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The *Drosophila* Serum Response Factor gene is required for the formation of intervein tissue of the wing and is allelic to *blistered*

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SUMMARY

The adult *Drosophila* wing is formed by an epithelial sheet, which differentiates into two non-neural tissues, vein or intervein. A large number of genes, many of them encoding components of an EGF-receptor signaling pathway, have previously been shown to be required for differentiation of vein tissue. Much less is known about the molecular control of intervein differentiation. Here we report that the *Drosophila* homolog of the mammalian Serum Response Factor gene (DSRF), which encodes a MADS-box containing transcriptional regulator, is expressed in the future intervein tissue of wing imaginal discs. In adult flies carrying only one functional copy of the DSRF gene, additional vein tissue develops in the wing, indicating that DSRF is required to spatially restrict the formation of veins. In mitotic clones lacking DSRF, intervein tissue fails to differentiate and becomes vein-like in appearance. Genetic and molecular evidence demonstrates that DSRF is encoded by the *blistered* locus, which produces ectopic veins and blistered wings when mutant. Our results show that DSRF plays a dual role during wing differentiation. It acts in a dosage-dependant manner to suppress the formation of wing veins and is required cell-autonomously to promote the development of intervein cells. We propose that DSRF acts at a key step between regulatory genes that define the early positional values in the developing wing disc and the subsequent localized expression of intervein-specific structural genes.

Key words: DSRF, MADS-box, *blistered*, *Drosophila*, wing development, vein-intervein fate

INTRODUCTION

The *Drosophila* wing is derived from a single epithelial sheet, the wing pouch, which folds at the future wing margin into a dorsal compartment and a ventral compartment; these adhere to one another and form an epithelial bilayer (for reviews see Cohen, 1993; Fristrom and Fristrom, 1993). The wing is organized into two major cell types, vein and intervein. During formation of the wing at metamorphosis, intervein cells differentiate a specific cytoskeletal support, the trans-alar array, which allows the formation of tight connections between the dorsal and ventral cell layers (Tucker *et al.*, 1986). After eclosion, intervein cells die leaving a double layer of transparent cuticle (Johnson and Milner, 1987). Vein cells are smaller than intervein cells, form a lumen after apposition of the dorsal and ventral layers, and remain alive after eclosion.

The vein-intervein network of the adult wing is organized in a stereotyped pattern (Waddington, 1940), which appears to be established by earlier positional information during the larval stages (Garcia-Bellido and Merriam, 1971; Zecca et al., 1995). Differentiation of veins and interveins occurs during metamorphosis (Fristrom *et al.*, 1993). Vein-intervein patterning is a powerful model system for studying how a cell chooses between two possible fates, and many mutations displaying modifications in the vein pattern have been identified (Lindsley and Zimm, 1992). Most of these mutants have been classified according to their loss-of-function phenotypes (Diaz-Benjumea and Garcia-Bellido, 1990; Garcia-Bellido and de Celis, 1992): (1) mutations in 'vein-promoting' genes (e.g. *veinlet* or *vein)* lead to the disappearance of portions of veins; (2) mutations in 'vein-suppressing' genes (e.g. *plexus* or *net*) lead to the formation of ectopic veins; and (3) 'thick veins' mutants (e.g. *Notch*, *Delta*) result in enlarged veins. Some of these genes are locally expressed in veins or interveins. For instance, *veinlet* (which is allelic to *rhomboid*) is expressed specifically in the future vein territories in the third instar wing disc, and loss-of-function mutations lead to the disappearance of the distal portion of the longitudinal veins (Sturtevant et al., 1993). Genetically, *veinlet* interacts with the ventrolateral group genes (*spitz*, *Star*, Ras; Bier et al., 1990; Mayer and Nüsslein-Volhard, 1988) and is proposed to potentiate the EGF receptor pathway in the vein territories (Sturtevant et al., 1993).

In this study, we describe the expression and function of the *Drosophila* homolog of the vertebrate Serum Response Factor (DSRF) during the development of the wing imaginal disc. SRF proteins are members of the MADS box family of transcriptional regulators (Shore and Sharrocks, 1995). The

mammalian counterpart (SRF) has been shown to be involved in induction of the c-*fos* promoter in response to serum growth factors (Treisman, 1994). We previously isolated the *Drosophila* homolog of the vertebrate SRF and found that the protein is expressed in a subset of tracheal cells and, during late larval stages, in the developing wing disc (Affolter et al., 1994). More recently, a P-element-induced loss-of-function mutation in *Drosophila* SRF (DSRF) was identified and called *pruned*^{1} because of its embryonic and larval phenotype in which all terminal branches of the trachea are missing (Guillemin et al., 1996). We now report that DSRF protein accumulates in the third instar wing imaginal disc exclusively in nuclei of intervein cells and is strictly required for the formation of these highly specialized cells. In addition, we show that *pruned*^{1} and other DSRF mutations are allelic to *blistered*, classical mutations which produce additional veins and blistered wings (Bridges and Morgan, 1919; Fristrom et al., 1994; Lindsley and Zimm, 1992). Furthermore, both DSRF and *blistered* null mutations cause a haploinsufficient ectopic vein phenotype. Our results demonstrate that DSRF plays a dual role during wing development; it is required for the restriction of veins to vein territories and for the terminal differentiation of intervein cells.

MATERIALS AND METHODS

Fly strains and genetics

The *pruned*^{1} allele was isolated in a screen for tracheal enhancer trap lines (Terminal-1, Samakovlis et al., 1996). It carries a P[lacZ, rosy^{+}] element and corresponds to strain l(2)03267 of the Spradling collection (Karpen and Spradling, 1992). The insertion is located in the 5′ untranslated region of the DSRF transcription unit; *pruned*^{1} behaves as a genetic null allele for the tracheal function (Guillemin et al., 1996). All *blistered* alleles were kindly provided by D. Fristrom (Fristrom et al., 1994 and unpublished results). Homozygous lethal excision alleles from a P-element insertion in *slbo* were generously provided by D. Eberl and L. M. Hall. The *slbo* transcription unit (Montell et al., 1992) maps approximately 8 kb upstream of the DSRF gene (Guillemin et al., 1996) and Southern blot experiments indicate that one of the deletions (DE1) removes at least the first exon of the DSRF gene (unpublished results). Other lethal excisions of the P-element insertion in *slbo*, as well as the small deletions C606, C470 and D707 provided by E. C. Raff (Kimble et al., 1990), were used to analyze the contribution of the different genes deleted in the overlapping region of the *Px*^{2} and *Px*^{4} *Plexate* deficiencies towards the formation of the haploinsufficient wing phenotype.

Homozygous *pruned*^{1} mitotic clones were generated essentially as described by Grieder et al. (1995). Adult wings were mounted as described by Sturtevant and Bier (1995) and photographed under bright-field optics at 40× magnification on a Zeiss Axiophot microscope. The *veinlet*, *vein* double mutant fly strain that was used is described by Diaz-Benjumea and Garcia-Bellido (1990).

Antibody and histochemical stainings

Third instar imaginal discs were dissected as described by Wagner-Bernholz et al. (1991) and immediately fixed for 30 minutes on ice in phosphate-buffered saline (PBS), freshly adjusted to 2 mM EGTA, 0.5% NP40 and 3.7% formaldehyde. Discs were washed 3× in PBS and permeabilized for 30 minutes at room temperature in 0.05% Triton X-100, 0.05% NP40, 0.05% DOC and 2 mg/ml BSA in PBS. After washing 3× in PBS, non-specific binding was blocked in solution A (0.1% Triton X-100, 2 mg/ml BSA in PBS). Antibody detection was performed as described by Affolter et al. (1994), except that all the washing and incubations were performed in solution A.

For histochemical staining, third instar discs were fixed for 20 minutes at room temperature in 0.1 M Pipes (pH 6.9), 20 mM EGTA, 1 mM $MgSO_4$ and 0.75% glutharaldehyde. Adult wings were fixed for 20 minutes at room temperature on a rotating arm, with 1 volume of fixation solution and two volumes of heptane. β-galactosidase activity was detected according to the procedure of Bellen et al. (1989), requiring 2 hours of incubation for the imaginal discs and overnight incubation for the adult wings.

Cloning of DSRF coding sequences

Template DNA for PCR amplification was prepared from homozygous *blistered* larvae or adult flies, as described by Wilson et al. (1989). To isolate DNA from homozygous lethal lines, *blistered* chromosomes were maintained over the T(2;3)L14,SM5-TM6B translocation (a gift of J. Botas) where the 2nd and 3rd chromosomes cosegregate, allowing the selection of non-tubby, homozygous *blistered* larvae. For amplification of coding sequences of the first exon, one oligonucleotide primed just upstream of the AUG initiator codon (5′-GATTTCGGTTTCGGGGTTGGG-3′), the other in the first intron (5′-CCTTGGATCTCTACGGCTCC-3′). The second exon was amplified with oligonucleotides priming in the first intron (5′-GTACTTACCACGATCCTCTGC-3′) and in the second intron (5′-GTGACCTAGATGAGCGTGGC-3′). PCR fragments were ligated directly into a T-tailed vector (pGEM-T, Promega) and sequenced with the primers used for PCR amplification.

RESULTS

DSRF is expressed in the future intervein tissue of the wing imaginal disc

We analyzed the expression of DSRF during imaginal disc development with an anti-DSRF antibody (Affolter et al., 1994). DSRF protein was distributed in a specific patched pattern in the wing disc of third instar larvae (Fig. 1B). Comparison with the fate map of the wing imaginal disc (Bryant, 1975; Fig. 1A) indicated that the patched staining was restricted to the wing pouch and the hinge region. Within the developing wing pouch, DSRF protein appears to be absent from the future wing margin, and from four stripes of cells extending at a right angle from the wing margin towards the proximal wing regions. As the intervein tissue of the wing is separated into five areas by the wing veins, the expression domains of DSRF suggested that the gene is expressed exclusively in those cells that correspond to the future intervein tissue. To determine whether DSRF protein was indeed restricted to intervein territories, the expression pattern of DSRF was compared to the A101 marker. A101 contains a P[lacZ] insertion (Bellen et al., 1989) in the *neuralized* gene and expresses β-galactosidase in sensory organs along the anterior wing margin and vein L3; it was used previously to show that *veinlet* expression is restricted to the future vein tissue of the wing disc (Sturtevant et al., 1993). Double staining for DSRF and β-galactosidase confirmed that in the wing pouch the middle stripe of cells that does not express DSRF corresponds to the anterior wing margin, and that one of the perpendicular stripes corresponds to L3 (Fig. 1C). In the adult wing, intervein areas B and C flank vein L3 on each side (for intervein nomenclature, see Fig. 1F). Consistent with this arrangement, the future vein L3 is located between the second and third patches of DSRF expression (Fig. 1C).

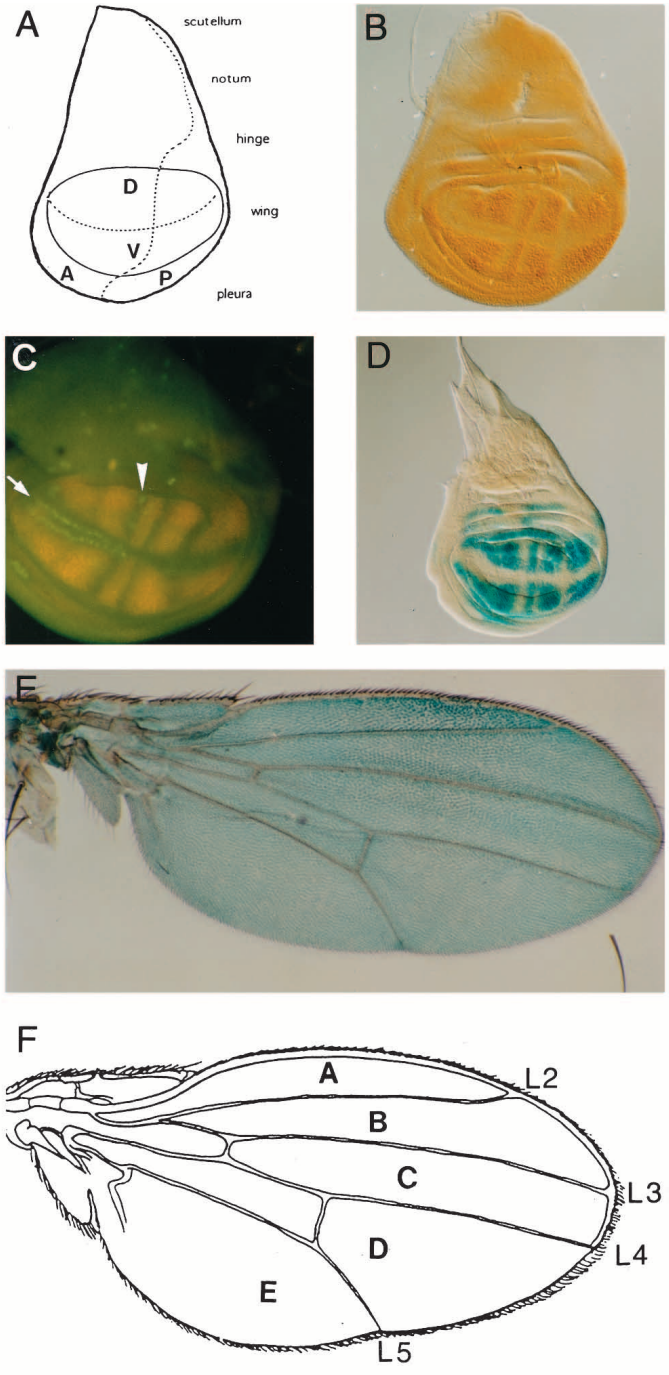

Fig. 1. DSRF expression in the wing imaginal disc. (A-D) Wing discs from late third instar, oriented dorsal up and anterior left. (A) Fate map of a wing imaginal disc (Bryant, 1975; Diaz-Benjumea and Cohen, 1993). The wing pouch is the oval domain that will give rise to the adult wing blade. The hinge is the proximal structures that fasten the wing blade to the body wall (Ng et al., 1995). (B) DSRF expression detected in a wing disc with a DSRF antiserum (brown peroxidase reaction product). Note the strong staining forming two series of five sectors in the future wing pouch, and the staining in the hinge region. (C) Immunofluorescent detection of DSRF (orange) and β-galactosidase protein (light green) in the A101 enhancer trap strain carrying a P[lacZ] insertion (Bellen et al., 1989) in the *neuralised* gene. β-galactosidase in A101 is expressed in all sensory mother cells in the wing, labeling the precursors for sensilla lining the future anterior wing margin (double row; arrow) and precursors of campaniform sensilla that lie on the third wing vein (arrowhead). (D) Histochemical staining for β-galactosidase activity detection using the *pruned*^{1} P[lacZ] insertion in the DSRF locus that directs expression of β-galactosidase in the nuclei. Note that the staining is identical to the DSRF antibody staining (compare with B and C), indicating that in the wing disc, this P[lacZ] insertion behaves as an enhancer trap of DSRF regulatory sequences. (E) β-galactosidase expression in the adult wing of the *pruned*^{1} enhancer trap insertion visualised by histochemical staining. Note the nuclear staining in the intervein areas, and its absence in the wing veins. Staining is restricted to intervein territories of the pouch and hinge. (F) Schematic representation of an adult wing. In accordance with the nomenclature of Garcia-Bellido et al. (1994), the intervein territories are designated A to E and the longitudinal veins L2 to L5.

A P[lacZ] insert in the DSRF gene called *pruned*^{1} mimicks endogenous DSRF protein expression in the tracheal system of the embryo and larva (Guillemin et al., 1996). We found that the pattern of β-galactosidase activity in wing imaginal discs was also virtually identical to DSRF expression (compare Fig. 1D with B, and C). Although intervein cells die soon after emergence, it is possible to detect β-galactosidase activity in the wings of newly hatched flies (Hama et al., 1990). Staining of wings from flies carrying one copy of the P[lacZ] insertion in DSRF showed that β-galactosidase activity was detected exclusively in the intervein tissue of the adult wing (Fig. 1E). Thus, DSRF expression is confined to the intervein territory of the third instar wing imaginal disc and, upon eclosion, is detectable in all intervein cells.

DSRF is essential for intervein differentiation

The highly specific expression pattern of DSRF in the wing disc suggested that DSRF might be necessary for the development of intervein tissue. Homozygous *pruned*^{1} mutants that carry a lethal P-element insertion in DSRF die during the larval period (Guillemin et al., 1996). To analyze the consequences of loss of expression of DSRF in the wing, we generated clones of homozygous *pruned*^{1} cells by FLP-mediated recombination. In these experiments, the *pruned*$^{+}$ chromosome carried a *forked*$^{+}$ bristle marker. Thus, homozygous *pruned*^{1} mutant clones could be identified by their *forked*$^{-}$ bristle phenotype under high magnification. Vein cells were distinguished from intervein cells by their color (vein cells are darker) and by their size (vein cells are smaller) (Fristrom et al., 1994; Garcia-Bellido and de Celis, 1992).

When FLP-mediated recombination was induced during late third instar, we observed many small *forked* mutant clones (Fig. 2A). When recombination was induced during late second/early third instar, we observed fewer yet larger clones (Fig. 2B), and when induced even earlier (during embryogenesis or first instar), the resulting wings were dark and small, and looked like tubes (Fig. 2C).

Fig. 2D,E shows a close up of the dorsal and ventral layers, respectively, of the same wing. Two individual *pruned*^{1} mutant clones (induced during late third instar in the intervein territory) are visible, one in the dorsal and the other in the ventral compartment. The mutant cells were smaller and darker than the surrounding intervein cells and resembled vein cells. In the case of the dorsal clone (right side, Fig. 2D), focusing on the underlying cells on the ventral side showed

that the size of these cells was the same as that of the surrounding intervein cells (Fig. 2E). Similarly, the dorsal cells overlying the ventral clone (left side Fig. 2E) were unaffected (Fig. 2E). Because only the *pruned*^{1} mutant cells were affected, we conclude that the DSRF gene behaves in a cell-autonomous manner.

Mutant clones induced earlier in development, during early third instar or before, gave larger clones (as expected) and mutant cells were again smaller and darker than the surrounding intervein cells, thus displaying vein-like properties. In addition, these large mutant regions formed blisters that were generally limited to the mutant tissue (data not shown). Because blisters formed even when the opposite cell layer differentiated as intervein, it appears that the formation of tight connections between dorsal and ventral cells of the wing blade requires intervein identity in cells of both compartments.

Our results demonstrate that DSRF acts cell-autonomously and is essential for the formation of intervein cells; in the absence of DSRF, intervein cells fail to differentiate and acquire vein cell morphology.

DSRF is allelic to *blistered*

blistered mutations lead to the formation of ectopic veins and blistered wings (Bridges and Morgan, 1919; Fristrom et al., 1994). The *blistered* locus is uncovered by Df(2R)*Px*^{2} and Df(2R)*Px*^{4}, which define the cytological region 60C5-6;60D1. Our previous mapping studies localized the DSRF transcription unit to the same chromosomal interval (Affolter et al., 1994). To analyze the relationship between these two genes, seven available *blistered* mutants (see Materials and Methods) were crossed to the *pruned*^{1} DSRF loss-of-function mutant. The mutant chromosomes failed to complement each other (Table 1). When transheterozygous with *pruned*^{1}, the viable *blistered* alleles *(bs10, bs11* and *bs12)* showed a wing phenotype that was stronger than the wing phenotype of the corresponding homozygous-viable *blistered* alleles (Fristrom *et al.*, 1994; Fig. 3 and Table 1). The homozygous-lethal *bs13*, *bs14*, *2R14* and *2R19* *blistered* alleles were lethal when transheterozygous with *pruned*^{1} (Table 1). *pruned*^{1} homozygotes die during second or third larval instar, and the same lethal period was observed for *bs14* and *2R14* homozygotes (Fristrom et al., 1994), as well as for transheterozygous combinations with *pruned*^{1} (Table 1). Homozygous *2R19* individuals died just at emergence, and the transheterozygotes with *pruned*^{1} died during the same period.

Thus, we find that *blistered* mutations do not complement the *pruned*^{1} DSRF loss-of-function mutation, suggesting that *blistered* alleles contain mutations in the DSRF gene.

Identification of DSRF point mutations in *blistered* alleles

To establish that the DSRF gene and *blistered* correspond to the same genetic locus, DSRF coding sequences from *blistered* alleles were cloned and sequenced. DSRF is a putative transcription factor of 450 amino acids containing a glutamine-rich region (residues 81 to 107) and an extended region of high sequence identity with mammalian SRF (residues 161 to 247), which includes the DNA binding MADS domain (residues 165 to 221) (Affolter et al., 1994). Oligonucleotides flanking the two first exons, which encode the N-terminal 278 residues, including the entire region of homology between DSRF and human SRF, were used to amplify *blistered* genomic DNA.

Point mutations were identified in three *blistered* alleles (see Fig. 4). In *bs14*, a nonsense mutation in codon 102 is predicted to give rise to a truncated protein lacking the MADS domain. The mutation in *2R19* leads to a stop codon just 3′ of the

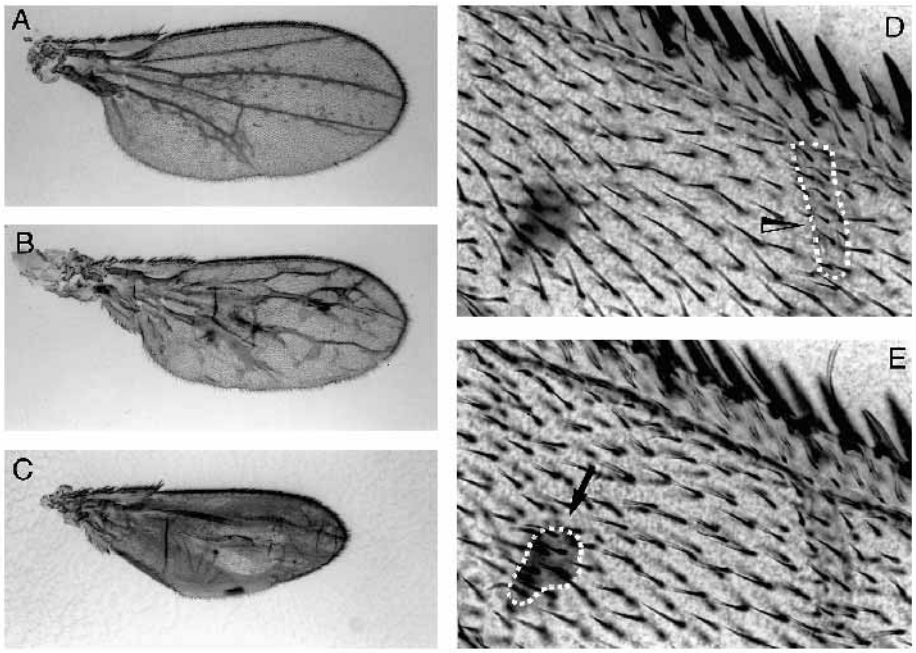

Fig. 2. DSRF is required for intervein tissue formation. Clones of homozygous *pruned*^{1}/*forked*$^{-}$ cells were generated by mitotic recombination and identified by their *forked*$^{-}$ bristle morphology. (A) Multiple small *pruned*^{1} clones were induced by recombination during late third instar. Clones in intervein regions appear as numerous small islets of vein-like tissue. (B) A similar effect is seen in the larger clones induced in early third instar. (C) Tube-like wings resulted when recombination was induced at early first instar and produced large clones. No contact between the ventral and the dorsal layers is evident. Nevertheless, tissues that have not undergone recombination are present (clear stain in the center of the wing) and the color and the size of these cells indicates that they have normal intervein identity. (D,E) Close-up view of the dorsal (D) and ventral (E) wing surface showing the cell autonomous effect of DSRF on intervein tissue formation. The dorsal cell layer can be identified by the presence of two rows of bristles in the wing margin, whereas the ventral cell layer of the wing margin shows only one row of bristles (Diaz-Benjumea and Cohen, 1993). The defects in a dorsal DSRF mutant clone (arrowhead in D) are restricted to the homozygous *pruned*^{1},*forked*$^{-}$ cells, which are smaller than the surrounding intervein cells. The size of the apposed cells on the ventral side (E) is not affected. Similarly, in a ventral clone (arrow in E), only the homozygous *pruned*^{1},*forked*$^{-}$ cells are affected, without any effect on the size of the apposed dorsal cells. No effects due to the removal of DSRF were observed in the wing cells other than intervein cells (data not shown).

Table 1. Wing phenotypes and genetic complementation tests between *pruned*[1] and *blistered* alleles

	blistered alleles						
Wing phenotype	*bs10*	*bs11*	*bs12*	*bs13*	*bs14*	*2R14*	*2R19*
bs/bs	Additional veins	Many additional veins	Tubes	(Lethal, 2nd or 3rd instar)	(Lethal, 2nd or 3rd instar)	(Lethal, 2nd or 3rd instar)	Tubes (lethal at eclosion)
bs/+	Normal	Normal	Normal	Normal	Few additional veins	Few additional veins	Few additional veins
bs/pruned[1]	Many additional veins	Many additional veins or tubes	Tubes	Tubes (lethal at eclosion)	(Lethal, 2nd or 3rd instar)	(Lethal, 2nd or 3rd instar)	Tubes (lethal at eclosion)

bs/bs is the phenotype of the homozygote *blistered* allele.
bs/+ is the wing phenotype of the heterozygote *blistered* allele.
bs/pruned[1] is the phenotype of the *blistered* allele over *pruned*[1].
'Additional veins' refers to wing phenotypes of varying intensity depending on the abundance of ectopic veins appearance. For instance, there are a few additional veins in *pruned*[1]/+ heterozygotes. 'Tube' indicates the absence of connection between the ventral and dorsal wing layers; the entire wing tissue seems to be composed exclusively by vein cells. Examples of these phenotypes are shown in Figs 3, 5. Homozygous lethal phase is indicated in parentheses. Lethalities of the *blistered* alleles were determined with the T(2;3)L14,SM5-TM6B translocation (see Materials and Methods). A larval stage was considered lethal if more than 90% of the *blistered* homozygotes died at that phase.

MADS-box coding sequence and eliminates an alpha-helix (αII) required for dimerization and interaction with accessory factors (Pellegrini et al., 1995). The viable *bs11* mutation causes a glycine to glutamate substitution within the MADS-box (Gly204Glu). The glycine residue is conserved in all vertebrate SRF and yeast orthologs, and X-ray crystal structure determination of the human SRF MADS domain bound to DNA (Pellegrini et al., 1995) reveals that this glycine is centered in a tight turn joining an alpha-helix (αI) involved in DNA binding and a beta-strand (βI) required for dimerization. These two critical secondary structural elements might be prevented from folding into an optimal native tertiary structure due to steric hindrance by the Gly204Glu substitution within the tight turn.

There is a strong correlation between the severity of the different *blistered* phenotypes and the predicted molecular defects in the encoded DSRF proteins. The single amino acid change in the DSRF MADS-box in *bs11* is sufficient to induce a homozygous wing phenotype, but causes neither haploinsufficiency (see below) nor larval lethality. Truncation of the DSRF protein (*2R19, bs14*) affects the function much more dramatically and causes a haploinsufficient wing phenotype and homozygous lethality. The most severe truncation, *bs14*, results in a tracheal phenotype similar to that of *pruned*[1] (see Guillemin et al., 1996).

In summary, *blistered* and DSRF mutations map in the same chromosomal location, lead to formation of ectopic veins when mutated (see also below), and do not complement each other. The DSRF loss-of-function mutation *pruned*[1] and the strong *blistered* alleles are homozygous lethal at the same developmental stages. These genetic results and the identification of point mutations in DSRF coding sequences from three *blistered* alleles demonstrate conclusively that the DSRF and *blistered* genes are the same.

DSRF function in intervein development is dosage-sensitive

Previous analysis have shown that a number of *Plexate* (*Px)* deficiencies delete the DSRF transcription unit (Affolter et al., 1994). *Px* deficiencies were isolated by virtue of their formation of ectopic veins in heterozygotes (Lindsley and Zimm, 1992). The finding that weak *blistered* mutations result in homozygous adult flies with ectopic veins prompted us to analyze the morphology of adult flies heterozygous for *blistered*/DSRF null mutations. Adult wings heterozygous for either a small deletion removing part of the DSRF gene (DE1; generously provided by D. Eberl and L. Hall; see Materials and Methods), the *pruned*[1] mutation, or several of the stronger *blistered* alleles, displayed ectopic veins similar to those seen in *Plexate* mutations (Fig. 5; see also figure legend). Although the haploinsufficient wing phenotypes due to DSRF loss-of-function mutations were somewhat weaker than the phenotypes generated by *Plexate* deficiencies, we noticed that both of them were more extreme in females and enhanced by cold (18°C) (data not shown; Lindsley and Zimm, 1992). To investigate whether the haploinsufficient wing phenotype observed in *Plexate* mutations was due primarily to the loss of DSRF function, we individually analyzed small deficiencies throughout the region deleted in both Px^2 and Px^4 (see Materials and Methods). None of these smaller deficiencies showed a haploinsufficient wing phenotype when crossed over a wild-type

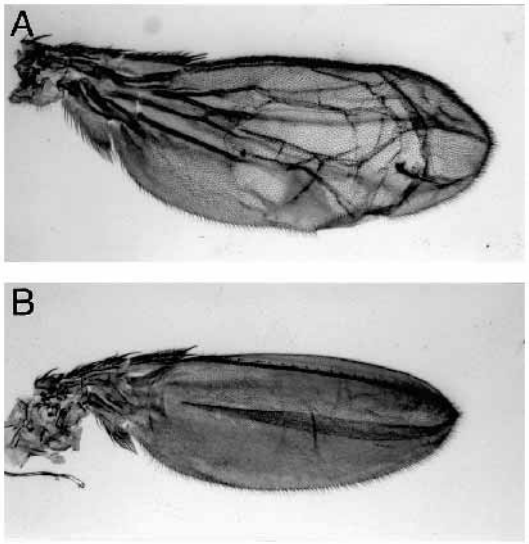

Fig. 3. The *pruned*[1] allele does not complement *blistered* alleles (compare with homozygous *blistered* phenotypes in Fristrom et al., 1994). (A) Adult wing of a *bs10* /*pruned*[1] transheterozygote, showing formation of many additional veins. (B) Formation of a tube-like wing in a *bs12* /*pruned*[1] transheterozygote.

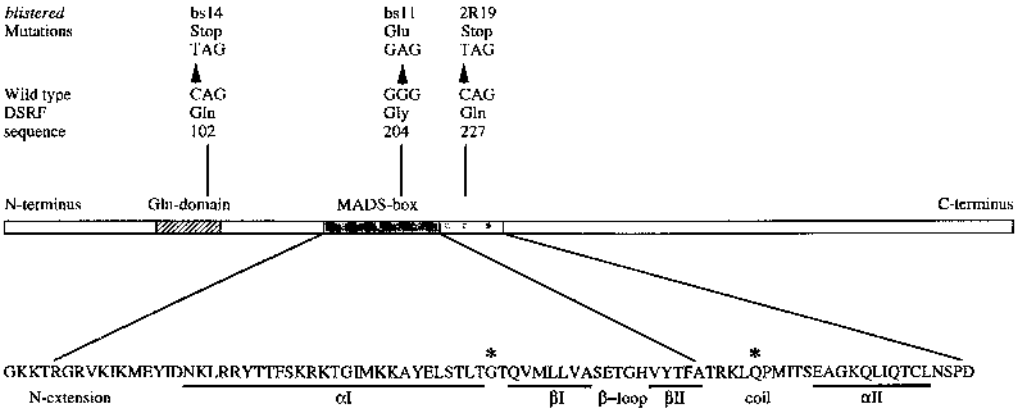

Fig. 4. Molecular analysis of *blistered* alleles. The glutamine-rich domain and the MADS domain of DSRF protein are depicted schematically. The MADS domain is composed of a highly conserved 'MADS-box' and a class-specific C-terminal one-third (Treisman and Ammerer, 1992). *Drosophila* SRF and human SRF proteins share 93% amino acid sequence identity within the MADS domain (Affolter et al., 1994). Secondary structure motifs indicated for the DSRF MADS domain are predicted from the crystal structure of the human SRF/DNA complex (Pellegrini et al., 1995). Asterisks mark the amino acid residues mutated in the *blistered* alleles *bs11* and *2R19*. Alterations in *blistered* alleles and their consequence for the DSRF reading frame are indicated.

chromosome (data not shown), or significantly modified the DSRF haploinsufficient phenotype in transheterozygous combination with *pruned*^{1} (data not shown). This demonstrates that many or most aspects of the *Plexate* haploinsufficient wing phenotype are attributable to the removal of one copy of the DSRF locus.

blistered/DSRF plays a dual role during wing development

As shown in the previous section, adult flies carrying only one functional copy of the *blistered*/DSRF gene develop additional vein tissue in the wing. Analysis of mitotic loss-of-function clones demonstrated that in the complete absence of *blistered*/DSRF function, intervein cells do not differentiate as such, but appear vein cell-like in morphology and in their inability to form tight connections with the apposed cells of the opposite compartment. Based on these phenotypes, one could postulate that the primary or even the sole function of *blistered*/DSRF during wing development is to suppress the activity of 'vein-promoting' genes in the intervein territory. To determine the relationship between these genes, we generated flies carrying mutations both in 'vein-promoting' genes (e.g. *veinlet*, *vein* double mutants; Diaz-Benjumea and Garcia-Bellido, 1990) and *blistered*/DSRF. In *veinlet*, *vein* mutant flies, wings lack veins and consist mostly of intervein cells (Fig. 6C,D). In contrast, vein-like cells cover the entire wing blade in the rare hatching adults homozygous for the *blistered 2R19* allele (Fig. 6E,F). In flies mutant for *veinlet*, *vein* and *blistered 2R19*, the size of the wing blade cells, their color and their adhesive properties are vein-like and correspond to those seen in flies mutant for *blistered*/DSRF only (compare Fig. 6G,H with E,F; see also legend). Thus, loss-of-function mutations in *blistered*/DSRF are epistatic to loss-of-function mutations in 'vein-promoting' genes with respect to epithelial wing cell morphology. This result argues strongly against the hypothesis that the sole function of DSRF/*blistered* is to suppress the activity of 'vein-promoting' genes in intervein territories. Taken together, the observations that *blistered*/DSRF mutations cause a haploinsufficient ectopic wing venation

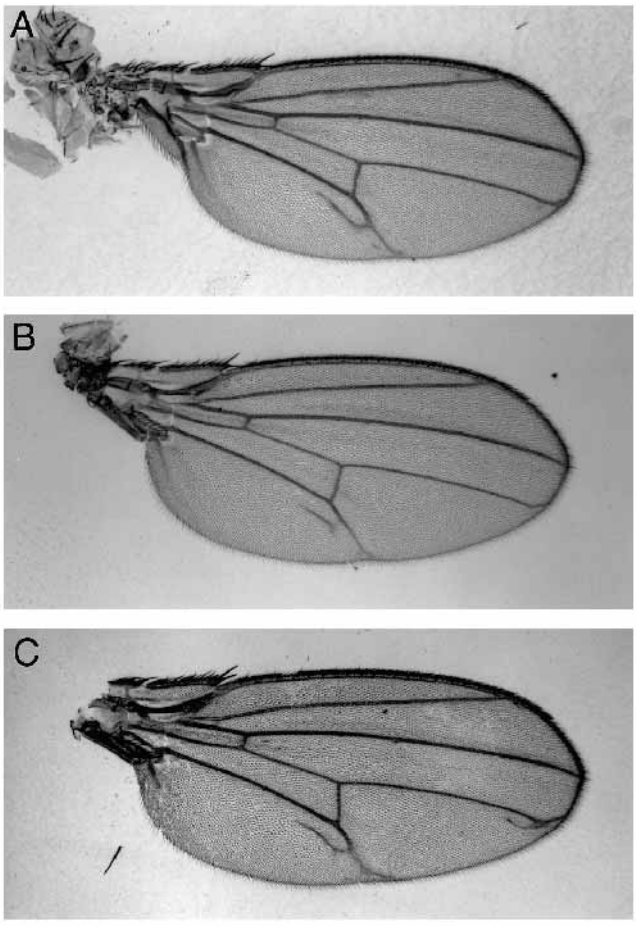

Fig. 5. DSRF requirement for intervein differentiation is dosage-dependant. (A) Heterozygote carrying a small deletion (DE1) that removes part of the DSRF locus (see Material and Methods). Note the additional vein tissue along vein L2, at the distal tip of L4, and below L5. (B) *pruned*^{1} heterozygote. (C) *bs14* heterozygote. Note in both cases the formation of additional vein tissue, as depicted in A.

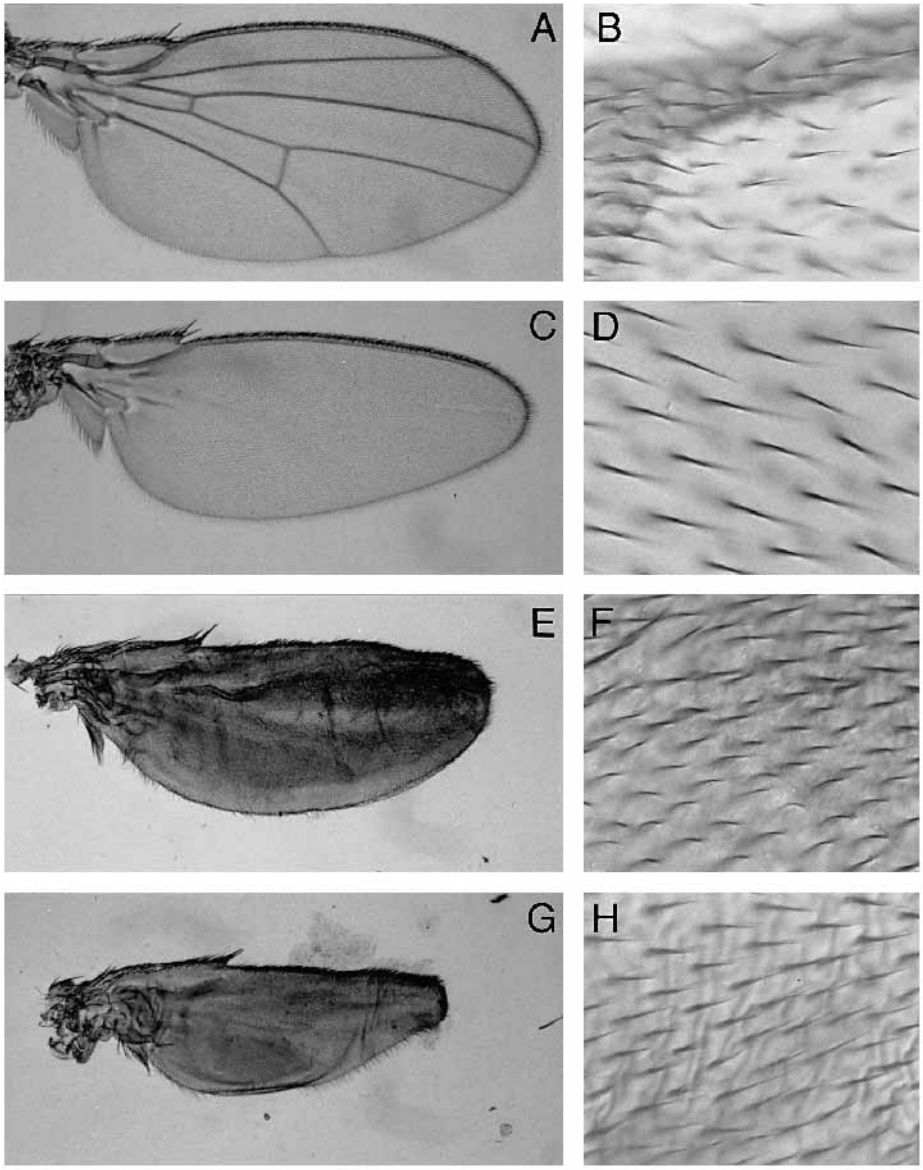

Fig. 6. Genetic interaction between *blistered* and *veinlet*, *vein*. Adult wings of wild-type flies (A) or of various mutant combinations (C,E,G) were photographed under the same conditions. The higher magnifications (B, D, F and H) correspond to the central part of the wing between the presumptive L3 and L4 longitudinal veins (see Fig. 1F). (A,B) Wild-type wing. (C,D) *veinlet*, *vein* homozygous double mutant wing. (E,F) Wing of an adult *blistered 2R19* homozygous escaper. (G,H) Wing of a *blistered 2R19*; *veinlet*, *vein* homozygous escaper. Note the similar pigmentation of the triple mutant wing (G) and of the *blistered 2R19* homozygous wing (E). To evaluate the cell size precisely, the cell density was measured in constant wing areas. The number of hairs was counted in five different pictures for each of the mutants. In each case, the cell density was similar in *blistered 2R19* and the triple mutants. As an additional criterion to compare the cell size between mutant and wild-type wing cells, the distances between a single hair and its five nearest neighbours was measured. Virtually identical values were obtained for wild-type vein cells, *blistered 2R19* homozygous tissue and *blistered 2R19*; *veinlet*, *vein* homozygous tissue. For the cells of wild-type intervein tissue and of the *veinlet*, *vein* wing blade tissue, similar values were also obtained.

phenotype, whereas the complete lack of *blistered*/DSRF function prevents the formation of intervein cells in different genetic contexts, suggest that DSRF plays a dual role during wing development. Two fully active copies of the *blistered*/DSRF gene are required to ensure that the formation of wing veins is limited to vein territories; in addition, DSRF protein is essential for the proper terminal differentiation of intervein cells.

DISCUSSION

DSRF is an early marker and a key regulator of intervein development

Extensive transplantation studies using imaginal disc fragments have shown that much of the information needed to pattern the adult wing appendage has been elaborated by the end of the third larval instar (Bryant, 1975). The availability of molecular probes has recently allowed these future structural territories to be visualized. In particular, the *veinlet* gene is exclusively expressed in the wing vein primordia in the developing wing pouch and is essential for the formation of distal vein structures (Sturtevant et al., 1993). Here we have shown that DSRF protein is expressed in the intervein territory of third instar wing discs and that DSRF function is strictly required for the differentiation of all intervein cells. To our knowledge, *blistered*/DSRF is the earliest known marker for the entire intervein territory of the developing wing disc, and the only gene known to be required in all intervein cells for the selection of their fate.

We do not know how the intervein-specific expression pattern of DSRF is set up in the third instar imaginal disc. It is possible that the DSRF gene is turned on throughout the wing pouch and hinge and that the activity of 'vein-promoting' genes suppresses DSRF expression in the vein territories. However, DSRF expression is only slightly affected in homozygous *veinlet*, *vein* third instar discs (data not shown), although such discs develop into veinless wings (Diaz-Benjumea and Garcia-Bellido, 1990). Conversely, *veinlet* expression is not altered in third instar wing imaginal discs of

bs11 mutant larvae, and expands only later during prepupal and pupal stages (Fristrom et al., 1994). These findings suggest that the expression patterns of both vein- and intervein-specific genes are set up for the most part independently, possibly by differential interpretation of similar positional information generated by earlier acting genes. Preliminary experiments indeed indicate that DSRF expression in distinct intervein regions is controlled by separate cis-acting DNA elements (Ute Nussbaumer, J.G., J.M. and M.A., unpublished results). The analysis of gene expression patterns in mutant backgrounds (see Sturtevant and Bier, 1995), as well as the detailed dissection of the cis-acting transcriptional control regions of vein- and intervein-specific genes, should provide more insight into the mechanisms that control these spatially restricted expression domains.

Transcriptional control of intervein differentiation

Our genetic and molecular analyses demonstrate that *blistered* mutations affect the structure and the function of the DSRF transcription unit. A detailed phenotypic and developmental analysis of *blistered* function in wing development has recently been reported (Fristrom et al., 1994). Using light and electron microscopy, *blistered* defects were first seen in the prepupal period as a failure of apposition of dorsal and ventral wing epithelia. Correspondingly, during definitive vein/intervein differentiation in the pupal period, the extent of dorso/ventral reapposition was found to be reduced, and intervein regions that failed to become apposed showed differentiated properties of vein cells (Fristrom et al., 1994). Our finding that *blistered* mutations affect the function of a MADS-box transcription factor suggests that differentiation of intervein cells requires transcriptional control, and that DSRF regulates certain target genes that suppress vein identity and promote intervein identity. A number of genes have been found to be expressed more strongly or exclusively in intervein cells during prepupal and/or pupal stages. These genes include *inflated* (*if*; codes for an integrin α chain) and *myospheroid* (*mys*; codes for an integrin β chain). Both integrin subunits are required for the tight apposition of dorsal and ventral surfaces, and mutations in *mys* and *if* result in adult wings with blisters (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990), a phenotype often associated with hypomorphic *blistered* mutations or homozygous mutant *blistered* clones. In addition, *blistered* and integrin mutations interact genetically (Fristrom et al., 1994; Wessendorf et al., 1992), and *mys* expression appears to be greatly reduced in *bs11* (D. Fristrom, personal communication). These results suggest that DSRF might directly control integrin expression at the transcriptional level during prepupal and/or pupal stages. In contrast to mutations in DSRF, intervein development appears normal in *mys* and *if*, suggesting that DSRF must also regulate other genes. One characteristic feature of intervein cells is the formation of a 'transalar cytoskeleton' and characteristic basal junctions (Fristrom et al., 1993; Tucker et al., 1986). One or several components of these structures might be under the control of DSRF. Interestingly, potential DSRF target sequences have been identified in the promoter region of the actin 5C gene (Bond-Matthews and Davidson, 1988), which is expressed during wing development (Petersen et al., 1985). The actin 5C gene encodes a cytoplasmic form of actin and might be involved in the formation of the transalar cytoskeleton. The identification of direct target genes of DSRF will certainly provide additional insight into the control of cell differentiation in the wing epithelium.

Is DSRF activity regulated by cell-cell signaling during wing morphogenesis?

The biochemical properties of human SRF and its DNA binding site SRE (Serum Response Element) have been analyzed extensively. The SRE has been shown to be a nuclear target of multiple signal transduction pathways and to be activated via various extracellular stimuli (Treisman, 1994). At the SRE, SRF is known to interact with a subset of Ets domain proteins, the activity of which is regulated via a phosphorylation cascade involving the ERK MAP-kinase. In addition, SRF can also be activated independently of its interaction with Ets proteins, as part of a novel Rho-mediated signaling pathway (Hill et al., 1995; Whitmarsh et al., 1995). The ERK-MAP kinase-mediated EGF receptor pathway is implicated in the formation of veins and in the control of cell size (Diaz-Benjumea and Hafen, 1994; Sturtevant et al., 1993). It is possible that the control of the size of intervein cells by the EGF pathway is mediated via DSRF. It is also possible that other, Rho-mediated signaling pathways positively induce intervein differentiation by triggering DSRF activity. DSRF function is required during a developmental period when the dorsal and ventral wing disc cells undergo a conversion from a folded single cell layer to a flat bilayered wing. During this process, each of four steps (apposition, adhesion, expansion and separation; see Fristrom et al., 1993) occurs twice, and cell-cell communication during these developmental events is well documented (Garcia-Bellido et al., 1994; Garcia-Bellido and de Celis, 1992). Consistent with the biochemical studies in vertebrate systems, the activity of DSRF, or of proteins that interact with it, could be modulated by these cell-cell interactions. In this context, it is worth mentioning that the *blistered* 2R19 allele, which encodes a protein containing the entire MADS-box but lacking C-terminal sequences of the MADS domain (i.e. αII), results in a haploinsufficiency and a complete failure to produce intervein tissue. Based on biochemical and structural evidence, such a protein might still be able to bind DNA targets but would fail to form ternary complexes with accessory factors (Mueller and Nordheim, 1991).

To test the hypothesis that DSRF function is modulated by distinct signal transduction pathways during wing morphogenesis in vivo, it will be crucial to identify target genes of DSRF and monitor their behavior in transgenic flies expressing dominant negative or constitutively activated forms of various signaling components. In parallel, the haploinsufficient phenotype of DSRF mutations should provide a simple and sensitive screening procedure for enhancers and suppressors of DSRF activity. The characterization of the genes corresponding to these modifiers should reveal more about the regulation of DSRF activity in vivo.

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REFERENCES

Affolter, M., Montagne, J., Walldorf, U., Groppe, J., Kloter, U., LaRosa, M. and Gehring, W. J. (1994). The *Drosophila* SRF homolog is expressed in a subset of tracheal cells and maps within a genomic region required for tracheal development. *Development* **120**, 743-753.

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Kurth Pearson, R. and Gehring, W. J. (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes Dev.* **3**, 1288-1300.

Bier, E., Jan, L. Y. and Jan, Y. N. (1990). *Rhomboid*, a gene required for dorso-ventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. *Genes Dev.* **4**, 190-203.

Bond-Matthews, B. and Davidson, N. (1988). Transcription from each of the *Drosophila act5C* leader exons is driven by a separate functional promoter. *Gene* **62**, 289-300.

Bridges, C. B. and Morgan, T. H. (1919). The second chromosome group of mutant characters. In *Contributions to the Genetics of* Drosophila melanogaster. pp. 123-304. Carnegie Institution of Washington, Washington, D.C.

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

Bryant, P. J. (1975). Pattern formation in the imaginal wing disc of *Drosophila melanogaster*: fate map, regeneration and duplication. *J. Exp. Zool.* **193**, 49-77.

Cohen, S. M. (1993). Imaginal disc development. In *The Development of* Drosophila melanogaster, Vol. II **(**ed. M. Bate and A. Martinez Arias), pp. 747-842. Cold Spring Harbor: Cold Spring Harbor Press.

Diaz-Benjumea, F. J. and Cohen, S. M. (1993). Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. *Cell* **75**, 741-752.

Diaz-Benjumea, F. J. and Garcia-Bellido, A. (1990). Genetic analysis of the wing vein pattern of *Drosophila*. *Roux's Arch. Dev. Biol.* **198**, 336-354.

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

Fristrom, D. and Fristrom, J. W. (1993). The metamorphic development of the adult epidermis. In *The Development of* Drosophila melanogaster, Vol. II **(**ed. M. Bate and A. Martinez Arias), pp. 843-897. Cold Spring Harbor: Cold Spring Harbor Press.

Fristrom, D., Gotwals, P., Eaton, S., Kornberg, T. B., Sturtevant, M., Bier, E. and Fristrom, J. W. (1994). *blistered:* a gene required for vein/intervein formation in wings of *Drosophila*. *Development* **120**, 2661-2671.

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., Cortes, F. and Milan, M. (1994). Cell interactions in the control of size in *Drosophila* wings. *Proc. Natl. Acad. Sci. USA* **91**, 10222-10226.

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

Garcia-Bellido, A. and Merriam, J. R. (1971). Genetic analysis of cell heredity in imaginal discs of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **65**, 2222-2226.

Grieder, N. C., Nellen, D., Burke, R., Basler, K. and Affolter, M. (1995). *schnurri* is required for *Drosophila* Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell* **81**, 791-800.

Guillemin, K., Groppe, J., Duecker, K., Treisman, R., Hafen, E., Affolter, M. and Krasnow, M. (1996). The *pruned* gene encodes the *Drosophila* serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development* **122**, 1353-1362.

Hama, C., Ali, Z. and Kornberg, T. B. (1990). Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. *Genes Dev.* **4**, 1079-1093.

Hill, C. S., Wynne, J. and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81**, 1159-1170.

Johnson, S. A. and Milner, M. J. (1987). The final stages of wing development in *Drosophila melanogaster*. *Tissue & Cell* **19**, 505-513.

Karpen, G. H. and Spradling, A. C. (1992). Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by single P element insertional mutagenesis. *Genetics* **132**, 737-753.

Kimble, M., Dettman, R. W. and Raff, E. C. (1990). The β3-tubulin gene of *Drosophila melanogaster* is essential for viability and fertility. *Genetics* **126**, 991-1005.

Lindsley, D. and Zimm, G. (1992). The Genome of *Drosophila melanogaster*. San Diego: Academic Press Inc.

Mayer, U. and Nüsslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. *Genes Dev.* **2**, 1496-1511.

Montell, D. J., Rorth, P. and Spradling, A. C. (1992). *slow border cells*, a locus required for a developmentally regulated cell migration during oogenesis, encodes *Drosophila* C/EBP. *Cell* **71**, 51-62.

Mueller, C. G. F. and Nordheim, A. (1991). A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. *EMBO J.* **10**, 4219-4229.

Ng, M., Diaz-Benjumea, F. J. and Cohen, S. M. (1995). *nubbin* encodes a POU-domain protein required for proximal-distal patterning in the *Drosophila* wing. *Development* **121**, 589-599.

Pellegrini, L., Tan, S. and Richmond, T. J. (1995). Structure of serum response factor core bound to DNA. *Nature* **376**, 490-498.

Petersen, N. S., Bond, B. J., Mitchell, H. K. and Davidson, N. (1985). Stage-specific regulation of actin genes in *Drosophila* wing cells. *Dev. Genet.* **5**, 219-225.

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D., Guillemin, K. and Krasnow, M. (1996). Branching morphogenesis of the *Drosophila* tracheal system occurs by a series of morphologically distinct but genetically coupled branching events. *Development* **122**, 1395-1407.

Shore, P. and Sharrocks, A. D. (1995). The MADS-box family of transcription factors. *Eur. J. Biochem.* **229**, 1-13.

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.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Op. Gen. Dev.* **4**, 96-101.

Treisman, R. and Ammerer, G. (1992). The SRF and MCM1 transcription factors. *Curr. Op. Gen. Dev.* **2**, 221-226.

Tucker, J. B., Milner, M. J., Currie, D. A., Muir, J. W., Forrest, D. A. and Spencer, M. -J. (1986). Centrosomal microtubule-organizing centres and a switch in the control of protofilament number for cell surface-associated microtubules during *Drosophila* wing morphogenesis. *Europ. J. Cell Biol.* **41**, 279-289.

Waddington, C. H. (1940). The genetic control of the wing development in *Drosophila*. *J. Genet.* **41**, 75-139.

Wagner-Bernholz, J. T., Wilson, C., Gibson, G., Schuh, R. and Gehring, W. J. (1991). Identification of target genes of the homeotic gene *Antennapedia* by enhancer detection. *Genes Dev.* **5**, 2467-2480.

Wessendorf, L. H. V., Wehrli, M., DiAntonio, A. and Wilcox, M. (1992). Genetic interactions with integrins during wing morphogenesis in *Drosophila*. *Cold Spring Harb. Symp. Quant. Biol.* **57**, 241-248.

Whitmarsh, A. J., Shore, P., Sharrocks, A. D. and Davis, R. J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**, 403-407.

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

Wilson, C., Kurth Pearson, R., Bellen, H. J., O'Kane, C. J., Grossniklaus, U. and Gehring, W. J. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Dev.* **3**, 1301-1313.

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

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

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