

## Genetic interactions and cell behaviour in *blistered* mutants during proliferation and differentiation of the *Drosophila* wing

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### SUMMARY

In this work, we analyse the *blistered* function in wing vein development by studying genetic mosaics of mutant cells, genetic interactions with other genes affecting vein development and *blistered* expression in several mutant backgrounds. *blistered* encodes for a nuclear protein homologous to the mammalian Serum Response Factor and is expressed in presumptive intervein cells of third larval instar and pupal wing discs. Clones of *blistered* mutant cells proliferate normally but tend to grow along veins and always differentiate as vein tissue. These observations indicate that vein-determined wing cells show a particular behaviour that is responsible for their allocation to vein regions. We observe strong genetic interactions between

*blistered*, *veinlet* and genes of the Ras signaling cascade. During disc proliferation, *blistered* expression is under the control of the Ras signal transduction pathway, but its expression is independent of *veinlet*. During the pupal period, *blistered* and *veinlet* expression become interdependent and mutually exclusive. These results link the activity of the Ras pathway to the process of early determination of intervein cells, by the transcriptional control of the *blistered* nuclear factor.

Key words: *blistered*, DSRF, Vein differentiation, Cell behaviour, Ras pathway, *Drosophila*

### INTRODUCTION

The adult wing of *Drosophila* consists of a bilayer of cells closely apposed by its basal face. Wings show a stereotyped pattern of five longitudinal and two transverse veins, which are cuticular sclerotizations enclosing hemolymph lacunae between dorsal and ventral wing surfaces. Veins have a characteristic corrugation, i.e. with either dorsal or ventral bulging, carry tracheae and nerves and provide structural support to the wing (García-Bellido and de Celis, 1992). The vein tissue is composed by cells that are smaller, more pigmented and more compacted than the intervein cells, which in turn form a transparent cuticle. This histotypic differentiation is closely coupled to the process of cell proliferation (González-Gaitán et al., 1994; de Celis et al., 1995). How the coordination of proliferation and specification of cell fates in the wing is achieved at genetic and molecular levels remains barely explored. Although a large group of mutations that affect the pattern of the veins in the wing of *Drosophila* has been described (García-Bellido and de Celis, 1992), only recently have the molecular elements acting in this process been identified.

In the wing, the *torpedo* (*top*) signaling cascade seems to be necessary for vein differentiation. *torpedo* encodes for the

*Drosophila* Epidermal Growth Factor (EGF) receptor tyrosine kinase homologue (DER) that activates a conserved signal transduction pathway. This pathway activates Ras and the Raf, MEK, MAPK cascade, which ultimately regulates the activities of specific transcription factors (see review by Perrimon, 1993). Viable mutant combinations and genetic mosaics of members of this pathway suppress vein formation (Díaz-Benjumea and Hafen, 1994). It is not known what signals activate the pathway, and how this implements the actual process of vein cell fate allocation and differentiation.

Genetic analyses have identified the putative transmembrane protein *Veinlet* as a specific regulator of DER signaling (Bier et al., 1990). Notably, *veinlet* viable mutants display a partial loss of the wing veins, which is greatly enhanced by mutations in DER or by mutations in genes required for Ras signaling. *veinlet* (*ve*; also known as *rhuboid* (*rho*)) is the earliest known gene to be expressed in the primordia of wing veins (Sturtevant et al., 1993, 1997) and genetic mosaics show that *veinlet* is required for vein differentiation, but does not affect cell proliferation (García-Bellido et al., 1994). Moreover, the ectopic expression of *veinlet* induces extra veins (Sturtevant et al., 1993).

In this paper, we focus on the *blistered* gene. Viable combinations of loss-of-function alleles of *blistered* show an

excess of vein tissue, the opposite phenotypes of those of *torpedo* and *veinlet* mutants. Moreover, in morphogenetic mosaics, cells homozygous for lethal alleles of *blistered* differentiate veins autonomously (Fristrom et al., 1994). *blistered* product is expressed in a pattern clearly associated to intervein regions and it encodes for the *Drosophila* homologue of the mammalian Serum Response Factor (SRF) (Montagne et al., 1996), a nuclear protein that regulates transcriptional activation in a complex with ETS transcription factors. Interestingly, the mammalian SRF activity seems to be regulated via a phosphorylation cascade involving the Ras pathway (Treisman, 1995).

We have studied the behaviour of *blistered* mutant cells in clones, and show that *blistered* is involved in cell fate allocation and differentiation, but does not participate in cell proliferation. The activity of *blistered* is necessary to specify cells to adopt an intervein fate. Its expression is repressed during larval stages in vein territories by Ras signaling. The differentiation of vein and intervein cells depends on the outcome of a fine-tuned balance between *blistered* and *veinlet* activities, which is achieved during pupal stages.

## MATERIALS AND METHODS

### Genetic variants

We have used the following genetic variants, described in Lindsley and Zimm (1992), unless otherwise stated. In the *blistered* locus, we use the loss-of-function alleles *bs<sup>2</sup>; Df(2R)Px<sup>1</sup>* and the *bs<sup>P1292</sup> [lac-Z, rosy<sup>+1</sup>]* insertion *l(2)03267* (Karpen and Spradling, 1992), also known as *pruned<sup>1</sup>* (Montagne et al., 1996). This insertion is a null allele of *blistered* and, although lethal in homozygosis (Guillemin et al., 1996), some escapers reach the adult stage. The Ras pathway mutants used were *top<sup>4A</sup>*, *top<sup>1</sup>*, *Elp<sup>B1</sup>*, (Díaz-Benjumea and García-Bellido, 1990b), *Sem*, *rl<sup>1</sup>* and *rl<sup>ems698</sup>*, (Díaz-Benjumea and Hafen, 1994) and *D-raf<sup>fl1-29</sup>* (Melnick et al., 1993). The alleles *ve<sup>1</sup>*, *ve<sup>M3</sup>*, *vn<sup>1</sup>*, *vn<sup>M2</sup>*, *vn<sup>ddrG436</sup>* (García-Bellido et al., 1994) and the Hs-*rho30A* transformant line as a source of ectopic *veinlet<sup>+</sup>* product (Sturtevant et al., 1993), were also employed. We used *emc<sup>1</sup>*, *emc<sup>P5C</sup>* (Garrell and Modolell, 1990), *px<sup>72</sup>* (Díaz-Benjumea and García-Bellido, 1990a), *Dp(3;Y;1)M2* (as *veinlet<sup>+</sup>* Duplication), *N<sup>55e11</sup>*, *fan<sup>d</sup>*, *Ax<sup>16172</sup>* and *Dl<sup>M1</sup>* (de Celis and Garcia-Bellido, 1994). For clonal analysis, we used *forked* (*f<sup>36a</sup>*) and the transgenes carrying the *f* wild-type allele designated as *P[f<sup>+</sup>]<sub>44</sub>*, *P[f<sup>+</sup>]<sub>52</sub>*, *P[f<sup>+</sup>]<sub>47</sub>* and *pawn* (*pwn<sup>1</sup>*) as cell markers (P. Martín and A. G. B., unpublished). *M(2)58F* was used for the *Minute<sup>+</sup>* experiments. For generation of clones in discs, we used a *D-raf<sup>fl1-29</sup>-FRT 10.1* recombinant and an *Ubiquitin GFP-FRT 10.1* line that provide ubiquitous expression of GFP throughout the imaginal discs (kindly provided by J. Duffy).

### Gal 4 and UAS lines

The MS-1096 GAL4 line (provided by I. Guerrero) is a homozygous viable insertion in the first chromosome of the GAL4 enhancer trap construct pGawB (Brand and Perrimon, 1993). MS-1096 GAL4 expression starts at early third instar stages on the dorsal wing pouch and expands later to the ventral surface (Capdevila and Guerrero, 1994).

UAS-KMRaf3.1 represents a dominant negative mutation in which the kinase catalytic domain has been inactivated (Baek et al., 1996). This DNA was introduced into the vector pUAST. Cloning details will be described elsewhere. UAS-DER, driving the expression of a wild-type DER was kindly provided by B. Shilo. UAS Ras1V12 was supplied by Alan Michelson.

### Generation of mitotic recombination clones

Mitotic recombination was either induced by X-rays (dose 1000 R;

300 R/minutes, 100 kV, 15 mA, 2 mm aluminium filter) or using the FRT/FLP system (Chou and Perrimon, 1992). In the first case, larvae were timed in hours after egg laying (AEL) and the clones initiated in separate experiments at 48-72 and 72-96 hours AEL. Mutant clones in the 2R arm were induced in male flies of the genotype *f<sup>36a</sup>; cn<sup>1</sup> bs<sup>P1292</sup>/ P[f<sup>+</sup>]<sub>44</sub> P[f<sup>+</sup>]<sub>52</sub> M(2)58F* or *f<sup>36a</sup>; pwn<sup>1</sup> top<sup>4A</sup> bs<sup>P1292</sup>/ P[f<sup>+</sup>]<sub>44</sub> P[f<sup>+</sup>]<sub>52</sub> M(2)58F* for the *Minute<sup>+</sup>* clones and *f<sup>36a</sup>; cn<sup>1</sup> bs<sup>P1292</sup>/pr<sup>1</sup> pwn<sup>1</sup> P[f<sup>+</sup>]<sub>44</sub> P[f<sup>+</sup>]<sub>47</sub>* for the twin analysis. For the induction of *D-raf* mutant clones, larvae aged 36±12 hours AEL of the genotype *D-raf<sup>fl1-29</sup> v FRT10.1/yw UbqGFP FRT10.1; HsFlp38* were heat shocked for 1 hour at 37°C, allowed to develop and then dissected at 100±12 hours AEL.

### Fly culture and staining procedures

Larvae and pupae of the appropriate genotypes were cultured at 25°C, unless otherwise stated, and timed in hours AEL or after puparium formation (APF). Heat shocks of 1 hour at 37°C were administered at 15 hours APF to Hs-*rho30A* stocks.

Vein nomenclature follows that of García-Bellido and de Celis, 1992.

In situ hybridisation was performed using digoxigenin-labelled *rho* RNA antisense probes following standard protocols for imaginal discs (Sturtevant et al., 1993; Lehner and O'Farrell, 1990). Anti-β-gal detection was done according to Cubas et al. (1991). Anti-*blistered* antibody, kindly provided by M. Affolter, was used at 1/300 dilution. Staining was detected using a Biorad MRC 1024 confocal microscope.

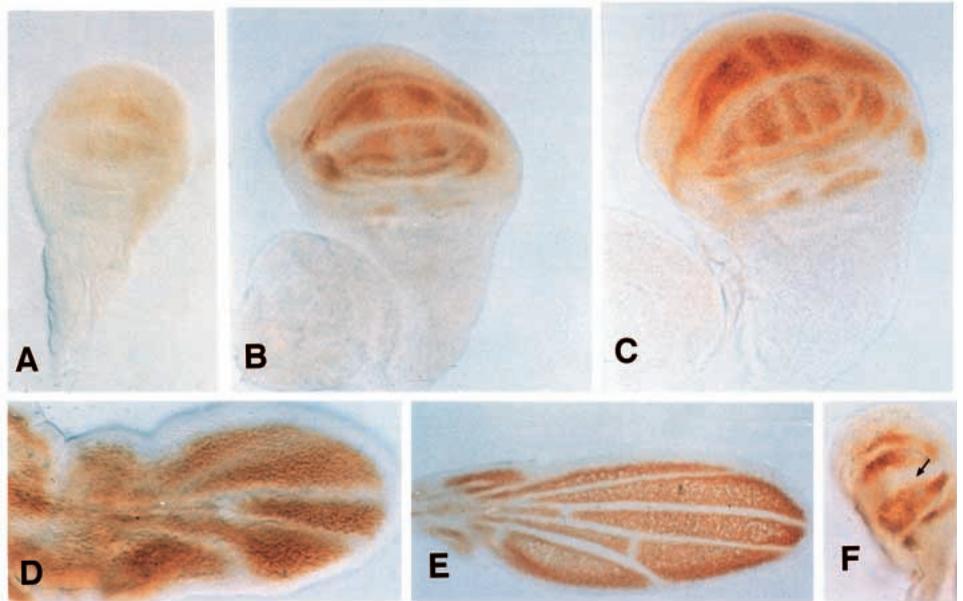
## RESULTS

### *blistered* is expressed during larval and pupal wing development

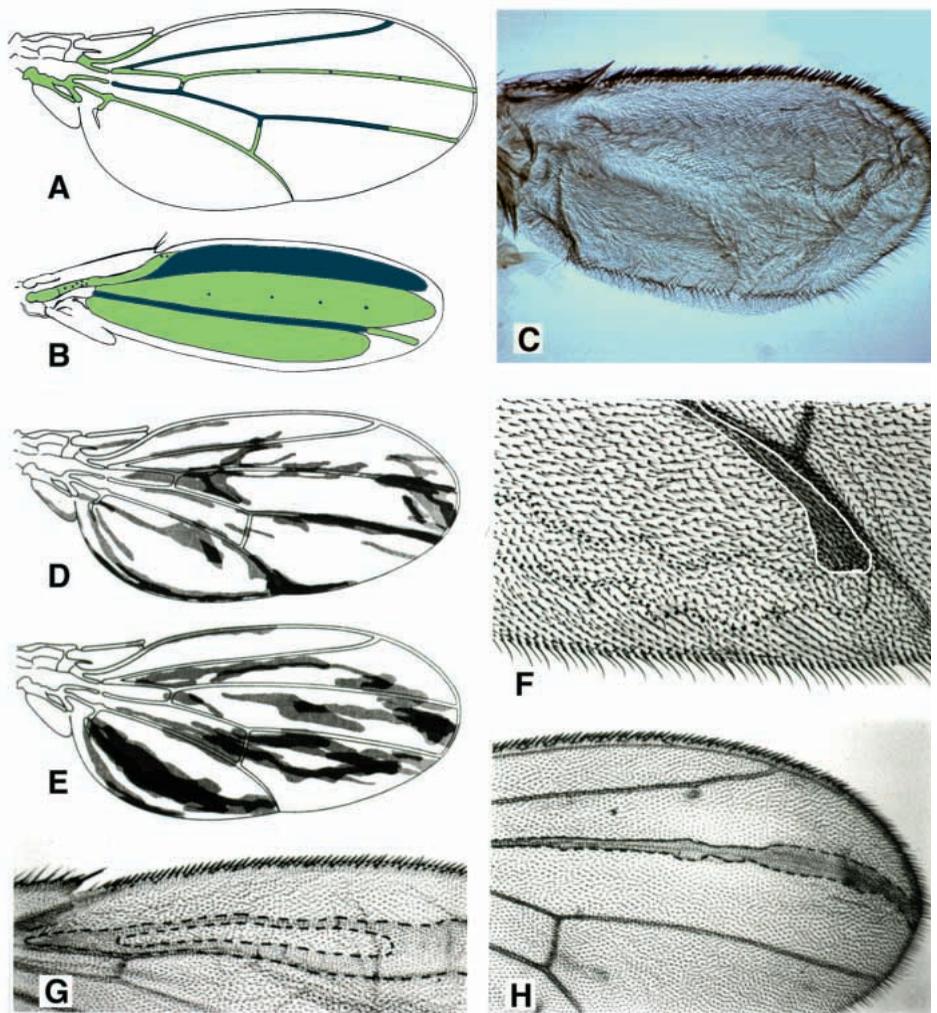
The pattern of expression of *blistered* in the third larval instar disc has been previously reported (Montagne et al., 1996). However, no detailed analysis of expression has been described. We first studied the evolution of this pattern from early larval to pupal development. In this analysis, we used a strain of *Drosophila* (*bs<sup>P1292</sup>*) that includes a *Plac-Z* insertion within the *blistered* gene and which shows a *lacZ* expression pattern in third instar imaginal wing discs indistinguishable from that observed with anti-Bs antibodies (Montagne et al., 1996; see below).

The expression of the *blistered* reporter is first detected in imaginal wing discs after 70-80 hours AEL (early third instar larva) (Fig. 1A). In this stage, it is expressed homogeneously at low levels throughout the wing pouch, except in the presumptive wing margin. Mid-third instar imaginal wing discs (80-100 hours AEL) reveal increasing *blistered* levels, except in the wing margin and three perpendicular stripes of cells corresponding to veins, where *blistered* expression begins to fade (Fig. 1B). At approximately the same developmental stage *veinlet* is expressed in stripes corresponding to the gaps in *blistered* expression (Sturtevant et al., 1997). The future veins L3, L4 and L5 will arise from these gaps. In late third instar imaginal wing discs (100-120 hours AEL), a further gap appears in the *blistered* expression, revealing the presence of the L2 vein (Fig. 1C). At this stage, we also detect a complex modulation of *blistered* expression in the hinge region, possibly corresponding to the proximal vein trunks and interveins. There is also no expression in the notum (Fig. 1C). These gaps in the expression of *blistered* become more conspicuous in everted discs of pupae (Fig. 1D,E) and, by 24-30 hours APF, all the

**Fig. 1.** *blistered* expression in wild-type wing discs. The pattern of *blistered* expression was monitored in *bs<sup>P1292</sup>P-lacZ* discs, from the second larval instar until pupae 24–30 hours APF, by antibody staining against  $\beta$ -gal. (A) Early third instar wing disc. Staining of the wing pouch is interrupted by a gap that correspond to the D/V boundary. (B) Wing disc from to mid third instar larva. At this stage, areas of weak staining corresponding to future veins are identifiable. (C) Late third instar wing disc. Intervein cells show increased levels of  $\beta$ -gal expression, whereas weak levels persist in the vein primordia. (D) Wing disc aged 4–7 hours APF. *blistered* staining is not detectable at the D/V boundary and is diminished within most vein primordia. Notice the complex pattern evolving in the hinge region and the anterior and posterior crossveins. (E) Wing at 24–30 hours APF. Final pattern of veins is apparent. Notice that vein width is about 6– to 8-cell diameters. (F) Late third larval instar haltere disc showing high levels of *blistered* expression in the presumptive pedicellum and scabellum, whereas the presumptive capitellum lacks staining (arrow).



**Fig. 2.** Analysis of the *bs<sup>P1292</sup>* allele in adult flies and morphogenetic mosaics. (A) Plot showing the corrugation of the veins in wild-type wing. Dark green represents ventrally corrugated veins, whereas light green marks dorsally corrugated regions. (B) Plot showing the corrugation observed in mutant *bs<sup>P1292</sup>* wings, using the same color code as in A. We were not able to identify unambiguously the corrugation of the regions showed in white. (C) Mutant wing of pharate adults homozygous for the *bs<sup>P1292</sup>* allele. The whole wing blade is transformed into vein tissue whereas the hinge region is almost intact. (D) Plot of *forked bs<sup>P1292</sup>* clones initiated at 48–72 hours AEL. Positions frequently occupied by clones are indicated as darker regions. Notice the concentration of clones in the proximity of vein positions. (E) Plot of *pawn* twin non-mutant control clones obtained in the same experiment, showing the normal distribution of clones at this stage. Clones appear to be excluded from veins. (F) Example of a clone *forked bs<sup>P1292</sup>* (white line) and its twin *pawn*  $+/+$  (dotted line). Mutant cells autonomously differentiate vein tissue. Note the wiggly border of the *pwn* clone, compared to the smooth contour of the mutant clone. (G) An example of a *bs<sup>P1292</sup> Minute<sup>+</sup>* clone that surrounds a patch of wild-type tissue. Clone contour is indicated by a dashed line. (H) Dorsal *bs<sup>P1292</sup> Minute<sup>+</sup>* clone running over L3 (dashed line). Control clones induced at this stage occupy an average of two intervein regions, whereas mutant cells are in this case trapped within veins.



interveins are apparent (Fig. 1E). *blistered* expression must be further refined after this stage, as the stripes lacking *blistered* are now about 6-8 cells wide, whereas in the adult wings they are only 3-5 cells wide (Montagne et al., 1996). In adult wings, all vein cells lack *blistered* expression, which is present in all the intervein cells (Montagne et al., 1996).

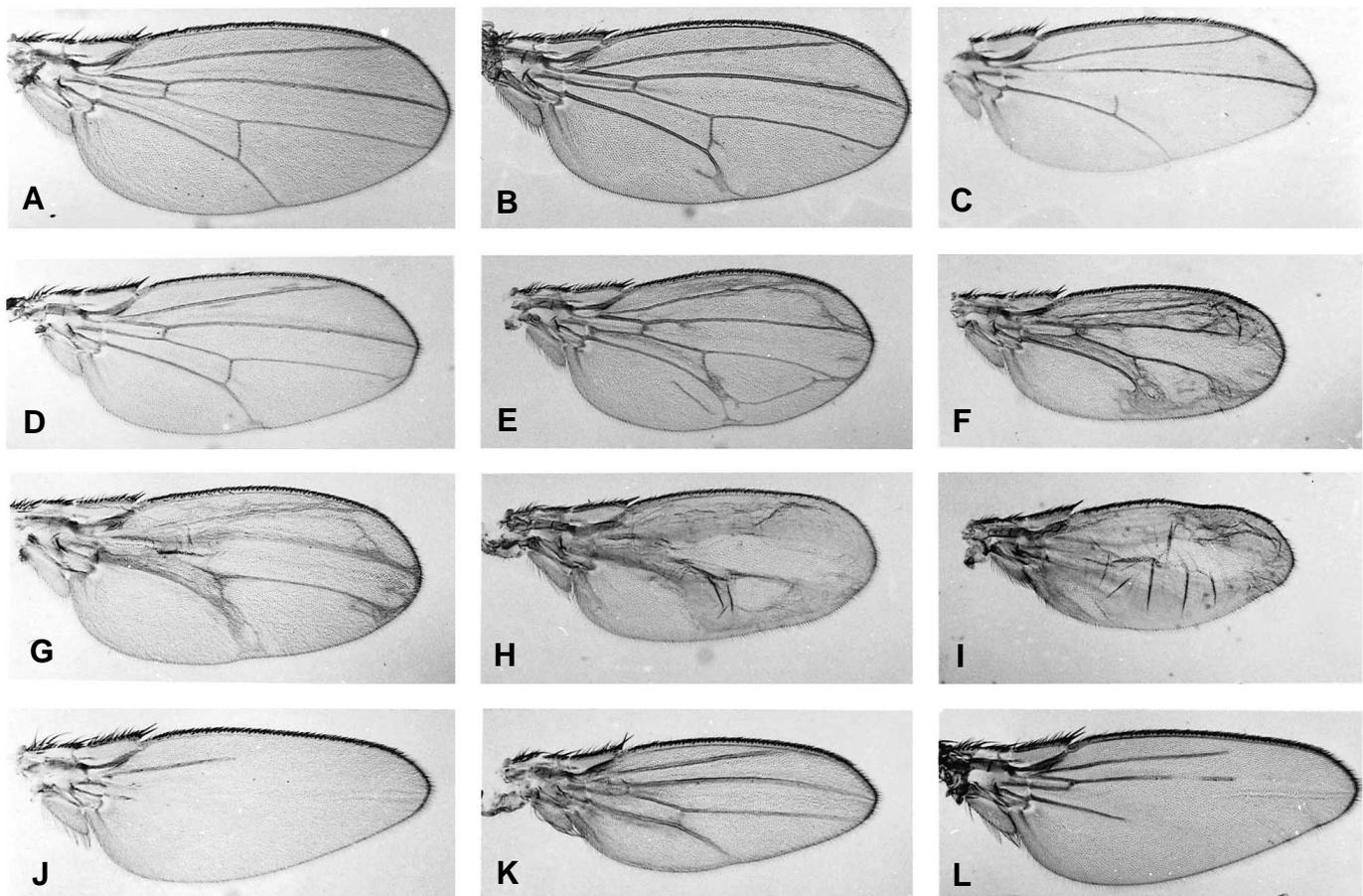
The only other imaginal disc to express *blistered* is the haltere. *blistered* is expressed at high levels in the region corresponding to the pedicellum and scabellum, but is not present in the presumptive capitellum, the homologous region to the wing pouch (Fig. 1F). It is thus tempting to speculate that the absence of transalar connections and lack of apposition of dorsal and ventral surfaces in the haltere capitellum might be related to this non-appearance of *blistered* expression, as occurs in the hollow wing veins.

We have also monitored the expression of  $\beta$ -galactosidase in third instar discs homozygous for the *bs-lac-Z* insertion and thus completely deprived of *blistered* function. These discs are normal in size and morphology. Their  $\beta$ -galactosidase pattern is identical to that observed in controls (data not shown), implying that at this stage *blistered* expression is independent of endogenous *blistered* activity.

### Corrugation pattern is not affected in the *blistered* null condition

In order to characterise the function of *blistered* in imaginal disc development, we examined the phenotype of flies mutant for the null allele *bs<sup>P1292</sup>*. Rare homozygous mutant escapers are found to die in the adult pharate stage and have been examined in search of cuticular defects. In the wing pouch of these animals, all cells differentiate as vein tissue, whereas both the wing margin sensory organs and the pattern of campaniform sensillae along the normal L3 are in the expected positions (Fig. 2B,C). However, some ectopic bristles and sensillae appear occasionally in internal regions of the wing. The proximal regions of the wing, corresponding to the hinge are barely affected. Other structures in the animal display no phenotypes, except the legs, which often show shortening of the tarsal segments and bent femurs (not shown).

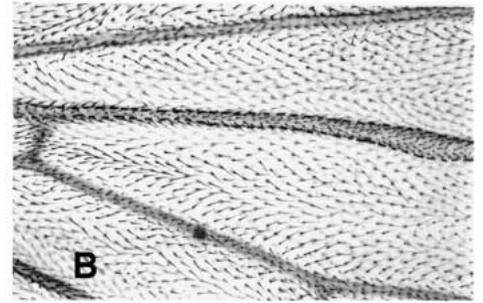
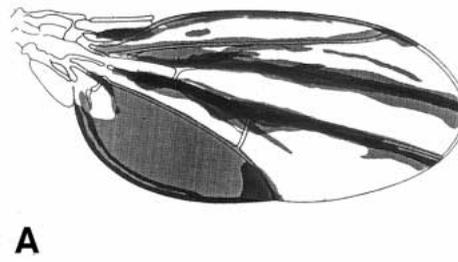
A constant feature of each adult vein is its characteristic dorsal or ventral bulging, namely, its corrugation. The corrugated side has thicker and more packed trichomes than the opposite side, and the cuticle is more pigmented. The pattern of corrugation is specific for each vein. In the wild-type condition, dorsal corrugation is found in the longitudinal veins



**Fig. 3.** Wing phenotypes resulting from genetic interactions between *blistered* and mutations affecting vein differentiation. (A) Wild-type adult wing. (B) *bs<sup>P1292</sup>/+* wing showing a typical phenotype, due to the *blistered* haploinsufficiency and consisting of small patches of ectopic veins in the L2 and L5 proximity. (C) *top<sup>4A</sup>/top<sup>1</sup>*. (D) *top<sup>4A</sup> bs<sup>P1292</sup>/top<sup>1</sup>*, in which L4 differentiation is rescued. (E) *Sem/+*. (F) *Sem/bs<sup>P1292</sup>*. (G) *Hs-rho30A/+* strong constitutive extravein phenotype found at 25°C. (H) *bs<sup>P1292</sup>/+; Hs-rho30A/+* at 25°C. (I) *bs<sup>P1292</sup>/bs<sup>2</sup>* wing, showing the most extreme viable *blistered* mutant phenotype. (J) *ve<sup>M3</sup> vn<sup>M2</sup>/ve<sup>1</sup> vn<sup>1</sup>*. (K) *bs<sup>P1292</sup>/bs<sup>2</sup>; ve<sup>M3</sup> vn<sup>M2</sup>/ve<sup>1</sup> vn<sup>1</sup>*, notice the wild-type vein pattern displayed. (L) *bs<sup>P1292</sup>/+; ve<sup>M3</sup> vn<sup>M2</sup>/ve<sup>1</sup> vn<sup>1</sup>*. All pictures were taken of females at the same magnification.

**Fig. 4.** Clonal analysis of the double mutant combination *top<sup>4A</sup> bs<sup>P1292</sup>*.

(A) Plot of frequencies of the recovered *Minute<sup>+</sup> top<sup>4A</sup> bs<sup>P1292</sup>* clones. Positions frequently occupied by clones are indicated as darker regions. Size of clones is reduced with respect to control *Minute<sup>+</sup>* clones and is similar to that of *top<sup>4A</sup> Minute<sup>+</sup>* clones (Díaz-Benjumea and García-Bellido, 1990b). Note the accumulation of clones observed in vein territories. (B) Dorsal *Minute<sup>+</sup> top<sup>4A</sup>*



*bs<sup>P1292</sup>* clone in the vein L3 (enclosed by a dashed line). The mutant clone differentiates as a vein and is composed of cells reduced in size with respect to the cells that correspond to vein tissue. The double mutant cells are smaller than either *blistered<sup>-</sup>* mutant cells or *torpedo<sup>-</sup>* cells alone, a fact interpreted as the superimposition of the *torpedo<sup>-</sup>* typical small cell phenotype to the *blistered<sup>-</sup>* condition.

L3, L5 and in the distal end of L4, whilst ventral corrugation is restricted to L2 and the proximal region of L4 (Fig. 2A). In *blistered* mutant wings, we observe corrugation throughout the wing blade. We observe the presence of broad territories showing dorsal or ventral corrugation separated by sharp boundaries. These areas are spatially arranged as in the wild-type condition (Fig 2B), suggesting that the positional cues controlling corrugation are independent of vein cell fate allocation. Interestingly, all the wing cells have information relative to acquisition of corrugation, even if they do not normally differentiate as veins.

#### Clonal analysis reveals that *blistered* function affects cell behaviour in larval stages

Fristrom and coworkers (1994) have shown by clonal analysis that all homozygous *blistered* mutant cells differentiate autonomously into a characteristic vein histotype regardless of where they lie in the wing blade, indicating that the *blistered* product is required autonomously for the differentiation of interveins. This effect has been attributed to a late function of *blistered* observed in pupal stages. However, we found that *blistered* is expressed from early third instar larval stages. In order to analyse the significance of this early expression, we studied the cellular behaviour of mutant cells in mosaic clones for the null allele *bs<sup>P1292</sup>*.

In a first experiment, we compared the behaviour of mutant *bs<sup>P1292</sup>* cells (marked with *forked (f)*) with that of *+/+* twin cells (marked with *pawn*) generated in the same recombination event. *blistered<sup>-</sup>* clones initiated between 48 and 72 hours AEL, at the onset of *blistered* expression, and have the same number of cells as their *+/+* twins (1.14 average ratio number mutant cells/number cells in control clone). This demonstrates that *blistered* function is not required for cell viability or cell division during larval stages. In addition, we notice that the position of mutant and wild-type twin clones is not equivalent, *blistered<sup>-</sup>* clones appearing preferentially within or along veins (78% abutting veins) while the twin clones (*+/+*) and clones, induced at the same age in control experiments, are more likely to lie entirely within intervein territories (only 36% abutting veins) (Fig. 2D,E). *blistered* mutant clones have a characteristic narrow and elongated shape, different from controls. Moreover, in intervein territory, the mutant clone contour is always sharply separated from the rest of the tissue by a smooth border, in contrast to control clones with indented borders (Fig. 2F). This is true both for clones running along veins and also

for those appearing in interveins, which also differentiate a vein histotype. These clonal results indicate that, in addition to vein differentiation, *blistered* also affects cell behaviour during the proliferation of the wing disc.

In a second experiment, we generated *Minute<sup>+</sup>* mosaics homozygous for the *bs<sup>P1292</sup>* allele in a *Minute (M)* heterozygous background. In these mosaics, *Minute<sup>+</sup>* cells outgrow the surrounding heterozygous territories (see Morata and Ripoll, 1975). Clones were induced by irradiation at two developmental stages, 48-72 and 72-96 hours AEL. Among the early induced clones, we observe large, but narrow, ones that run on the veins or along the wing margin (including the posterior margin, which in wild-type wings does not differentiate a vein histotype; Fig. 2H). Thus, these *Minute<sup>+</sup>* cells have the same abnormal behaviour as clones in the previous experiment, despite its great advantage in proliferation. In these animals, we also find rare clones that completely surround a patch of wild-type tissue which still differentiates intervein tissue (Fig. 2G). These clones, never observed in controls, reinforce the idea that fast proliferating *Minute<sup>+</sup> blistered* mutant cells are less able to occupy intervein territories than *blistered<sup>+</sup>* cells. Control *Minute<sup>+</sup>* clones initiated at 48-72 hours AEL fill an average of 2.3 intervein regions, with clone borders often running within intervein regions. Conversely, we find that the largest recovered *blistered<sup>-</sup> Minute<sup>+</sup>* clones occupy and fill only up to one intervein region. The borders of the clones always run along veins, as if mutant cells would be forced to remain within the intervein region where they originated, due to the presence of vein-intervein restriction border. These large mutant territories have nearly the same number of cells as non mosaic contralateral sib-wings, confirming that proliferation is not impaired by the loss of *blistered* function.

#### Genetic interactions reveal functional relationships between *blistered*, *veinlet* and *torpedo*

##### (A) Adult phenotypes

In order to establish functional relationships between *blistered* and other genes involved in vein patterning, we studied the genetic interactions of *blistered* viable mutant combinations with several mutations affecting vein development. All these results are summarised in Table 1.

We found strong, superadditive, interactions between *blistered* and mutations in genes of the Ras pathway in *Drosophila*: i.e., *DER (torpedo)*; Díaz-Benjumea and Hafén,

**Table 1. Genetic interaction between blistered and genes involved in vein patterning**

Combination	<i>bs<sup>P1292/+</sup></i> phenotype	Tester phenotype
<i>top<sup>4A/top<sup>1</sup></sup></i>	--	--
<i>r11/r1ems698</i>	--	--
<i>Sem/+</i>	+++	+++
<i>vn<sup>dddRG436/vn</sup></i>	--	--
<i>Hs-rho30A/+</i>	+++	+++
<i>Dp(3;Y;1)M2ve+</i>	+	+
<i>ve vn/+</i>	-	-
<i>ve<sup>M3</sup> vn<sup>M2</sup>/ve vn</i>	--	--
<i>emc<sup>1</sup>/emc<sup>P5C</sup></i>	++	++
<i>px<sup>72</sup></i>	+++	+++
<i>Ax<sup>16172</sup></i>	--	--
<i>N<sup>55e11/+</sup></i>	=	=
<i>fa<sup>nd</sup></i>	+	+
<i>DI<sup>M1</sup></i>	+++	+++

(First column) The variations (enhancements or suppressions) of the extra vein phenotype of *bs<sup>P1292/+</sup>* wings in different mutant backgrounds; (second column) how the phenotypes of different mutations are affected by *bs* haploinsufficiency (*bs<sup>P1292/+</sup>*).

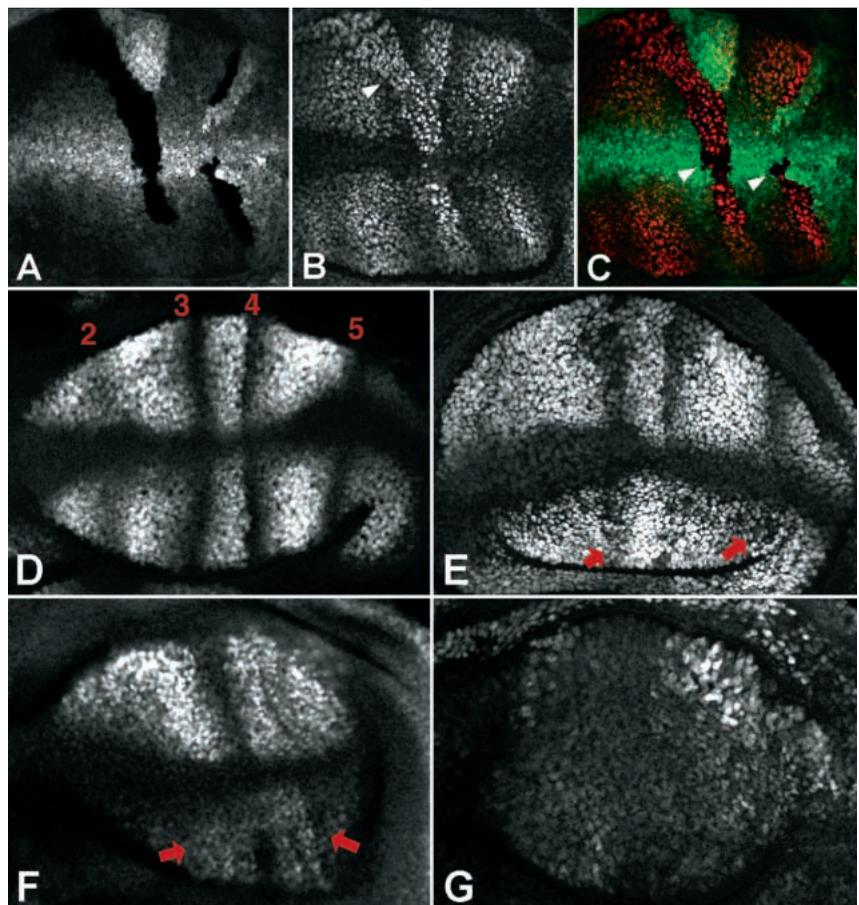
-, weak suppression; --, moderate suppression; ---, strong suppression; + weak enhancement; ++, moderate enhancement; +++, strong enhancement of the mutant vein phenotype; =, no variation.

1994), the MAPK (Mitogen-Activated Protein kinase; Bruner et al., 1994), *rolled* (*rl*), and a putative ligand of DER, *vein*, that codes for a neuregulin secreted protein (Simcox et al.,

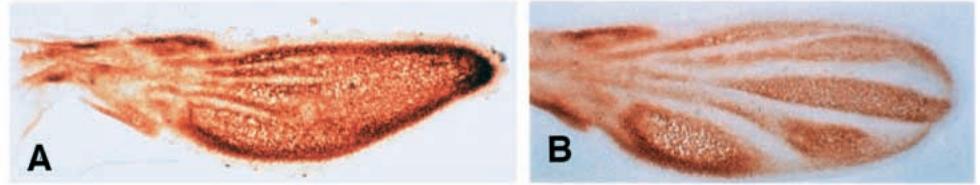
1996). Hemizyosity for *blistered* totally suppresses the lack of vein L4 phenotype of *torpedo*, *rolled* and *vein* homozygous mutants (Fig. 3C,D), while greatly enhances the amount of ectopic vein observed in the gain-of-function *rolled* allele *Sem* (Fig. 3E,F). *blistered* hemizyosity also suppresses the lack of veins in *veinlet* hypomorph conditions (see also Fristrom et al., 1994). Conversely, it dramatically enhances the amount of ectopic vein tissue obtained after ubiquitous expression of *veinlet<sup>+</sup>* product using the Hs-*rho* 30A construct, even when no heat-shock pulses are administered (Fig. 3G,H).

The observed interaction between *torpedo* and *blistered* in hypomorphic conditions led to the study of double mutants for strong alleles of both genes in mosaic clones. Double mutant clones were generated at 48-72 hours AEL for the *top<sup>4A</sup>* and *bs<sup>P1292</sup>* alleles, taking advantage also of the *Minute<sup>+</sup>* technique to favour cell viability. *top<sup>4A</sup>* clones appear with a reduced frequency, are smaller, narrower and more elongated than controls, and are composed of small cells that are unable to differentiate vein histotype, leaving a gap of intervein tissue wherever they touch a vein, except in the anterior wing margin vein (L1) (see below; Díaz-Benjumea and García-Bellido, 1990b). Double mutant *M<sup>+</sup>top<sup>4A</sup> bs<sup>P1292</sup>* tend to occupy vein territories like *bs<sup>P1292</sup>* clones (Fig. 4A), a preference never observed in *top<sup>4A</sup>* clones, but appear with a frequency and size similar to *top<sup>4A</sup>* controls. Double mutant cells differentiate autonomously, in all cases, into a pigmented, corrugated and compacted tissue with smaller cells than those characteristic of *torpedo* (Fig. 4B). The observation of these typical vein

**Fig. 5.** Ras pathway activity modify *blistered* expression in third-instar wing discs. (A) Wing disc showing three D-*raf<sup>11-29</sup>* mutant clones identified as patches devoid of GFP expression. (B) Pattern of expression of *blistered* monitored with anti-Blistered antibody in the same disc, note the patch of mutant cells expressing high levels of Blistered, despite its position over the L3 vein (arrowhead). (C) Superimposition of both images showing that the mutant cells autonomously overexpress Blistered except in the region corresponding to the D/V boundary (arrowheads). (D-G) The expression of Blistered protein in backgrounds in which different constructs are overexpressed under the control of the MS-1096 GAL4 line, which is mainly active in the dorsal side of the disc and is weakly expressed in the ventral one. (D) Control wild-type third instar wing disc, the region without *blistered* expression correspond to the veins. The numbers indicate the different veins in the adult wing. (E) Disc expressing a Raf dominant negative form. Blistered is overexpressed mainly in the dorsal compartment, which is also reduced in size. We observed high levels of Blistered in the presumptive veins regions (arrows). (F) Wing disc where Torpedo is overexpressed. We observe that the expression of *blistered* is suppressed mainly in the dorsal wing pouch (arrows). (G) *blistered* expression disappears almost completely when a constitutively active form of Ras is overexpressed.



**Fig. 6.** *blistered* expression in pupal wings of *veinlet<sup>-</sup> vein<sup>-</sup>* and *Hs-rho 30A* mutant background. (A) *bs-lacZ* expression in *ve<sup>M3</sup> vn<sup>M2</sup>/ve vn* pupal wings dissected 30 hours APF. The expression of *blistered* occupies the distal parts of all vein territories and is also found at higher levels within the extant veins of the proximal region than in wild-type veins (compare to Fig. 1E). (B) *blistered* expression in *Hs-rho30A/+* pupal wing dissected 30 hours APF, heat shocked at 37°C for 1 hour at 15 hours APF. Width of stripes deprived of expression and corresponding to vein tissue are dramatically increased, while general staining levels are clearly diminished within the intervein regions (compare to Fig. 1E). In both A and B, positions void of *blistered* staining coincide precisely with veins present in the corresponding mutant adult wings (Fig. 3L,H, respectively).



features allow us to conclude that this tissue has a vein histotype indicating that the *blistered* extra vein phenotype is epistatic to *torpedo* lack of veins.

We also test the ability of *blistered* mutations to suppress the phenotype observed in extreme mutant viable combinations of other loci that show a total lack of veins. For this purpose, we use several *veinlet vein* combinations that prevent vein differentiation (Díaz-Benjumea and García-Bellido, 1990a). Strong *veinlet vein* combinations are epistatic to weak *blistered* alleles and show an incomplete correction of the lack of veins phenotype (i.e. *bs<sup>P1292/+</sup>; ve<sup>M3</sup> vn<sup>M2</sup>/ve vn* and *bs<sup>2</sup>; ve vn* (Fig. 3L; Fristrom et al., 1994). The strong viable *bs<sup>P1292</sup>/bs<sup>2</sup>* combination displays almost exclusively vein tissue (Fig. 3I). However, the triple mutant combination *bs<sup>P1292</sup>/bs<sup>2</sup>; ve<sup>M3</sup> vn<sup>M2</sup>/ve vn* show an almost wild-type vein pattern (Fig. 3K).

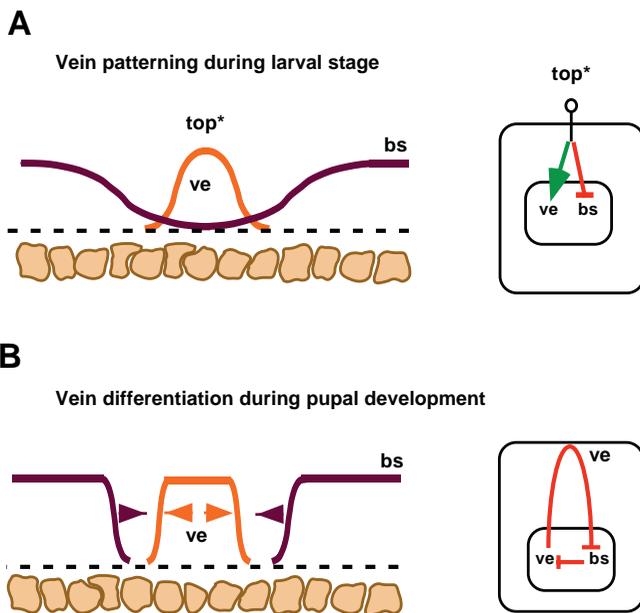
Furthermore, for stronger allelic combinations of *blistered*, the excess of veins is epistatic to the *ve vn* phenotype (Montagne et al., 1996). Taken together, these experiments indicate that, in mutant combinations, the final amount of vein tissue is the result of a balance between the antagonistic effects of *veinlet/vein* and *blistered* in vein promotion/suppression.

*blistered* also presents strong interactions with other mutants such as *Notch (N)* or *Delta (Dl)* which affect vein width (de Celis and García-Bellido, 1994) (see Table 1). *blistered* hemizyosity enhances the thickened vein phenotype of *Dl<sup>M1</sup>* and *N<sup>55e11</sup>/fa<sup>nd</sup>* and restores the veins that are missing in *Ax<sup>16172</sup>* males.

**(B) Gene expression patterns**

To determine if the suppression of *blistered* activity necessary for vein differentiation depends on its transcriptional repression, we studied the *blistered* pattern of expression in different mutant backgrounds affecting vein development.

We analysed the pattern of expression of *blistered* in wing cells mutant for a null allele of *D-Raf*. We induced mutant mitotic recombination clones marked as patches of tissue devoid of GFP expression, by using the FRT/FLP system (see Materials and Methods). Although proliferation is impaired in these cells and the size of clones is reduced with respect to controls, we scored patches of 100-200 cells that show always high levels of *blistered* when they occupy vein territories (Fig. 5A-C). Interestingly, this does not occur in the stripe of 4-6 cells corresponding to the D/V boundary, where we have not detected any *blistered* upregulation. Removal of *D-Raf* activity results in loss of vein differentiation in adult wings, except in the anterior part of the wing margin, where vein tissue normally differentiates (not shown) (see also Díaz-Benjumea and Hafen, 1994). To confirm the role of the Ras pathway repressing *blistered* expression, we ectopically expressed dominant negative or activated forms of members of the Ras pathway using the UAS/Gal 4 system (Brand and Perrimon, 1993) (see Materials and Methods). In wing discs expressing a Raf dominant negative protein (UAS-KMRaf3.1) (Fig. 5E) under the control of the MS-1096 GAL4, *blistered* is misexpressed in vein territories at high levels throughout the wing pouch, mainly in dorsal areas, where Gal4 expression is enhanced, thus differing from control wings (Fig. 5D). Conversely, the overexpression of Torpedo (UAS-DER) (Fig. 5F), which at high concentration has constitutive activity, or activated Ras protein (UAS-Ras1V12) (Fig. 5G) suppresses most of the *blistered* expression in the wing pouch. Taken together, these data indicate that the role of the Ras pathway during normal vein patterning is to repress *blistered* transcription within presumptive vein territories.



**Fig. 7.** Model of *blistered*, *veinlet* and *torpedo* function in vein formation. (A) During larval development, we postulate that there exists a landscape of positional values responsible for *blistered* and *veinlet* expression allocation. Ras pathway activity is necessary to repress *blistered* and to activate *veinlet* in vein domains. (B) In pupal stages, *veinlet* and *blistered* expression is interdependent and mutually exclusive, and thus the two products repress each other at the transcriptional level. This mechanism guarantees the proper allocation of each cell to a vein or intervein fate, refining thereby the vein pattern.

To study whether the observed antagonism between *blistered* and *veinlet/vein* activities operates on the transcriptional control of *blistered* and/or *veinlet* during vein development, we first examined the pattern of expression of *blistered* in loss-of-function ( $ve^{M3} \text{ } vn^{M2}/ve \text{ } vn$ ) and gain-of-function (by means of the *Hs-rho30A* line) conditions for *veinlet*. In both backgrounds, *blistered* pattern in larval discs is indistinguishable from wild type (data not shown), indicating that the expression of *blistered* is not affected by changes in *veinlet* and *vein* levels at this stage (see also Montagne et al., 1996). However, in  $ve^{M3} \text{ } vn^{M2}/ve \text{ } vn$  pupal wings as early as 4 hours APF, *blistered* expression begins to invade the distal part of the wing, in regions normally occupied for veins, differing from controls (data not shown). At 27 hours APF, high levels of *blistered*, equivalent to those present in intervein regions, are visible in the distal and medial part of the wing in regions where the veins appear in controls, in close correspondence with the patches of vein which are eliminated in the mutant wing (Figs 6A, 3L). Conversely, after ectopic expression of *veinlet*<sup>+</sup>, large patches of tissue, which closely correspond to ectopic veins in the adult, lack *blistered* expression (Figs 6B, 3G) (see Materials and Methods). These results demonstrate that, in pupal stages, but not before, *blistered* expression is dependent on *veinlet* activity.

We also analysed the effects of different *blistered* levels in the pattern of expression of *veinlet*. The few recovered homozygous escapers of the null allele  $bs^{P1292}$  show a normal wild-type pattern of *veinlet* expression in third larval instar (data not shown, see Fristrom et al., 1994), suggesting that initiation of *veinlet* expression is independent of *blistered* at this stage.

## DISCUSSION

### Role of *blistered* during wing disc proliferation

Previous work point out that *blistered* function is involved in the process of vein differentiation and it operates only in pupal stages (Fristrom et al., 1994). We show here that *blistered* is expressed in the presumptive interveins in early third larval instar and that its activity is necessary for the allocation of cell fates in the wing disc.

The function of *blistered* is to promote intervein over vein differentiation. This requirement seems to be still in place up to late pupal stage, as clones of 4–5 cells generated in late third larval instar also differentiate vein tissue autonomously (Montagne et al., 1996). In the twin clonal analysis and mutant wings, pattern elements other than veins (i.e. corrugation and sensory organs) are always correctly positioned, which indicates that the mechanisms controlling the positioning of sensory organs and corrugation are independent of vein histotypic differentiation. Analysis of *blistered* null mutant wings shows that proliferation of *blistered* cells is not impaired. This is confirmed by the finding that mutant clones have the same number of cells as the twin controls. Thus, *blistered* function does not affect cell division or the general patterning of wing structures, but only the specification of vein and intervein tissues.

When clones of *blistered*<sup>−</sup> cells are generated at the onset of *blistered* expression, they become abnormally distributed,

appearing concentrated along or within veins. In twin experiments, the mutant cells always keep contact with the twin wild-type clone, indicating that no cell migration occurs, and that the abnormal distribution detected may be due to a process of preferential cell reallocation or positional rearrangement of each recently divided mutant cell (Milán et al., 1996). The early initiated *Minute*<sup>+</sup> clones, which in control experiments can fill whole compartments and may have borders within intervein regions, in *blistered*<sup>−</sup> mutant remain trapped between veins (occupy only one intervein region), with clone borders running along veins.

Preferential allocation to veins of cells with vein specification can be understood if patterning and proliferation are two interdependent processes. Presumptive veins first appear early in disc development associated with clonal restriction borders separating units of proliferation, i.e. interveins (González-Gaitán et al., 1994). According to the Entelechia model (see de Celis et al., 1995), the proliferation of the disc would be driven by differences in positional values between neighbouring cells. Maximal positional values locate along clonal restrictions, where veins will arise, decreasing gradually towards intervein positions. As proliferation proceeds the differences get levelled. Intercalar growth will continue while discernible differences in positional values persists between neighbouring cells. At the end of the proliferation process, value differences between cells will be minimal. Cell fates are specified during this process of proliferation and those cells with the highest positional values will differentiate as vein tissue.

*veinlet* and *blistered* are expressed in patterns that appear to be coupled to the postulated positional values and their function could be ligated to the interpretation of positional information in the process of differential fate allocation of vein and intervein cells. *blistered*<sup>−</sup> clones appear preferentially within or along veins. Clones of *veinlet*<sup>+/veinlet</sup><sup>+</sup> cells growing in a *veinlet* heterozygous background, also grow close to the veins (García-Bellido et al., 1994). Similarly, *Notch*<sup>−</sup> clones, which cause to cells differentiate autonomously to vein, also survive along veins (de Celis and García-Bellido, 1994). Thus, cells not expressing *blistered* or expressing *veinlet* will be determined early in third larval instar to acquire vein histotype, which is associated with maximal positional values, leading to cell reallocation to veins after each division. Thus, veins are formed by cells generating the highest positional values. The preferential rearrangement of cells may reflect the differential expression of adhesion molecules. Studies on reaggregation of cell dissociates (García-Bellido, 1966, 1972) show in fact that preferential adhesion properties allow the reconstruction of partial patterns by cells not yet differentiated but positionally labelled, suggesting that the developing discs cells contain precise positional information, related to morphogenesis.

### Initiation of vein patterning

Several lines of evidence show that the Ras pathway plays a key role in the process of vein formation (Díaz-Benjumea and García-Bellido, 1990b). Most of the known genes affecting vein development are linked genetically or molecularly to the activity of the Ras pathway, as is the case of *veinlet*, *Star*, *argos* and *vein* (Sturtevant et al., 1993; Heberlein et al., 1993; Sawamoto et al., 1994; Schweitzer et al., 1995; Simcox et al., 1996). We have found strong interactions in viable double

mutant combinations in which both *blistered* and the Ras pathway activities are affected. In all cases, both mutant phenotypes appear to be mutually suppressed, indicating that the two wild-type functions act antagonistically. Double mutant cells for *torpedo* and *blistered* differentiate as veins, thus locating *blistered* downstream of the Ras pathway.

The Ras pathway activity appears to be necessary for the continued downregulation of *blistered* transcription in presumptive vein cells. We have shown that *blistered* is indeed transcriptionally repressed in combinations where activated forms of Ras pathway members are overexpressed by means of the UAS/Gal4 system. Conversely, *blistered* is found ectopically expressed in veins if Ras signaling is impaired, in mitotic clones mutant for *D-Raf*, and by the overexpression of dominant negative forms of Raf or DER. Interestingly, in none of these experiments, have we found *blistered* overexpression along the wing margin, which correlates with the observation that *top* clones never prevent the differentiation of the L1 vein. This suggests that *blistered* is not repressed by the Ras signaling cascade at the D/V boundary and that, from the generative point of view, the L1 vein is different from the rest of veins.

The regulation of *blistered* function by the Ras pathway occurs in the veins of *Drosophila* at the level of transcription, a result not predicted in view of the links proposed between this signaling cascade and the SRF (the *blistered* homologue) in other systems, where its regulation activity seems to operate by phosphorylation events (Treisman, 1995). *blistered* reporter expression in a *blistered* null mutant background is indistinguishable from wild type, and this excludes that *blistered* repression in the veins would depend on an autoregulatory loop initiated after early phosphorylation of Blistered protein, induced by the Ras pathway.

The low viability corresponding to the *torpedo* phenotype is retained in double mutant clones for *blistered* and *torpedo*, suggesting that Ras pathway activity has other targets controlling proliferation independently of *blistered*.

The positional cues responsible for *blistered* repression within vein territories appear to be also responsible for the activation of *veinlet* in its normal pattern. Combinations in which the Ras pathway activity is lowered show reduced levels of *veinlet* expression in third larval instar (E. Martin-Blanco, E. Noll, J. Duffy, F. Roch, A. Baonza and N. Perrimon, unpublished data) while, in combinations in which there is hyperactivity of the pathway, we observe ectopic expression of *veinlet* at this stage (A. Baonza, unpublished results). Our results show that the initiation of the *veinlet* pattern of expression is directed by cues independent of *blistered* function; in fact, we observe a wild-type pattern of *veinlet* in wing discs of null mutants for *blistered*. We also found that *blistered* expression is totally independent of *veinlet* activity at this stage, as the overexpression of *Veinlet* from a heat-shock promoter during the third larval instar has no effects on *blistered* pattern (data not shown). We suggest that the role of the Ras pathway in vein development is the transcriptional repression of *blistered* and the transcriptional activation of *veinlet* in a localised pattern (Fig. 7A).

### Pupal development

The proposed network of interactions at work in the larval period is altered during pupal development. Here *veinlet* and *blistered* expression become interdependent and mutually exclusive. During pupation, *blistered* is repressed by *veinlet*, as

is demonstrated by the reduction of *blistered* expression in pupal wings expressing ectopic *veinlet* product, and the converse overexpression of *blistered* detected in presumptive veins of *ve vn* mutant discs, which do not differentiate veins by themselves. We propose a scenario in which, in wild-type discs, high levels of *veinlet* within veins maintain the repression of *blistered* in these cells.

The observation that, in *blistered* mutants, *veinlet* is overexpressed in pupal wing discs (Fristrom et al., 1994; Sturtevant and Bier, 1995) suggests that during this period *veinlet* expression continues to be repressed by *blistered*. Whereas other factors determine the correct positioning of *veinlet* in third larval instar, in pupal stages *blistered* seems to be a key factor determining *veinlet* repression.

A mutual repression mechanism can account for the sensitive interactions that we report between *blistered* and *veinlet*. We strongly favour the hypothesis that an accurately tuned balance between *blistered* and *veinlet* acts refining the boundary between vein and intervein territories (Fig. 7B). In double mutant combinations, the amount of vein tissue depends on the strength of the alleles employed and is proportional to the relative levels of both *blistered* and *veinlet* activities. Moreover, high levels of *veinlet*<sup>+</sup> product, produced by the *Hs-rho30A* line or the *Dp veinlet*<sup>+</sup>, enhance the excess of veins phenotype of *bs<sup>P1292</sup>/+* flies, which by themselves have an impaired vein-repressing *blistered* activity. The equilibrium proposed for *blistered-veinlet* interaction must be sensitive to changes in *veinlet* product expression during third larval instar. Thus, *blistered*, in hemizygous condition, suppresses the loss of L4 of *vein* mutants, which corresponds to the absence of the L4 *veinlet* stripe in mutant *vein* discs (Sturtevant and Bier, 1995). Furthermore, it also dramatically enhances the amount of ectopic vein tissue in those mutant backgrounds where *veinlet* is overexpressed in third larval instar, such as in *Sem* or *plexus* (unpublished data). *Notch* and *Delta* loss-of-function mutations have been also shown to widen the stripes of *veinlet* expression (Sturtevant and Bier, 1995) and, as expected, we found strong superadditive interactions between *blistered* loss-of-function and mutations in these loci. Further studies will be necessary to address whether *Notch* function affects *blistered* expression by itself or if the observed interactions can be explained as an indirect effect on *blistered* due to the ectopic expression of *veinlet* in *Notch* or *Delta* mutant discs.

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