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Integrins modulate Sog activity in the *Drosophila* wing

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SUMMARY

Morphogenesis of the *Drosophila* wing depends on a series of cell-cell and cell-extracellular matrix interactions. During pupal wing development, two secreted proteins, encoded by the *short gastrulation* (*sog*) and *decapentaplegic* (*dpp*) genes, vie to position wing veins in the center of broad provein territories. Expression of the Bmp4 homolog *dpp* in vein cells is counteracted by expression of the Bmp antagonist *sog* in intervein cells, which results in the formation of straight veins of precise width. We screened for genetic interactions between *sog* and genes encoding a variety of extracellular components and uncovered interactions between *sog* and *myospheroid* (*mys*), *multiple edematous wing* (*mew*) and *scab* (*scb*), which encode βPS, αPS1 and αPS3 integrin subunits, respectively. Clonal analysis reveals that integrin mutations affect the trajectory of veins inside the provein domain and/or their width and that misexpression of *sog* can alter the behavior of cells in such clones. In addition, we show that a low molecular weight form of Sog protein binds to αPS1βPS. We find that Sog can diffuse from its intervein site of production into adjacent provein domains, but only on the dorsal surface of the wing, where Sog interacts functionally with integrins. Finally, we show that Sog diffusion into provein regions and the reticular pattern of extracellular Sog distribution in wild-type wings requires *mys* and *mew* function. We propose that integrins act by binding and possibly regulating the activity/availability of different forms of Sog during pupal development through an adhesion independent mechanism.

Key words: *Drosophila*, Sog, Integins, Bmps

INTRODUCTION

Vein differentiation is controlled by groups of genes that act in two developmentally distinct phases. During mid-third larval instar and early prepupal stages, the positions of vein territories are defined within the monolayer of wing imaginal disc cells (reviewed by Bier, 2000). In the second phase of vein development, during pupal stages, the vein versus intervein cell fate choice is resolved among cells in broad vein competent domains of a bilayered wing primordium. This refinement step is mediated in part by lateral inhibitory signals elaborated by presumptive vein cells. At the same time that lateral inhibition limits the width of veins, vein continuity signals act along the axis of the vein to promote their formation in straight continuous lines. Genes such as *net* (Brentrup et al., 2000) and *blistered* (Roch et al., 1998), which govern intervein development, are also required for restricting vein development to appropriate cells. In addition, the activity of intervein genes, which are required for intersurface adhesion, such as those coding for the integrins, must be excluded from veins to permit the non-adherent strips of cells in the veins to form open channels between the two wing surfaces (Brown, 2000). Interestingly, some combinations of integrin mutants have been found to generate ectopic veins; however, the mechanisms that underlie this phenotype are not understood (Brower and Jaffe, 1989; Brown et al., 1989; Zusman et al., 1993; Zusman et al., 1990).

During early pupal development, Decapentaplegic (Dpp), the *Drosophila* homolog of vertebrate Bmp2/Bmp4, and the Bmp-binding protein Short gastrulation (Sog), the homolog of vertebrate chordin, function antagonistically to ensure the formation of straight continuous veins (Haerry et al., 1998; Lecuit et al., 1996; Sturtevant and Bier, 1995; Yu et al., 1996; Zecca et al., 1995). Throughout this period, *dpp* is expressed in vein primordia, while *sog* is expressed in a complementary intervein pattern (Yu et al., 1996). Sog also opposes Bmp signaling during dorsoventral patterning of the early embryo, which involves zygotic (Biehs et al., 1996; Francois et al., 1994; Marques et al., 1997) as well as maternal (Araujo and Bier, 2000) functions of this pathway. *sog* encodes a secreted molecule with domains resembling thrombospondin and procollagen (Francois and Bier, 1995; Francois et al., 1994) and has been shown to bind Dpp (Ross et al., 2001). It has been suggested that regulated cleavage of Sog generates different forms with distinct activities (Yu et al., 2000). Cleavage at three sites by the metalloprotease Tolloid (Tld) inactivates Sog (Marques et al., 1997), while alternative cleavage at a different site, which occurs in the presence of the co-factor Twisted Gastrulation (Tsg), results in the production of truncated forms of Sog referred to as Supersog, which antagonize a broader spectrum of Bmp activities than intact Sog (Yu et al., 2000). Chordin is also subject to proteolysis by Xolloid, the vertebrate homolog of Tolloid (Piccolo et al., 1997). Because all of these molecules interact extracellularly, it

is important to understand how the activities and localization of these factors are regulated in the extracellular milieu.

Binding of growth factors to specific proteins or to the ECM is one type of mechanism for regulating the availability or dispersion of growth factors in different developmental contexts. ECM proteins may sequester growth factors in an inactive form, as well as modulate cellular responses to them (Streuli, 1999). Several ECM proteins such as Collagen, Fibronectin, Thrombospondin, Noggin and Chordin bind to TGFβ or to members of the bone morphogenetic protein (Bmp) subfamily (Piccolo et al., 1996; Taipale and Keski-Oja, 1997; Zimmerman et al., 1996). Such binding may activate or reduce growth factor activity and/or availability. Several extracellular matrix molecules and their receptors have been described in *Drosophila* (for a review, see Brown, 2000). Among these proteins, integrins are expressed during embryogenesis, larval and pupal stages and perform functions including muscle attachment, morphogenesis of the midgut, and adhesion between the two surfaces of the wing (Brabant et al., 1996; Brower et al., 1995a; Brower et al., 1995b; Martin-Bermudo and Brown, 1996; Roote and Zusman, 1995; Wilcox et al., 1989). During pupal development, integrins are expressed in intervein cells and perform a central role in regulating apposition (i.e. alignment and adhesion) of the dorsal and ventral surfaces of the wing (Brabant et al., 1996; Fristrom et al., 1993; Wilcox et al., 1989). Three integrin subunits are known to be required for adhesion between the two wing surfaces: βPS integrin, encoded by the *myospheroid* (*mys*) gene, is expressed on both wing surfaces during pupal development; and two α-integrins, αPS1, encoded by the *multiple edematous wing* (*mew*) gene, and αPS2, encoded by the *inflated* (*if*) gene, are expressed on the dorsal and ventral wing surfaces, respectively, during early wing development (Brabant et al., 1996; Brower et al., 1995b). Functional integrin molecules are composed of one β-subunit combined with one of the α-subunits, and consist of a large extracellular domain and a small cytoplasmic tail. Mutations in any of these integrin genes cause blisters in the adult wing, a phenotype characteristic of a lack of apposition between the wing surfaces (Brower et al., 1995b; Brown et al., 1989; Wilcox et al., 1989; Zusman et al., 1990).

In this report, we show that in addition to their well established adhesive function, integrins play another role during pupal vein development to modulate Bmp signaling. This modulation of Bmp activity may be mediated, at least in part, by integrins binding and/or regulating the activity or diffusion/distribution of the Sog protein. Genetic analysis indicates that the role of integrins in modulating Bmp signaling involves the well studied βPS and αPS1 subunits, and another less extensively characterized αPS3 subunit (Grotewiel et al., 1998; Stark et al., 1997), which we show is also expressed in dorsal cells of the pupal wing. We find that Sog diffuses into provein domains from adjacent intervein cells, but does so only on the dorsal surface. Moreover, we find that this dorsal specific diffusion of Sog into provein regions depends on the activity of both βPS and αPS1 integrin subunits. We discuss these results in light of current models for regulated Sog processing and recycling.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles were used in this study.

collagenIV a1: *DCg1*^{234}, *Cg25c*^{GDB};
collagenIV a2: *vkg*^{BLK}, *vkg*^{SAL}, *vkg*^{ICO}, *vkg*^{RML}, *vkg*^{177}, *vkg*^{228};
laminin: *lam*A$^{6\text{-}36}$, *lam*A$^{9\text{-}32}$, *lam*A^{216}, *lam*A^{25};
integrins: *if*^{3}, *if*^{B2}, *if*^{k27e}, *mew*^{M6}, *mew*^{498}, *mys*^{1}, *mys*^{ts1}, *mys*^{XB87}, *mys*^{nj42}, *mys*^{XR04}, *scb*^{1}, *scb*^{2}, *vol*^{1}, *vol*^{2};
dally: *dally*^{gem}, *dally*^{P2}, *dally*$^{\Delta P527}$;
dachsous: *ds*^{33k}, *ds*^{1};
stranded at second: *sas*^{15};
decapentaplegic: *dpp*^{shv};
glass bottom boat: *gbb*-60A^{1}, *gbb*-60A^{4};
thick veins: *tkv*^{1}, *tkv*^{8};
saxophone: *sax*^{8};
tolloid: *tld*$^{68\text{-}62}$;
tolloid-related: *tlr*$^{\Delta\text{-}41}$;
Df 3R *slo*^{3}, which deletes both *tld* and *tlr*.

Detailed characteristics of all alleles can be found in FlyBase. Enhancer piracy *sog* lines (e.g. *sog*^{EP2}, *sog*^{EP3}, *sog*^{EP7}, *sog*^{EP8}, *sog*^{EP9}, *sog*^{EP11}) are described elsewhere (Yu et al., 1996).

Production and analysis of mitotic clones

Clones of cells mutant for X-linked genes were induced by mitotic recombination in animals homozygous for FRT 18A and heterozygous for FRT18A *mys* or FRT18A *mew* chromosomes. *mys* clones were generated using the allele *mys*^{XB87} and the marker *multiple wing hair* (*mwh*), by use of *mwh*- flies containing a copy of the *mwh* gene on the first chromosome. Clones were generated in a wild-type background or in an Enhancer Piracy *sog* line (*sog*^{EP}) background, as below. *sog*^{EP} lines drive transgenic *sog* expression in vein primordia.

$$\frac{mys^{XB87}\ \text{FRT 18A}}{\text{FM7a}};\frac{sog^{EP7}}{sog^{EP7}};\frac{mwh}{mwh}\times\frac{mwh(+)\ \text{FRT 18A}}{\text{Y}};\frac{\text{Flp}}{\text{Flp}};\frac{mwh}{mwh}.$$

Eggs were collected for 24 hours and aged for 48 hours before the heat shock in order to generate predominantly small clones (<100 cells). First instar larvae were heat shocked for 15 minutes at 37°C. Unmarked clones generated with the same *mys*^{XB87} FRT line produced similar phenotypes. Twenty-seven dorsal clones, four ventral clones, and four dorsal and ventral clones were analyzed in detail.

mew clones were generated using the allele *mew*^{M6} and scored using the bristle and trichome marker *forked* (*f*^{36a}). Clones were generated in a wild-type background or in a *sog*^{EP} background as indicated below:

$$\frac{mew^{M6}\ f\ \text{FRT 18A}}{\text{FM7c}};\frac{sog^{EP7}}{sog^{EP7}}\times\frac{\text{FRT 18A}}{\text{Y}};\frac{\text{MKRS Flp}}{\text{TM6}}.$$

Eggs were collected and aged as described above. First instar larvae were heat shocked for three 1 hour intervals at 37°C, with 30 minute recovery periods in between. Twenty-seven dorsal clones, seven ventral clones, and five dorsal and ventral clones were analyzed in detail.

scb clones were generated using the allele *scb*^{1} and scored using the bristle and trichome marker *pawn* (*pwn*). The *scb*^{1} allele was recombined with FRT 42D and clones were induced in animals homozygous for FRT 42D and heterozygous for *scb* chromosomes. Clones were generated in a wild-type background or in an *sog*^{EP} background as indicated below:

$$\frac{scb^{1}\ pwn\ \text{FRT 42D}}{\text{CyO}};\frac{sog^{EP11}}{\text{TM3}}\times\frac{\text{Flp}}{\text{Y}};\frac{\text{FRT 42D}}{\text{FRT 42D}}.$$

Eggs were collected and aged as described above. First instar larvae were heat shocked for three 1 hour intervals at 37°C, separated by 45 minute recovery periods. Eighteen dorsal clones, nine ventral clones, and five dorsal and ventral clones were analyzed in detail.

In situ hybridization and immunohistochemistry

In situ hybridization was performed using digoxigenin-labeled antisense RNA probes and visualized as a blue alkaline phosphatase precipitate (O'Neill and Bier, 1994). Immunohistochemistry was performed as described by Sturtevant et al. (Sturtevant et al., 1993). Sog protein was detected using polyclonal 8B as primary antibody (1:500) (Srinivasan et al., 2002), anti-rabbit HRP as secondary antibody (1:2000, Jackson Laboratories), and visualized using the rhodamine TSA kit (NEB). For Sog and Integrin double labels, Sog protein was detected with anti-8B antiserum as above, CF.6G11 monoclonal antiserum was used for βPS integrin (1:500) and DK.1A4 monoclonal antiserum was used for αPS1 (1:500) (Brower et al., 1984), and detected with secondary anti-mouse Alexa 488 (Molecular Probes). Images were analyzed either on a Zeiss Axiovert 135, collected digitally with Axiocam or on a LSM 510 Meta Zeiss Confocal Microscope.

Immunoprecipitation and immunoblotting

Co-immunoprecipitation was based on procedures described by Brower (Brower, 1984), with minor modifications. Wings of pupae taken 20-24 hours after puparium formation (APF) were rapidly dissected from the pupal cases, homogenized with a pestle in ice cold lysis buffer (10 mM Tris pH 8.1/75 mM NaCl/0.5 mM $MgCl_2$/0.5 mM $CaCl_2$/0.25% NP40/0.25% BSA/0.01% NaN_3/1 mM PMSF and protease inhibitor cocktail – Complete, Boehringer Mannheim), and left for 30 minutes on ice with occasional rocking. After brief centrifugation (10 minutes at 10,000 ***g***) to pellet non-homogenized tissue, supernatants were incubated overnight at 4°C with protein A Sepharose bound integrin antibodies. Unbound supernatants where collected as 'unbound' sample and 4× SDS sample buffer was added. Beads were washed twice with lysis buffer and stripped of bound proteins by two rounds of acid elution with 200 mM glycine (pH 3.0) generating 'bound 1' and 'bound 2' samples to which 4×SDS sample buffer was added. All samples were boiled before running in 10% SDS-PAGE gels and transferred by electroblotting to nitrocellulose membranes. Membranes were blocked in Tris/NaCl/0.3% BSA 0.1% Tween 20 and incubated in primary antibody (anti-Sog 8A at 1:500 dilution) followed by incubation in HRP-conjugated anti-rabbit secondary antibody (Sigma, at 1:5000 dilution) and developed using Supersignal (Pierce) according to manufacturer's instructions. For detection of co-immunoprecipitated integrins used as control, membranes were stripped of antibodies using 200 mM glycine (10 minutes at room temperature), incubated in biotin-conjugated Concanavalin A, treated in Vectastain AB system and visualized by chemiluminescence as above. The 8A anti-Sog antiserum was raised against a small peptide fragment that included CR1 and the first part of the stem and the antibody recognizes a *sog* construct on western blots that contains the stem but not CR1.

Antibodies were covalently attached to protein A Sepharose beads by incubating them with beads overnight at 4°C. After three washes in cold PBS and two washes in 2 M sodium borate (pH 9.0), antibodies were crosslinked to the beads by addition of 5 mg/ml DMP. Beads were incubated for 30 minutes at room temperature, washed in 0.2 M triethanolamine pH 8.0, and incubated for 2 hours. After equilibrating in PBS, beads were stored at 4°C with 0.01% sodium azide. Beads were washed in PBS before use. Antibodies used were: CF.6G11 (monoclonal for βPS integrin); DK.1A4 (monoclonal for αPS1) (Brower et al., 1984); CF.2C7 monoclonal (Wilcox et al., 1981) for αPS2; and polyclonal α*vol* (*volado*, also known as *scab*) for αPS3 (Grotewiel et al., 1998).

Mounting fly wings

Wings from adult flies were dissected in isopropanol and mounted in Canada Balsam mounting medium.

Microsequencing

N-terminal microsequencing was used to ensure the identity of the protein band co-immunoprecipitated with αPS1 integrin antibodies. Immobilon instead of Nitrocellulose membranes were used, and BSA was omitted in the homogenization buffer. Protein bands were transferred from SDS-PAGE onto Immobilon membranes, followed by N-terminal microsequencing by Edman degradation. Microsequencing revealed contaminating proteins in the band recognized by the Sog antibody; however, we were able to detect the sequence GV(X)EGR(X)H(XX)L(XX)EE(X). A Blast search for short sequences aligning to this sequence found that it aligns to the N-terminal region of the Sog protein (**GV**T**EGR**R**H**AP**L**MF**EE**S).

RESULTS

Integrin mutants modify the effect of *sog* overexpression

Ectopic expression of *sog* in the wing results in the truncation of longitudinal veins and/or crossveins as a result of inhibition of Bmp signaling during pupal stages (Yu et al., 1996). We have previously described a line of flies (*sog*^{EP7}) in which a *sog* transgene is expressed in pupal vein primordia (Yu et al., 1996) as a consequence of the transgene bearing P-element having inserted next to a genomic enhancer [an effect we have termed enhancer piracy (Noll et al., 1994)]. *sog*^{EP7} flies have truncated L4 and L5 wing veins, a meandering L2 vein, and/or ectopic vein material in the vicinity of L2 (Fig. 1C). Consistent with previous analysis of *sog* during pupal development, mutant alleles of *dpp* and *tkv* can modify *sog*^{EP7} phenotypes (Yu et al., 1996) (Table 1).

In the course of screening for additional mutations that modified the effect of ectopic *sog* expression, we identified interactions with several genes encoding cell-cell or cell-extracellular matrix adhesion molecules. Based on these initial findings, we tested for transheterozygous genetic interactions between *sog*^{EP7} and mutants in components of the extracellular matrix or their receptors to identify candidate ECM proteins that might regulate Sog activity or diffusion (Table 1). Among the mutants scored, alleles of α and β-integrins showed consistent enhancement or suppression of *sog*^{EP7} phenotypes (Table 1, Fig. 1C-F). Strong alleles of *myospheroid* (*mys*) enhanced *sog*^{EP7} phenotypes (Fig. 1D), whereas alleles of *multiple edematous wing* (*mew*) suppressed these phenotypes (Fig. 1E). Importantly, the peak period of interaction between a heat shock *mys* construct and *sog*^{EP7} is between 20 and 28 hours after puparium formation (apf) (Table 1), which is the same time window for interaction between *sog* and the dpp^{shv} allele (Yu et al., 1996). By contrast, no interactions were observed with alleles of *inflated* (*if*). The *mys* and *mew* interactions were also tested with several other *sog*^{EP} lines as well as for several integrin alleles (Table 1). Alleles of *laminin* A, described as an extracellular ligand for αPS1 (encoded by *mew*), also strongly enhanced *sog*^{EP} vein truncations. In addition, we found that alleles of *scab* (or *volado*), which encodes an αPS3 subunit, enhance *sog*^{EP} phenotypes (Fig. 1F). Other extracellular modifiers of *sog*^{EP} phenotypes included alleles of genes encoding *Drosophila* Collagen IV and Selectin. The basis for these latter interactions will not be considered further in this report.

We also observed genetic interactions between integrins and other Bmp signaling components. For example, the hypomorphic β-integrin allele *mys*^{nj42} suppresses the thickened vein phenotype of the *tkv*^{1} allele of the Dpp receptor *thick veins* (Fig. 1G,H; Table 1). Similarly, decreasing the level of *scb* (in *scb*^{1} *tkv*^{1}/*tkv*^{1} flies)

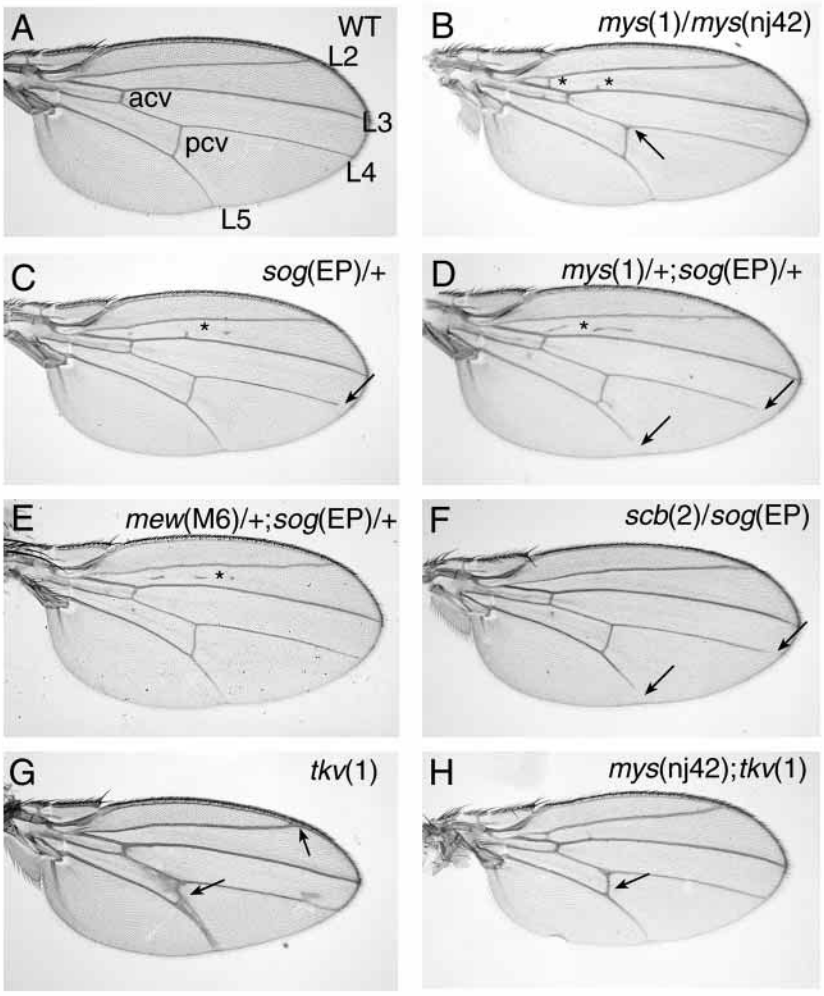

Fig. 1. Components of the Bmp pathway interact genetically with integrins. (A) A wild-type wing. Longitudinal L2, L3, L4 and L5 veins, and anterior (acv) and posterior (pcv) crossveins are indicated. Anterior is towards the top, and proximal towards the left. (B) As previously reported, certain allelic combinations of *myospheroid*, such as mys^{1}/mys^{nj42}, produce wings with ectopic vein material (asterisks) or vein thickening (arrow). Escapers from mys^{XR04}/mys^{nj42} exhibit both ectopic veins and vein truncations (not shown). (C) The enhancer piracy-*sog* line sog^{EP7} induces small distal truncations of the L4 vein (arrow). Asterisk indicates ectopic vein material. (D) The mys^{1} null allele enhances the sog^{EP7} truncation phenotype (arrows), while the mew^{M6} null allele suppresses this phenotype (E). Note that the mys^{1} and mew^{M6} alleles both enhance the amount of ectopic vein material between L2/L3 (asterisk). (F) A scb^{2} null allele also enhances vein truncation; however, no ectopic veins are seen between L2/L3. (G) Thickened veins are produced in tkv^{1} mutants (arrows). This phenotype is suppressed by the βPS allele mys^{nj42} (H).

suppresses the tkv^{1} phenotype (not shown). In addition, *dpp* and *gbb* alleles enhance sog^{EP} phenotypes as do reduced levels of *tld* and *tok*, which encode highly related metalloproteases (Table 1). This latter observation is interesting in light of the fact that *tld* and *tok* can collaborate to either degrade (Marques et al., 1997) or process Sog into more broadly active Bmp inhibitory forms in embryos and pupae (Yu et al., 2000).

Ectopic *sog* expression alters the behavior of clones lacking βPS and αPS1 integrins

Because decreasing the dose of *mys* enhanced sog^{EP} phenotypes, we examined the effect of complete loss of *mys* function in a sog^{EP7} background by producing *mys*-null clones. Large mys^{-} clones generated by FLP-FRT-mediated recombination frequently induce blisters due to non-apposition of the dorsal and ventral surfaces of the wing. In small mys^{-} clones, however, blisters are not observed and veins appear normal although the two wing surfaces remain unapposed within the center of the clone (Brabant et al., 1996). When similar small mys^{-} clones are generated in a sog^{EP7} background, a different phenotype is observed in which veins become ill-defined and broadened (e.g. four or five cells across compared with two or three cells in diameter in wild type) wherever the clones cross or abut longitudinal veins or the posterior crossvein (Fig. 2), and is observed in clones consisting of as few as 20 cells. The ability of mys^{-} clones to induce vein broadening non-autonomously in neighboring cells occurs only at very short range as clones displaced by three or more cell diameters from veins have a wild-type phenotype. Dorsal and ventral mys^{-} clones can induce non-apposition of the wing surfaces in both wild-type and sog^{EP7} backgrounds, consistent with the fact that βPS integrin is expressed on both surfaces of the wing during larval and pupal development (Brabant et al., 1996; Brower et al., 1995a). Vein broadening in an sog^{EP7} background, however, is observed only in dorsal clones, indicating that this phenotype is not simply a secondary consequence of an adhesion defect. The restriction of mys^{-} vein phenotypes to the dorsal surface also suggests that there is a dorsally expressed α-integrin, which acts in conjunction with β-integrin during vein development.

As in the case of mys^{-} clones, large null mew^{-} clones result in wing blisters (Brabant et al., 1996). Consistent with *mew* being expressed exclusively on the dorsal surface of the pupal wing, only dorsal mew^{-} clones produce a phenotype. Large mew^{-} null clones generated in a sog^{EP7} background produce similar blistered phenotypes. In contrast to these adhesion defective phenotypes, smaller mew^{-} clones (e.g. <100 cells) generated in a sog^{EP7} background alter vein formation, but do so in a different way than observed for small mys^{-} clones. Such mew^{-} clones located in the proximity of veins bend and displace the veins towards the clone, which then run along and outside the clone boundary (Fig. 3A-C). This vein shifting phenotype of mew^{-} clones is observed for all longitudinal veins. However, clones that cross over a vein, and therefore lack *mew* expression in intervein cells on both sides of the vein, generate no phenotype (Fig. 3D), neither do clones generated at a distance of three or more cells from a vein (not shown). A different vein thickening phenotype is associated with clones generated in the vicinity of crossveins (Fig. 3E).

Table 1. *sog* interacts genetically with genes coding ECM molecules

Gene/allele	Interaction with *sog* (EP7)	Interaction with *sog* (EP11)	Molecule encoded	Wing expression pattern
LamA(25)	EE	EE	Laminin	All wing, veins after apposition
LamA(6-36)	EEE	EEE		
LamA(9-32)	EEE	EEE		
LamA(216)	EE	EE		
DCg1(234)	S	S	Collagen IV a1	All wing, veins after apposition
Cg25c(GDB)	S	S		
vkg(BLK)	0	0	Collagen IV a2	ND
vkg(SAC)	0	0		
vkg(ICO)	0	0		
vkg(228)	0	0		
vkg(177)	0	0		
mys(1)	EE	EE	βPS integrin	Both wing surfaces; interveins
mys(XB87)	EE	EE		
mys(nj42)	E	E		
mys(nj42)/*mys*(nj42)	EE	EE		
mys(XR04)	0	O		
hs-βPS*	S	S	βPS under control of a hs promoter	NA
mew(498)	S	S	αPS1 integrin	Dorsal wing surface; interveins
mew(M6)	S	S		
if(k27e)	0	0	αPS2 integrin	Ventral wing surface; interveins
if(B2)	0	0		
if(3)	0	0		
scb(1)†	EE	EE	αPS3 integrin	Pupal wing (this report)
scb(2)	EE	EE		
vol(1)	E	E		
vol(2)	E	E		
ds(33k)	0	0	Cadherin	ND
ds(1)	0	0		
dally(Gem)	0	0	Proteoglycan	Imaginal wing disc
dally(P2)	0	0		
dally(ΔP527)	0	0		
sas(15)	0	0	Fibronectin type III repeats	Imaginal discs
fw(1)	E	E	Selectin	ND
tig(X)	0	0	Ligand for *if*	ND
tig(A1)	0	0		
tld(68-62)	0	0	Metalloprotease	Imaginal wing disc
Df3R *slo*(3)‡	EE	EE		
tok(Δ-41)	E	E	Metalloprotease	Imaginal wing disc
tkv(1)	EE	EE	Bmp receptor	All wing, stronger at vein border
tkv(8)	S	S		
sax(8)	0	0	Bmp receptor	Imaginal wing disc
dpp(shv)	EE	EE	Bmp family member	Pupal wing veins
gbb(1)	E	0	Bmp family member	Imaginal wing disc
gbb(4)	E	0		
rho (ve)	EE	EE	Membrane protein, Egfr signalling	Pupal wing veins

Alleles of genes coding for ECM molecules or their receptors were tested for genetic interactions with *sog* misexpression lines. Among the *Drosophila* genes encoding ECM proteins, we tested those for which a wing phenotype and/or expression in the wing had been previously reported. Interactions were scored against the enhancer piracy *sog* lines EP7 and EP11 (Yu et al., 1996), and classified as no interaction (0), enhancement (E) or suppression (S) of the sog^{EP} vein truncation phenotype. In the case of enhancer mutations, the number of Es indicates the strength of the interaction. sog^{EP7} induces ectopic expression of *sog* in vein domains during pupal development. Interacting alleles were also crossed to enhancer piracy lines EP9, EP8, EP2 and EP3. In all such cases, we observed consistent types of interactions. Unless cited, modifications of the sog^{EP} phenotype were similar for both the L4/L5 truncation and L2 wandering phenotypes.

*The strongest effects were observed when pupae were heat shocked at 4-8 hours after fertilization and 20-28 hours after fertilization, which coincide with periods of apposition between wing surfaces (Fristrom et al., 1993). Phenotypes were compared with those observed in sog^{EP7}/+ flies heat shocked under the same conditions.

†*scb* only enhances the vein truncation phenotype.

‡The deficiency Df 3R slo^3 uncovers *tld* and *tok*.

NA, not applicable; ND, not determined or not analyzed through all stages.

As expected, based on the dorsal specific expression of *mew*, ventral mew^- clones have no affect even in a sog^{EP7} background (Fig. 3F). We conclude that mew^- clones act non-autonomously to promote vein development in adjacent longitudinal provein cells. These phenotypes suggest that αPS1βPS integrin may normally play a role in positioning

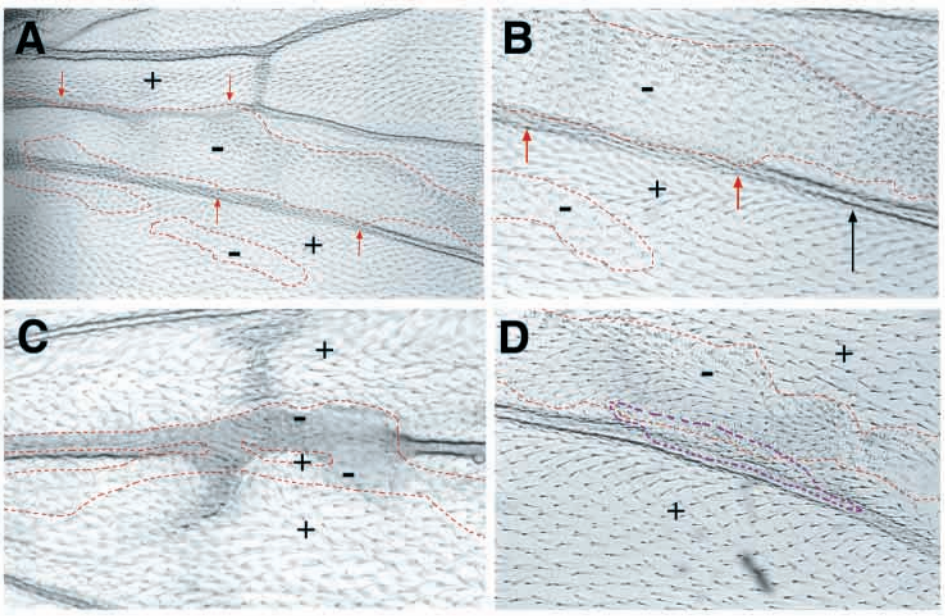

Fig. 2. Wing veins are poorly defined in βPS⁻ mutant clones. *mys*^{XB87} clones were generated in a *sog* misexpression background (*sog*^{EP7}) using FLP/FRT-mediated recombination and were recognized by the marker *multiple wing hair* (*mwh*). (A) Dorsal clones that cross over or lie adjacent to longitudinal veins, such as L4 and L5 induce the formation of veins with diffuse boarders (arrows indicate limits of the vein phenotype). Veins in these regions are less compact, less pigmented and wider than normal veins. (B) A higher magnification view of the wing in A showing that veins broaden within two cell diameters from the border of *mys*^{XB87} clones, but are unaffected when displaced by greater distances from the clones (e.g. compare vein phenotypes at red versus black arrows). (C) Two dorsal clones running over L3 induce thickened and diffuse vein sections. (D) A ventral clone adjacent to L5 has no effect on vein formation. Broken red lines indicate the limits of dorsal clones; broken purple lines indicate ventral clones; + indicates heterozygous or wild-type tissue; – indicates homozygous mutant clones.

veins by regulating the levels or activity of Sog at the vein/intervein border.

As mentioned above, the phenotypes generated by *mys*⁻ and *mew*⁻ clones in a *sog*^{EP7} background differ with respect to their effects on vein formation. As α and β-integrin subunits bind to form complexes, the *mys*⁻ clone phenotype may disrupt the formation of a complex composed of *mew* and an additional dorsally expressed integrin α-subunit. This alternative α-subunit is unlikely to be αPS2 (=*if*) because *if* is expressed only on the ventral surface of the wing and *if* mutant alleles do not modify the *sog*^{EP7} phenotype.

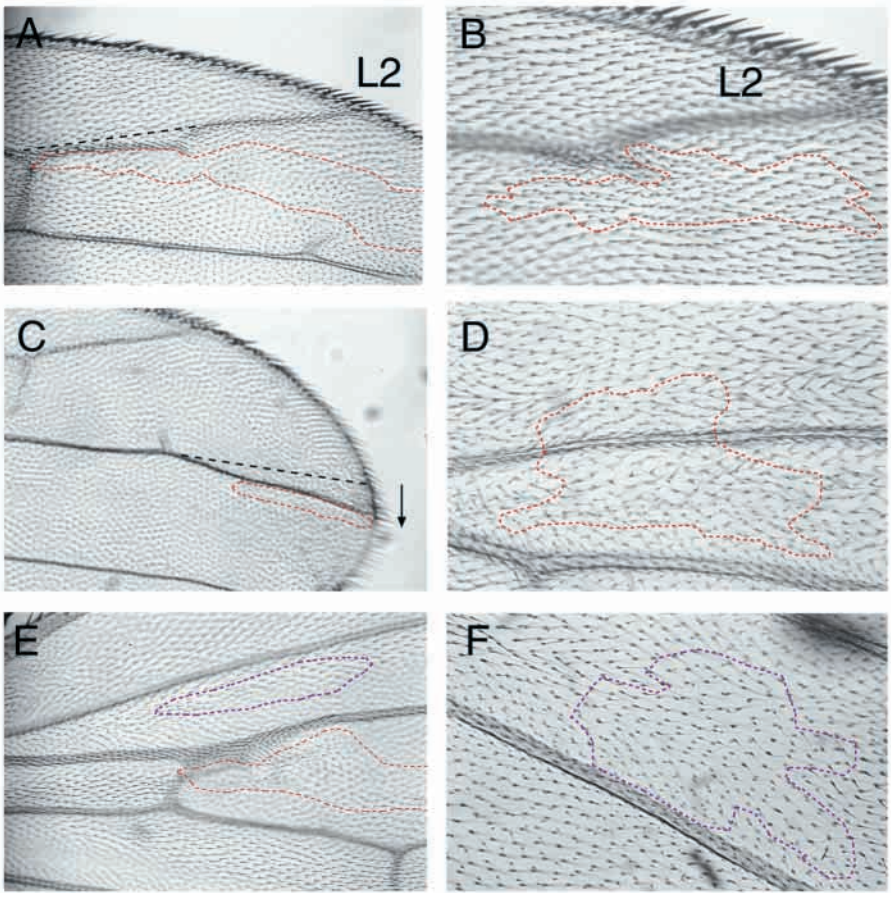

Fig. 3. Wing veins deviate toward nearby *mew*⁻ clones. *mew*^{M6} clones were generated in a *sog* misexpression background (*sog*^{EP7}) using FLP/FRT-mediated recombination and were scored by the marker *forked* (*f*). (A) A dorsal *mew*^{M6} clone adjacent to the L2 vein displaces the vein towards the clone. The broken black line indicates the normal trajectory of the L2 vein. (B) A small *mew*^{M6} clone adjacent to a distal region of L2 has a similar vein deviating effect. (C) A *mew*^{M6} clone between veins L3 and L4 alters the course of the L3 vein. The distance between the tips of L2 and L3 is increased as a function of L3 being displaced posteriorly (arrow) towards the *mew*^{M6} clone. The broken black line indicates the approximate location where the L3 vein would normally form. (D) A *mew*^{M6} clone that straddles the L2 vein by several cells on each side of the vein does not significantly disrupt the course of the vein. (E) A *mew*^{M6} clone adjacent to the posterior crossvein induces formation of ectopic vein material between the normal vein and the clone. (F) Ventral *mew*^{M6} clones adjacent to veins have no effect. Broken red lines indicate the limits of dorsal clones, broken purple lines indicate ventral clones.

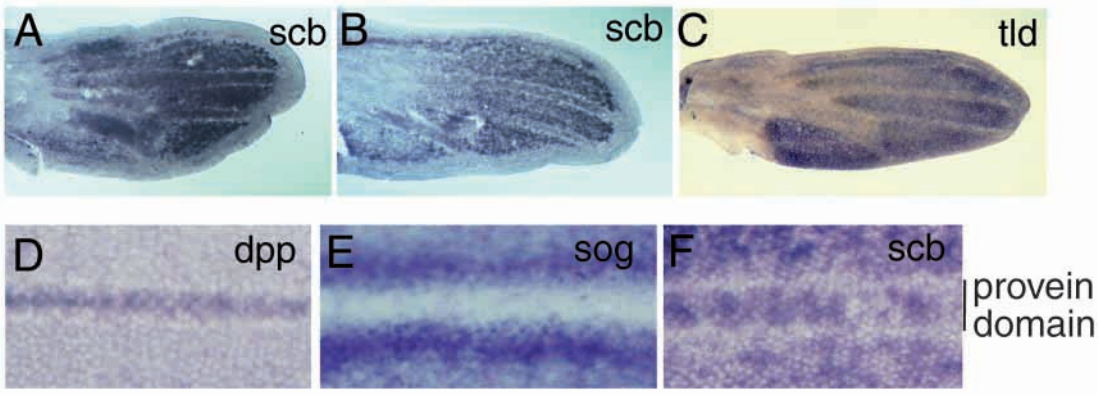

Fig. 4. *scab* is expressed in pupal intervein cells. *scb* expression during vein development was monitored by in situ hybridization using a *scb* antisense RNA probe. *scb* expression is absent in larval wing imaginal discs (not shown). (A) At 20 hours apf, *scb* expression is observed in intervein and vein cells. (B) At 25 hours apf *scb* expression in the interveins is maintained while vein expression becomes weaker. Metalloproteases that cleave Sog are expressed in intervein cells as revealed by RNA probes for *tld* (C) and *tok* (not shown). (D) *dpp* is expressed in the center of provein domains during pupal development, as shown for the L3 vein, while *sog* is expressed in the intervein cells (E), with peak expression often observed at the border of the provein territory. (F) High magnification view of *scb* expression in the L3 provein domain at 25 hours apf, showing that staining is excluded from the outermost provein area.

αPS3 is expressed dorsally in the pupal wing and regulates vein formation

It has not yet been reported whether the α-integrin *scb* is expressed in the wing or functions during wing development. Because *scb* alleles interacted genetically with *sog*^{EP7}, we examined the pattern of *scb* expression during larval and pupal wing development by in situ hybridization. No *scb* expression was detected during larval and prepupal stages (data not shown); however, a dynamic pattern of *scb* expression emerges in 20-30 hour pupal wings (Fig. 4A,B). *scb* expression, which is restricted to the dorsal surface of the wing at all times, is initiated most intensely in intervein regions in the vicinity of L2 and L3 and then rapidly expands to encompass all intervein cells. Subsequently, *scb* expression is also observed within the provein domain, initially at high levels and then tapering off after 25 hours of pupal development (Fig. 4B,F).

We examined the requirement for *scb* during wing development by producing *scb*$^{-}$ clones in an otherwise wild-type background. Small dorsal *scb*$^{-}$ clones result in a phenotype not previously observed for PS integrin mutants in which clones that touch or cross over a vein by a few cells broaden the vein from two or three cells to four or five cells across (Fig. 5B,C). The vein thickening phenotype of *scb* clones is observed for all longitudinal veins, as well as for the posterior crossvein. In contrast to *mys*$^{-}$ clones generated in a *sog*^{EP7} background, which result in irregular broadened veins, the veins associated with *scb*$^{-}$ clones generated in a wild-type background are well defined. In some cases, small *scb* clones (<20 cells) are restricted to the center of veins and can split the vein into two vein-like territories separated by a central less pigmented intervein-like area (Fig. 5D). This phenotype is

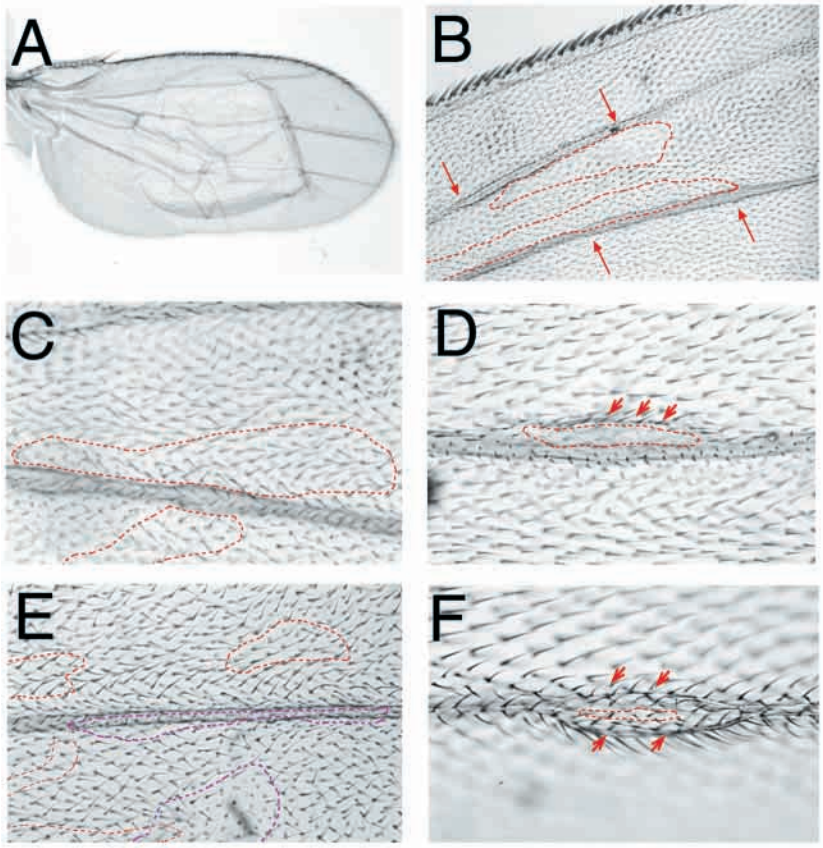

Fig. 5. *scb*$^{-}$ clones alter the width or course of wing veins. *scb*^{1} clones were generated using FLP/FRT mediated recombination and scored by the marker *pawn*. (A) Large clones generate blistered wings, a phenotype characteristic of other integrin^{-} mutants. (B) Two *scb*^{1} clones adjacent to veins L2 and L3 result in broadening of the adjacent veins (regions between arrows indicate affected sections of veins), which comprise wild-type cells. (C) A high magnification view of two *scb*^{1} dorsal clones surrounding the L3 vein, which are associated with a thickened vein segment (four cells wide). (D) A dorsal *scb*^{1} clone located in the center of the L3 provein region divides the vein into two branches, which avoid the clone (arrows). (E) A wing containing several *scb*^{1} clones in the proximity of L3. A ventral clone running over the vein has no effect. (F) A dorsal *scb*^{1} clone generated in a *sog*^{EP11} background lying inside the provein domain splits the L2 vein into two well defined branches. Arrows indicate veins formed outside the clonal boundary (indicated by broken red lines).

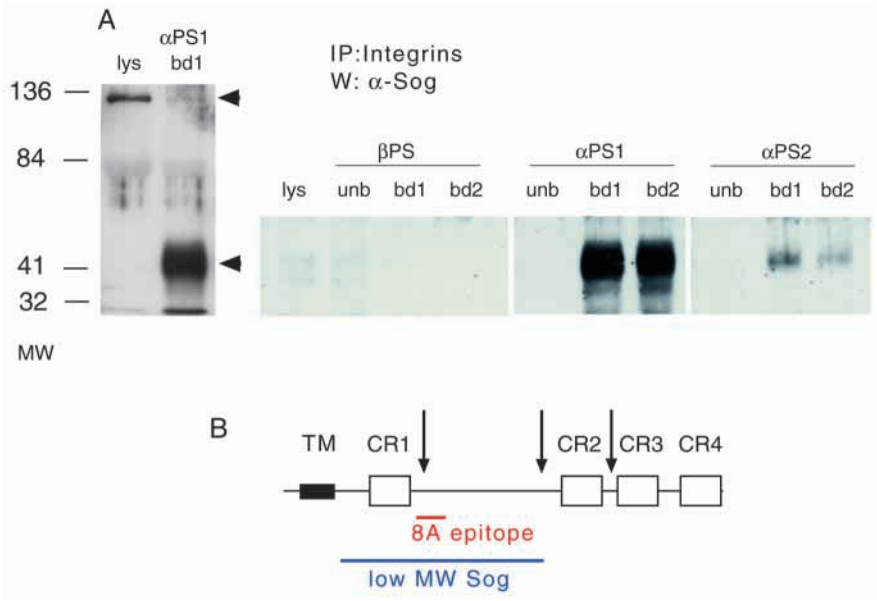

Fig. 6. A truncated form of Sog binds to αPS1 integrin. Co-immunoprecipitation of Sog with various anti-integrin antibodies. Protein lysates were prepared from wild-type pupae (24 hours apf) and then incubated with protein A-Sepharose bound anti-βPS, anti-αPS1 or anti-αPS2 antibodies. Lysates (lys), unbound supernatants (unb) and bound (bd1 and bd2) protein samples were run on 10% SDS-PAGE, immunoblotted and detected by the polyclonal 8A anti-Sog antiserum, which recognizes an epitope following CR1. (A) The Sog antibody reacts strongly with a large 120 kDa fragment in pupal lysates. A smaller 50 kDa reactive fragment is also present at very low levels. After co-immunoprecipitation with anti-αPS1, the 50 kDa band is greatly enriched and small amounts of the full-length band are detected (arrowhead). Sog protein does not co-immunoprecipitate with the anti-βPS antibody, but does co-immunoprecipitate to a much lesser extent with αPS2. No binding was observed for short or full-length Sog with the protein A-Sepharose beads alone. (B) The structure of Sog protein indicating the transmembrane domain (TM), four cysteine repeats (CR1-CR4) and putative Tolloid cleavage sites (arrows). The blue bar indicates the predicted fragment corresponding to the 50 kDa Sog band that co-immunoprecipitates with anti-αPS1. The red bar indicates the location of the epitope recognized by the 8A anti-Sog antibody.

accentuated when *scb*$^{-}$ clones are generated in a *sog*^{EP7} background (Fig. 5F) and is similar to phenotypes observed with clones of lateral inhibitory mutants (Garcia-Bellido and de Celis, 1992; Sturtevant and Bier, 1995). When *scb*$^{-}$ clones of similar size are generated at a distance from veins, however, they have no effect. In the few large *scb*$^{-}$ clones we have recovered (>200 cells), dorsal clones were associated with blisters characteristic of integrin mutants (Fig. 5A), although no phenotype was observed for any ventral *scb*$^{-}$ clones (Fig. 5E), consistent with the dorsally restricted expression of *scb*.

Sog interacts physically with αPS1βPS integrin

One explanation for the observed genetic interactions between *sog* and integrin mutations is that Sog physically binds to an integrin(s) subunit(s). To test for physical interactions between Sog and integrins we performed co-immunoprecipitation experiments (Fig. 6). During pupal development, *sog* is expressed in intervein cells and produces a full-length protein species of 120 kDa as well as several lower molecular weight forms of 76, 60, 50 and 42 kDa detected by immunoblotting with the 8A anti-Sog antiserum, which detects an epitope located immediately after the CR1 domain (Yu et al., 2000). We prepared protein extracts from hand dissected pupal wings, performed immunoprecipitations with antibodies against βPS, αPS1 or αPS2 integrins, ran the precipitates on SDS-PAGE, and immunoblotted with the 8A anti-Sog antiserum. These experiments revealed that a 50 kDa Sog protein co-precipitates efficiently with αPS1 and weakly with αPS2 (Fig. 6A), but not with αPS3 (not shown) or with β-integrin (Fig. 6A). A small amount of full-length Sog was also co-precipitated with αPS1. To confirm that the 50 kDa immunoprecipitated protein recognized by the Sog antibody was indeed a fragment of Sog, we isolated the reactive Sog band, subjected it to N-terminal microsequencing and found that it corresponds to an N-terminal fragment of the Sog protein (see Materials and Methods). Given the size of the band on SDS-PAGE (e.g. 50 kDa), this Sog fragment is predicted to include the epitope immediately following the CR1 domain recognized by the 8A antiserum (Yu et al., 2000) and terminate before the second cysteine repeat (CR2) (Francois et al., 1994). The basis for the binding between Sog and αPS1 may be related to the binding between a vertebrate α-integrin and thrombospondin, which are similar to *Drosophila* αPS1 and the CR domains of Sog, respectively (Francois et al., 1994; Guo et al., 2000; Hynes and Zhao, 2000). The failure of Sog to co-precipitate with βPS is surprising as ligand binding surfaces of integrins typically span both the α and β-subunits (Calderwood et al., 1997; Humphries, 2000; Sonnenberg, 1993). Such an α-chain-specific association could either result from an interaction between Sog and αPS1 outside of the ligand binding site of αPS1, or may reflect an indirect interaction with αPS1 mediated by another extracellular matrix protein or cell surface receptor.

Despite the clear genetic interaction between *sog* and *scb*, we have not been able to detect a physical binding between Sog and αPS3 as we have observed between Sog and αPS1. The lack of observed binding between Sog and αPS3 could result from a difficulty in detecting an alternatively processed form of Sog bound to αPS3, or may reflect an indirect mode of action of *scb* (e.g. by altering the abundance of interacting integrins such as αPS1βPS or by binding to a different Bmp inhibitor).

Integrins regulate the distribution of Sog protein in the pupal wing

It has been observed that *sog* mRNA is confined to intervein cells during pupal development (Yu et al., 1996) (Fig. 4E). As Sog protein diffuses during early embryonic development (Srinivasan et al., 2002), however, we wondered whether Sog might also travel from its intervein site of production into the provein region during pupal development. We stained pupal

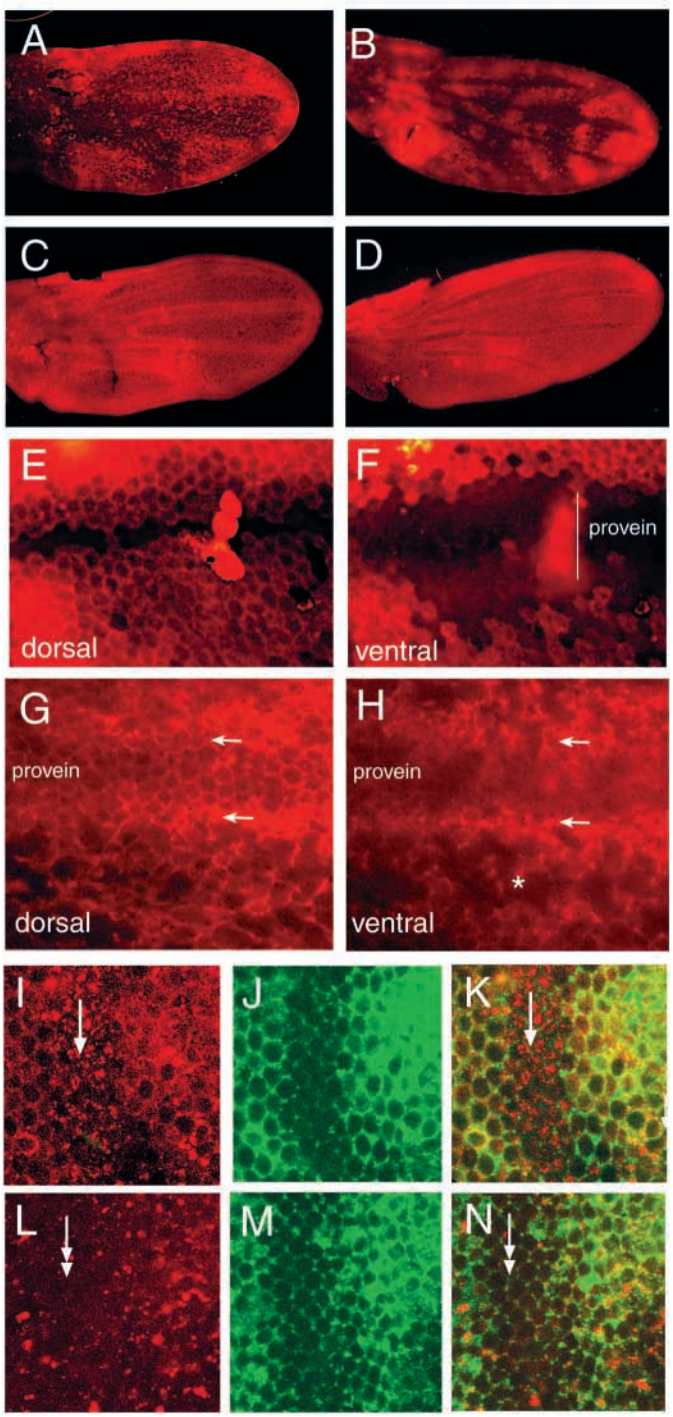

Fig. 7. Sog protein diffuses into the provein territory. Staining with the 8B anti-Sog antiserum reveals that Sog protein is initially expressed in a patchy intervein pattern at 18-22 hours apf (A). At this stage, there are more labeled intervein cells on the dorsal than ventral surface. (B) At 22-26 hours apf, Sog staining is still patchy but expands evenly to all intervein cells towards the end of this period. Staining is also visible in provein domains on the dorsal surface of the wing. (C) At 26-30 hours apf, Sog protein is present throughout the entire provein domain on the dorsal surface of veins, with increased staining at the provein/intervein border. (D) Later (>30 hours apf), anti-Sog staining again becomes restricted to intervein cells. Hemocytes running through the center of the vein also label. Staining fades away in subsequent stages. (E) High magnification of the L3 vein of a 24-26 hours apf pupal wing shows that Sog protein is present in provein cells on the dorsal surface of the wing except for the most central cells. This pattern is not synchronous for all veins. (F) On the corresponding ventral surface of the same region no provein (bar) staining is observed. (G) High magnification of the L3 vein of a wing as in C, showing Sog protein localized over the entire vein competent domain of all veins on the dorsal surface, but excluded from the provein regions of veins on the ventral (H) surface. Arrows indicate increased staining at the provein/intervein border. Note that the texture of reticular staining in intervein regions (asterisk) on the dorsal surface is different from the more punctate staining on the ventral surface of the wing. (I-N) High magnification confocal images of a 22-26 hours apf wild-type wing double labeled for Sog (red) and βPS integrin (green). This optical section, which is focused on the basolateral region of the dorsal wing epithelium, reveals that Sog (I,K) and integrin receptors (J,M) are co-localized, staining the cell perimeter. Sog staining is also observed inside intervein cells and entering the provein area (arrows, I,K), where integrins are absent. No Sog staining inside the provein area is observed on the ventral wing epithelium (L-N), as shown by the double arrows (L,N).

wings with the 8B anti-Sog antiserum (Srinivasan et al., 2002) and observed a dynamic pattern of Sog protein distribution, which includes vein competent domains as well as intervein cells (Fig. 7). Anti-Sog staining is initially patchy (around 20 hours apf), stronger on the dorsal surface and mostly restricted to intervein cells (Fig. 7A). Shortly thereafter (22-26 hours apf), Sog staining spreads into provein cells on the dorsal surface of the wing, at which point it is excluded only from the most central vein-proper cells (Fig. 7B,E). On the corresponding ventral surface, however, Sog staining remains excluded from the entire provein region throughout pupal development (i.e. up to 34 hours apf) (Fig. 7F,H). Between 26 and 30 hours apf, Sog staining fills all the provein domains on the dorsal surface, with increased levels of staining observed at the provein/intervein border (Fig. 7C,G). At 30 hours apf, Sog staining diminishes overall and becomes restricted to intervein cells and hemocytes running in the middle of the vein (Fig. 7D). As *sog* mRNA is detectable only in intervein cells during the examined pupal period, we conclude that Sog protein must be delivered to cells within the provein territory on the dorsal surface by some form of passive diffusion or active transport.

Because diffusion of Sog into provein domains is restricted to the dorsal surface of the wing where integrins interact with Sog, we asked whether they play a role in regulating the distribution of Sog protein on the dorsal surface of pupal wings. We generated marked *mys*$^{-}$ or *mew*$^{-}$ clones in an otherwise wild-type background and examined Sog staining (Fig. 8). In control wings, double-labeling with anti-β integrin and anti-Sog antisera confirmed that the dorsally restricted pattern of reticular Sog staining extends beyond β-integrin staining into provein domains (Fig. 7I-N). By contrast, Sog staining has a patchy intracellular appearance in dorsal *mys*$^{-}$ clones (Fig. 8A-F), and is excluded from wild-type provein cells on the dorsal surface that are adjacent to *mys*$^{-}$ clones. In such cases where *mys*$^{-}$ clones are located on one side of a provein domain, Sog is still able to enter the provein region from the opposite *mys*$^{+}$ side of the same vein (Fig. 8A-C). These results demonstrate that *mys* is required for diffusion or

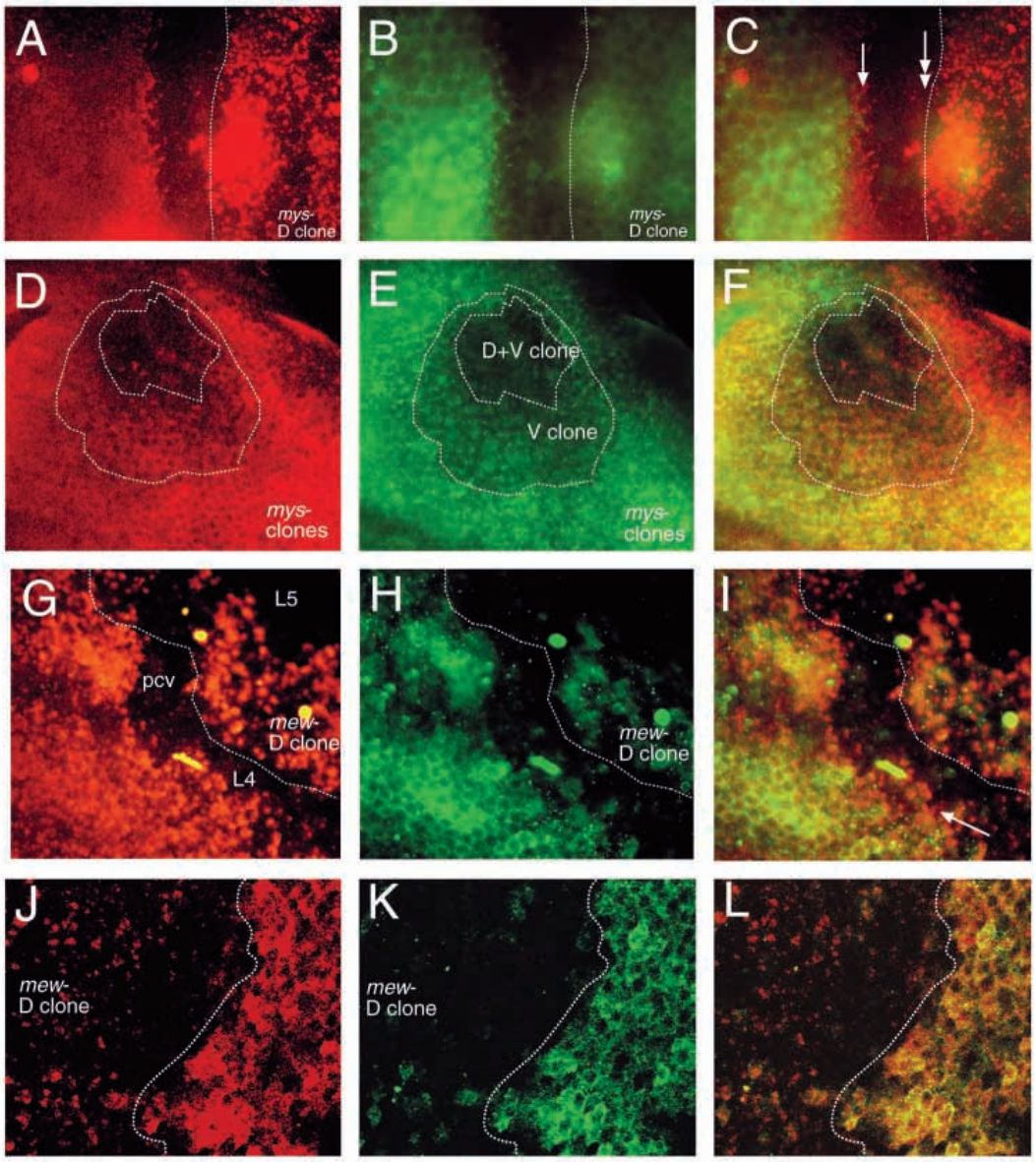

Fig. 8. Integrins regulate Sog protein distribution on the dorsal wing surface. The distribution of Sog and integrin proteins was examined in wings containing integrin$^-$ clones. In all panels, integrin staining is green and Sog staining is red. *mys*$^-$ clones were generated to analyze the distribution of Sog protein in the absence of β-integrin (A-F). Clones generated on the dorsal surface induce a patchy pattern of Sog distribution (A-C). In addition (C), adjacent to a dorsal *mys*$^-$ clone Sog protein does not enter the provein area (double arrow), while on the opposite side of the same vein, Sog enters the vein competent domain (arrow). Integrin staining defines the limit of the intervein territory (B,C). A ventral *mys*$^-$ clone does not alter the distribution Sog (D-F), while inside a small dorsal and ventral clone, Sog staining is reduced and patchy in appearance. (G-L) *mew*$^-$ clones result in similar effects on Sog protein distribution. (G-I) A dorsal *mew*$^-$ clone running between L4 and L5 veins induces patchy Sog distribution. The arrow in I indicates that Sog enters the provein area on the opposite side of the vein. A high magnification confocal optical section localized at the basolateral region of the dorsal wing epithelium reveals that Sog (J,L) and αPS1 (K,L) co-localize at the intervein area where the integrin is expressed, while a Sog is distributed in a patchy fashion at the same cell level inside a *mew*$^-$ clone.

transport of Sog into the vein competent domain. Consistent with the observations discussed above in which only dorsally located integrin$^-$ clones can alter the course of veins, we find that only dorsal *mys*$^-$ clones modify Sog distribution in the pupal wing (Fig. 8D-F). We observed similarly altered Sog staining within *mew*$^-$ clones on the dorsal wing surface resulting in punctate rather than reticular staining and lack of Sog diffusion into the provein region (Fig. 8G-L). These results demonstrate that the βPS and αPS1 integrins play an important role in determining the distribution of Sog protein in the pupal wing.

DISCUSSION

In this study, we have provided three primary lines of evidence that integrins play an important role in regulating Bmp signaling in provein regions of the pupal wing. First, we showed that integrin$^-$ clones generated on the dorsal surface of the wing alter the trajectory and/or width of adjacent veins. Second, we found that a truncated form of Sog present in pupal wings binds to αPS1. Finally, we observed that diffusion of Sog into provein domains, which is restricted to the dorsal surface of the wing, depends on integrin function. Cumulatively, these results strongly suggest that the ability of Sog to diffuse or to be transported into provein regions on the dorsal surface depends on an interaction with integrins.

Integrins regulate Sog distribution in the pupal wing

Consistent with Sog interacting genetically with integrins to alter the course of veins on the dorsal surface of the wing, we found that the αPS1 and βPS-integrins are required for the diffusion or transport of Sog from dorsal intervein cells where *sog* mRNA is expressed into adjacent provein regions. As

αPS1 binds Sog, this physical interaction may contribute to regulating the distribution of Sog. The 8B anti-Sog antiserum, which recognizes Sog protein in intervein cells and inside the provein domain, detects an epitope located near the second cystein repeat (CR2). Consequently, Sog fragments that diffuse or that are delivered to provein cells must be either full length, which weakly binds to αPS1 in co-immunoprecipitation experiments, or fragments that contain CR2. The truncated Supersog-like fragment that binds strongly to αPS1 in co-immunoprecipitation experiments should not be recognized by the 8B antiserum. Therefore, integrins may differentially regulate the distribution of Sog fragments on the dorsal surface of the pupal wing, restraining the movement of broad spectrum Bmp inhibitory Sog fragments (such as Supersog-like molecules) and allowing or mediating transport of other fragments to provein cells, such as full-length Sog, which also has a vein inhibitory function. Unfortunately, it is not possible currently to examine the diffusion of Supersog-like fragments directly because the 8A antiserum is not suitable for staining pupal wings. These findings suggest that integrins regulate the delivery or diffusion of active Sog protein from intervein cells into the vein competent domain. In contrast to the dorsally restricted functions of integrins required for vein development, the previously analyzed adhesive functions of integrins depends on subunits functioning on both surfaces of the wing.

Integrins modulate Sog activity in the wing

There are several possible mechanisms by which interaction with integrins could modulate Sog activity in pupal wings. It has been previously shown that elevated *sog* expression results in vein truncation, while misexpression of *dpp* induces ectopic veins, indicating that *sog* restricts vein formation by opposing Bmp signals emanating from the center of the vein (Yu et al., 1996). One possibility is that such a Bmp inhibitory form(s) of Sog must interact with integrins in order to diffuse or be transported into provein domains (on the dorsal surface of the wing only). This hypothesis would be consistent with the finding that veins appear to be attracted to integrin$^-$ clones. Such a vein repulsive form(s) of Sog would presumably act as a Bmp antagonist.

According to the simple model in which integrins are essential for delivering a Bmp inhibitory form of Sog to provein cells, one would expect that integrin$^-$ and *sog*$^-$ clones would generate similar phenotypes in which veins deviated towards the mutant clones and/or broadened within them. However, *sog*$^-$ clones induce meandering of veins (Yu et al., 1996), which show only a weak tendency to track along the outside of *sog*$^-$ clones (B.N. and E.B., unpublished), in contrast to integrin$^-$ clones, which bend or widen veins in a more dramatic fashion. One possible explanation for the differences between the *sog*$^-$ and integrin$^-$ phenotypes is that there are several different endogenous forms of Sog in pupal wings (Yu et al., 2000), which might exert opposing activities. If multiple Sog fragments exert effects on vein development, some providing repulsive and others attractive activities on vein formation, the differences between the behaviors of *sog*- and integrin- clones could be explained by a repulsive (Bmp inhibitory) form(s) of Sog selectively requiring an interaction with integrins. The possibility that a positive Bmp-promoting activity of Sog might also be present that acts as a vein attractant has precedent in that a positive Sog activity has been proposed to explain a requirement for Sog in activating expression of the Dpp target gene *race* in early embryos (Ashe and Levine, 1999). Structure/function studies of Sog have also revealed a potential Bmp promoting form of Sog, which is longer than Supersog forms (K. Yu and E.B., unpublished). According to this model, altering the balance between repulsive and attractive Sog activities would generate different vein phenotypes. In the total absence of *sog*, both repulsive and attractive activities would be lost, generating a mild meandering vein phenotype in which neither attraction nor repulsion clearly dominated, as is observed in *sog*$^-$ clones (Yu et al., 1996). If an interaction with integrins were required only for production or delivery of Bmp inhibitory forms of Sog into the vein, then integrin$^-$ clones, which still contain the Bmp-activating forms of Sog, could exert a net attractive influence on veins, leading to more pronounced deviation of veins toward the clones. This hypothesis is consistent with vein phenotypes we have observed associated with integrin$^-$ clones that cross over veins or run along both sides of the vein, such as narrowing, bending and wandering of veins which are similar to phenotypes observed in correspondingly located *sog*$^-$ clones. The existence of different Sog fragments bearing opposing activities would also explain the different phenotypes we obtain upon ectopic Sog expression in some sog^{EP} lines (Yu et al., 1996), such as sporadic ectopic vein material between L3 and L2 and meandering L2 veins in addition to vein loss in other areas.

Another possible explanation for the differences between the *sog*$^-$ and integrin$^-$ phenotypes is that integrins may regulate the activity of extracellular signals in addition to Sog. One hint of such an activity is that when a *scb*$^-$ clone falls within the provein area, the vein splits around the border of the clone in a cell autonomous fashion. As this later phenotype is enhanced by ectopic *sog* expression in veins (e.g. in a *sog*^{EP} background), αPS3 may normally promote Bmp signaling within the vein. Although the identity of such potential targets is unknown, candidates would include Bmps (e.g. Dpp or Gbb) or Bmp receptors. Further analysis will be needed to explain the basis for the different behaviors of *sog*$^-$ and integrin$^-$ clones, as well as the variations observed in different integrin$^-$ clones.

In summary, we propose that Sog fragments with differential activities may regulate vein formation. The vein bending phenotype observed in the absence of αPS1 would result from a remaining attractive Sog activity that outweighs the activity of a repulsive form of Sog, which can no longer be delivered from intervein cells (Fig. 9). As βPS integrin forms heterodimers with both αPS1 and αPS3 (Brower et al., 1984; Stark et al., 1997) *mys* would be expected to be required for the activity of both αPS chains. Consistent with this expectation, the phenotype of *mys*$^-$ clones (i.e. broad poorly defined veins) resembles a hybrid of those observed for *mew*$^-$ and *scb*$^-$ clones.

Do integrins regulate endocytosis of Sog?

Endocytosis has been shown to play an important role in the establishment of Bmp activity gradients. The endocytic pathway has been implicated in transport of Dpp between cells by transcytosis during larval wing development (Entchev et al., 2000; Teleman and Cohen, 2000) and for vectorial transport of Wg during mid-embryogenesis (Dubois et al., 2001; Moline et al., 1999). During early embryonic development, formation of

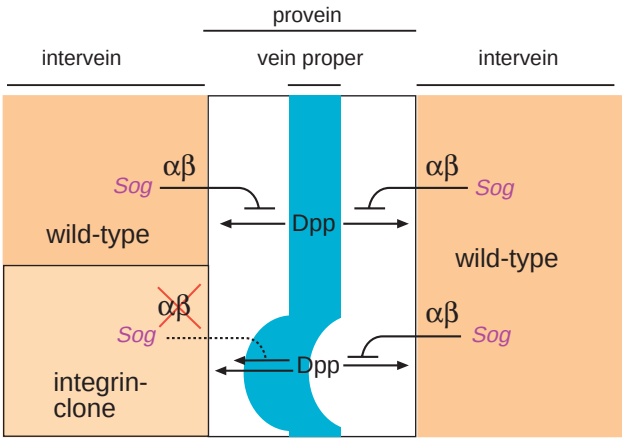

Fig. 9. A model for Sog/integrin interactions in the wing. The primary proposed function of αPS1βPS integrin in modulating Sog activity in the wing. In wild-type wings, αβ integrin heterodimers enhance the delivery or diffusion of an active Bmp inhibitory form of Sog into the provein domain. This non-autonomous source of Sog limits peak Bmp signaling to the center of the provein territory. In the absence of αPS1βPS, a repulsive form of Sog protein is unable to enter the provein territory, while a remaining unaffected Bmp promoting Sog activity (not shown) attracts the vein towards the mutant clone.

a Sog protein gradient in dorsal regions also relies on the action of Dynamin (Srinivasan et al., 2002), although in this pre-blastoderm context, it has been proposed that endocytosis limits the dorsal diffusion of Sog, which is essential for the partitioning of the dorsal ectoderm into epidermis and amnioserosa. Recently, deRobertis' group has shown that vertebrate α3β Integrin binds to the *Xenopus* Sog counterpart Chordin in vitro, leading to endocytosis of Chordin (E. deRobertis, personal communication).

Although we have not directly addressed whether integrins regulate Sog endocytosis in this current study, the altered distribution of Sog within integrin$^-$ clones is suggestive of such a role. Reticular Sog staining, which outlines the cell perimeter is lost in integrin$^-$ clones on the dorsal surface, leaving only a punctate intracellular staining. This mis-localization of Sog implicates integrins in internalizing and/or trafficking of Sog to the cell surface. Because appropriately located integrin$^-$ clones also block the accumulation of Sog in adjacent provein domains, the observed defects in Sog distribution between the surface and the cytoplasm may underlie the failure to deliver Sog to vein competent cells. The endocytic pathway could promote the transport of Sog to provein cells by a mechanism similar to that proposed to be involved in the transport of Dpp along the AP axis during larval stages (Entchev et al., 2000; Teleman and Cohen, 2000). Alternatively, endocytosis could function to limit Sog diffusion as is the case during embryogenesis (Srinivasan et al., 2002). According to this latter scenario, integrins would normally prevent or reduce Sog endocytosis because integrins are necessary for delivery of Sog to provein cells. Integrins have been shown to play a direct role in endocytosis of viral particles and in mediating membrane traffic through the endocytic cycle (de Curtis, 2001; Triantafilou et al., 2001). Indirect mechanisms for integrin-mediated endocytosis may also exist that would not involve endocytosis of the integrin receptor itself, but of other components that regulate Sog trafficking. Further analysis will be necessary to investigate whether *Drosophila* integrins regulate delivery of Sog to endocytic vesicles or transport of Sog through the endocytic pathway to adjacent cells.

Do integrins regulate other pathways required for vein development?

The modulatory effect of integrins on Sog activity described in this paper are likely to be mediated by *dpp* and/or *gbb* signaling because existing evidence indicates that Sog is a dedicated modulator of Bmp signaling. In addition, the phenocritical period for *mys* and *sog* interaction coincides with that for interaction between *sog* and *dpp* (Yu et al., 1996). On the one hand, we cannot exclude the existence of an additional role of integrins in regulating vein formation through another pathway, such as the Egf and Notch pathways, which have been shown to exert important roles on vein development (de Celis et al., 1997; de Celis and Garcia-Bellido, 1994; Garcia-Bellido and de Celis, 1992; Guichard et al., 1999; Huppert et al., 1997; Martin-Blanco et al., 1999; Sturtevant and Bier, 1995). On the other hand, the integrin$^-$ clonal phenotypes described in this manuscript are observed only on the dorsal surface and all known components of the Egfr pathway promote vein development on both surfaces of the wing (Diaz-Benjumea and Garcia-Bellido, 1990; Diaz-Benjumea and Hafen, 1994; Guichard et al., 1999).

We also found that *mys*^{nj42} and *scb*^{1} suppress the thickened vein phenotype of *tkv*^{1} mutants, which raises the possibility of a direct interaction between integrins and a Bmp receptor involved in wing vein development. The vein splitting and vein thickening *scb*$^-$ clonal phenotypes are reminiscent of *tkv* mutant phenotypes, which derive from a positive requirement for Bmp signaling for vein formation inside the vein competent domain and a negative ligand titrating function that limits the range of Bmp diffusion into the intervein territory adjacent to the provein domain (de Celis, 1997). The fact that *scb* is expressed in both vein and intervein territories is consistent with a dual action of *scb*. Additional experiments will be necessary to investigate whether *scb* plays a direct role in modulating Bmp receptor activity.

Interactions with the extracellular matrix may help shape morphogen gradients

Diffusion of putative growth factors and the shaping of their activity gradients have been the focus of intense interest since Allan Turing formulated the concept of morphogens (Turing, 1952). Recently, several groups have described mechanisms to explain how soluble factors can create morphogen gradients. These include degradative proteolysis and a retrieval role for endocytosis in creating the early embryonic Sog gradient (Srinivasan et al., 2002), regulated endocytosis of *wingless* (Strigini and Cohen, 2000), extracellular transport of Wg in membrane bound argosomes (Greco et al., 2001), planar transcytosis [as is required for Dpp movement in the wing imaginal disc (Entchev et al., 2000; Teleman and Cohen, 2000)], and the formation of thin cell extensions (cytonemes) that deliver Dpp over several rows of cells (Ramirez-Weber and Kornberg, 1999). Protein-protein interactions in the extracellular milieu, such as those described here, may also be capable of modulating the magnitude and spatial pattern of

Bmp activity, working independently or in conjunction with other mechanisms.

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REFERENCES

Araujo, H. and Bier, E. (2000). sog and dpp exert opposing maternal functions to modify toll signaling and pattern the dorsoventral axis of the Drosophila embryo. *Development* **127**, 3631-3644.

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

Biehs, B., Francois, V. and Bier, E. (1996). The Drosophila short gastrulation gene prevents Dpp from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Dev.* **10**, 2922-2934.

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

Brabant, M. C., Fristrom, D., Bunch, T. A. and Brower, D. L. (1996). Distinct spatial and temporal functions for PS integrins during Drosophila wing morphogenesis. *Development* **122**, 3307-3317.

Brentrup, D., Lerch, H., Jackle, H. and Noll, M. (2000). Regulation of Drosophila wing vein patterning: net encodes a bHLH protein repressing rhomboid and is repressed by rhomboid-dependent Egfr signalling. *Development* **127**, 4729-4741.

Brower, D. L., Brabant, M. C. and Bunch, T. A. (1995a). Role of the PS integrins in Drosophila development. *Immunol. Cell Biol.* **73**, 558-564.

Brower, D. L., Bunch, T. A., Mukai, L., Adamson, T. E., Wehrli, M., Lam, S., Friedlander, E., Roote, C. E. and Zusman, S. (1995b). Nonequivalent requirements for PS1 and PS2 integrin at cell attachments in Drosophila: genetic analysis of the alpha PS1 integrin subunit. *Development* **121**, 1311-1320.

Brower, D. L. and Jaffe, S. M. (1989). Requirement for integrins during Drosophila wing development. *Nature* **342**, 285-287.

Brower, D. L., Wilcox, M., Piovant, M., Smith, R. J. and Reger, L. A. (1984). Related cell-surface antigens expressed with positional specificity in Drosophila imaginal discs. *Proc. Natl. Acad. Sci. USA* **81**, 7485-7489.

Brown, N. H. (2000). Cell-cell adhesion via the ECM: integrin genetics in fly and worm. *Matrix Biol.* **19**, 191-201.

Brown, N. H., King, D. L., Wilcox, M. and Kafatos, F. C. (1989). Developmentally regulated alternative splicing of Drosophila integrin PS2 alpha transcripts. *Cell* **59**, 185-195.

Calderwood, D. A., Tuckwell, D. S., Eble, J., Kuhn, K. and Humphries, M. J. (1997). The integrin alpha1 A-domain is a ligand binding site for collagens and laminin. *J. Biol. Chem.* **272**, 12311-12317.

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

de Celis, J. F. and Garcia-Bellido, A. (1994). Roles of the Notch gene in Drosophila wing morphogenesis. *Mech. Dev.* **46**, 109-122.

de Celis, J. F., Bray, S. and Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing. *Development* **124**, 1919-1928.

de Curtis, I. (2001). Cell migration: GAPs between membrane traffic and the cytoskeleton. *EMBO Rep.* **2**, 277-281.

Diaz-Benjumea, F. J. and Garcia-Bellido, A. (1990). Behaviour of cells mutant for an EGF receptor homologue of Drosophila in genetic mosaics. *Proc. R. Soc. Lond. B Biol. Sci.* **242**, 36-44.

Diaz-Benjumea, F. J. and Hafen, E. (1994). The sevenless signalling cassette mediates Drosophila EGF receptor function during epidermal development. *Development* **120**, 569-578.

Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E. and Vincent, J. P. (2001). Regulated endocytic routing modulates wingless signaling in Drosophila embryos. *Cell* **105**, 613-624.

Entchev, E. V., Schwabedissen, A. and Gonzalez-Gaitan, M. (2000). Gradient formation of the TGF-beta homolog Dpp. *Cell* **103**, 981-991.

Francois, V. and Bier, E. (1995). Xenopus chordin and Drosophila short gastrulation genes encode homologous proteins functioning in dorsal-ventral axis formation. *Cell* **80**, 19-20.

Francois, V., Solloway, M., O'Neill, J. W., Emery, J. and Bier, E. (1994). Dorsal-ventral patterning of the Drosophila embryo depends on a putative negative growth factor encoded by the short gastrulation gene. *Genes Dev.* **8**, 2602-2616.

Fristrom, D., Wilcox, M. and Fristrom, J. (1993). The distribution of PS integrins, laminin A and F-actin during key stages in Drosophila wing development. *Development* **117**, 509-523.

Garcia-Bellido, A. and de Celis, J. F. (1992). Developmental genetics of the venation pattern of Drosophila. *Annu. Rev. Genet.* **26**, 277-304.

Greco, V., Hannus, M. and Eaton, S. (2001). Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* **106**, 633-645.

Grotewiel, M. S., Beck, C. D., Wu, K. H., Zhu, X. R. and Davis, R. L. (1998). Integrin-mediated short-term memory in Drosophila. *Nature* **391**, 455-460.

Guichard, A., Biehs, B., Sturtevant, M. A., Wickline, L., Chacko, J., Howard, K. and Bier, E. (1999). rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during Drosophila wing vein development. *Development* **126**, 2663-2676.

Guo, N., Templeton, N. S., Al-Barazi, H., Cashel, J. A., Sipes, J. M., Krutzsch, H. C. and Roberts, D. D. (2000). Thrombospondin-1 promotes alpha3beta1 integrin-mediated adhesion and neurite-like outgrowth and inhibits proliferation of small cell lung carcinoma cells. *Cancer Res.* **60**, 457-466.

Haerry, T. E., Khalsa, O., O'Connor, M. B. and Wharton, K. A. (1998). Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila. *Development* **125**, 3977-3987.

Humphries, M. J. (2000). Integrin structure. *Biochem. Soc. Trans.* **28**, 311-339.

Huppert, S. S., Jacobsen, T. L. and Muskavitch, M. A. (1997). Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. *Development* **124**, 3283-3291.

Hynes, R. O. and Zhao, Q. (2000). The evolution of cell adhesion. *J. Cell Biol.* **150**, F89-F96.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

Marques, G., Musacchio, M., Shimell, M. J., Wunnenberg-Stapleton, K., Cho, K. W. and O'Connor, M. B. (1997). Production of a DPP activity gradient in the early Drosophila embryo through the opposing actions of the SOG and TLD proteins. *Cell* **91**, 417-426.

Martin-Bermudo, M. D. and Brown, N. H. (1996). Intracellular signals direct integrin localization to sites of function in embryonic muscles. *J. Cell Biol.* **134**, 217-226.

Martin-Blanco, E., Roch, F., Noll, E., Baonza, A., Duffy, J. B. and Perrimon, N. (1999). A temporal switch in DER signaling controls the specification and differentiation of veins and interveins in the Drosophila wing. *Development* **126**, 5739-5747.

Moline, M. M., Southern, C. and Bejsovec, A. (1999). Directionality of wingless protein transport influences epidermal patterning in the Drosophila embryo. *Development* **126**, 4375-4384.

Noll, R., Sturtevant, M. A., Gollapudi, R. R. and Bier, E. (1994). New functions of the Drosophila rhomboid gene during embryonic and adult development are revealed by a novel genetic method, enhancer piracy. *Development* **120**, 2329-2338.

O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 870, 874-875.

Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L. and de Robertis, E. M. (1997). Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* **91**, 407-416.

Piccolo, S., Sasai, Y., Lu, B. and de Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

Ramirez-Weber, F. A. and Kornberg, T. B. (1999). Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs. *Cell* **97**, 599-607.

Roch, F., Baonza, A., Martin-Blanco, E. and Garcia-Bellido, A. (1998). Genetic interactions and cell behaviour in blistered mutants during proliferation and differentiation of the Drosophila wing. *Development* **125**, 1823-1832.

Roote, C. E. and Zusman, S. (1995). Functions for PS integrins in tissue adhesion, migration, and shape changes during early embryonic development in Drosophila. *Dev. Biol.* **169**, 322-336.

Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* **410**, 479-483.

Sonnenberg, A. (1993). Integrins and their ligands. *Curr. Top. Microbiol. Immunol.* **184**, 7-35.

Srinivasan, S., Rashka, K. E. and Bier, E. (2002). Creation of a Sog morphogen gradient in the Drosophila embryo. *Dev. Cell* **2**, 91-101.

Stark, K. A., Yee, G. H., Roote, C. E., Williams, E. L., Zusman, S. and Hynes, R. O. (1997). A novel alpha integrin subunit associates with betaPS and functions in tissue morphogenesis and movement during Drosophila development. *Development* **124**, 4583-4594.

Streuli, C. (1999). Extracellular matrix remodelling and cellular differentiation. *Curr. Opin. Cell Biol.* **11**, 634-640.

Strigini, M. and Cohen, S. M. (2000). Wingless gradient formation in the Drosophila wing. *Curr. Biol.* **10**, 293-300.

Sturtevant, M. A. and Bier, E. (1995). Analysis of the genetic hierarchy guiding wing vein development in *Drosophila*. *Development* **121**, 785-801.

Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila* rhomboid gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.

Taipale, J. and Keski-Oja, J. (1997). Growth factors in the extracellular matrix. *FASEB J.* **11**, 51-59.

Teleman, A. A. and Cohen, S. M. (2000). Dpp gradient formation in the Drosophila wing imaginal disc. *Cell* **103**, 971-980.

Triantafilou, K., Takada, Y. and Triantafilou, M. (2001). Mechanisms of integrin-mediated virus attachment and internalization process. *Crit. Rev. Immunol.* **21**, 311-322.

Turing, A. (1952). The chemical basis of morphogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Soc.* **237**, 37-72.

Wilcox, M., Brower, D. L. and Smith, R. J. (1981). A position-specific cell surface antigen in the drosophila wing imaginal disc. *Cell* **25**, 159-164.

Wilcox, M., DiAntonio, A. and Leptin, M. (1989). The function of PS integrins in Drosophila wing morphogenesis. *Development* **107**, 891-897.

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

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

Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. *Development* **121**, 2265-2278.

Zimmerman, L. B., de Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.

Zusman, S., Patel-King, R. S., Ffrench-Constant, C. and Hynes, R. O. (1990). Requirements for integrins during Drosophila development. *Development* **108**, 391-402.

Zusman, S., Grinblat, Y., Yee, G., Kafatos, F. C. and Hynes, R. O. (1993). Analyses of PS integrin functions during Drosophila development. *Development* **118**, 737-750.