Context-dependent relationships between the BMPs *gbb* and *dpp* during development of the *Drosophila* wing imaginal disk

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SUMMARY

The *Drosophila* BMP5/6/7/8 homolog, *glass bottom boat* (*gbb*), has been shown to be involved in proliferation and vein patterning in the wing disk. To better understand the roles for *gbb* in wing development, as well as its relationship with the *Drosophila* BMP2/4 homolog *decapentaplegic* (*dpp*), we have used clonal analysis to define the functional foci of *gbb* during wing development. Our results show that *gbb* has both local and long-range functions in the disk that coincide both spatially and functionally with the established functions of *dpp*, suggesting that both BMPs contribute to the same processes during wing development. Indeed, comparison of the mutant phenotypes of *dpp* and *gbb* hypomorphs and null clones shows that both BMPs act locally along the longitudinal and cross veins to affect the process of vein promotion during pupal development, and long-range from a single focus along the A/P compartment boundary to affect the processes of disk proliferation and vein specification during larval development. Moreover, we show that duplications of *dpp* are able to rescue many of the phenotypes associated with *gbb* mutants and clones, indicating that the functions of *gbb* are at least partially redundant with those of *dpp*. While this relationship is similar to that described for *dpp* and the BMP *screw* (*scw*) in the embryo, we show that the mechanisms underlying both local and long-range functions of *gbb* and *dpp* in the

wing are different. For the local foci, *gbb* function is confined to the regions of the veins that require the highest levels of *dpp* signaling, suggesting that *gbb* acts to augment *dpp* signaling in the same way as *scw* is proposed to do in the embryo. However, unlike *scw*-dependent signals in the embryo, these *gbb* signals are not transduced by the Type I receptor *saxophone* (*sax*), thus, the cooperativity between *gbb* and *dpp* is not achieved by signaling through distinct receptor complexes. For the long-range focus along the A/P compartment boundary, *gbb* function does not appear to affect the high point of the *dpp* gradient, but, rather, appears to be required for low points, which is the reciprocal of the relationship between *dpp* and *scw* in the embryo. Moreover, these functions of *gbb* also do not require the Type I receptor *sax*. Given these results, we conclude that the relationships between *gbb* and *dpp* in the wing disk represent novel paradigms for how multiple BMP ligands signal during development, and that signaling by multiple BMPs involves a variety of different inter-ligand relationships that depend on the developmental context in which they act.

Key words: *gbb*, *dpp*, BMP, Imaginal disk, Wing, Pattern formation, *Drosophila*

INTRODUCTION

In the course of development, cells within a developing tissue receive many different kinds of signals, mediated by a variety of signaling systems, and the cells are able to integrate and coordinate these signals into actions appropriate to their role in the developmental program. Studies on most of these systems have shown that proper signaling involves multiple ligands and multiple receptors. For example, at least three types of ligands have been described for the epidermal growth factor (EGF) receptor: transforming growth factor (TGF)α proteins, Neuregulins and Amphiregulins, all of which contain the canonical cysteine-rich EGF-repeat, yet each has different effects on receptor function (Moghal and Sternberg, 1999; Wells, 1999). Perhaps most diverse in this regard is the TGFβ

signaling system. In vertebrates, TGFβ superfamily members include at least 16 ligands, many combinations of which are expressed in overlapping patterns within the same tissue. These ligands signal through heteromeric receptor complexes consisting of Type I and Type II receptors that are also diverse (Hogan, 1996; Massagué, 1998). This kind of complexity within a single signaling system raises the question of how cells distinguish between specific ligands or ligand-receptor pairs, and how these different combinations influence the development of the organism.

The TGFβ superfamily is comprised of more than 25 structurally related members that have been grouped into four families, TGFβs, bone morphogenetic proteins (BMPs), Activins and MIS. All of the members are produced as pro-protein dimers consisting of an N-terminal pro domain and a

C-terminal ligand domain, the latter of which is cleaved from the pro region during secretion to release the biologically active ligand dimer. Studies in a number of systems have shown that the signaling potential of these ligands may be regulated in a number of different ways. For example, the ligands are subject to regulation by extracellular antagonists such as Chordin, Noggin and Follistatin, which act directly on the ligands thereby preventing their interaction with receptors (Piek et al., 1999). Ligand function can also be affected by the formation of heterodimers that may have properties distinct from their corresponding homodimers (Yu et al., 1987; Petraglia, 1989).

In *Drosophila*, as in vertebrates, there are multiple TGFβ superfamily ligands and multiple receptors. The three characterized ligands, all members of the BMP family, are the BMP2/4 homolog *dpp*, the BMP5/6/7/8 homolog *gbb*, and the more distantly related *screw* (*scw*) (Padgett et al., 1987; Wharton et al., 1991; Arora et al., 1994). The BMP receptors include two Type I receptors, *thick veins* (*tkv*) and *saxophone* (*sax*), and a single Type II receptor, *punt* (*put*) (Nellen et al., 1994; Brummel et al., 1994; Letsou et al., 1995). *dpp* is a central figure in all characterized BMP signaling events in *Drosophila*, and has been implicated in numerous functions throughout the life cycle of the fly. In two of these functions, specifically, dorsal-ventral patterning in the embryo and anteroposterior (A/P) patterning in the wing disk, it has been proposed that *dpp* has morphogenetic properties in that multiple cell fates are specified as a function of different levels of *dpp* activity (Podos and Ferguson, 1999).

In the embryo, *dpp* acts in combination with *scw* to specify pattern elements in the dorsal epidermis through a gradient of BMP signaling whose high point lies along the dorsal midline (Ferguson and Anderson, 1992; Wharton et al., 1993; Neul and Ferguson, 1998; Nguyen et al., 1998). According to the current model, formation of this activity gradient depends on three features of the system: specificity of each ligand for a different receptor complex, strict dependency of Scw signaling on *dpp*, and antagonism of Scw activity by *short gastrulation* (*sog*), the *Drosophila* ortholog of Chordin. In brief, it is thought that Dpp signals uniformly throughout the dorsal 40% embryo (where the *dpp* RNA is expressed) through a receptor complex composed of Tkv and Put. Scw is thought to signal through a receptor complex composed of Sax and Put, and this signaling is limited to the dorsal regions of the embryo in two ways. First, Scw signaling is strictly dependent on Dpp signaling, and thus, while *scw* is expressed throughout the embryo, Scw signaling only occurs in the dorsal 40% of the embryo where *dpp* is expressed. Notably, this dependency does not require the formation of Scw:Dpp heterodimers, as restriction of *scw* expression to ventral cells does not compromise its ability to act in conjunction with Dpp signaling to generate a normal dorsal-to-ventral gradient. Second, Scw activity is negatively regulated by a gradient of Sog diffusing dorsally from its site of expression in the ventral ectoderm, such that Scw activity is highest along the dorsal midline and grades off ventrally. As Scw signaling acts to augment Dpp signaling, the highest levels of BMP signaling in the embryo lie along the dorsal midline, where Scw activity is highest, and the levels grade off ventrally (Podos and Ferguson, 1999).

In the wing disk, *dpp* has a number of developmentally and genetically separable functions. Throughout larval development, *dpp* is expressed in a narrow band of cells that lie just anterior to the A/P compartment boundary (Masucci, et al., 1990; Posakony et al., 1991). From this localized site of expression, *dpp* acts long range across the disk to promote disk proliferation, predominantly during early larval development (Spencer et al., 1982; Burke and Basler, 1996), and specification of vein territories during later larval development (deCelis et al., 1996; Sturtevant et al., 1997). It has been proposed that this 'stripe' of expression serves as a localized source for a gradient of *dpp* activity that activates the expression of target genes *spalt* (*sal*) and *optomotor blind* (*omb*) with respect to different activity thresholds (Lecuit et al., 1996; Nellen et al., 1996). Mutations in the disk region of *dpp* (i.e. *dpp^d* alleles) affect these functions, and give rise to small disks in mutant larvae (Spencer et al., 1982), or, in adult viable combinations, to small wings that show loss of vein and intervein territories (Segal and Gelbart, 1985; deCelis, et al., 1996). Similar phenotypes have been observed by clonal analysis with null and *dpp^d* alleles (Posakony, et al., 1991), and, as mutant wings can be recovered showing phenotypes far from the site of the clone, these studies confirm that *dpp* acts non-autonomously (i.e. at a long range) from this focus along the A/P compartment boundary.

During pupal development, *dpp* ceases to be expressed along the A/P compartment boundary, and novel transcription of *dpp* is detected along the lengths of the presumptive veins (Yu, et al., 1996; deCelis, 1997). At this stage of development, *dpp* is thought to contribute to the process of vein promotion whereby vein and intervein tissues in the wing are defined and refined. Mutations in the shortvein region of *dpp* (i.e. *dpp^s* alleles) affect this function, and, in adult viable combinations show truncation of the distal tips of the longitudinal veins and loss of the crossveins (Segal and Gelbart, 1985). Based on clonal analyses with null and *dpp^s* alleles, it has been shown that the vein loss associated with the mutant clones respects the clone boundaries, indicating that, for this function, *dpp* acts more or less autonomously (Posakony et al., 1991; deCelis, 1997). Thus, in contrast to its long-range functions during larval development, *dpp* appears to act locally during pupal development to promote the vein fate.

The developmental events that require *dpp* during wing development do not involve *scw*, which is not expressed after the embryonic stages (Arora et al., 1994), but may involve *gbb*. *gbb* is broadly expressed in the wing disk (Khalsa et al., 1998), and *gbb* mutants show phenotypes that are to some extent similar to the wing phenotypes of *dpp* (Khalsa et al., 1998; Wharton et al., 1999). Despite these similarities, the nature of the relationship between *dpp* and *gbb*, and how these two BMPs interact to pattern the wing properly is not clearly understood. Indeed, while a previous study based on the overexpression of *gbb* and *dpp* in combination with dominant-negative receptor constructs suggested that the relationship between *gbb* and *dpp* in the wing was similar to that of *scw* and *dpp* in the embryo (Haerry et al., 1998), these results were not entirely consistent with corresponding loss-of-function data (Khalsa et al., 1998), and prompted a more detailed analysis of *gbb* function in wing development.

We present a detailed clonal analysis of *gbb* in the wing. Our results show that, like *dpp*, *gbb* has two different types of foci in the disk, local and long range, and these foci correlate both spatially and functionally with the local and long-range functions of *dpp*. This coincidence of the foci of *gbb* and *dpp* in the disk indicates that the two BMPs act from the same sites

to regulate disk proliferation and vein specification in the larval imaginal disk, and vein promotion in the pupal wing. Function-by-function comparisons of the phenotypes of *gbb* mutants and null clones with the phenotypes of *dpp* and *sax* clones clearly demonstrates that the relationship between *gbb* and *dpp* in the wing is not only different from that proposed for *dpp* and *scw* in the embryo, but that the relationship between *gbb* and *dpp* depends on the developmental process they affect. These results provide evidence that there is not a single type of relationship between different BMPs that is co-opted into a variety of different developmental contexts, but rather that the BMPs have evolved relationships that are specific to the developmental context in which they act.

MATERIALS AND METHODS

Drosophila strains

Flies were raised on standard *Drosophila* cornmeal-yeast medium at 25°C unless otherwise indicated. The amorphic *gbb* alleles, *gbb¹* and *gbb²*, and hypomorphs, *gbb³* and *gbb⁴*, have been described elsewhere (Wharton et al., 1999). The *gbb* mutant chromosomes used in this report are derivatives of the original mutagenized chromosomes. The *dp cn bw gbb¹* chromosome was recombined with an isogenic *pk cn* stock to generate *pk cn gbb¹* which was used to generate the *FRT-G13 sha^IN gbb¹* lines used for the clonal analysis. The *pk cn gbb¹* chromosome was in turn recombined with an isogenic *b pr cn bw* stock to generate the *b pr cn bw gbb¹*, which was then recombined with Oregon R to generate the *bw gbb¹* chromosome. We have shown that the lethality of this chromosome is specific to *gbb*, as we can rescue it to viability with a single copy of a *gbb⁺* transgene. For *gbb²*, the mutagenized *dp b cn gbb²* chromosome was recombined with *b pr cn bw* to generate *b pr cn bw gbb²* chromosome that was then used to generate the *FRT-G13 sha^IN bw gbb²* chromosome. The *b pr cn bw gbb⁴* chromosome was recombined with Oregon R to generate *bw gbb⁴* and *cn bw gbb⁴* chromosomes.

For the rescue experiments with additional copies of *dpp*, recombinants were made directly between two *dpp* duplications, *Dp(2;2)B16* and *Dp(2;2)DTD48* (Wharton et al., 1993), and the *pk cn gbb¹* and *b pr cn bw gbb⁴* chromosomes. For *gbb* mutant clones bearing additional copies of *dpp*, double recombinants were isolated directly from a cross of *FRT-G13 sha^IN bw gbb²* × *FRT-G13 Dp(2;2)DTD48* to generate the stock *FRT-G13 sha^IN Dp(2;2)DTD48 bw gbb²*. For *gbb*-null clones produced in a background carrying three copies of the *dpp* locus, *Dp(2;2)DTD48* was recombined onto a chromosome carrying *FRT-G13 sha^IN* to generate the *FRT-G13 Dp(2;2)DTD48* which was, in turn, recombined with *FRT-G13 M(2)53* to generate *FRT-G13 Dp(2;2)DTD48 M(2)53*.

For *dpp* clones, the *dpp^H46 ck¹³ FRT-40A bw* and *Dp(2;2)B16 dp πM-36F FRT-40A* stocks were generated by recombination from the crosses *dpp^H46 Sp cn bw* X *P[y⁺]25D ck¹³ FRT-40A* (Bloomington) and *Dp(2;2)B16 dp cl cn bw* × *2πM FRT-40A* (T. Xu), respectively.

All other strains are listed in FlyBase (http://fly.ebi.ac.uk:7081).

Clonal analysis

Clones were generated using the FLP/FRT technique (Xu and Rubin, 1993) in the progeny of the following crosses:

y w¹¹¹⁸; FRT-G13 sha^IN gbb¹/SM6a X *y w* hs-FLP1; FRT-G13 2πM*

w hs-FLP1; FRT-G13 M(2)53/CyO X *y w¹¹¹⁸; FRT-G13 sha^IN gbb¹/SM6a*

w hs-FLP1; Dp(2;2)B16 dp πM FRT-40A/CyO X *dpp^H46 ck¹³ FRT-40A/CyO23,dpp⁺*

w hsFLP1; FRT-G13 M(2)53/CyO X *FRT-G13 sha^IN Dp(2;2)DTD48 bw gbb²/SM6a*

w hsFLP1; FRT-G13 Dp(2;2)DTD48/CyO X *FRT-G13 sha^IN gbb¹/SM6a*

w hsFLP1; FRT-G13 Dp(2;2)DTD48 M(2)53/CyO X *FRT-G13 sha^IN gbb¹/SM6a*

w hs-FLP1; FRT-G13 M(2)53/CyO X *y w¹¹¹⁸; FRT-G13 sax⁴ sha^IN/SM6a*

Crosses were made in bottles, brooded every 12 or 24 hours, aged for 24 hours, and then heat-shocked for 2 hours at 37°C. Wings of flies of appropriate genotype were mounted in DPX mountant (EM Sciences) and analyzed on a Nikon Microphot-FXA photomicroscope. Images were collected with a SPOT-RT color digital camera (Diagnostic Instruments). For each experiment, at least 500 clones were mapped and analyzed, and in some cases, for example, for the *dpp* clones affecting the posterior cross vein and the large *sax* clones occupying the anterior or posterior compartments, many more were scored for these particular characteristics.

Rescue studies

For rescue of *gbb¹/gbb⁴* transheterozygotes, crosses of *pk cn gbb¹/SM6a, Dp(2;2)B16 dp cn gbb¹/SM6a, Dp(2;2)DTD48 gbb¹/SM6a* to *b pr cn bw gbb⁴/SM6a* and a cross of *Dp(2;2)B16 dp cn gbb¹/SM6a* to *Dp(2;2)DTD48 bw gbb⁴/SM6a* were scored for the presence of *Cy⁺* progeny. The statistic 'percent of expected' was calculated by dividing the total number of *Cy⁺* progeny by half the total number of *Cy* progeny. Pharate and pupal lethals were scored 2 days after the last eclosed progeny were collected. For each cross more than 1000 progeny were scored from multiple broods. For rescue of *gbb¹* homozygotes crosses of *pk cn gbb¹/SM6a, Dp(2;2)B16 dp cn gbb¹/SM6a, Dp(2;2)DTD48 gbb¹/SM6a* to *bw gbb¹/SM6a* and a cross of *Dp(2;2)B16 dp cn gbb¹/SM6a* to *Dp(2;2)DTD48 gbb¹/SM6a* were scored.

RESULTS

gbb wing phenotypes and clonal analysis

We have previously provided evidence that *gbb* plays a role in the development of the wing imaginal disk (Khalsa et al., 1998; Wharton et al., 1999). Amorphic alleles of *gbb*, e.g. *gbb¹* and *gbb²*, are larval/pupal lethals, and the mutant larvae have variably reduced wing imaginal disks. Hypomorphic alleles of *gbb*, e.g. *gbb⁴*, are semi-viable as homozygotes and *in trans* to null alleles (see Fig. 6; Wharton et al., 1999). The wings of the mutant flies are reduced in size compared with wild type and show defects in wing vein patterning (Fig. 1). The weakest vein defect observed is the specific loss of the posterior cross vein (PCV). Moderate phenotypes, which are most commonly observed, show loss of the PCV as well as truncations of the distal tips of longitudinal veins L4 and L5 (Fig. 1B, compare with Fig. 1A). The most severe wing phenotype shows complete loss of L5 except for the most proximal portion, and loss of the PCV and distal tip of L4 common to the weaker phenotypes (Fig. 1C). These phenotypes suggest a number of possible roles for *gbb* in wing development: the small disk and wing phenotypes suggest a role in disk proliferation, while the venation defects suggest a role in either the establishment of vein territories or wing vein promotion.

To better understand the role that *gbb* plays in these developmental functions, we have used clonal analysis to look for functional foci of *gbb* in the wing disk. Clones were generated in both wild type and *Minute* backgrounds, and the results from both analyses reveal a number of general features of *gbb* mutant clones. First, when compared with marked,
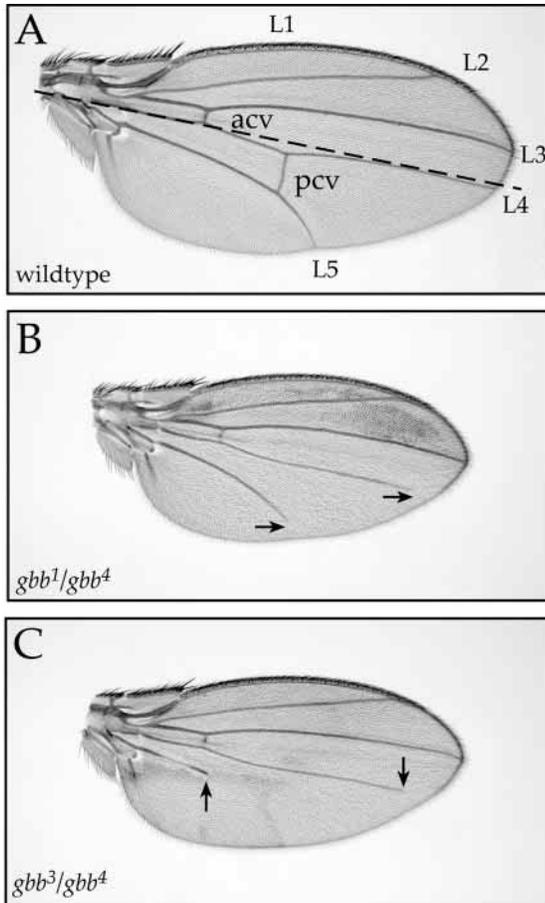
Fig. 1. Wing phenotypes of *gbb*. (A) A wild-type wing. The five longitudinal veins L1-L5 are indicated, as well as the two crossveins, the anterior crossvein (acv) and posterior crossvein (pcv). The A/P compartment boundary is indicated with a broken line. (B) A *gbb¹/gbb⁴* wing illustrating a moderate *gbb* phenotype. The PCV is absent as well as the distal tips of L4 and L5 (arrows). (C) A *gbb³/gbb⁴* wing showing the most severe phenotype produced by the *gbb* hypomorphic alleles. The PCV is absent, L5 is truncated almost to the base and L4 is truncated distally (arrow). Note that in both B and C, the overall wing size is reduced compared with the wild-type wing in A.

wild-type clones induced in parallel, the presence of a null allele of *gbb* has no effect on clone frequency, clone distribution or clone size (data not shown). Thus, *gbb* is neither cell lethal, either generally or within specific regions of the disk, nor does it have a significant autonomous effect on cell growth or proliferation. Second, the regions of the wing where *gbb* clones are associated with mutant phenotypes are fairly restricted. Thus, despite the broad expression of *gbb* in the wing disk (Khalsa et al., 1998), its function is limited to specific areas. Third, mutant phenotypes are observed only in clones or regions of clones where there is a dorsoventral overlap of mutant tissue. Mutant clones entirely confined to either the dorsal or the ventral surface of the wing – even if the clone occupies the entire anterior or posterior compartment – do not show mutant phenotypes. In the following discussion, we refer to clones with a dorsoventral overlap as 'double-sided' clones.

gbb is required locally for vein promotion in the posterior compartment

Double-sided *gbb* clones that encompass the entire posterior compartment show loss of the PCV as well as loss of the distal quarter of L5 (Fig. 2A), consistent with the phenotypes of *gbb* hypomorphs (compare with Fig. 1B). Smaller clones covering either just the PCV or just the distal tip of L5 also show loss of the corresponding vein (data not shown, Fig. 2E), indicating that these two foci are independent of one another.

Both the PCV and L5 foci have only short range effects on the veins, and thus constitute 'local' requirements for *gbb*. For the PCV, in double-sided clones that cover only the anterior or posterior half of the PCV, the vein is absent in the mutant cells and stops either precisely at the boundary of the dorsoventral overlap or within two to three cells of it (Fig. 2B,C,G). Notably, the PCV can stop on either the wild-type or the mutant side of the clone boundary, indicating that this effect is not simply a consequence of *gbb* product diffusing from wild-type cells into mutant tissue. Interestingly, this partial loss of the PCV requires that the double-sided clone include at least one of its two junctions with the longitudinal veins. Double-sided clones that occupy half of the L4/L5 intervein but stop short of the L4 or L5 vein do not affect the PCV (Fig. 2D). These latter clones suggest that *gbb* may not be required uniformly along the length of the PCV, but rather more strongly near the junctions with the longitudinal veins and less so in the intervein between them.

For the distal tip of L5, double-sided clones that fall within the distal quarter of L5 show loss of the vein only within the mutant tissue with the vein stopping within two to three cells of the dorsoventral overlap of the clone (Fig. 2F). Paradoxically, the distal tip of L5 can also be lost in association with clones covering proximal L5, even if the distal quarter of the vein is wild type for *gbb* function on the ventral, dorsal or even both surfaces (Fig. 2G). Such clones imply a degree of long-range non-autonomy for the L5 focus. However, as clones of this type invariably cover part of the vein that is normally absent in larger clones covering all of L5, we would predict that such clones should be associated with a gap between the proximal boundary of the focus and the boundary of the dorsal-ventral overlap of the clone. It is possible that such short gaps in the middle of the vein interfere with the differentiation of vein tissue more distally, and consequently result in the deletion of the entire distal tip.

In addition to the loss of the PCV and the distal tip of L5, we have noted that hypomorphic *gbb* alleles also affect the distal tip of L4 (Fig. 1B), yet, *gbb* clones that occupy the entire posterior compartment have little or no effect on this vein (Fig. 2A). This difference is also a reflection of the local non-autonomy of *gbb* in the disk. While posterior clones show little or no effect on L4, we do observe loss of L4 when double-sided mutant tissue covers the regions both anterior and posterior to the vein (data not shown). As L4 lies just posterior to the A/P compartment boundary (Fig. 1A), L4 loss is only observed in the statistically rare instances when clones are induced in both the anterior and posterior compartments that happen to fall next to one another along the distal tip of L4. As such, the failure to lose L4 in posterior clones, though the vein is entirely within mutant tissue, is presumably due to wild-type *gbb* product diffusing locally from the anterior compartment to compensate for its loss in the posterior.

Fig. 2. Phenotypes associated with *gbb* clones in the posterior compartment. In all cases, the boundaries of the dorsal-ventral overlap of the clone are shown with broken lines. (A) A large *gbb* null clone in the posterior compartment shows loss of the PCV and the distal tip of L5, as seen in the *gbb* hypomorphs, but not the distal tip of L4. (B) A *gbb* null clone including half of the PCV and the L4/PCV junction. The PCV terminates within two to three cells inside of the clone boundary. (C) A *gbb* null clone mutant for the posterior half of the PCV and including the L5/PCV junction. In this case the PCV terminates two to three cells outside of the clone boundary. Note also that the proximal region of L5 is mutant and is truncated before reaching the margin, even though the tissue more distally is wild type for *gbb* function. (D) A *gbb* null clone that does not include the junctions between the PCV and L4 or L5. Although a significant part of the vein is mutant for *gbb*, the PCV is not interrupted. (E) Loss of *gbb* only affects the distal quarter of L5 even if more of the vein is covered in the clone (arrow, compare with Fig. 2A). In clones that cross L5 within this distal quarter (F), L5 is truncated at the clone boundary (arrow). (G) Clones that cover proximal L5 up to within the distal quarter truncate the vein as if all of L5 were mutant (vertical arrow), even if the distal most part of the wing is wild type for *gbb*. Note that this clone also truncates the PCV (horizontal arrow), but in this case, the vein terminates just outside the clone boundary.
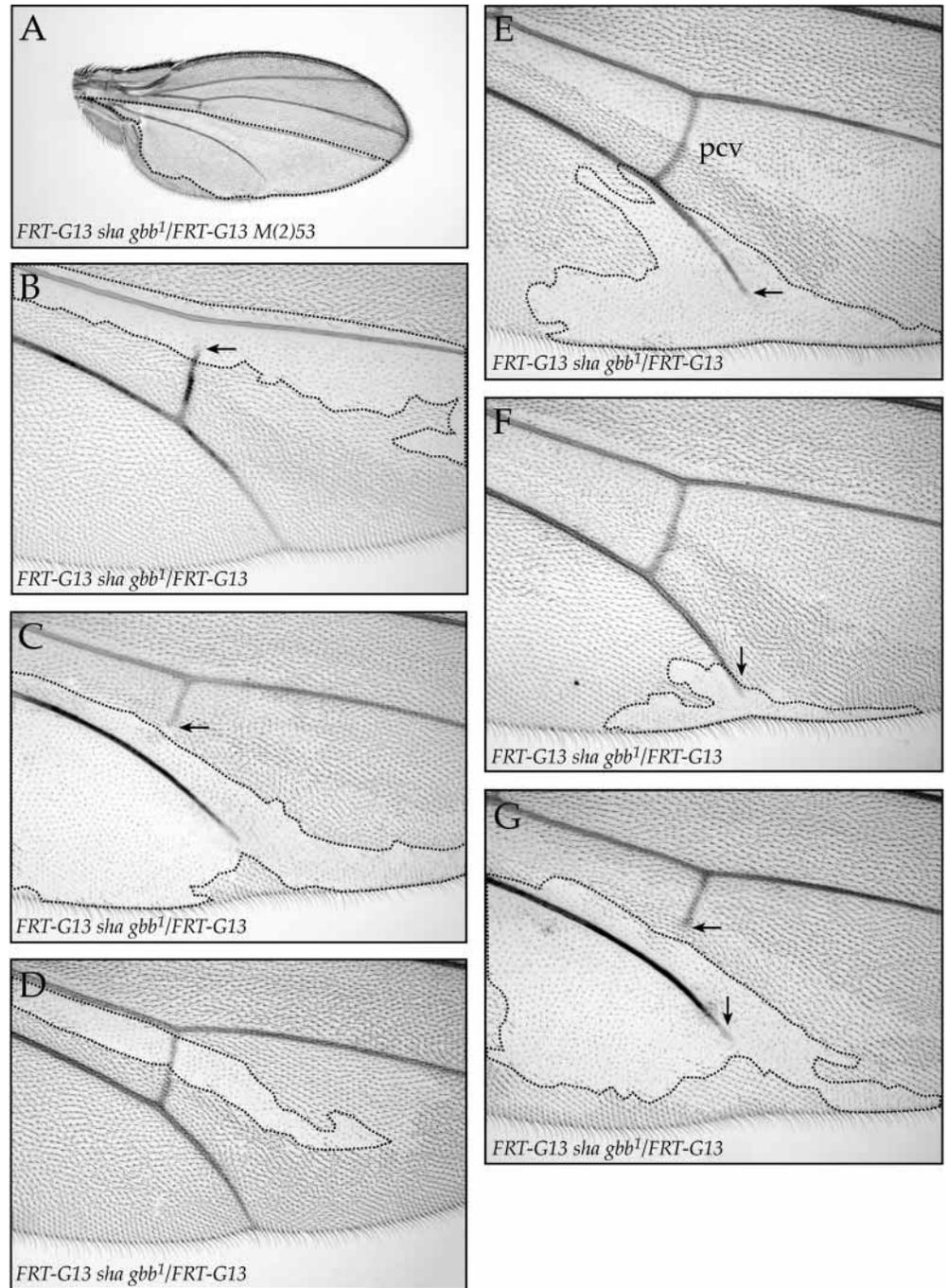
From these data, we conclude that there are three independent foci of *gbb* function in the posterior compartment, along the length of the PCV and the distal quarters of L4 and L5. The phenotypes associated with clones overlying these foci are confined to them, and have only short range effects on their respective veins. As such, they reflect local requirements for *gbb*, and most probably reflect a role for *gbb* in vein promotion.

gbb is required along the A/P compartment boundary for disk proliferation and to specify L5

The phenotypes of *gbb* hypomorphic mutations have little or no effect on the patterning of veins in the anterior compartment

(Fig. 1B,C; Khalsa et al., 1998; Wharton et al., 1999), and this is also true for null *gbb* clones. Double-sided clones covering the entire anterior compartment exhibit no defects in the costal vein or longitudinal veins L1, L2 or L3 (Fig. 3A). Thus, *gbb* is not required locally for promotion of veins in the anterior compartment. However, such clones are associated with a reduction in the overall size of the wing blade and loss of all but the most proximal region of L5 (Fig. 3A,B).

The difference in wing size associated with these clones can be seen clearly in comparisons of wings of the same fly, one of which has a large anterior clone, and the other not. In such situations, the wing lacking the clone serves as a size control

Fig. 3. Phenotypes associated with *gbb* clones in the anterior compartment. In all cases, the boundaries of the double-sided region of the clone are shown with broken lines. (A) A null *gbb* clone encompassing the entire anterior compartment. The wing is reduced in size and lacks all of distal L5. (B) Another null *gbb* clone encompassing the entire anterior compartment where L5 extends to the junction with the PCV (arrow). (C,D) Comparisons between wings bearing large anterior *gbb* clones (at left) and the wild type opposing wing from the same fly (at right). (C) Control wings with a *sha* marked clone encompassing the entire anterior compartment. The ratio of left:right (L:R) wing in this case is 0.98. (D) *gbb* mutant anterior clones result in a dramatic reduction in overall wing size. The ratio of L:R is 0.70.
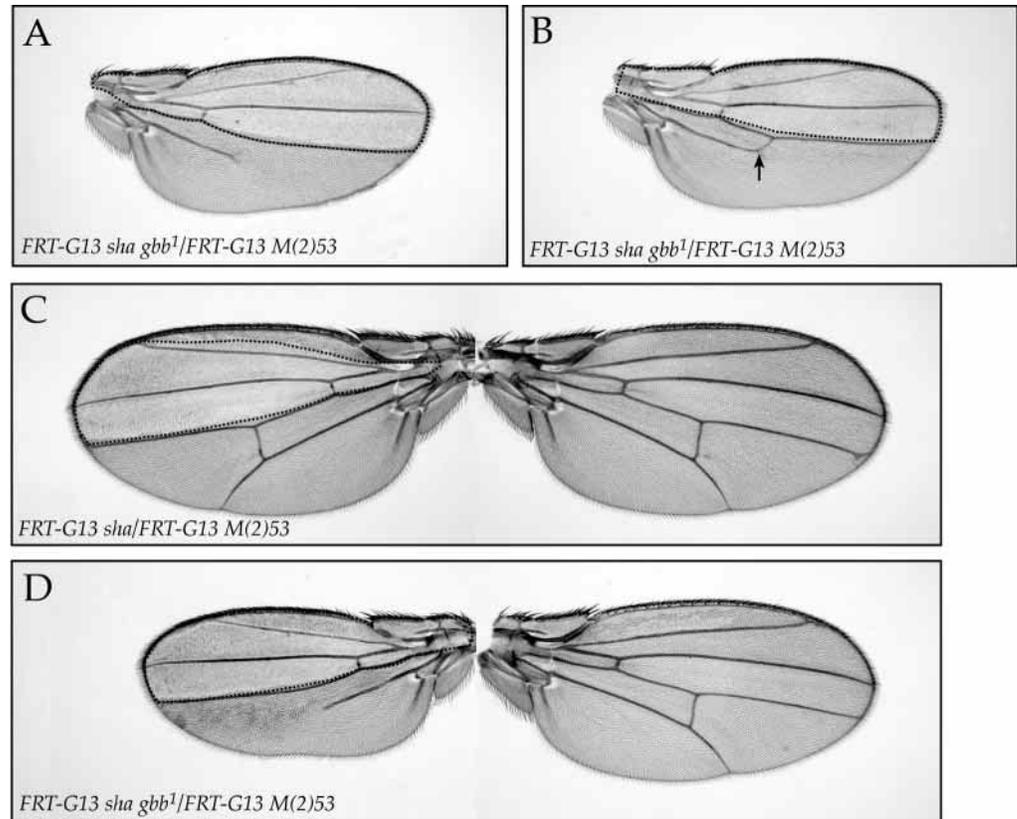
for the wing with the clone. Wild-type left and right wings differ in area by no more than 2%, and we observe the same range of variability in flies with a wild type double-sided anterior clone on one of its two wings (Fig. 3C). By contrast, wings with a *gbb* mutant double-sided anterior clone show approximately a 30% reduction in overall wing size (Fig. 3D). Notably, both the anterior and posterior compartments show a reduction in size in these wings. For the pair in Fig. 3D, the overall left:right (L:R) wing ratio is 0.70, for the anterior compartments, the ratio is 0.74, and for the posterior compartments 0.69. Thus, *gbb* clones in the anterior compartment affect wing size over the entire wing blade.

Large anterior *gbb* clones are also associated with a loss of most of L5. The truncation of L5 is mildly variable, and may or may not be accompanied by the loss of the PCV. In the most severe cases, as in Fig. 3A, L5 is truncated back beyond the point of its junction with the PCV, and in these instances the PCV is always absent. In weaker examples, L5 extends further distal and abruptly turns up to make a right angle junction with L4 (Fig. 3B). Such veins are presumably chimeras of L5 and the PCV. Notably, although most of L5 is absent in these wings, the intervein between L4 and L5 is still present.

Fine mapping of this *gbb* focus indicates that the requirements for proliferation and specification of L5 map to the same region of the anterior compartment: just anterior to the A/P compartment boundary. Double-sided clones covering the region between longitudinal veins L1 and L3, show no effect on overall wing size, or on patterning of L5 (Fig. 4A,B). Similarly, double-sided clones that occupy only the region between L3 (but not including L3) and L4 are wild-type in size and pattern (Fig. 4E). By contrast, double-sided clones with an

anterior border in the intervein between L2 and L3 and a posterior border running the length of the A/P compartment boundary show the mutant phenotypes associated with clones covering the entire anterior compartment (Fig. 4C,D). Thus, the anterior focus for *gbb* falls in a broad band of cells that lie just anterior to the A/P compartment boundary.

From these data we conclude that there is a single focus for *gbb* in the anterior compartment, lying just anterior to the A/P compartment boundary, from which *gbb* affects wing size and specification of the L5 vein territory. The phenotypes associated with clones overlying this focus are not confined to it, but affect either the whole wing blade (wing size) or vein structures far from the site of the clone (L5). As such, this focus constitutes a long-range requirement for *gbb* in wing disk patterning. The wing size phenotype reflects a role in disk proliferation and the loss of L5 a role in specification of vein territories. Significantly, the sum of the phenotypes associated with anterior and posterior *gbb* clones is essentially the same as the most severe wing phenotypes associated with the *gbb* hypomorphs (Fig. 1C), indicating that all of the *gbb* wing disk functions are recapitulated in the phenotypes of the hypomorphic alleles.

Coincidence of *gbb* and *dpp* foci in the wing

The four *gbb* foci we have mapped correspond in location and function with the established foci for *dpp* in the disk. We have shown that *gbb* is required locally for vein promotion at the distal tips of L4 and L5. Mutations in the shortvein region of *dpp* (i.e. *dpp^s* alleles) also result in loss of the distal tips of the longitudinal veins, though the phenotypes are more severe than what is observed for *gbb*. Weak *dpp^s* alleles show truncations
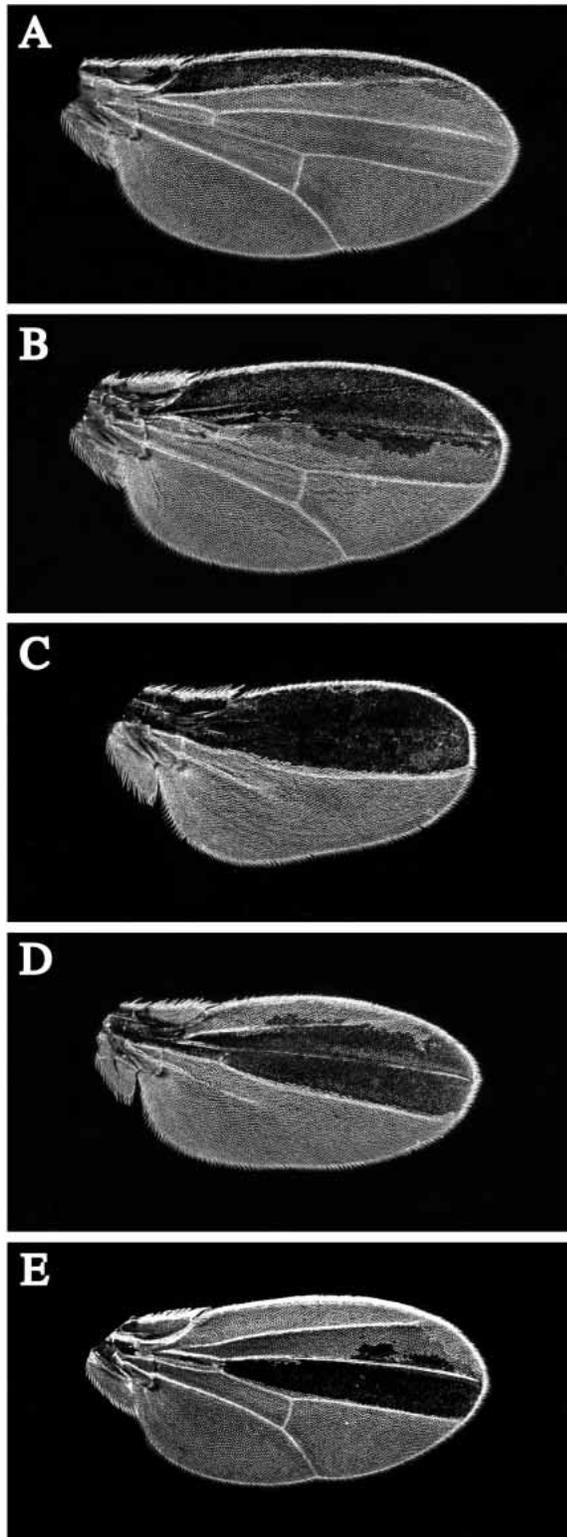
Fig. 4. Fine mapping of the *gbb* focus in the anterior compartment. Dark field photomicrographs of wings bearing double-sided *gbb* null clones (dark patches). Clones falling between the anterior margin (L1) and L3 (A,B) have no effect on L5 or overall wing size. Similarly, clones entirely contained within L3 and L4 are wild type (E). Effects on wing size and L5 specification are observed in clones that occupy the entire anterior compartment (C) or all of the region between the L2/L3 intervein and L4 (D).
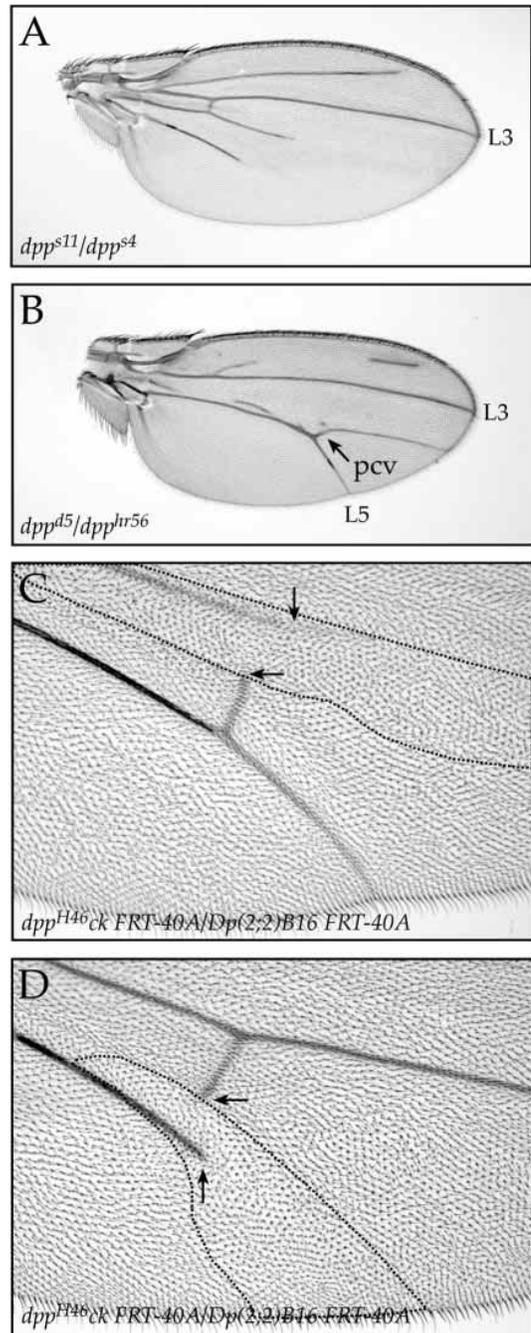

Fig. 5. Phenotypes associated with local and long-range requirements of *dpp*. (A) A *dpp^{s11}/dpp^{s4}* wing illustrating a typical shortvein phenotype. In this case, the distal ends of L2, L4 and L5 are truncated. These phenotypes are correlated with local vein promotion requirements for *dpp*. (B) A *dpp^{d5}/dpp^{hr56}* wing showing a weak disk phenotype. Defects include failure to specify L3 and L4, as well as loss of the intervein between L4 and L5. This phenotype is very similar to that associated with clones of the *dpp* target gene *sal* (de Celis et al., 1996). (C) A *dpp* null clone showing the local effect on promotion of the PCV (compare with Fig. 2B). In this case, the PCV terminates precisely on the clone boundary (horizontal arrow). *dpp* has a more pronounced effect than *gbb* on L4, in this case truncating the vein back to the site of the PCV (vertical arrow). (D) A *dpp* null clone showing effects on the posterior PCV as well as L5. Again, *dpp* shows a local effect on the PCV but has a more pronounced effect on L5 than *gbb* (arrows).

of distal L4 and L5 to half their normal length, complete loss of the PCV, and loss of distal L2 in the anterior compartment (Fig. 5A; Segal and Gelbart, 1985). Consistent with a previous clonal analysis, which showed that *dpp* was required for the distal half of L5 (Posakony, et al., 1991), we find that in *dpp* null clones, *dpp* is required locally along at least half the length of L4 and L5 (Fig. 5C,D). Thus, the *gbb* foci represent a subset of the regions of the longitudinal veins that require *dpp*. Moreover, as the sites of the *gbb* foci correspond to those regions of L4 and L5 most sensitive to the loss of *dpp*, we conclude that *gbb* is required to achieve maximal levels of BMP signaling at the distal tips of the veins.

The local focus for *gbb* along the PCV is also a focus for *dpp* function. As no allele of *dpp* results in the specific loss of the PCV, and as previous clonal analyses (Posakony et al., 1991; deCelis, 1997) did not show a specific effect of *dpp* on the PCV, we have generated null *dpp* clones specifically over the PCV to determine if such clones show the same behaviors as the *gbb* clones described above. We find that, indeed, null *dpp* clones covering all or part of the PCV show the same behavior as *gbb* clones: the *dpp* clones have no effect on the PCV unless there is a dorsoventral overlap of mutant tissue, and double-sided clones that cover half of the PCV show loss of the vein in the mutant tissue up to or within two to three cells of the clone boundary (Fig. 5C,D). Thus, *gbb* and *dpp* are both required for promotion of the PCV. As such, we conclude that *gbb* and *dpp* act together to achieve the levels of BMP signaling required for this vein.

The anterior focus of *gbb* is associated with two different functions, disk proliferation and L5 specification, and this focus coincides precisely with the major focus for *dpp* in the disk that has been implicated both in proliferation (Burke and Basler, 1996) and in specification of wing vein territories (deCelis et al., 1996; Sturtevant et al., 1997). Expression of *dpp* in this focus is regulated by *cis*-acting sequences in the disk region of the *dpp* gene (Blackman et al., 1991), and *dpp* alleles that affect this region (i.e. *dpp^d* alleles) exhibit phenotypes that can be related to those of our *gbb* clones. The most severe *dpp^d* alleles are pupal lethals (Spencer et al., 1982), and the mutant larvae have small disks very similar to those of *gbb* null larvae (Khalsa et al., 1998). Weaker *dpp^d* allelic combinations are adult viable, and the wings of these flies are also reduced in size. However, the *dpp* phenotypes are more severe than those associated with *gbb* clones. The weakest *dpp^d* heteroallelic combinations give rise to 'winglets' that may be no more than one tenth the size of a normal wing (Spencer et al., 1982), and it is only in heteroallelic combinations of *dpp^d* and *dpp^{hr}* alleles that wings are produced of comparable size with those with null *gbb* clones (Fig. 5B; Segal and Gelbart, 1985; deCelis, 1997). Thus, while it is clear that *gbb* and *dpp* both contribute to proliferation in the wing disk, *dpp* has a much more profound effect, suggesting that the role of *gbb* in this process may be facilitatory.

The specific loss of L5 associated with the anterior focus is the only *gbb* wing phenotype which cannot be correlated with a phenotype of *dpp*. That *dpp* is involved in the specification of vein territories has been clearly established (deCelis et al., 1996; Sturtevant et al., 1997). *dpp* function along the A/P compartment boundary is required for expression of the transcription factor *sal* (Lecuit et al., 1996; Nellen et al., 1996), and this gene is required for specification of L2 and the

intervein between L4 and L5 (deCelis et al., 1996). Indeed, the phenotypes of weak *dpp^d*/*dpp^{hr}* combinations as well as clones of *sal* consist of a loss of L2 in the anterior compartment and loss of the intervein between L4 and L5 in the posterior compartment (Fig. 5B; deCelis, 1997). However, as anterior clones of *gbb* affect neither of these structures, our data are not consistent with a role for *gbb* in contributing to the maximal levels of BMP signaling along the A/P compartment boundary. Rather, as the structures affected in *gbb* clones lie further away from the source than the domain of *sal* expression, it appears that *gbb* is required for the low points of BMP gradient at sites far from the A/P compartment boundary. As such, the *gbb* and *dpp* phenotypes are not the same because the *dpp* hypomorphs affect the high point of the gradient, and less so the low points, while *gbb* mutations affect the low points of the gradient and not the high point.

Duplications of *dpp* rescue phenotypes associated with *gbb* mutants and clones

To better understand the relationship between *gbb* and *dpp* in the disk, we tested for suppression of the *gbb* mutant phenotypes by additional doses of the *dpp* locus. As suggested above, one possible function for *gbb* may be to augment the levels of BMP signaling provided by Dpp. As such, we would expect that raising the level of *dpp* expression in the disk would compensate for the loss of *gbb*. That is, if we increase the levels of *dpp* activity at those sites where our clonal analysis indicates that *gbb* is active, we should be able to suppress the corresponding *gbb* mutant phenotypes. To do this, we took advantage of two duplications of the *dpp* locus, *Dp(2;2)B16* and *Dp(2;2)DTD48*, to generate *gbb* mutant flies bearing three or four copies of the *dpp* locus (see Materials and Methods).

As the hypomorphic *gbb* mutations are to some degree sensitive to genetic background (Khalsa et al., 1998), it was necessary to demonstrate that any rescue associated with the *Dp(dpp) gbb* recombinants was due to the additional copies of *dpp* rather than other modifying factors on the chromosome. To show this, we tested for the ability of extra copies of the *dpp* gene to rescue the lethality associated with hypomorphic and amorphic *gbb* alleles and found that additional doses of *dpp* do show a dose-dependent rescue of *gbb* lethality (Fig. 6A,B). For *gbb^1*/*gbb^4* transheterozygotes, 2% of expected are viable to adulthood, with three copies of *dpp*, 25% or 30% are viable, depending on which of the two duplications was used, and with four copies, 75%. Moreover, although four copies of *dpp* cannot rescue the lethality of *gbb^1* homozygotes, we did observe a dose-dependent rescue of the lethal phase from larval to pupal lethal. For *gbb^1* homozygotes, 10% of the expected class form pupae, with three copies of *dpp*, 30% or 60% depending on the duplication, and with four copies, 80% of expected form pupae. Thus, in both these assays we see a dose-dependent rescue of *gbb* phenotypes with additional copies of the *dpp* locus.

To examine the effects of additional doses of *dpp* on the phenotypes associated with specific *gbb* foci, we have generated clones that are both null for *gbb* and carry four copies of the *dpp* gene (see Materials and Methods). Duplications of *dpp* are able to rescue the distal tip of L5 in posterior clones (Fig. 6C,D), and while we cannot use clonal analysis to assay the effects of additional doses of *dpp* on the distal tip of L4, we do see rescue of this phenotype in

Fig. 6. *dpp* duplications rescue *gbb* lethality and phenotypes associated with *gbb* null clones. (A,B) Graphs showing rescue of *gbb¹/gbb⁴* transheterozygotes (A) and *gbb¹/gbb¹`* homozygotes (B) to adulthood (black bars) and to pupal/pharate stage (hatched bars). *dpp* duplications cannot rescue *gbb* null larvae to adulthood, but there is a dramatic rescue of larval lethality to pupal/pharate lethality (B). (C-E) Phenotypes associated with clones both mutant for *gbb* and carrying four copies of *dpp*. (C) A large posterior clone. Despite the rescue of *gbb* lethality and small disk phenotypes, additional doses of *dpp* fail to rescue PCV loss (arrow), even in clones confined to the posterior compartment. (D) High magnification of the wing in C showing the distal tip of L5. A *gbb* null clone covering this same region would show loss of the distal quarter of L5 (arrow, compare with Fig. 2G), but with four copies of *dpp*, the vein is rescued to the margin. Anterior clones mutant for *gbb* and carrying four copies of the *dpp* gene (E) show rescue of both wing size and loss of L5 (arrow).
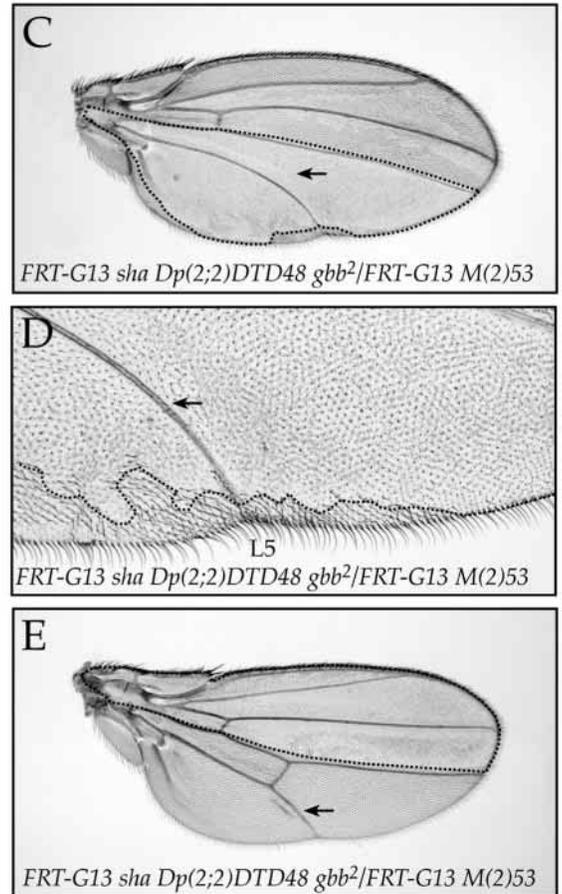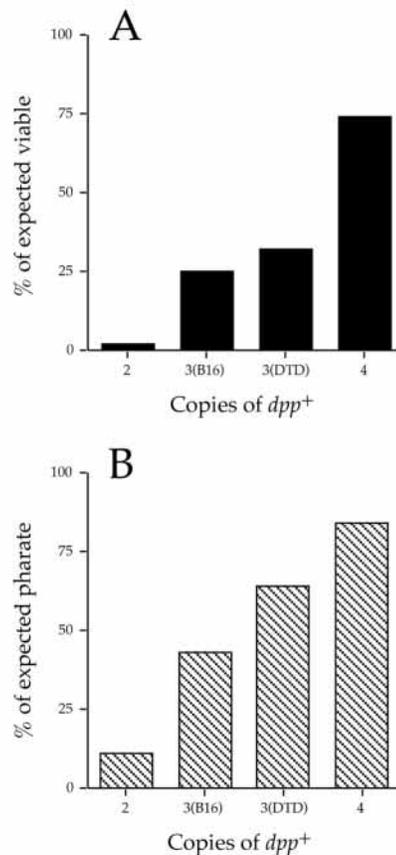
gbb¹/gbb⁴ transheterozygotes bearing four copies of *dpp* (data not shown). Thus, for the L4 and L5 vein promotion foci, additional doses of *dpp* are able to rescue the *gbb* mutant phenotypes. By contrast, we never observe rescue of the PCV by *dpp* duplications in either *gbb* mutants or clones (Fig. 6C). This result suggests that either *gbb* and *dpp* act independently at this focus, or that the four doses of *dpp* are not sufficient to compensate for the loss of *gbb*. In favor of the latter hypothesis, it is worth noting that the PCV is the wing structure that is most sensitive to loss of *gbb*, and it is not clear that this is the case for *dpp*. As such, it is possible that the relationship between *gbb* and *dpp* is reversed in this case, and *gbb* is the central figure in PCV promotion while the role of *dpp* is secondary.

The truncation of L5 associated with the anterior focus can also be rescued with additional doses of *dpp* (Fig. 6E). Given that this function of *gbb* seems to reflect a requirement in extending the range of BMP signaling in the disk, we can account for this result in two possible ways. On the one hand, *gbb* may simply act to augment the levels of *dpp* signaling at the low points of the *dpp* gradient. As such, the additional doses of *dpp* increase these levels and compensate for the loss of *gbb*. Alternatively, *gbb* may be required for signaling in regions beyond the normal limit of the spread of *dpp* across the disk. In this case, the rescue by *dpp* would reflect an increase in the spread of *dpp*, owing to the higher levels of *dpp* at the source along the A/P compartment boundary.

In the above clonal experiments, while it is possible to confine the clones carrying four copies of *dpp* to the known

sites of *gbb* foci in the wing, because of the method employed to make the clones, the wing cells outside the clone carry three copies of *dpp* rather than the wild-type two copies. As such, it is possible that the rescue of *gbb* phenotypes we observe is due to this additional copy of *dpp* in the background and not the four copies within the clones. This is a particularly relevant issue with regard to the long-range anterior focus for which the responding cells in the posterior compartment all carry three copies of *dpp*. To control for this, we performed the 'reciprocal' experiment to the one above and generated *gbb* null clones that carry the wild-type two copies of *dpp* in a background that carries three copies. We found that, in all cases, three copies of *dpp* outside of the clones could not rescue either local or long-range phenotypes associated with *gbb* clones (data not shown). For the anterior focus, this result demonstrates that the rescue of L5 that we observed in the clones carrying four copies of *dpp* is strictly due to the additional copies of *dpp* within the focus along the A/P compartment boundary. Moreover, as this focus corresponds to the early stripe expression of *dpp* in the disk, the result provides further evidence that the loss of L5 in *gbb* mutants and clones identifies a vein specification function associated with the global patterning functions of *dpp*, and not a vein promotion function.

sax does not transduce *gbb*-dependent signals in the wing disk

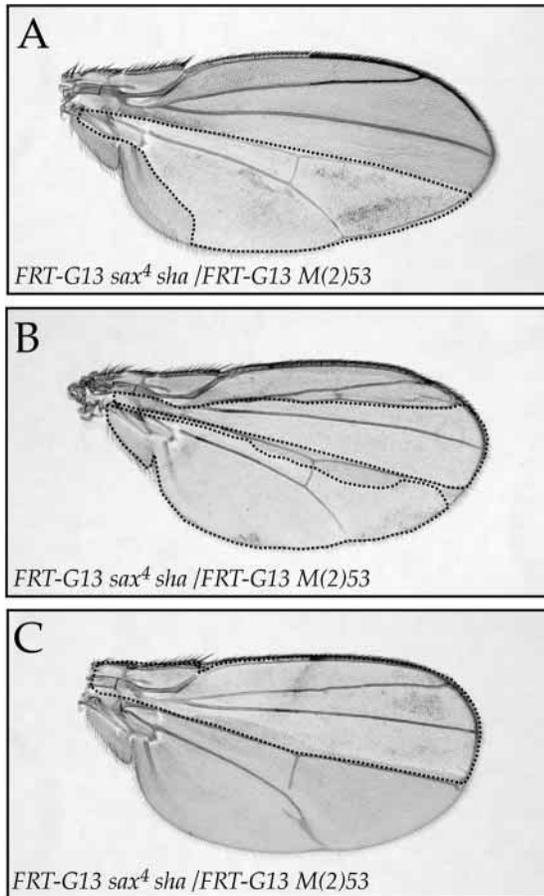In the embryo, cooperative signaling by *dpp* and *scw* are

Fig. 7. Phenotypes associated with *sax* null clones. (A) A large *sax* null clone occupying most of the posterior compartment. Unlike *gbb* clones, this clone is not associated with loss of L4, L5 or the PCV. (B) In this wing, two independent clones encompass most of the posterior compartment and half of the anterior compartment. Note that the clone boundary running along the middle of the anterior compartment is associated with an ectopic vein. (C) A large *sax* null clone occupying the entire anterior compartment. Like the posterior clone in A there is no effect on venation; however, the wing is reduced in size as has been shown previously (Singer et al., 1997).

required for elaboration of the pattern in the dorsal ectoderm (Neul and Ferguson, 1998; Nguyen et al., 1998). According to the current model, this cooperation is achieved by *dpp* and *scw* signaling through different receptor complexes composed of *tkv* and *put*, and *sax* and *put*, respectively. Given that many of the relationships we observe between *dpp* and *gbb* are similar to the relationships between *dpp* and *scw* in the embryo, we were interested in determining if the *gbb*-dependent signals we had characterized in the wing were also transduced by *sax*. In a previous clonal analysis, it has been shown that large *sax* null clones resulted in reduced wing size, blunting of the wing tip, ectopic venation and mis-patterning of the anterior wing margin (Singer et al., 1997). However, as many of these phenotypes appeared to be associated with the creation of discontinuities in the BMP gradient, we were interested in establishing the phenotype of null *sax* clones occupying the entire anterior or posterior compartments, and comparing these phenotypes with those of our *gbb* clones. We have found that

large *sax* null clones that occupy the entire anterior or posterior compartment give rise to essentially normal wings (Fig. 7A,C), though anterior clones are associated with a slight reduction in wing size. Notably, the ectopic vein and margin bristle phenotypes that have been described previously (Singer et al., 1997) are not observed in clones that encompass an entire compartment, but only when the clone boundary subdivides a compartment (Fig. 7B), indicating that it is not the loss of *sax*, per se, but the discontinuity between *sax*⁺ and *sax*⁻ cells that results in this phenotype. Given these results, we conclude that the *gbb* signals that we have characterized in the wing are not transduced by *sax*.

DISCUSSION

We have used clonal analysis to map foci for *gbb* in the developing wing imaginal disk. Our results show that *gbb* has two distinct types of functions: local and long range. The local foci are confined to the posterior compartment, and affect the promotion of the PCV and the distal tips of the longitudinal veins L4 and L5. The long-range focus lies in the anterior compartment comprising a broad band of cells along the A/P compartment boundary and affects disk proliferation and the specification of L5. These *gbb* foci are coincident with the foci for *dpp* in the disk, and many of the phenotypes associated with the *gbb* clones are rescued by additional copies of the *dpp* locus. Thus, *gbb* and *dpp* contribute to the same functions in the disk and *gbb* functions are to some extent redundant with those of *dpp*. Comparison of the foci and phenotypes of *gbb* and *dpp* mutants and clones indicates that the relationship between *gbb* and *dpp* is different for different functions. For promotion of distal tips of L4 and L5, *gbb* function is restricted to those areas that require the highest levels of *dpp* signaling, and as these phenotypes can be rescued with additional copies of *dpp*, we conclude that *gbb* is required to augment the levels of *dpp* signaling. For promotion of the PCV, the case is not so clear. We have shown that both *gbb* and *dpp* are required for PCV promotion. However, as *dpp* duplications do not rescue this phenotype, it is possible that *gbb* and *dpp* act independently or that the contribution of *gbb* to this process is sufficiently great that it cannot be compensated for by the additional doses of *dpp*. The requirement for *gbb* in the specification of L5 is not consistent with an augmentation of *dpp* signaling, as *gbb* mutants and clones do not affect structures specified by the high point of the *dpp* gradient. Rather, *gbb* clones affect structures far from the source along the A/P compartment boundary, suggesting that *gbb* signaling contributes to the low levels of BMP signaling at the extremes of the gradient.

We have noted that mutant phenotypes are observed only in *gbb* clones when the mutant tissue encompasses the entirety of the focus on both the dorsal and ventral surfaces of the wing. For example, clones that occupy the dorsal-anterior quadrant of the wing exhibit no defects in the patterning or size of the wing, while clones that occupy both the dorsal-anterior and ventral-anterior quadrants affect both these aspects of wing development (see Fig. 3). One explanation for this phenomenon is that Gbb exhibits long-range non-autonomy in the disk, and, in fact, there is some evidence for this, as we have found that small patches of wild-type cells along the A/P

compartment boundary in the context of a large mutant clone are able to rescue loss of L5 completely in the posterior compartment (data not shown). However, *gbb* clearly does not act in a broadly non-autonomous fashion in all of its functions: *gbb* clones that cover the PCV or distal L5 exhibit vein defects that respect the clone boundaries indicating that the presumptive vein cells within the clone cannot be rescued by the wild-type Gbb present in the adjacent cells (see Fig. 2). For these functions, the 'rescue' observed in single-sided clones implies pattern regulation occurring between the two wing surfaces. Indeed, it has long been asserted that there are signaling events between the dorsal and ventral surfaces of the wing as it has been shown for several genes that loss of veins on one surface can be compensated for by the wild-type pattern in the opposing surface (Garcia-Bellido and de Celis, 1992). The requirement for dorsal-ventral overlap that we have observed with *gbb* mutant clones is indicative of such a signaling mechanism, and given these results, as well as those from previous studies that have shown a requirement for dorsal-ventral overlap in clones of *dpp* and *sog* (Posakony et al., 1991; Yu et al., 1996; deCelis, 1997), it is plausible that the BMPs themselves might be responsible for mediating these signaling processes.

Gbb activity is localized to specific foci

Perhaps the most striking result from our clonal analysis is that the requirements for *gbb* in the wing disk are localized even though the gene is widely expressed. This result implies that Gbb activity is in some way restricted post-transcriptionally. Two models seem the most likely to account for this effect. First, as we have shown that all *gbb* foci are coincident with sites of *dpp* expression in the disk, it is possible that Gbb and Dpp form heterodimers, and that Gbb is only active in this form. Heterodimer formation has been documented for a number of different TGFβ superfamily members, and in some cases heterodimers and homodimers have been shown to have

distinct properties. For example, heterodimers of BMP2 or BMP4 and BMP7 are much more potent in the induction of ventral mesoderm and bone induction than their respective homodimers (Isreal et al., 1996; Nishimatsu and Thompsen, 1998). Activins and Inhibins illustrate a different relationship: the homodimeric Activins having the opposite biological effects of the heteromeric Inhibins (Yu et al., 1987; Petraglia, 1989).

An alternative model is that the restriction of *gbb* function in the disk is achieved through local activation of Gbb homodimers, which may be achieved by specific agonists expressed within the foci or antagonists expressed everywhere else. Possible agonists include the *Drosophila* BMP-1 homologs *tolloid* and *tolkin* (Shimell et al., 1991; Nguyen et al., 1994; Finelli et al., 1995), or *Drosophila* homologs of the subtilisin-like proprotein convertases or furins, that are thought to be involved in the cleavage of BMP pro-proteins into the active ligand (Cui et al., 1998; Constam and Robertson, 1999). In addition, the recently characterized secreted protein *crossveinless 2* (*cv-2*) may act as an agonist of BMP signaling specifically in the presumptive crossveins (Conley et al., 2000, see below). The antagonist *sog* is a likely candidate for restricting BMP activity during pupal development (i.e. for vein promotion functions) as it has been shown to be expressed in all intervein cells at this time (Yu et al., 1996). Moreover, there is some evidence that *sog* function in the wing may specifically antagonize *gbb* (Yu et al., 2000), and thus may very well account for the restriction of *gbb* function to the presumptive veins.

Different functions employ different relationships between BMPs

Our clonal analysis has identified four processes that require *gbb* during wing development, disk proliferation, specification of the L5 vein territory, promotion of the PCV and promotion of the longitudinal veins L4 and L5. Based on the criteria of

Table 1. Comparison of features of Scw:Dpp and Gbb:Dpp relationships for different developmental functions

	Embryonic D/V patterning	Disk proliferation‡	Vein territory specification‡	Vein promotion*	
				L4/L5	PCV
Dpp>receptor§	Dpp>Tkv	Dpp>Tkv	Dpp>Tkv	Dpp>Tkv	Dpp>Tkv
Scw/Gbb>receptor¶	Scw>Sax	Gbb>(Tkv/Sax)?**	Gbb>Tkv	Gbb>Tkv	Gbb>Tkv
Heterodimer formation‡‡	No	Possible	Possible	Possible	Possible
Scw/Gbb required for Dpp§§	High point	?	Low points	High levels	High levels BMP
Scw/Gbb defect rescued by dpp¶	Yes	Yes	Yes	Yes	No

*Vein promotion functions of *dpp* correspond to a number of independent foci along L2, L3, L4, L5 and the PCV, those of *gbb* correspond to the three independent, locally acting foci presented in this work, i.e. along the PCV and at the distal tips of L4 and L5.

‡Disk proliferation and vein territory specification are both functions of the same *gbb* and *dpp* focus, i.e. along the A/P compartment boundary.

§D/V patterning: Neul et al., 1998; Ngyuen et al., 1998.

Disk proliferation: Burke and Basler, 1996; Singer et al., 1997.

Vein territory specification: Podos and Furguson, 1999.

Vein promotion: de Celis, 1997.

¶Neul et al., 1998; Ngyuen et al., 1998. A requirement for *tkv* in *gbb* singaling events in the wing has been suggested based on interaction studies between *gbb* and *tkv* hypomorphs (Khalsa et al., 1998).

**For the proliferation function, it is clear that all four genes, *gbb*, *dpp*, *tkv* and *sax* are required, but given the nature of the phenotype, it is not possible to distinguish the relationships between them.

‡‡Based on the requirement for *scw* or *gbb* to be expressed in the same cells as *dpp*; *scw* (Ngyuen et al., 1998).

§§Based on the correspondence between *gbb*/*scw* null phenotypes and *dpp* hypomorphs. For the embryonic D/V and vein territory functions, *dpp* is thought to act in a gradient, thus the second BMP contributes to either the 'high point' or 'low points' of that gradient; for the vein promotion functions, *gbb* only contributes to the relative 'levels' of BMP signaling. For the PCV promotion function, it is not clear if Dpp or Gbb is the central player, thus, for this function, it is more precise to say that Gbb is required for high levels of BMP signaling (see text; Neul et al., 1998; Ngyuen et al., 1998).

¶Assay is different for *scw* versus *gbb* functions: for *scw*, injection of dpp+ mRNA was used (Neul et al., 1998; Ngyuen et al., 1998), while in the present studies, a *dpp* duplication was used.

comparisons of *gbb* clone phenotypes with *dpp* and *sax* phenotypes, the ability for the *gbb* mutant phenotypes to be rescued by additional copies of *dpp*, and the spatial requirements for *gbb* during wing development, it is clear that each of these functions employs a different relationship between *dpp* and *gbb*, and each of these relationships is distinct from that which has been established for *dpp* and *scw* in the embryo (Table 1).

Some of the features of *dpp* and *gbb* function indicate consistent distinctions between *gbb*-dependent functions in the wing and *scw*-dependent functions in the embryo. For example, as discussed above, the explicit coincidence of *dpp* expression and *gbb* functions in the wing disk raises the possibility that heterodimer formation may play a role in some or all of the wing functions of *dpp* and *gbb*, while this does not appear to be the case for *dpp* and *scw* in the embryo (Nguyen et al., 1998). In particular, the absolute requirement for both *gbb* and *dpp* in PCV promotion, as evidenced by the failure of *dpp* duplications to rescue PCV loss in *gbb* mutants and clones, is entirely consistent with a mechanism requiring Gbb:Dpp heterodimers. Similarly, comparison of *sax* clone phenotypes with those of *dpp* and *gbb* indicate that *sax* is not required for the transduction of *gbb* signals in the wing, with the possible exception of the disk proliferation function. This is in contrast to the situation in the embryo, where *sax* is proposed to be a dedicated receptor for *scw*.

We have also observed distinctions in the relationships between *dpp* and *gbb* in different wing functions. Comparing the specification of the L5 territory and promotion of the distal tips of L4 and L5, *gbb* acts differently to modulate the activity of *dpp*. In the case of the vein promotion functions, *gbb* is required for maximal levels of BMP signaling at the distal tips of L4 and L5 – which is similar to what has been described for *scw* and *dpp* in the embryo. By contrast, for the specification of L5 during larval development, g*bb* is required for the specification of fates at the low points of the BMP gradient. Indeed, as the *gbb* clone phenotypes do not reflect the phenotypes of the *dpp* target gene *sal* (which requires maximal levels of BMP signaling for its expression), it follows that the expression of this gene, and thus the high point of the BMP gradient, is normal in wings bearing *gbb* null clones in the anterior compartment. This relationship is quite distinct from that of *dpp* and *scw* in the embryo.

The promotion of the PCV, while similar in many ways to the longitudinal vein promotion functions, is distinct in that it is one *gbb* function in the wing that cannot be rescued by additional copies of the *dpp* gene. It is relevant here that hypomorphic mutations of *gbb* and *dpp* show distinct phenotypes with regard to PCV promotion. For *gbb*, it is clear that the PCV is the structure most sensitive to a reduction in *gbb* activity as the weakest alleles show specific loss of it. By contrast, weak shortvein alleles show truncations of the distal tips of the L2, L4 and L5, but the PCV is intact (Segal and Gelbart, 1985). This suggests that for PCV promotion the relationship between *dpp* and *gbb* may be reversed, and *gbb* may play the more central role. This notion is supported by analysis of the distribution of the phosphorylated from of the Smad protein Mad (pMad), which can be detected in the presumptive PCV before the localized expression of *dpp* is detected at this site by in situ hybridization (Conley et al., 2000). Conley et al. have suggested that the localized

expression of *cv-2* in the presumptive PCV cells may account for the early appearance of pMad in this vein. It is tempting to speculate that *cv-2* may localize the activity of *gbb* to the presumptive PCV, which results in the subsequent activation of *dpp* expression.

Given these different functions and the different relationships between BMP ligands specific to each, it is evident that there is not a 'canonical' relationship between BMP2/4- and BMP5/6/7/8-like molecules that is co-opted like a cassette into different developmental contexts. Rather, it seems that specific relationships have evolved between the two types of ligands that fulfill particular functional requirements during development. Moreover, as many of the distinctions appear to be occurring at the level of ligand activation, distribution, and ligand-receptor interactions, it follows that extracellular modulation of BMP ligands plays a major role in the establishment of these particular relationships. Identifying and understanding the roles of such extracellular factors will be key to understanding the molecular mechanisms underlying these different signaling events.

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