCES

Aono, A., Hazama, M., Notoya, K., Taketomi, S., Yamasaki, H., Tsukuda, R., Sasaki, S. and Fujisawa, Y. (1995). Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer. *Biochem. Biophys. Res. Commun.* **210**, 670-677.

Araujo, H., Negreiros, E. and Bier, E. (2003). Integrins modulate Sog activity in the Drosophila wing. *Development* **130**, 3851-3864.

Arora, K. and Nusslein-Volhard, C. (1992). Altered mitotic domains reveal fate map changes in Drosophila embryos mutant for zygotic dorsoventral patterning genes. *Development* **114**, 1003-1024.

Arora, K., Levine, M. S. and O'Connor, M. B. (1994). The screw gene encodes a ubiquitously expressed member of the TGF-beta family required for specification of dorsal cell fates in the Drosophila embryo. *Genes Dev.* **8**, 2588-2601.

Ashe, H. L. and Levine, M. (1999). Local inhibition and long-range enhancement of Dpp signal transduction by Sog. *Nature* **398**, 427-431.

Balemans, W. and Van Hul, W. (2002). Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Dev. Biol.* **250**, 231-250.

Baron, M., O'Leary, V., Evans, D. A., Hicks, M. and Hudson, K. (2000). Multiple roles of the Dcdc42 GTPase during wing development in Drosophila melanogaster. *Mol. Gen. Genet.* **264**, 98-104.

Bier, E. (2000). Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Dev.* **10**, 393-398.

Binnerts, M. E., Wen, X., Cante-Barrett, K., Bright, J., Chen, H. T., Asundi, V., Sattari, P., Tang, T., Boyle, B., Funk, W. et al. (2004). Human Crossveinless-2 is a novel inhibitor of bone morphogenetic proteins. *Biochem. Biophys. Res. Commun.* **315**, 272-280.

Brummel, T. J., Twombly, V., Marques, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massague, J., O'Connor, M. B. and Gelbart, W. M. (1994). Characterization and relationship of Dpp receptors encoded by the saxophone and thick veins genes in Drosophila. *Cell* **78**, 251-261.

Butler, S. J. and Dodd, J. (2003). A role for BMP heterodimers in roof plate-mediated repulsion of commissural axons. *Neuron* **38**, 389-401.

Chang, C., Holtzman, D. A., Chau, S., Chickering, T., Woolf, E. A., Holmgren, L. M., Bodorova, J., Gearing, D. P., Holmes, W. E. and Brivanlou, A. H. (2001). Twisted gastrulation can function as a BMP antagonist. *Nature* **410**, 483-487.

Coffinier, C., Ketpura, N., Tran, U., Geissert, D. and De Robertis, E. M. (2002). Mouse Crossveinless-2 is the vertebrate homolog of a Drosophila extracellular regulator of BMP signaling. *Mech. Dev.* **119**, S179-S184.

Coles, E., Christiansen, J., Economou, A., Bronner-Fraser, M. and Wilkinson, D. G. (2004). A vertebrate crossveinless 2 homologue modulates BMP activity and neural crest cell migration. *Development* **131**, 5309-5317.

Conley, C. A., Silburn, R., Singer, M. A., Ralston, A., Rohwer-Nutter, D., Olson, D. J., Gelbart, W. and Blair, S. S. (2000). Crossveinless 2 contains cysteine-rich domains and is required for high levels of BMP-like activity during the formation of the cross veins in Drosophila. *Development* **127**, 3947-3959.

de Celis, J. F. (1997). Expression and function of *decapentaplegic* and *thickveins* during the differentiation of the veins in the *Drosophila* wing. *Development* **124**, 1007-1018.

de Celis, J. F. (2003). Pattern formation in the Drosophila Wing: The development of veins. *BioEssays* **25**, 443-451.

De Robertis, E. M. and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu. Rev. Cell Dev. Biol.* **20**, 285-308.

Decotto, E. and Ferguson, E. L. (2001). A positive role for Short gastrulation in modulating BMP signaling during dorsoventral patterning in the *Drosophila* embryo. *Development* **128**, 3831-3841.

Denholm, B., Brown, S., Ray, R. P., Ruiz-Gomez, M., Skaer, H. and Hombria, J. C. (2005). crossveinless-c is a RhoGAP required for actin reorganisation during morphogenesis. *Development* **132**, 2389-2400.

Dillon, R. and Othmer, H. G. (1999). A mathematical model for outgrowth and spatial patterning of the vertebrate limb bud. *J. Theor. Biol.* **197**, 295-330.

Dorfman, R. and Shilo, B. Z. (2001). Biphasic activation of the BMP pathway patterns the Drosophila embryonic dorsal region. *Development* **128**, 965-972.

Eldar, A., Dorfman, R., Weiss, D., Ashe, H., Shilo, B. Z. and Barkai, N. (2002). Robustness of the BMP morphogen gradient in Drosophila embryonic patterning. *Nature* **419**, 304-308.

Eldar, A., Rosin, D., Shilo, B. Z. and Barkai, N. (2003). Self-enhanced ligand degradation underlies robustness of morphogen gradients. *Dev. Cell* **5**, 635-646.

England, J. and Cardy, J. (2005). Morphogen gradient from a noisy source. *Phys. Rev. Lett.* **94**, 078101.

Ferguson, E. L. and Anderson, K. V. (1992). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the Drosophila embryo. *Cell* **71**, 451-461.

Finelli, A. L., Xie, T., Bossie, C. A., Blackman, R. K. and Padgett, R. W. (1995). The tolkin gene is a tolloid/BMP-1 homologue that is essential for Drosophila development. *Genetics* **141**, 271-281.

Francois, V., Solloway, M., O'Neill, J. W., Emery, J. and Bier, E. (1994). Dorsal-ventral patterning of the Drosophila embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes Dev.* **8**, 2602-2616.
Genova, J. L., Jong, S., Camp, J. T. and Fehon, R. G. (2000). Functional analysis of Cdc42 in actin filament assembly, epithelial morphogenesis, and cell signaling during Drosophila development. *Dev. Biol.* **221**, 181-194.
Gray, A. M. and Mason, A. J. (1990). Requirement for activin A and transforming growth factor–beta 1 pro-regions in homodimer assembly. *Science* **247**, 1328-1330.
Hassel, S., Schmitt, S., Hartung, A., Roth, M., Nohe, A., Petersen, N., Ehrlich, M., Henis, Y. I., Sebald, W. and Knaus, P. (2003). Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes. *J. Bone Joint Surg. Am.* **85**, 44-51.
Holley, S. A., Neul, J. L., Attisano, L., Wrana, J. L., Sasai, Y., O'Connor, M. B., De Robertis, E. M. and Ferguson, E. L. (1996). The Xenopus dorsalizing factor noggin ventralizes Drosophila embryos by preventing DPP from activating its receptor. *Cell* **86**, 607-617.
Jasuja, R., Allen, B. L., Pappano, W. N., Rapraeger, A. C. and Greenspan, D. S. (2004). Cell-surface heparan sulfate proteoglycans potentiate chordin antagonism of bone morphogenetic protein signaling and are necessary for cellular uptake of chordin. *J. Biol. Chem.* **279**, 51289-51297.
Kamimura, M., Matsumoto, K., Koshiba-Takeuchi, K. and Ogura, T. (2004). Vertebrate crossveinless 2 is secreted and acts as an extracellular modulator of the BMP signaling cascade. *Dev. Dyn.* **230**, 434-445.
Khalsa, O., Yoon, J. W., Torres-Schumann, S. and Wharton, K. A. (1998). TGF-beta/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the Drosophila wing. *Development* **125**, 2723-2734.
Kishigami, S. and Mishina, Y. (2005). BMP signaling and early embryonic patterning. *Cytokine Growth Factor Rev.* **16**, 265-278.
Kolle, G., Georgas, K., Holmes, G. P., Little, M. H. and Yamada, T. (2000). CRIM1, a novel gene encoding a cysteine-rich repeat protein, is developmentally regulated and implicated in vertebrate CNS development and organogenesis. *Mech. Dev.* **90**, 181-193.
Larrain, J., Brown, C. and De Robertis, E. M. (2003). Integrin-alpha3 mediates binding of Chordin to the cell surface and promotes its endocytosis. *EMBO Rep.* **4**, 813-818.
Lecuit, T. and Cohen, S. M. (1998). Dpp receptor levels contribute to shaping the Dpp morphogen gradient in the *Drosophila* wing imaginal disc. *Development* **125**, 4901-4917.
Lin, J., Patel, S. R., Cheng, X., Cho, E. A., Levitan, I., Ullenbruch, M., Phan, S. H., Park, J. M. and Dressler, G. R. (2005). Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. *Nat. Med.* **11**, 387-393.
Little, S. C. and Mullins, M. C. (2004). Twisted gastrulation promotes BMP signaling in zebrafish dorsal-ventral axial patterning. *Development* **131**, 5825-5835.
Marques, G., Musacchio, M., Shimell, M. J., Wunnenberg-Stapleton, K., Cho, K. W. and O'Connor, M. B. (1997). Production of a DPP activity gradient in the early Drosophila embryo through the opposing actions of the SOG and TLD proteins. *Cell* **91**, 417-426.
Mason, E. D., Konrad, K. D., Webb, C. D. and Marsh, J. L. (1994). Dorsal midline fate in Drosophila embryos requires twisted gastrulation, a gene encoding a secreted protein related to human connective tissue growth factor. *Genes Dev.* **8**, 1489-1501.
Matsui, M., Mizuseki, K., Nakatani, J., Nakanishi, S. and Sasai, Y. (2000). Xenopus kielin: A dorsalizing factor containing multiple chordin-type repeats secreted from the embryonic midline. *Proc. Natl. Acad. Sci. USA* **97**, 5291-5296.
Mizutani, C. M., Nie, Q., Wan, F. Y., Zhang, Y. T., Vilmos, P., Sousa-Neves, R., Bier, E., Marsh, J. L. and Lander, A. D. (2005). Formation of the BMP activity gradient in the Drosophila embryo. *Dev. Cell* **8**, 915-924.
Moser, M., Binder, O., Wu, Y., Aitsebaomo, J., Ren, R., Bode, C., Bautch, V. L., Conlon, F. L. and Patterson, C. (2003). BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. *Mol. Cell. Biol.* **23**, 5664-5679.
Neul, J. L. and Ferguson, E. L. (1998). Spatially restricted activation of the SAX receptor by SCW modulates DPP/TKV signaling in Drosophila dorsal-ventral patterning. *Cell* **95**, 483-494.
Newfeld, S. J., Wisotzkey, R. G. and Kumar, S. (1999). Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. *Genetics* **152**, 783-795.
Nguyen, M., Park, S., Marques, G. and Arora, K. (1998). Interpretation of a BMP activity gradient in Drosophila embryos depends on synergistic signaling by two type I receptors, SAX and TKV. *Cell* **95**, 495-506.
Nguyen, T., Jamal, J., Shimell, M. J., Arora, K. and O'Connor, M. B. (1994). Characterization of tolloid-related-1: a BMP-1-like product that is required during larval and pupal stages of Drosophila development. *Dev. Biol.* **166**, 569-586.
Oelgeschlager, M., Larrain, J., Geissert, D. and De Robertis, E. M. (2000). The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature* **405**, 757-763.
Oelgeschlager, M., Reversade, B., Larrain, J., Little, S., Mullins, M. C. and De Robertis, E. M. (2003). The pro-BMP activity of Twisted gastrulation is independent of BMP binding. *Development* **130**, 4047-4056.
Parker, L., Stathakis, D. G. and Arora, K. (2004). Regulation of BMP and activin signaling in Drosophila. *Prog. Mol. Subcell Biol.* **34**, 73-101.
Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L. and De Robertis, E. M. (1997). Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* **91**, 407-416.
Podos, S. D., Hanson, K. K., Wang, Y. C. and Ferguson, E. L. (2001). The DSmurf ubiquitin-protein ligase restricts BMP signaling spatially and temporally during Drosophila embryogenesis. *Dev. Cell* **1**, 567-578.
Posakony, L. G., Raftery, L. A. and Gelbart, W. M. (1990). Wing formation in Drosophila melanogaster requires decapentaplegic gene function along the anterior-posterior compartment boundary. *Mech. Dev.* **33**, 69-82.
Ralston, A. and Blair, S. S. (2005). Long-range Dpp signaling is regulated to restrict BMP signaling to a crossvein competent zone. *Dev. Biol.* **280**, 187-200.
Ray, R. P. and Wharton, K. A. (2001). Context-dependent relationships between the BMPs gbb and dpp during development of the Drosophila wing imaginal disk. *Development* **128**, 3913-3925.
Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* **410**, 479-483.
Schier, A. F. and Talbot, W. S. (2005). Molecular genetics of axis formation in zebrafish. *Annu. Rev. Genet.* **39**, 561-613.
Schmid, B., Furthauer, M., Connors, S. A., Trout, J., Thisse, B., Thisse, C. and Mullins, M. C. (2000). Equivalent genetic roles for bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. *Development* **127**, 957-967.
Scott, I. C., Blitz, I. L., Pappano, W. N., Imamura, Y., Clark, T. G., Steiglitz, B. M., Thomas, C. L., Maas, S. A., Takahara, K., Cho, K. W. et al. (1999). Mammalian BMP-1/Tolloid-related metalloproteinases, including novel family member mammalian Tolloid-like 2, have differential enzymatic activities and distributions of expression relevant to patterning and skeletogenesis. *Dev. Biol.* **213**, 283-300.
Scott, I. C., Blitz, I. L., Pappano, W. N., Maas, S. A., Cho, K. W. and Greenspan, D. S. (2001). Homologues of Twisted gastrulation are extracellular cofactors in antagonism of BMP signalling. *Nature* **410**, 475-478.
Serpe, M., Ralston, A., Blair, S. S. and O'Connor, M. B. (2005). Matching catalytic activity to developmental function: Tolloid-related processes Sog in order to help specify the posterior crossvein in the *Drosophila* wing. *Development* **132**, 2645-2656.
Shimell, M. J., Ferguson, E. L., Childs, S. R. and O'Connor, M. B. (1991). The Drosophila dorsal-ventral patterning gene tolloid is related to human bone morphogenetic protein 1. *Cell* **67**, 469-481.
Shimmi, O. and O'Connor, M. B. (2003). Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. *Development* **130**, 4673-4682.
Shimmi, O., Ralston, A., Blair, S. S. and O'Connor, M. B. (2005a). The crossveinless gene encodes a new member of the Twisted gastrulation family of BMP-binding proteins which, with Short gastrulation, promotes BMP signaling in the crossveins of the Drosophila wing. *Dev. Biol.* **282**, 70-83.
Shimmi, O., Umulis, D., Othmer, H. and O'Connor, M. B. (2005b). Facilitated transport of a Dpp/Scw heterodimer by Sog/Tsg leads to robust patterning of the Drosophila blastoderm embryo. *Cell* **120**, 873-886.
Singer, M. A., Penton, A., Twombly, V., Hoffmann, F. M. and Gelbart, W. M. (1997). Signaling through both type I DPP receptors is required for anterior-posterior patterning of the entire Drosophila wing. *Development* **124**, 79-89.
Srinivasan, S., Rashka, K. E. and Bier, E. (2002). Creation of a Sog morphogen gradient in the Drosophila embryo. *Dev. Cell* **2**, 91-101.
Sutherland, D. J., Li, M., Liu, X. Q., Stefancsik, R. and Raftery, L. A. (2003). Stepwise formation of a SMAD activity gradient during dorsal-ventral patterning of the Drosophila embryo. *Development* **130**, 5705-5716.
Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of Dpp activity in *Drosophila* wing imaginal discs. *Mol. Cell* **5**, 59-71.
Vilmos, P., Sousa-Neves, R., Lukacsovich, T. and Marsh, J. L. (2005). The *crossveinless* gene of Drosophila represents a new family of Twisted Gastrulation-like modulators of BMP signaling. *EMBO Rep.* **6**, 262-267.
Wang, Y. C. and Ferguson, E. L. (2005). Spatial bistability of Dpp-receptor interactions during Drosophila dorsal-ventral patterning. *Nature* **434**, 229-234.
Wharton, K. A., Cook, J. M., Torres-Schumann, S., de Castro, K., Borod, E. and Phillips, D. A. (1999). Genetic analysis of the bone morphogenetic protein-related gene, gbb, identifies multiple requirements during Drosophila development. *Genetics* **152**, 629-640.
Xie, J. and Fisher, S. (2005). Twisted gastrulation enhances BMP signaling through chordin dependent and independent mechanisms. *Development* **132**, 383-391.

Yu, K., Sturtevant, M. A., Biehs, B., Francois, V., Padgett, R. W., Blackman, R. K. and Bier, E. (1996). The Drosophila decapentaplegic and short gastrulation genes function antagonistically during adult wing vein development. *Development* **122**, 4033-4044.

Yu, K., Srinivasan, S., Shimmi, O., Biehs, B., Rashka, K. E., Kimelman, D., O'Connor, M. B. and Bier, E. (2000). Processing of the Drosophila Sog protein creates a novel BMP inhibitory activity. *Development* **127**, 2143-2154.

Yu, K., Kang, K. H., Heine, P., Pyati, U., Srinivasan, S., Biehs, B., Kimelman, D. and Bier, E. (2004). Cysteine repeat domains and adjacent sequences determine distinct bone morphogenetic protein modulatory activities of the Drosophila Sog protein. *Genetics* **166**, 1323-1336.

Zakin, L. and De Robertis, E. M. (2004). Inactivation of mouse Twisted gastrulation reveals its role in promoting Bmp4 activity during forebrain development. *Development* **131**, 413-424.