Dual function of the Drosophila Alk1/Alk2 ortholog  
Saxophone shapes the Bmp activity gradient in the wing imaginal disc

Erdem Bangi and Kristi Wharton*

Wing patterning in Drosophila requires a Bmp activity gradient created by two Bmp ligands, Gbb and Dpp, and two Bmp type I receptors, Sax and Tkv. Gbb provides long-range signaling, while Dpp signals preferentially to cells near its source along the anteroposterior (AP) boundary of the wing disc. How each receptor contributes to the signaling activity of each ligand is not well understood. Here, we show that while Tkv mediates signals from both Dpp and Gbb, Sax exhibits a novel function for a Bmp type I receptor: the ability to both promote and antagonize signaling. Given its high affinity for Gbb, this dual function of Sax impacts the function of Gbb in the Bmp activity gradient more profoundly than does Dpp. We propose that this dual function of Sax is dependent on its receptor partner. When complexed with Tkv, Sax facilitates Bmp signaling, but when alone, Sax fails to signal effectively and sequesters Gbb. Overall, our model proposes that the balance between antagonizing and promoting Bmp signaling varies across the wing pouch, modulating the level and effective range, and, thus, shaping the Bmp activity gradient. This previously unknown mechanism for modulating ligand availability and range raises important questions regarding the function of vertebrate Sax orthologs.

KEY WORDS: Bmp signaling, Dpp, Gbb, Tkv, Sax, Bmp type I receptor, Wing imaginal disc, Morphogen

INTRODUCTION

Positional information that is crucial for the assignment of different cell fates is often provided by graded outputs of one or more of a number of signaling pathways (i.e. Tgfβ/Bmp, Wg/Wnt and Hh/Shh). Activity gradients formed by bone morphogenetic proteins (Bmps), a subfamily of the Tgfβ superfamily of signaling molecules, are crucial for many patterning processes in both vertebrates and invertebrates (Green, 2002). Tgfβ superfamily members elicit their effects by binding to heteromeric receptor complexes composed of two types of transmembrane serine/threonine kinase receptor proteins; type I and type II (Luo and Lodish, 1996; Weis-Garcia and Massagué, 1996). Biochemical evidence, as well as structural data, indicate that the basic signaling unit consists of a single ligand dimer in contact with two type I and two type II transmembrane serine/threonine kinase receptors (Kirsch et al., 2000a; Kirsch et al., 2000c). Upon ligand binding, the constitutively active type II receptor phosphorylates the type I receptor (Wrana et al., 1994; Luo and Lodish, 1996) that then phosphorylates a cytoplasmic effector molecule belonging to a family of transcriptional regulators, the receptor-mediated Smads (R-Smads) (Eppert et al., 1996; Hoodless et al., 1996; Attisano and Wrana, 2002). Phosphorylated R-Smads form heteromeric complexes with a related co-Smad, and the R-Smad/co-Smad complex translocates into the nucleus, where it, with other transcriptional regulators, influences target gene transcription (Massagué and Wotton, 2000; Moustakas et al., 2001; ten Dijke and Hill, 2004).

Patterning of the Drosophila wing imaginal disc requires a Bmp activity gradient. The graded activation of Bmp signaling along the AP axis can be visualized using an antibody that recognizes the phosphorylated form of Mad (pMad), the Drosophila R-Smad (Tanimoto et al., 2000). Cells within the wing pouch respond to graded levels of pMad by activating three Bmp target genes, spalt (sal), optomotor blind (omb) and vestigial (vg), at different distances from the AP boundary (Lecuit et al., 1996; Nellen et al., 1996; Minami et al., 1999; Kirkpatrick et al., 2001), and by repressing brinker (brk) expression, a direct Bmp target (Muller et al., 2003). Proper patterning of the adult wing has been known for some time to require the Bmp ligand encoded by decapentaplegic (dpp) and, more recently, a second Bmp encoded by glass bottom boat (gbb) (Spencer et al., 1982; Ray and Wharton, 2001a). dpp is expressed in a stripe of anterior compartment cells abutting the AP compartment boundary (Blackman et al., 1991) and gbb is expressed more broadly throughout much of the wing pouch (Khalasa et al., 1998). Despite its broad expression, loss-of-function studies indicate that Gbb produced by cells along the AP boundary is absolutely required for patterning (Ray and Wharton, 2001a). In the absence of Gbb, Dpp exhibits only short-range signaling and the breadth of the Bmp activity gradient requires gbb function, indicating that Gbb provides the long range nature of the Bmp activity gradient (Bangi and Wharton, 2006). A reduction of Dpp, however, profoundly influences the peaks of the gradient and, thus, cell fate specification in the central domain.

The two Bmp type I receptors, Thick veins (Tkv) and Saxophone (Sax), have different requirements in wing patterning. Although sax is reported to be ubiquitously expressed in the wing pouch (Brummel et al., 1994), tkv, which is regulated by both Hh and Bmp signaling, shows a more complex expression pattern with higher expression in the posterior compartment and a downregulation near the AP boundary (Funakoshi et al., 2001; del Alamo Rodriguez et al., 2004). Tkv is essential for all Bmp signaling in the wing disc based on the fact that eliminating tkv function leads to the absence of pMad (Tanimoto et al., 2000) (L. Soares and K.W., unpublished). However, although Sax is not absolutely required for wing patterning (Ray and Wharton, 2001a), the reduction in sal
expression seen in small sax-null clones (Singer et al., 1997) indicates that Sax must contribute in some way to the mediation of Bmp signals in the wing imaginal disc.

Previous experiments suggested that Gbb signaling is primarily mediated by Sax, whereas Dpp is primarily mediated by Tkv, based on the fact that the wing phenotype associated with overexpression of Gbb or Dpp is better suppressed by co-expression of the dominant-negative (DN) receptor, DN-Sax or DN-Tkv, respectively (Haerry et al., 1998). These respective suppressive phenomena are similar to demonstrated preferred binding affinities, i.e. Dpp and its vertebrate ortholog Bmp2 have high binding affinity for Tkv, and Gbb, or its vertebrate ortholog Bmp7, cannot compete with Bmp2 for Tkv binding (Penton et al., 1994). A similar receptor-ligand binding preference has been observed among the vertebrate orthologs (Yamashita et al., 1995; Nishioto et al., 1996; Chalaux et al., 1998; Ebisawa et al., 1999; Piek et al., 1999).

Curiously, other data do not agree with the proposal that Sax is the primary mediator of Gbb during wing patterning, despite the likelihood that Gbb and Sax interact with high affinity. Primarily, unlike what would be expected of a receptor and its ligand, loss of sax function does not result in a wing phenotype similar to the loss of gbb function (Singer et al., 1997; Khalsa et al., 1998; Ray and Wharton, 2001a). Furthermore, reducing sax gene dose does not enhance the gbb partial loss of function wing phenotype, whereas a similar reduction in tkv gene dose clearly compromises Gbb signaling (Khalsa et al., 1998). These inconsistencies prompted us to examine the role of Sax as a potential mediator of Gbb signaling more closely.

If the functional data suggest, Sax is not absolutely necessary for mediating Gbb signals, what role does Sax play in wing patterning? We found in the complete absence of sax function that alterations in pMad distribution and target gene expression were instead consistent with an increase in Gbb activity, not a loss of Gbb signaling. In addition to this apparent antagonistic function, our experiments also revealed that Sax positively mediates Gbb signaling. To account for both positive and negative functions of Sax, we propose a mechanism for signal transduction that depends on the dual function mediation of Dpp and Gbb by the Tkv, but also on the dual function of Sax as a modulator of ligand availability. In both Drosophila and vertebrates, a different mechanism for modulating ligand activity has been shown to involve secreted molecules in other Bmp-dependent processes (Massagué and Chen, 2000; Mityazono, 2000). The modulation of Tgfβ/Bmp ligand availability by a signaling receptor has not been observed before, and we discuss the implications of this dual role for Sax in shaping the wing disc Bmp activity gradient.

MATERIALS AND METHODS

Drosophila melanogaster strains and crosses
Flies were raised at 25°C unless otherwise indicated. gbb

expression was achieved using the Gal4-UAS system (Brand and Perrimon, 1993). Progeny overexpressing gbb in a background compromised for signaling component genes were generated by yw/+/+; UASgbb/TM3.Sb X w; A9-Gal4 (4 spre/take/M or Med). Wings from the following genotypes were mounted (DPX, EM sciences) and scored: w; A9-Gal4/4 w; +/+;/++ UASgbb/+ versus w; A9-Gal4/4 w; Bl/+; +/+;/++ UASgbb/+ and w; A9-Gal4/4 w; UASgbb/+ versus w; A9-Gal4/4 w; +/+ UASgbb/+.

tkv and sax overexpression

Wings were scored at different levels by raising y w/ w; A9UASX X w; A9-Gal4, w; MS1066-Gal4 or w; 1348-Gal4 at 25°C or 29°C, and in a gbb mutant background: wings of the w 2+Br/+; w; UASbx v gbb/+ females were scored, where 2 + indicates A9 or MS1066. Wings of all progeny were scored. Different levels of tkv overexpression were achieved by crossing w/ w; UAS tkv X w; A9r w; AUS tkv +/+ at 25°C or 29°C. For each cross, wings from both sexes and with one versus two copies of UAS tkv were scored.

Clonal analysis

sax-null clones were generated in the following genotypes: (1) for adult wing, whsFLP 2/2; w; FRT40/gbb Dsh/FRTG14; (2) in gbb mutant background, whsFLP 2/2; w; FRT40/gbb Dsh/FRTG14 sax Dsh gbb, and (3) with brk-lacZ in wing discs, w; brk-lacZ/FRTG14; FLP 2/2; FRT40/gbb Dsh sax Dsh/FRTG14 Mabi-GFP+ P2. Clones were induced by heat-shock (37°C) at various times after egg lay to generate different size clones. A recessive marker, shavenoid (Dsh), which removes or reduces the trichomes on the wing blade (Lawrence et al., 1986), was used to identify wing clones.

Immunohistochemistry

sax 4/4 Df(2R)H23, Df(2R)H23/+ and gbb 4/4 larvae with brk-lacZ were selected making use of CyO,GFP. Everted larvae were fixed in 4% formaldehyde for 10 minutes, blocked in 1% NGS and 0.1% Triton-X in PBS for an hour and incubated overnight at 4°C with mouse anti-Galactosidase antibody (Cappel, 1:3000) or rabbit anti-PS1 (anti-pMad, 1:2000). Secondary antibodies (Alexa 488, 594 and 633, Molecular Probes) were used at 1:3000. Discs were mounted in 80% glycerol, 0.5% N-propyl galate in PBS. Within an experiment, fluorescence images were captured at identical settings with a Leica TCS-SP2-405 confocal microscope, 3D reconstructions were made using DeskVOX (Jürgen Schulze in the Computer Science Department at Brown University (http://www.caltech.edu/~jschulze/)). Fluorescence intensity profiles were obtained using Metamorph across the regions of the wing disc shown in each figure.

RESULTS

Sax antagonizes Gbb signaling during wing development

Overexpression of Bmp ligands, Gbb or Dpp, throughout the wing imaginal disc results in a range of wing phenotypes (Fig. 1A,F), the severity of which depends not only on the level of ligand but also on the level of different downstream signaling components that are crucial for mediating Bmp signals (see Fig. 1I). As phenotypic severity correlates directly with the level of ligand overexpressed, as well as the level of downstream components, the resulting phenotype can be used as an in vivo readout of Bmp signaling levels. By manipulating the levels of wild-type Sax or Tkv in the presence of excess ligand we used this signaling assay to test the receptor preference of Dpp and Gbb.

Based on the results and interpretations of previous experiments using DN receptors (Haerry et al., 1998), increasing levels of wild-type Sax were expected to facilitate Gbb signaling and enhance this phenotype. Similarly, reducing Sax levels should suppress the gbb overexpression wing phenotype by lowering the number of receptors available for the increased level of Gbb ligand to signal. However, in striking contrast to these predictions, we found that

DEVELOPMENT

3296 RESEARCH ARTICLE

Development 133 (17)
overexpression of wild-type Sax strongly suppressed gbb overexpression (Fig. 1A,B). Thus, increasing Sax levels appears to inhibit Bmp signaling attributed to excess Gbb. To ensure that Gbb overexpression did not saturate the signaling system by limiting factors essential for Bmp signaling and thus, indirectly prevent Sax from further increasing signaling, we tested the effect of overexpressing Tkv. Increasing Tkv levels enhanced the gbb overexpression phenotype (Fig. 1C), clearly demonstrating that the ability of Sax to suppress Gbb signaling in this assay cannot be explained simply by the limitation of a general Bmp signaling component, but rather, reflects an ability of excess Sax to block Gbb signaling.

Making use of a cell culture assay for Bmp signaling (Muller et al., 2003), we found that transfection of Drosophila S2 cells with DNA encoding Dpp or Gbb stimulated signaling in a dose-dependent manner, making use of endogenously expressed components (see Fig. S1 in the supplementary material). Co-transfection of constructs encoding Dpp and Tkv or Gbb and Tkv led to an increase in signaling, while co-transfection with Sax led to reduced signaling consistent with our in vivo studies indicating that Sax is able to block Bmp signaling.

In order to test whether the antagonistic behavior of Sax (revealed by the co-overexpression experiments in the wing and in cell culture) reflects an inherent inability of endogenous Sax receptor to mediate a Gbb signal when ligand and receptor are overexpressed, we assayed for the effect of increased Gbb on reduced levels of endogenous Tkv or Sax (Fig. 1D,E). Reducing tkv dose strongly suppresses gbb overexpression (Fig. 1E) in agreement with our previous data that showed a reduction in tkv dose enhanced the gbb loss of function wing phenotype (Khalsa et al., 1998). By contrast, reducing the dose of sax enhances the gbb overexpression phenotype (Fig. 1D), indicating that a reduction in endogenous Sax receptor leads to a further increase in Gbb signaling. Taken together, these findings suggest that excess Gbb signaling is mediated by endogenous Tkv but antagonized by endogenous Sax.

In order to test whether Sax also antagonizes Dpp function, we took a similar approach and, as expected, reduction of tkv gene dose resulted in a strong suppression of the dpp overexpression phenotype (Fig. 1F,H). A mild suppression was also seen when the sax gene dose was lowered (Fig. 1G), indicating that lowering endogenous Sax compromises excess Dpp signaling. The strong suppression of dpp overexpression by reduced Tkv levels supports the current proposal that Tkv is the primary mediator of Dpp signaling. The ability of tkv/+ to efficiently suppress gbb overexpression indicates that Gbb signaling is also dependent on endogenous Tkv. As indicated by studies on the relative binding affinities of Dpp and Gbb for Tkv and Sax (see Fig. S2 in the supplementary material), both Dpp and Gbb are sensitive to Tkv levels and the higher sensitivity of Dpp is consistent with its higher affinity. Endogenous Sax, however, can antagonize the ability of Gbb to signal. No other Bmp signaling component was found to behave antagonistically towards Gbb signaling (Fig. 1I).
Antagonizing function of Sax limits the range of the Bmp activity gradient

From the experiments presented thus far, several important conclusions can be drawn: (1) increasing Sax levels in the developing wing inhibits Gbb signaling caused by excess ligand activity; (2) the ability of Sax to block Gbb signaling is also evident when endogenous levels of Sax are reduced; (3) Sax is the only Bmp signaling component tested thus far that antagonizes Gbb signaling; and (4) although Tkv shows a preference for Dpp, both Gbb and Dpp signals are mediated by Tkv. These conclusions predict that in the complete absence of Sax, Bmp signaling should still occur. Furthermore, when Sax levels are lowered, a change in Bmp signaling activity (pMad) should be evident given the potential for ligand availability to be affected. We examined the distribution of pMad and the expression of the Bmp target gene brk in wing discs with varying doses of sax to get a better understanding of how Sax levels may effect Bmp signaling globally (Fig. 2).

In agreement with an antagonistic role for Sax in Bmp signaling, wing discs derived from animals completely lacking sax function (sax2/Df(2R)H23) show an increase in pMad in the peripheral wing pouch (Fig. 2B). Consistent with this expansion in pMad, brk expression is abolished from essentially all cells of the wing pouch in these sax mutant discs (Fig. 2B′). Interestingly, even in heterozygous wing discs (Df(2R)H23/+ ) a slight expansion of pMad was evident with a concordant increase in brk repression in the wing pouch compared with wild type (Fig. 2C).

As one would predict for a morphogen gradient, the Bmp activity (pMad) gradient is very sensitive to the ligand levels that generate it. When gbb dose is halved (gbb1/+), we consistently saw in a sampling of discs a slight narrowing of pMad distribution and a corresponding failure to repress brk expression in the more central domain of the wing pouch (Fig. 2D). Taken together, the fact that the profile of the pMad gradient changes with a corresponding expansion or retraction of brk repression as the dose of sax or gbb changes, indicates that the ability of endogenous Sax to antagonize Gbb signaling affects the shape of the Bmp activity gradient. The fact that obvious patterning defects are not apparent in the adult wings of sax or gbb heterozygotes indicates that the developing wing is able to self-regulate with time at some level across the disc at either the total amount of ligand produced (L. Soares and K.A.W., unpublished) or at the level of signaling thresholds required for fate specification.

Sax as a signaling receptor in the wing

Although global changes in Sax levels reveal its novel antagonistic function, they also show a lowering in pMad levels near the AP boundary where it normally peaks in wild-type discs (Fig. 2B). These observations indicate that Sax plays some role in mediating Bmp signals, consistent with the reduction in sal expression seen in small sax clones and the ability of such clones to generate pattern duplications of L2 and L5 (Singer et al., 1997). If the anterior border of the sax clone falls between L2 and L3, an ectopic L2 may form at the anterior boundary of the clone (Fig. 3A) consistent with the idea of a signal threshold for pattern specification.
that reducing Bmp signaling in the clone would lead to a shift in the gradient, and thus, a change in target gene expression, such that cells at the border take on a fate typical of those closer to the AP boundary (Fig. 3F). Yet, we previously showed that sax clones encompassing an entire compartment had little effect on the overall wing pattern, suggesting that global removal of Sax does not compromise Bmp signaling (Ray and Wharton, 2001b). Given this apparent contradiction, we looked more closely at the ability of Sax to promote signaling and investigated the possibility that Sax could both promote, as well as antagonize signaling in vivo.

We found that the effect of globally removing sax function depends on the dose or level of gbb function. Complete loss of sax can enhance a gbb partial loss-of-function wing phenotype (see Fig. S3 in the supplementary material), indicating that Sax must mediate some Gbb signaling. This requirement was only revealed when gbb function is compromised. Small sax clones near the AP boundary show a reduced level of pMad (Fig. 4B,C). This reduction in pMad provides a direct readout for the requirement of Sax for signaling and indicates that endogenous Tkv alone cannot compensate for the loss of Sax to maintain wild-type levels of signaling. The level of Bmp signaling must be below what is necessary to repress brk expression in some sax mutant cells (Fig. 4C). Those that are near the AP boundary are less likely to show ectopic brk, indicating that loss of sax function must not impact Bmp signaling as significantly in these cells as in the periphery of the wing disc. An interesting feature of these small sax clones is the increase in pMad levels in cells immediately outside the clone, clearly evident from pMad intensity profiles taken across the sax clone (Fig. 4C,E-H). This increase in Mad-mediated signaling is consistent with the idea that ligand produced by the cells of the sax null clone is no longer trapped or bound and is able to move to and signal in surrounding cells. Thus, it is possible to visualize both the antagonistic and positive signaling functions of Sax in the wing disc. Close examination of the effect of small sax clones on adult wing pattern indicates that clones near but anterior to L2, result in the formation of an ectopic vein adjacent their anterior border (Fig. 3B). This phenotype cannot be explained in the same way as the clone shown in Fig. 3A, by a reduction in Sax-mediated Bmp signaling within the clone, as the level of signaling in this region of the wing disc should already be below the level of Bmp activity necessary for the specification of L2 (Fig. 3G). However, analogous to disc clones, if the loss of Sax allowed Gbb that would have been unavailable or bound, to now signal to adjacent cells, a local increase in pMad could generate target gene expression sufficient for L2 specification.

Fig. 3. Ectopic veins produced by sax clones depend on gbb function. In a wild-type background (A-C), sax-null clones produce an ectopic vein, while in a gbb+/gbb− mutant background (D) sax-null clones fail to produce vein duplications. Higher magnification of clones: A′-D′, bright field; A″-D″, dark field. sax clones are marked with sha93. Elimination of trichomes on both surfaces of the wing (i.e. a double-sided clone) appears black in dark field. (A,B,D) Anterior border of each double-sided clone (white arrowhead), ectopic vein (black arrowhead). (C) Posterior border of clone with anterior border between L2 and L3 produces an ectopic vein at the anterior border of the clone. (B) sax clone with its anterior border between L1 and L2 produces an ectopic vein anterior to L2. (C) A sax clone in the posterior compartment that generated an ectopic L5 outside the clone boundary. (D) sax clone in a genetic background reduced for gbb function is very similar in position and shape to that shown in B but it lacks an ectopic vein. (E-H) Schematics depicting effect of sax clone (white box) on graded pMad (purple) in the anterior compartment of wing discs. x-axis indicates distance from Bmp source near the AP boundary. y-axis indicates level of pMad or Bmp signaling activity. (E) Relative domains of kni, sal,omb and brk expression and the position of L2 primordia (thick blue line). (F-H) Interpretation of clone in A,B,D, respectively. (F,G) Loss of Sax results in a reduction in Bmp signaling (gray arrows) within the clone and an increase in Gbb (blue), which influences the level of Bmp signaling in surrounding cells (black arrows). (H) Increased availability of mutant Gbb protein (pale blue) is not sufficient to raise pMad levels to the point necessary for ectopic L2 specification.
(Fig. 3G). Consistent with this interpretation, we see that sax clones often exhibit non-autonomous effects such as an ectopic vein forming in wild-type tissue outside the boundaries of the clone (Fig. 3C). This non-autonomous behavior is not expected from the loss of a membrane-bound receptor molecule and must involve a substance, such as a secreted ligand, that can move from the cells lacking Sax to influence surrounding tissue.

If the localized increase in Bmp signaling is indeed due to an increase in available Gbb, then ectopic veins associated with sax clones should not form when the total level of functional Gbb available for signaling is reduced. Null sax clones induced in a genetic background compromised for Gbb function that are posterior to L5 or anterior to L2 never (>50) lead to vein duplications (Fig. 3D). Thus, the formation of such ectopic veins is sensitive to Gbb function and any increase in mutant Gbb protein due to the loss of Sax does not produce levels of pMad high enough to specify an ectopic L2. Together, these data reveal that endogenous Sax must normally limit the amount of endogenous Gbb available for signaling and that local changes in Sax levels can impact the Bmp activity gradient and, thus, wing patterning.

**DISCUSSION**

The data presented here clarifies the respective roles of Sax and Tkv in mediating Bmp signaling during wing patterning. Our analysis shows that Tkv is responsible for mediating both Dpp and Gbb signals, and that Sax has a much more complex role in wing patterning than previously appreciated; Sax not only promotes signaling but also antagonizes signaling by limiting the availability of primarily the Gbb ligand. Both the antagonistic and signal promoting functions of Sax were revealed not only by gain-of-function studies but importantly, also by loss-of-function analyses. Loss of the antagonistic function of endogenous sax is evident: (1) as a broadening of the pMad profile when the wing disc completely lacks sax function (Fig. 2B); and (2) as a non-autonomous increase in pMad levels in wild-type cells abutting the boundary of sax null clones (Fig. 3C, Fig. 4B,C). Loss of Sax-mediated signaling itself is evident: (1) in sax mutant discs as a reduction in the peak pMad levels along the AP boundary (Fig. 2); and (2) in sax clones as a cell-autonomous reduction in pMad accumulation (Fig. 4). Gain-of-function or overexpression studies indicate that the balance of Sax and Tkv levels in wing disc cells is crucial for proper signaling and, thus, wing patterning. Altogether, our results indicate that Sax is important in modulating Bmp signaling across the wing disc by both mediating and blocking Bmp signals, and, thus, shaping the Bmp activity gradient. How can the novel function of Sax as an antagonist be reconciled at the molecular level with the ability of Sax to promote signaling?

**Dual function of Sax likely depends on its receptor partner**

Given that Tkv is required for all Bmp signaling in the wing disc, the simplest explanation for the fact that Sax signaling appears to depend on the presence of Tkv is that Sax can only promote signaling in a receptor complex also containing Tkv. Three different forms of Bmp receptor complexes can potentially form in wing disc cells, those composed of two type II receptor molecules and either two Tkv, two Sax or one molecule of each: Tkv-Tkv, Sax-Sax and Tkv-Sax (Fig. 5A). Overexpressing Tkv or Sax in wing disc cells enabled us to shift the balance between the relative levels of these two molecules, artificially enriching for the formation of receptor complexes homomeric for type I molecules Tkv-Tkv or Sax-Sax. Disrupting the balance of endogenous Tkv to Sax levels by overexpressing Sax immediately reveals the antagonistic function of Sax, consistent with the idea that excess Sax could be sequestering ligand in Sax-Sax receptor complexes which signal either very poorly or not at all. However, overexpression of Tkv, enriching for Tkv-Tkv complexes with high affinity for Dpp and lower affinity for Gbb, leads to increased signaling given sufficient ligand. The third receptor complex, Tkv-Sax, probably accounts for the contribution of Sax to the promotion of Bmp signaling and probably signals in vivo more efficiently than Tkv-Tkv, based on the fact that pMad levels are lower inside clones devoid of Sax than the pMad levels seen in cells at an equivalent position along the AP axis elsewhere on the disc (see Fig. 4, Fig. 5B). Loss of Tkv, by definition, eliminates signaling by both Tkv-Tkv and Tkv-Sax, leaving only Sax-Sax containing receptor complexes, which are clearly unable to elicit a pMad-mediated signal on their own. Thus, our model predicts that removing Sax function results in two opposing consequences: (1) a reduction in total Bmp signaling caused by loss of Tkv-Sax complexes; and (2) an increased availability of Bmp ligand and potential signaling caused by loss of Sax-Sax complexes (Fig. 5C). Several biochemical studies support the putative existence of functional Sax-Tkv receptor complexes. Heteromeric complexes involving different vertebrate type I receptors have been shown to contribute to a single signaling receptor complex (Gilboa et al., 2000; Kirsch et al., 2000b) and in Drosophila S2 cells both Sax and Tkv appear to be necessary to produce a synergistic signal (Shimmi et al., 2005).
Sax both promotes and antagonizes Bmp signaling

**Fig. 5. Model of Bmp signaling and the effect of Sax modulation.** (A) A model reconciling both the antagonistic and the signaling functions of Sax in wing disc cells. Dpp and Gbb have different binding preferences (indicated by the thickness of the arrows) for receptor complexes with different combinations of type II receptors. Tkv-Sax receptor complexes contribute more significantly to signaling (indicated by the thickness of the black arrow) than Tkv-Tkv, whereas Sax-Sax complexes fail to phosphorylate Mad. (B) tkv-null cells lack p-Mad. (C) sax-null clones with receptor complexes solely of the Tkv-Tkv type are not as efficient at signaling as wild-type cells. (D) Wild-type cells adjacent to sax-null cells exhibit a higher level of pMad than normal. The antagonistic function of Sax is preferentially directed at Gbb and the high affinity of Gbb for Sax ensures that in the absence of Sax, a region of the Gbb pool is available for signaling not only in the mutant cells via Tkv-Tkv but also in wild-type neighboring cells. (E) Gene expression domains for brk (green) and omb (orange) in the wing pouch of wild-type (top) and sax-null wing disc (bottom). Gbb derived from cells along the AP boundary (black vertical line) is required to repress brk expression in the lateral wing pouch of both compartments (Bangi and Wharton, 2006). Sax limits the range of Gbb by acting antagonically on the ability of Gbb to signal; thus, in the absence of Sax, the range of Gbb signaling expands, repressing brk expression throughout the wing pouch.

It is important to note that increasing wild-type Tkv levels in the presence versus absence of excess ligand results in very different phenotypic outcomes. In contrast to Sax, increasing Tkv in the presence of excess ligand leads to a larger increase in Bmp signaling. However, at endogenous ligand levels, as Tkv levels are experimentally increased, we see a loss of Bmp signaling that is indicative of the preference of Tkv for binding Dpp over Gbb (Fig. S2). Clearly, both Gbb and Dpp become limiting in the presence of excess Tkv, with low level Tkv overexpression preferentially limiting Dpp-dependent signaling, while higher levels of overexpression limit both. Clearly, although overexpression of ligand and receptor together reveals a significant difference in the signaling ability of Tkv and Sax, overexpression of receptor alone in the absence of increased ligand appears to reflect only receptor ligand-binding preference.

Such experimental manipulations of Tkv levels can lead to the loss of Bmp signaling by limiting the range of Bmp signaling, but unlike sax, loss of endogenous tkv function never leads to an increase in Bmp signaling. Furthermore, there is no indication that Tkv is required for or involved in the antagonistic function of Sax. At endogenous levels, Sax-Sax complexes, unlike Tkv-Tkv or Tkv-Sax complexes, appear to modulate the range of Bmp signaling by sequestering ligand without any associated signaling, and, thus, Sax identifies a new previously unrecognized Bmp modulator whose signaling ability appears to depend on which receptor it partners.

**Ligand specificity of different receptor complexes in wing disc**

The fact that both Dpp and Gbb are dependent on Tkv for signaling has significant implications regarding the Bmp activity gradient, given that removal of Tkv at any point along the gradient results in the loss of both Gbb and Dpp signaling, not just Dpp signaling. When both ligands are present at similar levels, the higher affinity of Dpp for Tkv means the contribution of Dpp to total Bmp signaling will be more significant than that of Gbb, and movement of Dpp across the wing disc will be affected more strongly by Tkv than that of Gbb. Thus, Gbb should and does contribute more significantly to the low points of the Bmp activity gradient (Ray and Wharton, 2001b; Bangi and Wharton, 2006), especially as competition with Dpp for binding to Tkv will also be lower in these regions.

Our findings from receptor and ligand overexpression experiments suggest that both the antagonistic and signal promoting functions of Sax impact Gbb signaling most significantly because of their preferential interaction. For example, although localized loss of Sax from the peripheral cells of the wing pouch leads to ectopic induction of brk, loss in more central cells does not, suggesting that the relative contribution of Sax to overall Bmp signaling is less in the central cells where Tkv must contribute more significantly given the higher level of Dpp near the AP boundary. The greater contribution of Sax to total signaling in the more peripheral cells of the wing pouch is consistent with its higher affinity for Gbb and the long-range nature of Gbb versus Dpp (Bangi and Wharton, 2006). Similarly, removal of Sax from just anterior compartment cells results in brk repression in both the anterior and posterior compartments (data not shown) suggesting that in the absence of Sax, anteriorly expressed Gbb can signal to the posterior-most cells of the wing pouch to effectively repress brk expression beyond its normal domain. This result indicates that endogenous Sax normally functions to not only restrict the level of Gbb signaling but also the range of Gbb. The role that Sax plays in promoting Gbb function, in particular, is detected only when sax
function is completely eliminated and gbb function is also significantly compromised (see Fig. S3 in the supplementary material).

Given that Tkv is also required for mediating Gbb signals, of the two proposed receptor complexes that could mediate Gbb signaling (Tkv-Tkv and Tkv-Sax), which is preferentially used by Gbb in wild-type cells? It is clear that Tkv-Sax complexes are not obligatory for Gbb signaling as Gbb signaling is not abolished in sax mutants. The fact that removing Sax does not cause a gbb loss-of-function phenotype indicates that enough Gbb is made available by the loss of Sax antagonism and can signal to compensate for losing that region of total signaling that Sax normally promotes. The fact that pMad levels within a sax clone are lower then endogenous levels indicates that signaling in the clone cells containing only Tkv-Tkv is less efficient than the neighboring cells that have wild-type levels of both Sax and Tkv (Fig. 5D).

A synergy has been observed between co-expressed constitutively active (CA) Tkv and Sax in the early embryo (Neul and Ferguson, 1998; Nguyen et al., 1998) and between Tkv and Sax in S2 cells in response to Dpp-Swc heterodimers, as only Dpp homodimers are able to signal efficiently in the absence of Sax (Shimm et al., 2005). We have detected a likely, albeit minimal, contribution of Dpp-Gbb heterodimers to long-range wing patterning (Bangi and Wharton, 2006) making it is possible that Tkv-Sax complexes could respond to Dpp-Gbb heterodimers and such complexes could be particularly efficient at signaling. Given the dual function of Sax, the relative levels of Sax to Tkv are likely to be crucial for establishing a synergistic interaction. The ability of Tkv-Sax containing complexes to mediate ligand homodimers has not yet been determined in vivo and it is also not yet completely clear if the antagonism by Sax can affect heterodimers as well as homodimers. Our data indicate that the ability of Sax to promote signaling must reside with Tkv-Sax-containing complexes and the strong contribution of Gbb to the low points of the gradient with a minimal contribution by Dpp leaves open the possibility that Dpp-Gbb can signal, in addition to Gbb-Gbb, to cells far from the AP boundary.

### Sax as a general modulator of Bmp signaling

Overexpression studies in the follicle cells of the Drosophila ovary produce the same results as those described in Fig. 1, indicating that the ability of Sax to block Gbb signaling is not limited to the developing wing (F. Cernilogar, R. Ray and K.W., unpublished). However, in contrast to studies in the wing disc, loss of sax from the follicle cells, as well as the embryonic midgut and neuromuscular synapse produces mutant phenotypes indicative of a loss of ligand function (Nellen et al., 1994; Twombly et al., 1996; Khalsa et al., 1998; Rawson et al., 2003). It is possible that the contribution of Sax to signal promotion in these tissues may be stronger than its antagonistic function. The phenotypic outcome of sax loss of function in a particular process probably depends on the relative numbers of Sax-Sax and Sax-Tkv complexes on the cell surface and the relative binding affinity of a given Bmp ligand for these two complexes. What regulates the composition of type I receptors in a signaling complex is not yet known.

### Possible molecular explanations for unique role of Sax receptor

The ability of the Sax to block Bmp signaling may reflect its requirement to have input from another molecule to activate its kinase domain. When activated by in vitro mutagenesis, Sax and its vertebrate orthologs Alk1/Alk2 (AcvR1 and AcvR1 – Mouse Genome Informatics) are able to phosphorylate Bmp specific R-Smads (Chen and Massagué, 1999), but ligand-induced activation of Sax or Alk1/2 kinase has not been reported. Interestingly, a ligand-induced Bmp receptor complex containing Alk2 and ActRII is unable to phosphorylate Smad1 (Macias-Silva et al., 1998). Furthermore, Alk1 has been shown to require a different type I receptor (Alk5) to activate its kinase domain (Goumans et al., 2003). Although Macias-Silva et al. (Macias-Silva et al., 1998) suggest that the Alk2/ActRII complex might be unstable in vitro, it is also possible that activation of Alk2 (and of its Drosophila ortholog Sax) may depend on its partner type I receptor and/or which ligand is bound, or some other protein. Although Gbb fails to activate Sax-Sax, perhaps another Bmp ligand (i.e. Scw) can. Similarly, endoglin, related to the co-receptor betaglycan (Lopez-Casillas et al., 1993; Lopez-Casillas et al., 1994; Letamendia et al., 1998), could be important in modulating Alk1-dependent signaling given that mutations in either gene give rise to hereditary hemorrhagic telangiectasia (van den Driesche et al., 2003). Sax may require a different type I receptor partner, i.e. Tkv, to activate its kinase or transduce a signal, and such a requirement may be a universal feature of the Alk1/Alk2/Sax subgroup of Bmp type I receptors.

### Role of Sax as an antagonist for the establishment of a robust Bmp activity gradient

The robustness of morphogen gradients may depend on negative-feedback mechanisms to buffer against environmental and genetic fluctuations. Clearly, Sax plays a crucial role in modulating the range of the Bmp activity gradient from analysis at both the level of Bmp-dependent target gene expression and the final pattern of the adult wing. The identification of the antagonistic nature of a Bmp type I receptor to modulate signaling activity by sequestering ligand without transducing a signal provides a new mechanism that contributes to the robustness of the Bmp activity gradient. We propose that the dual function of Sax is crucial for buffering the wing disc Bmp activity gradient against local fluctuations in ligand levels (environmental, genetic or experimentally induced). Whether this mechanism of signal modulation is evolutionarily conserved remains to be determined, but the fact that the vertebrate Sax orthologs Alk1 and Alk2 have been shown biochemically to exhibit antagonistic behaviors in vitro is interesting. Detailed analysis of these orthologs in developmental contexts will be crucial to determine whether the robustness of vertebrate Bmp activity gradients also depends on the modulation of ligand availability by specific receptors.

We thank Jen Shagensky for outstanding technical assistance during the final stages of this project; R. Ray during the initial stages; M. Affolter; B. Hartmann and G. Pyrowolakis for their assistance and support in setting up the reporter activity assay; P. ten Dijke for kindly providing anti-PS1; E. Matusin and M. O’Connor for sharing fly stocks; M. McKeown for thoughtful discussions; and members of Wharton laboratory for critically reading the manuscript. This work was supported in part by an American Cancer Research Award #RG52DC-98790, a NIH/NCRR COBRE award 1P20OR15578-01 and a NIH 1R01GM068118-01 (K.W.). A Richard B. Solomon Faculty Research Award to K.W. provided partial support to E.B.

### Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/17/3295/DC1

### References


of decapentaplegic, a member of the TGF-β family in Drosophila. Development 111, 657-666.


del Alamo Rodriguez, D., Terriente Felix, J. and Diaz-Benjumea, F. J. (2005). Hedgehog creates a spatially restricted activation of the SAX receptor and heterodimerization with the type II receptor are both required for DPP/TKV signaling in Drosophila embryos depending on synergistic signaling by two type I receptors, SAX and TKV. Cell 95, 495-506.