Development 124, 1007-1018 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV7514

Expression and function of *decapentaplegic* and *thick veins* during the differentiation of the veins in the *Drosophila* wing

Jose F. de Celis

Department of Genetics, University of Cambridge, Cambridge CB2 4TH, UK

(e-mail: jdc@mole.bio.cam.ac.uk)

SUMMARY

The differentiation of the veins in the *Drosophila* wing involves the coordinate activities of several signal transduction pathways, including those mediated by the transmembrane receptors Torpedo and Notch. In this report, the role of the signalling molecule Decapentaplegic during vein differentiation has been analysed. It is shown that *decapentaplegic* is expressed in the pupal veins under the control of genes that establish vein territories in the imaginal disc. Decapentaplegic, acting through its receptor Thick veins, activates vein differentiation and restricts expression of both *veinlet* and the Notch-ligand *Delta* to the developing veins. Genetic combinations between mutations that increase or reduce *Notch*, *veinlet* and *decapentaplegic* activities suggest that the maintenance of the vein differentiation state during pupal development involves cross-regulatory interactions between these pathways.

Key words: *decapentaplegic*, *thick veins*, vein differentiation, cell interactions

INTRODUCTION

Cell differentiation involves the regulated expression of specific sets of transcription factors, such as the basic helix-loop-helix proteins of the MyoD family in muscle differentiation (Olson, 1990) or the proneural Achaete and Scute proteins during the establishment of neural precursors (Campuzano and Modolell, 1992; Ghysen et al., 1993). In many cases, the activity of specific signal transduction pathways directs the expression of regulatory proteins involved in the differentiation of cell types in precise patterns (Woods and Bryant, 1992; Greenwald and Rubin, 1992).

In the wing of *Drosophila*, two classes of structures, sensory organs and veins, differentiate in characteristic positions. The veins are formed by stripes of dorsal and ventral cells that are more compact and differentiate more pigmented cuticle than intervein cells. The veins correspond to clonal restrictions in the wing, suggesting that their positioning is related to the proliferation of the wing imaginal disc (Gonzalez-Gaitan et al., 1994; Milan et al., 1996). In wing discs of third instar larvae the expression of several genes is restricted to presumptive vein regions (Sturtevant et al., 1993; Heberlein et al., 1993), indicating that veins are already genetically specified. After eversion of the imaginal disc, dorsal and ventral wing surfaces adhere to each other and interactions between dorsal and ventral vein territories lead to the refinement of vein differentiation (Garcia-Bellido, 1977). At this stage the expression of different cell adhesion molecules becomes restricted to either vein or interveins (Fristrom et al., 1993; Murray et al. 1995).

The patterning and differentiation of the veins involves the activities of many genes, some of them components of different signal transduction pathways (Garcia-Bellido and de Celis, 1992). In the third instar wing disc the expression of *veinlet* (*ve*) is initiated in proximodistal stripes of cells that correspond to the presumptive veins (Sturtevant et al., 1993). The Veinlet protein appears to mediate an increase in the activity of the tyrosine kinase receptor, Torpedo (*top*; Sturtevant et al., 1993), and mutations in *top* itself, as well as in several genes belonging to the *top* pathway, result in the absence of veins (Diaz-Benjumea and Garcia-Bellido, 1990a; Diaz-Benjumea and Hafen, 1994). Conversely, mutations in which the activity of *top* or any of its downstream genes is increased, result in thicker and ectopic veins (Diaz-Benjumea and Garcia-Bellido, 1990a; Brunner et al., 1994). The formation of the veins also depends on the activity of the Notch pathway (Artavanis-Tsakonas et al., 1995), which restricts vein differentiation within broader 'vein competent' regions (de Celis and Garcia-Bellido, 1994b).

In addition to *Notch* and *torpedo*, the formation of veins requires the function of the gene *decapentaplegic* (*dpp*), as indicated by the phenotypes of particular *dpp* alleles (Segal and Gelbart, 1985). *dpp* is the best studied member of the Transforming growth factor β (TGFβ) superfamily in *Drosophila* (St. Johnston et al., 1990) and its function is required in processes as diverse as dorsoventral patterning in the embryo (Ferguson and Anderson, 1992; Wharton, et al., 1993), mesoderm specification (Staehling-Hampton and Hoffmann, 1994; Frasch, 1995), gut development (Panganiban et al., 1990) and growth and patterning of all imaginal discs (Posakony et al., 1991; Held et al., 1994). Recently, Dpp receptors that mediate most of the embryonic functions of Dpp have been isolated. They are encoded by the genes *thick veins* (*tkv*), *saxophone* (*sax*) and *punt* (*pnt*) (Xie et al., 1994; Brummel et al., 1994; Penton et al.,

1994; Letsou et al., 1995; Ruberte et al., 1995), and correspond to transmembrane proteins with serine-threonine kinase activity that belong to the previously identified TGFβ type I (Tkv and Sax) and type II (Punt) receptors (Massague et al., 1994; Wrana et al., 1994). The genetic structure of *dpp* is complex, with large *cis*-regulatory regions that direct *dpp* expression in particular tissues (St. Johnston et al., 1990). Two classes of *dpp* alleles specifically affect the development of the imaginal discs; dpp^{disc} alleles (dpp^{d}) affect the characteristic expression of *dpp* in anterior cells along the anteroposterior compartment boundary, and result in a failure in disc growth (Masucci et al., 1990; Blackman et al., 1991). Another class of alleles, *short vein* (dpp^{s}) affects the differentiation of the veins, causing incomplete veins which fail to reach the wing margin (Segal and Gelbart, 1985). Paradoxically, mutations that reduce the activity of the Dpp receptor *tkv*, result in opposite phenotypes, with the differentiation of ectopic vein tissue and thicker veins (Terracol and Lengyel, 1994).

In this paper the function of *dpp* and *tkv* during vein differentiation has been analysed, with particular emphasis on the relationships between them and between *dpp*/*tkv* and other signal transduction pathways affecting the same process. It is shown that *dpp* is expressed in the developing veins during pupal development, where it is required for the differentiation of the veins. *dpp* and *tkv* mutations interact with *Notch* and *ve* alleles, and alter the vein restricted expression of *ve* and the

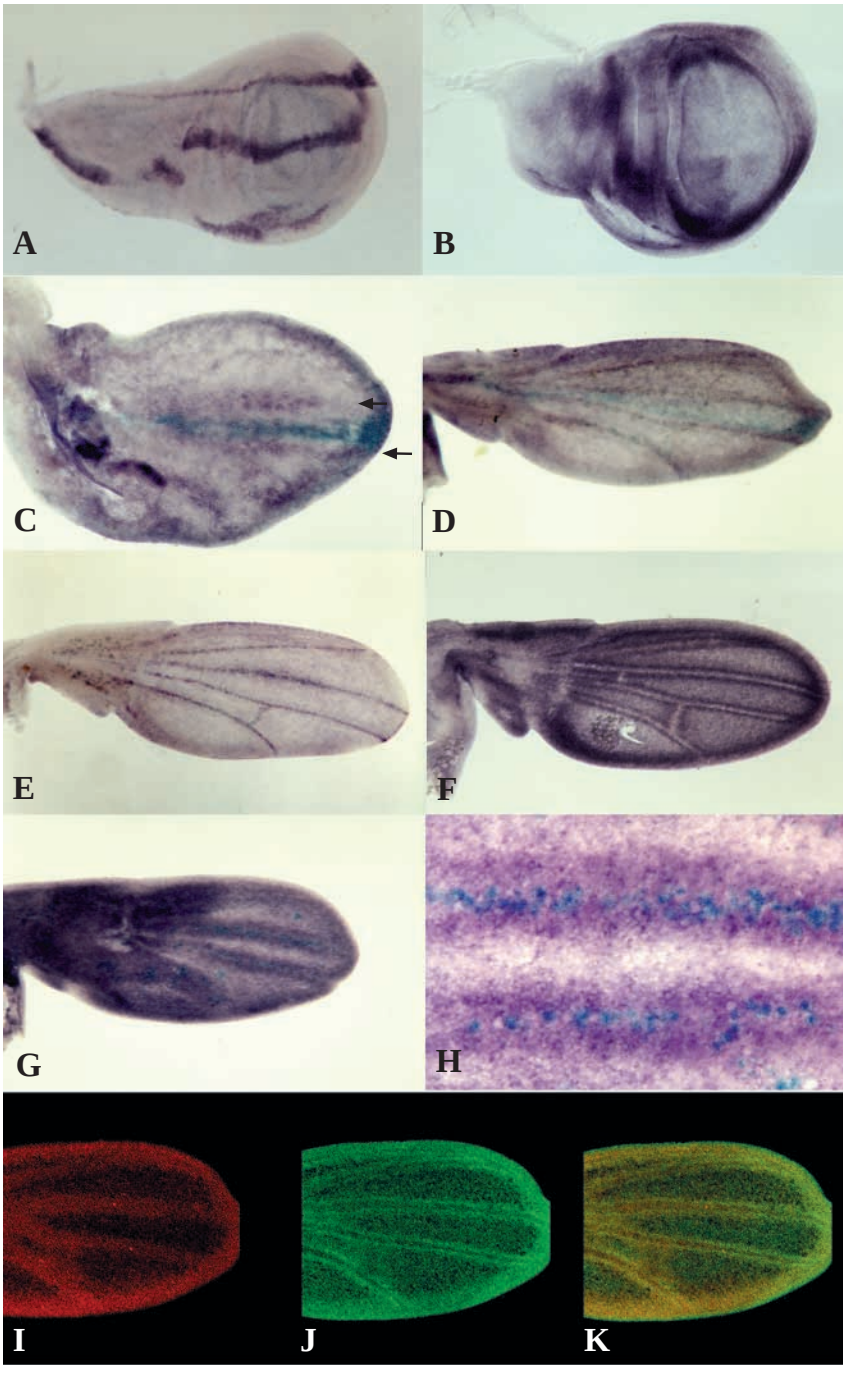

Fig. 1. Expression patterns of *dpp* and *tkv* in the wing imaginal disc and in the pupal wing. (A) *dpp* expression in wing discs from late third instar larva. (B) *tkv* expression in a wing disc of similar age showing reduced levels of expression in a central territory of the presumptive wing blade and along the dorsoventral boundary. (C,D) X-gal staining to reveal Notch expression in the reporter line N^{MLz} (blue) is mainly restricted to a broad domain localised between the veins LIII and LIV at 16 (C) and 30 (D) hours after puparium formation. The expression of *dpp* at 16 hours after puparium formation is recognisable in two broad stripes corresponding to the developing veins LIII and LIV (arrows in C), and is restricted to all longitudinal veins in pupal wings at 30 hours (D). (E-F) *dpp* (E) and *tkv* (F) expression in pupal wings 24-28 hours after puparium formation. *tkv* expression (F) is modulated with respect to the developing veins, with maximal expression at both sides of the vein. (G-H) X-gal staining (blue) to detect β-gal expression directed by 8.5 kb of *short vein* regulatory regions (shvLZ), and RNA expression of *tkv* (purple) show that higher levels of *tkv* transcription occurs at both sides of the stripe of *dpp* expressing cells. All the expression patterns related to the veins in the pupal wing occurs in the dorsal and ventral components of every vein. (I-K) Double staining to reveal β-gal expression in the shvLZ line (red in I) and Notch protein (green in J) and the merged image (K) in 20- to 24-hour old pupal wings, confirming that the stripes of *dpp* expression occurs in the developing veins.

Notch ligand Delta. Finally, *dpp* is able to trigger the vein differentiation program when it is inappropriately expressed in the pupal wing, leading to ectopic expression of *ve* and *Delta*, and to the formation of ectopic veins. These results suggest that the differentiation of the veins is a progressive process that involves sequential and coordinate activities of Torpedo, Notch and Dpp signal transduction pathways.

MATERIALS AND METHODS

Drosophila strains

The following alleles have been used. At the *dpp* locus the *short vein* alleles dpp^{s22}, dpp^{s4} and dpp^{s8} (Segal and Gelbart, 1985), at the *tkv* locus the hypomorphic, viable allele tkv^{1} and two lethal alleles, one null (tkv^{IIB}), the other antimorphic (tkv^{IO}) (Terracol and Lengyel, 1994; Nellen et al., 1994; Penton et al., 1994); at the *Notch* locus the loss-of-function allele fa^{nd} and the gain-of-function allele Ax^{28}; at the *veinlet* locus (also named *rhomboid* after the embryonic phenotype of its lethal alleles; Bier et al., 1990) the viable allele ve^{1} (Sturtevant et al., 1993) and the viable allele of *vein* (*vn*) vn^{1} (Simcox et al., 1996). Transgenic lines used were as follows. *3A* (activated *ras-2* under the regulation of the actin promoter; Bishop and Corces, 1988), *rho30* (*ve* under hs promoter; Sturtevant et al., 1993), *UAS-dpp* (Staehling-Hampton and Hoffmann, 1994), *UAS-tkv** (a constitutively active form of Tkv; Lecuit et al., 1996), *UAS-IMP* (Sweeney et al., 1995), *UAS-E(spl)mβ* (de Celis et al., 1996a) and the GAL4 lines GAL-580 and GAL4-1348. Clones of Dpp- and Tkv*-expressing cells were generated by FLP-mediated excision of a *lacZ* flip-out casette in flies of genotype *FLP1.22*/+; *Ubx>lacZ>dpp*/+ and *FLP1.22*/+; *Ubx>lacZ>tkv**/+ as described by Lecuit et al. (1996). Two reporter lines were also used: a construct in which an 8.5 kb *Eco*RI fragment of the *short vein* regulatory region (St Johnston et al. 1990) is fused to β-gal (shvLZ, a gift from J. Botas), and a *lacZ* insertion in the Notch locus (N^{MLz}) that reproduces the expression pattern of *Notch* (data not shown). The phenotypic analysis of genetic combinations was carried out in flies raised at 25ºC, with the exception of those involving the *UAS-dpp* which were done at 18ºC. Wings were mounted in lactic acid/ethanol (1:1) and photographed using a Zeiss axiophot microscope.

Generation of dpp^{s22} clones

dpp^{s22} clones were generated by X-ray-induced mitotic recombination. Larvae were irradiated (dose 1000 R; 300 R/min, 100 kv, 15 mA, 2 mm Al filter) 48-72 hours after egg laying (AEL). Clones were scored in males of genotype f^{36a}; dpp^{s22}/*M(2)Z Ins*$[f^{+}]$*30B*. Mitotic recombination proximal to the f^{+} insertion produces homozygous dpp^{s22} mutant cells labelled with the cell marker *forked* (*f*). 23 dorsal, 14 ventral and 6 dorsal and ventral clones were analysed.

In situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation with digoxigenin-labelled DNA probes to imaginal discs were performed as described previously for both imaginal discs (Cubas et al., 1991) and pupal wings (Sturtevant et al., 1993). In situ hybridisation with digoxigenin RNA labelled probes was carried out using the same protocols, but the hybridisation step and washes were at 55°C. The following DNA probes were used: 2 kb *Eco*RI fragment from a *dpp* cDNA clone (St. Johnston et al., 1990), 1.4 kb *Eco*RI/*Cla*I from a *tkv* cDNA clone (Nellen et al., 1994), 3 kb *Eco*RI from a *Dl* cDNA clone (Vassin et al., 1987), 0.7 kb *Hin*dIII/*Not*I from c-*mβ*-14a (Delidakis and Artavanis-Tsakonas, 1992), 0.8 kb *Bgl*II/*Kpn*I from a *Notch* cDNA clone (Kidd et al., 1986). *dpp* and *ve* RNA probes were synthesised from appropiate cDNA clones.

Immunocytochemistry with rabbit anti-β-galactosidase (Cappel), mouse monoclonal anti-Notch (Fehon et al., 1991) mouse anti-IMP (Sweeney et al., 1995) were carried out as described by Cubas et al. (1991). Secondary antibodies were from Jackson Immunological Laboratories (used at 1/250).

RESULTS

decapentaplegic and *thick veins* are differentially expressed in vein territories during pupal development

The effects of *dpp* and *tkv* mutations in vein formation prompted an analysis of their expression at different stages during wing development. In the wing imaginal disc, *dpp* and *tkv* expression patterns have no apparent relationship with presumptive vein territories. The expression of *dpp* is restricted to a narrow stripe of anterior cells abutting the anteroposterior compartment boundary (Fig. 1A; Sanicola et al., 1995), where

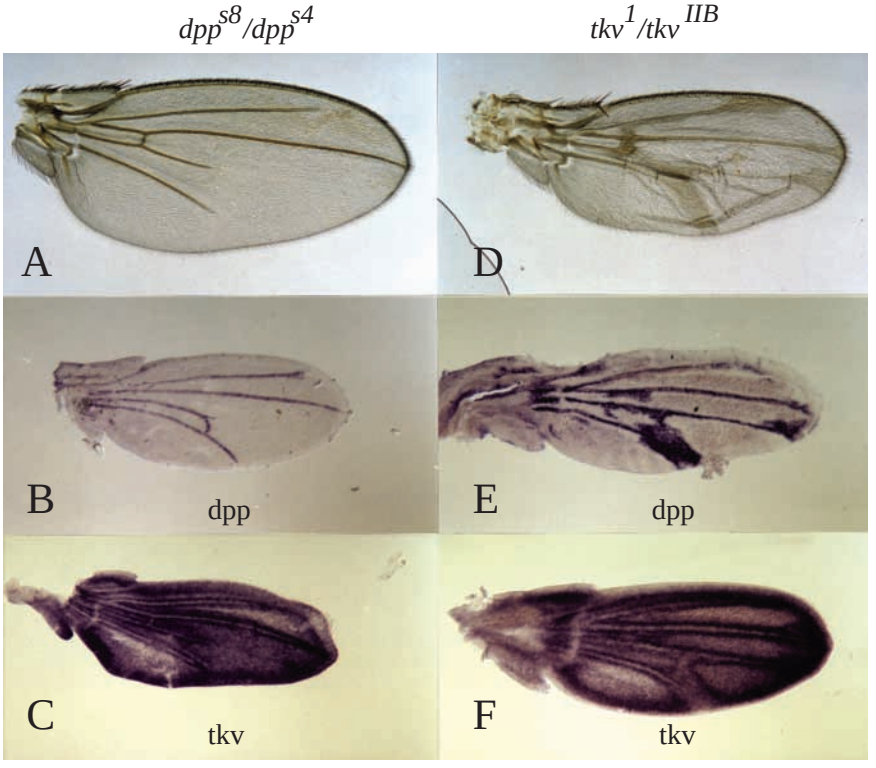

Fig. 2. Effects of dpp^{s} and *tkv* mutations on *dpp* and *tkv* expression. (A) Wings of genotype dpp^{s4}/dpp^{s8} lose the distal ends of LIV and to a lesser extent LV (A). (B-C) The extent of vein tissue loss in dpp^{s4}/dpp^{s8} correlates with the absence of *dpp* expression in the corresponding regions of the pupal wing (B), and with the lack of modulation in *tkv* expression in the same places (C). (D) Phenotype of tkv^{1}/tkv^{IIB} flies. (E-F) In pupal wings of this genotype, *dpp* expression is detected in the sites corresponding to the regions where extra vein tissue differentiates (E). The expression of *tkv* in the same genotype is affected at the same locations (F), where intermediate levels of *tkv* are present, mainly in distal LV vein. Pupal wings were 24-28 hours after puparium formation.

dpp it is required for the growth of the disc (Posakony et al., 1991). The expression of *tkv* is wide-spread, but clear differences in levels of expression can be recognised. Minimal expression of *tkv* occurs in a central region of the disc that includes both the stripe of *dpp*-expressing cells and the dorsoventral boundary, whereas maximal expression is observed in more anterior and posterior domains of the presumptive wing blade, pleuras and thorax (Fig. 1B; Brummel et al., 1994). *dpp* and *tkv* expression in wild-type discs does not change during the first 8 hours after puparium formation (not shown), but subsequently becomes related to the differentiating veins (Fig. 1C and data not shown). *dpp* transcription appears in broad stripes of dorsal and ventral cells along the presumptive veins 12-16 hours after puparium formation (Fig. 1C), and latter becomes restricted to the developing veins (Fig. 1D,E,I-K). At approximately the same time *tkv* expression is modulated with respect to the veins, with minimal expression within the vein, and higher expression at both the anterior and posterior boundaries of every vein (Fig. 1F-H). The expression of *dpp* and *tkv* occurs in both the dorsal and ventral vein components. The relationship between the developing veins and expression of *dpp* and *tkv* suggests that they are involved in vein formation at the pupal stage. In addition, the coincidence between low levels of *tkv* expression and the regions where *dpp* is expressed in the wing disc and in the pupal veins (Fig. 1G,H) suggests that the expression of *tkv* is reduced in cells that produce or are exposed to Dpp. A similar relationship between *dpp* expression and lower levels of *tkv* transcription is also manifest in leg and eye-antenna discs (Brummel et al., 1994 and data not shown).

decapentaplegic and *thick veins* regulate each other and mediate vein differentiation

Viable dpp^s and *tkv* alleles cause loss and gain of vein tissue, respectively (Fig. 2A,D; Segal and Gelbart, 1985; Terracol and Lengyel, 1994). The expression of *dpp* and *tkv* in the third instar wing disc is not modified by dpp^s and *tkv* mutations that result in strong modifications of the vein pattern (data not shown), suggesting that the effect of these mutations on vein differentiation occur after the veins have been specified in the imaginal disc. In dpp^s combinations (dpp^{s4}/dpp^{s8}) there is a good correlation between the veins that are absent in the adult wing and the disappearance of *dpp* expression in the corresponding presumptive vein regions in the pupal wing (Fig. 2A,B). These observations are compatible with dpp^s mutations affecting *cis*-regulatory regions (St. Johnston et al., 1990) required to activate *dpp* in presumptive vein cells in the pupal wing. In addition, the absence of *dpp* always occurs symmetrically in both the dorsal and ventral components of the affected veins. The expression of *tkv* in dpp^s pupal wings remains at high levels in the regions of the developing veins where *dpp* is absent (Fig. 2C), indicating a causal relationship between the presence of Dpp and the reduction of *tkv* transcription in the developing veins. The expression of *tkv* in *tkv* viable heteroallelic combinations (tkv^1/tkv^{IIB}) is only modified in regions where ectopic veins differentiate (Fig. 2D,F). Interestingly, *dpp* is ectopically expressed in pupal wings of *tkv* mutants, mainly in the sites where ectopic vein tissue differentiates in *tkv* wings (Fig. 2E).

The effects of *tkv* mutations on *dpp* expression suggest a feedback mechanism in which Tkv represses *dpp* expression. This observation helps to explain the paradox that mutations in *dpp* eliminate veins while mutations in its receptor cause ectopic vein formation. Thus, the ectopic veins that differentiate in *tkv* mutants could be a consequence of ectopic Dpp acting on Tkv functional products present in cells with reduced Tkv activity. In addition, it appears that Tkv is also necessary to activate vein differentiation. Thus double mutant combinations between dpp^s and *tkv* result in strong dpp^s phenotypes in which the differentiation of *tkv* ectopic veins is suppressed, and, in addition, the loss of veins typical of dpp^s is strongly

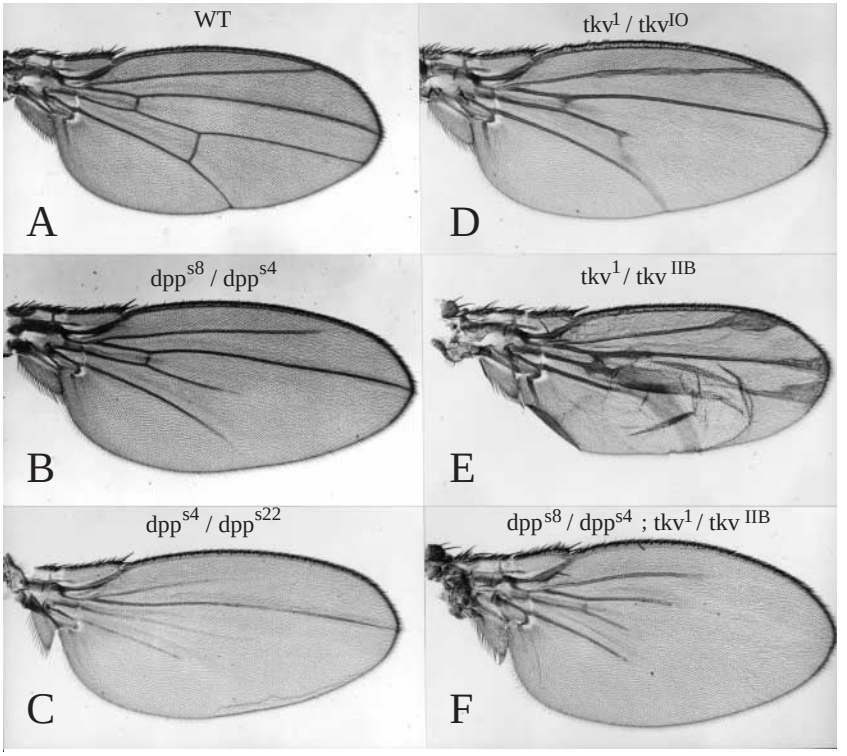

Fig. 3. Wing phenotypes resulting from modifications of *dpp* and *tkv* activity. (A) Wild-type wing showing the position of the longitudinal veins (LII, LIII, LIV and LV from anterior to posterior). (B-C) Weak dpp^s heteroallelic combinations (dpp^{s4}/dpp^{s8}) result in the absence of only the distal ends of the veins LII, LIV and LV (B), whereas stronger combinations (dpp^{s4}/dpp^{s22}) eliminate most of the veins LII, LIV and LV (C). (D-E) The combination between the *tkv* viable hypomorphic allele tkv^1 and the null allele tkv^{IIB} results in variable thickening of the veins, particularly in regions close to the cross veins and in the distal ends of all longitudinal veins (E). (D) The combination between the same viable allele and the antimorphic allele tkv^{IO} (Terracol and Lengyel, 1994; Nellen et al., 1994) cause weaker vein thickening, and in addition the absence of distal stretches of LIV. (F) In the combination dpp^{s4} tkv^1/dpp^{s8} tkv^{IIB} the *tkv* phenotype is suppressed (compare with E), and the *shv* phenotype is strongly enhanced (compare with B).

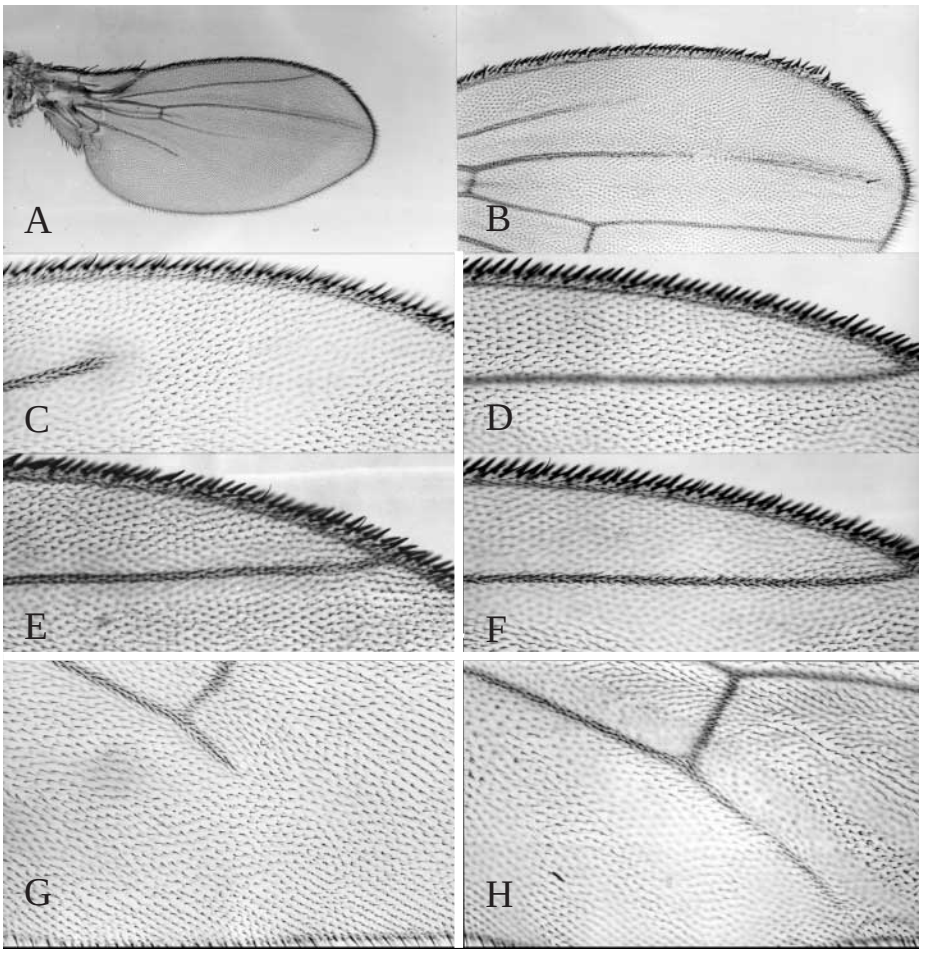

Fig. 4. Mosaic analysis of dpp^{s22}. (A-B) Examples of large dorsoventral dpp^{s22} clones occupying all the posterior (A) and anterior (B) compartments, and causing loss of veins. (C-F) Effects of different clones on the differentiation of the ventral LII vein. Dorsoventral clones eliminate LII distally (C), whereas dorsal (D) or ventral (E) clones do not affect the differentiation of this vein. (F) is focussed in the ventral LII vein that differentiates despite the presence of a large clone in the dorsal surface (D). (G-H) The dorsal vein LV is eliminated only when clones extend to both the dorsal and ventral surfaces (G, only dorsal surface in focus), and differentiate when clones occupy only the dorsal component of the vein (H). In C, D and E, all the regions in the frame are formed by mutant cells, and in G and H mutant clones form the relevant veins.

enhanced (Fig. 3B,E,F), reproducing the pattern of vein loss observed in stronger dpp^{s} heteroallelic combinations (Fig. 3C). This model is compatible with the observation that *tkv* heteroallelic combinations that result in further reductions in the levels of Tkv functional products (tkv^{1} and the antimorphic *tkv* allele tkv^{IO}) cause the elimination of veins (Fig. 3D; Terracol and Lengyel, 1994). The interpretation that Tkv has a dual function during vein differentiation, repressing *dpp* in intervein cells neighbouring the vein, and activating vein differentiation in vein cells, is reinforced by the behaviour of *tkv* mutant clones: they prevent vein differentiation autonomously, but induce vein differentiation in adjacent wild-type cells (Burke and Basler, 1996), consistently with the postulated repression of *dpp* by Tkv.

To confirm that the effects of *dpp* in vein differentiation are due to the localised expression of *dpp* in pupal veins and not to its earlier expression in anterior cells in the disc, a mosaic analysis of a strong dpp^{s} allele (dpp^{s22}) was carried out. It is expected that posterior as well as anterior dpp^{s22} clones would affect vein formation. Clones of $Minute^{+}$ cells homozygous for the dpp^{s22} allele are of normal size and do not modify the overall patterning of the wing (Fig. 4A,B). When clones are restricted to only one wing surface (dorsal or ventral) both anterior and posterior clones have minimal effects on vein differentiation (Fig. 4D-F,H). However, anterior and posterior dpp^{s22} clones induced before the segregation of dorsal and ventral compartments consistently remove the distal regions of LII and cause gaps in LIII in anterior dorsoventral clones, and remove most of LIV and LV in posterior dorsoventral clones (Fig. 4A-C,G). The comparison of the dpp^{s22} phenotype in dorsal, ventral and dorsoventral clones indicates that the presence of wild-type cells in either the dorsal or ventral wing surface is enough to implement vein differentiation in both surfaces, suggesting that *dpp* expression in one surface is sufficient to provide dpp function to the other surface.

The expression pattern of *dpp* and the phenotype of dpp^{s} alleles in viable combinations or in genetic mosaics indicates that the localised expression of *dpp* during pupal development mediates vein differentiation. Thus it is expected that ectopic *dpp* expression or Tkv activation during this period would result in the differentiation of ectopic veins. This was tested using the GAL4 system (Brand and Perrimon, 1993) to drive ectopic expression of *dpp* and an activated, ligand independent, Tkv receptor (Tkv*, Lecuit et al., 1996) in the pupal wing. In GAL4-580/+; UAS-dpp/+ flies (580-dpp) raised at 18ºC during larval development and at 25ºC during pupal development, there is ectopic expression of *dpp* in the pupal wing in two broad domains, one anterior and other posterior (Fig. 5C). As a consequence, the expression of *tkv* is strongly repressed in the same territories (Fig. 5D), and the resulting adult wing differentiates ectopic vein tissue in the places that correspond to the domain of ectopic *dpp* expression (Fig. 5B). As expected, the phenotype

of 580-dpp flies is temperature-sensitive, being weaker when pupal development occurs at 17ºC (Fig. 5A). When *dpp* is expressed in all intervein cells during pupal development (using the line GAL4-1348, Fig. 6A), most wing cells differentiate as vein (Fig. 6B). Ectopic expression of activated Tkv also leads to the formation of ectopic veins, both using GAL4-580 (data not shown) and GAL4-1348 (Fig. 6C,F). The formation of ectopic vein tissue can also be induced in mosaics of cells that express Dpp or Tkv* (Fig. 6G-I). Although in these experiment clones are not labelled, there is a good correlation between the time of clone induction and the size of the ectopic veins (Fig. 6G-I).

Genetic interactions between dpp/tkv and veinlet

The activity of early-acting vein promoting genes might be required to activate *dpp* and *tkv* expression in the developing veins in the pupa. The pathway mediated by the tyrosine kinase receptor Torpedo is involved in the early positioning of vein territories in the imaginal disc, and the localised expression of its putative ligand Vein and the transmembrane protein Veinlet are important components of this function (Sturtevant et al., 1993; Sturtevant and Bier, 1995; Simcox et al., 1996; Schnepp et al., 1996). Thus, a regulatory mutation that removes *ve* expression in the developing veins (ve^1; Sturtevant et al., 1993) causes the loss of the distal ends of all longitudinal veins (Fig. 7A; Diaz-Benjumea and Garcia-Bellido, 1990b), and the genetic combination ve^1 vn^1 eliminates all longitudinal veins (Fig. 7J; Diaz-Benjumea and Garcia-Bellido, 1990b), presumably because of a strong reduction in Top activation in vein territories. Conversely, ectopic expression of *ve* in the disc and pupal wing (as in rho^{30} heterozygous flies) results in the differentiation of ectopic and thicker veins (Fig. 7D; Sturtevant et al., 1993). The resulting phenotypes of genetic combinations between *ve*, *dpp* and *tkv* alleles are presented in Fig. 7. These results can be summarised as follows: ve^1 suppresses the differentiation of the thicker veins of *tkv* alleles and results in fewer veins in combination with both *tkv* and dpp^s (Fig. 7B,C). The ectopic veins typical of *rho30* are suppressed in combination with dpp^s, even in regions where dpp^s does not show any obvious phenotype (Fig. 7E); conversely, the differentiation of thicker veins in *rho30*/+ is strongly increased in combination with *tkv* (Fig. 7F). The ve^1 vn^1 phenotype is not modified in combinations with either dpp^s or *tkv* (data not shown), but vein tissue differentiates when *dpp* is expressed ectopically in ve^1 vn^1; *580-dpp* flies (Fig. 7J-L). In pupal wings of this genotype, *ve* expression is absent in the wing blade (data not shown), indicating that Dpp activity is able to trigger vein differentiation even in the absence of *ve*. The interactions between dpp^s and *tkv* mutations and a transgenic line in which an activated version of *D-ras2* (*ras2**) is expressed ubiquitously under the control of the actin promoter were also analysed. *ras2** flies differentiate ectopic veins (Fig. 7G), and in *ras2** pupal wings *ve* is ectopically expressed in the proximity of the veins (data not shown). As with *rho30*, the phenotype of *ras2** flies is suppressed in combination with dpp^s (Fig. 7H) and increased in combinations with *tkv* mutations (Fig. 7I). To further investigate the epistatic relationships between Top activity and Dpp signalling, a dominant negative Draf1 (raf^{DN}, a gift from E. Martin) and Tkv* were expressed in the same cells using the GAL4 system (Fig. 6C,E). These flies (GAL4-1348/+; UAS-tkv*/UAS-raf$^{DN)}$ have a weaker phenotype of ectopic vein differentiation than their control siblings (GAL4-1348/+; UAS-tkv*/+), suggesting that Top and Tkv activities co-operate in promoting vein differentiation.

The adult vein patterns observed in genetic combinations between *ve*, dpp^s and *tkv* are correlated with similar changes in the expression patterns of *ve*, *dpp* and *tkv* in mutant pupal wings (Fig. 8). Thus, *dpp* and *tkv* are not expressed in ve^1 vn^1 wings (Fig. 8A,C), indicating that their expression in vein territories is activated by Top. In *rho30*/+ and *ras2** pupal wings, *dpp* is expressed ectopically at the sites where ectopic *ve* occurs (Fig. 8B and not shown), suggesting that *ve* is involved in the control of *dpp* expression in the developing veins. These transcriptional effects are reciprocal: *ve* expression is eliminated in the affected veins of dpp^s wings (Fig. 8D), and ectopically expressed in *tkv* pupal wings (Fig. 8E), and in *580-dpp* and *1348-tkv** pupal wings (Figs 5E, 8F), indicating that *dpp*

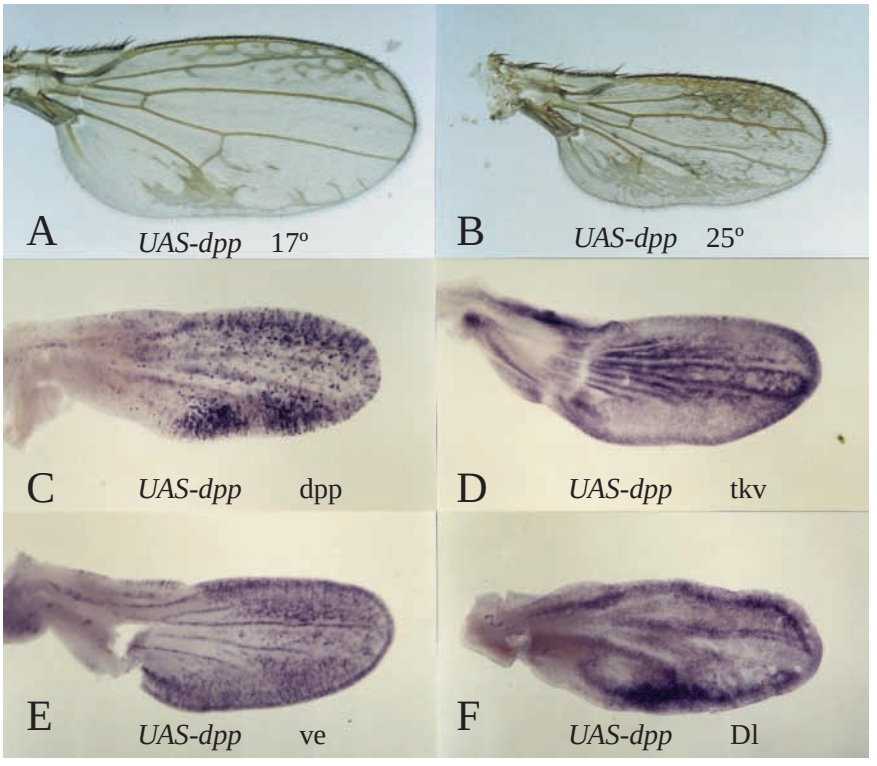

Fig. 5. Effects of ectopic *dpp* expression in the pupal wing on vein differentiation. (A-B) The ectopic expression of *dpp* in the pupal wing in the combination *GAL4-580/+*; *UAS-dpp/+* results in the differentiation of ectopic veins when development occurs at 17ºC (A). This phenotype is increased when pupa are maintained at 25ºC until eclosion (B). (C) In $GAL\text{-}4^{580}$/+; *UAS-dpp*/+ pupal wings (24-28 APF) maintained during pupal development at 25ºC, *dpp* is ectopically expressed in anterior and posterior domains. (D) The transcription of *tkv* is down-regulated in the places where *dpp* is present at the same stage. (E-F) The expression of *ve* (E) and *Dl* (F) is activated as a consequence of *dpp* ectopic expression. Pupal wings were 24-28 hours after puparium formation.

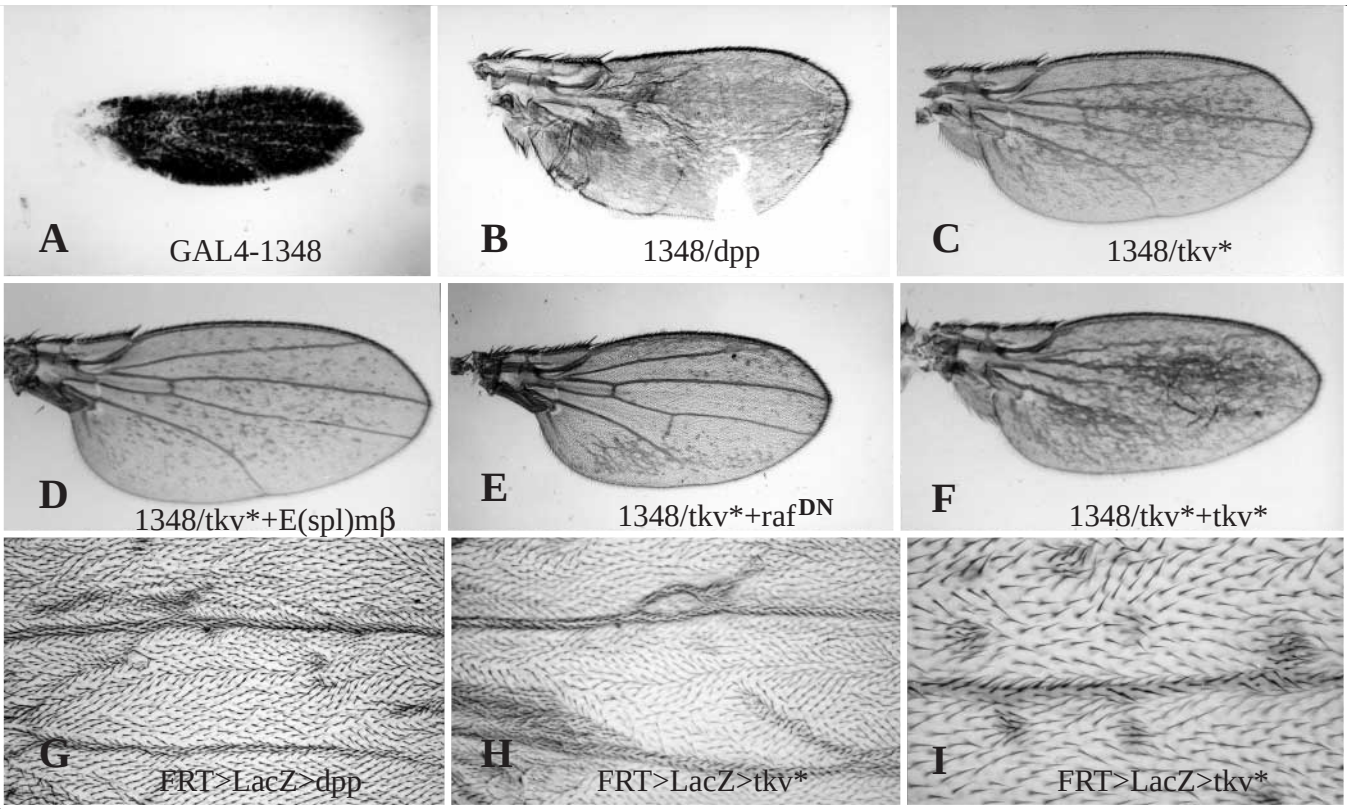

Fig. 6. Effects of ectopic Tkv activated receptor (Tkv*) in vein differentiation. (A) Expression pattern of the GAL4 line 1348 occurs in most intervein cells during pupal development (here GAL4-1348/+; UAS-IMP/+ 25 hours old pupa stained with anti-IMP), and is not expressed in the imaginal disc (not shown). (B-D) The combinations GAL4-1348/+; UAS-dpp/+ (1348/dpp; B), GAL4-1348/+; UAS-tkv*/+ (1348/tkv*; C) and GAL4-1348/+; UAS-tkv*/UAS-tkv* (1348/tkv*+tkv*; D) produce the differentiation of ectopic vein tissue throughout the wing blade. (E-F) The presence of one copy of either UAS-E(spl)mβ (GAL4-1348/UAS-E(spl)mβ; UAS-tkv*/+; D) or UAS-raf^{DN} (GAL4-1348/+; UAS-tkv*/UAS-raf^{DN}; E), reduces but does not supress completely the extra vein differentiation of ectopic Tkv*. (G-I) FLP induced clones of *dpp* (G) and *tkv**-expressing cells result in the formation of ectopic veins. Although clones are not labelled in these experiments, the size of the ectopic veins is smaller when clones are induced later (compare H and I). Clones were induced in flies *FLP1.22/+; Ubx>FRTβgal>dpp* (G) and *FLP1.22/+; Ubx>FRTβgal>tkv** (H,I) with a 10 minute heat shock at 37°C at 72-96 hours AEL (G-H) and 96-120 hours AEL (I).

activity is involved in the regulation of *ve* expression in the developing veins. The phenotypic interactions between *top*/*ve* and *dpp* genetic variants, and the requirements of *ve* and *dpp* functions for *dpp* and *ve* expression respectively, suggest that *dpp* and *ve* transcription are linked to each other: Top function would be required to activate *dpp*, and subsequently Dpp activity would maintain the expression of *ve* in the veins.

Genetic interactions between the dpp/tkv and the Notch pathway during vein differentiation

The function of the *Notch* pathway is required for the veins to differentiate their normal thickness; mutations that reduce the activity of *Notch* result in thicker veins, whereas mutations that increase *Notch* activity cause the loss of veins (de Celis and Garcia-Bellido, 1994a,b). *Notch* function is also required during pupal development, since temperature shifts experiments at this stage with N^{ts} alleles alter vein thickness (Shellenbarger and Mohler, 1978). The relationships between *Notch* and *dpp*/*tkv* were analysed using genetic combinations between mutations that modify the activities of these pathways (Fig. 9). In combinations between dpp^s and *Notch* loss-of-function alleles, the dpp^s loss of vein phenotype is epistatic, and the vein thickening typical of the *Notch* allele is eliminated (Fig. 9A,B). Conversely, *Notch* gain-of-function alleles that lead to truncation of veins (*Abruptex*, *Ax*) result in very pronounced vein loss in combination with both dpp^s and *tkv* mutations (Fig. 9D-F). The thickening of veins caused by *tkv* is suppressed in combinations with *Ax* mutations (Fig. 9F), and strongly enhanced in combination with *Notch* loss-of-function alleles (Fig. 9C). Finally the extent of extra-vein differentiation caused by ectopic expression of Tkv* (Fig. 6C) is strongly reduced when the Enhancer of split gene *mβ* is expressed in the same cells during pupal development (Fig. 6D), suggesting that Dpp/Tkv* and the Notch downstream gene *E(spl)mβ* have antagonistic effects on common target genes during vein differentiation, a good candidate being the gene *ve* (Sturtevant and Bier, 1995).

The relationship between Notch and Dpp activities in vein development is also manifest in the effects of *dpp* mutations on the transcription of the Notch ligand *Delta* (*Dl*) and the Notch target gene *E(spl)mβ*. In normal pupal wings the expression of *Dl* and *E(spl)mβ* occurs in vein territories (J. de Celis, S. Bray and A. Garcia-Bellido, unpublished data). In dpp^s mutations, *Dl* and *E(spl)mβ* expression are absent in the affected vein regions (not shown), and both *Dl* and *E(spl)mβ* transcription are activated when Dpp or tkv* are ectopically expressed in pupal wings (Figs 5F, 8G-I and data not shown). These results indicate

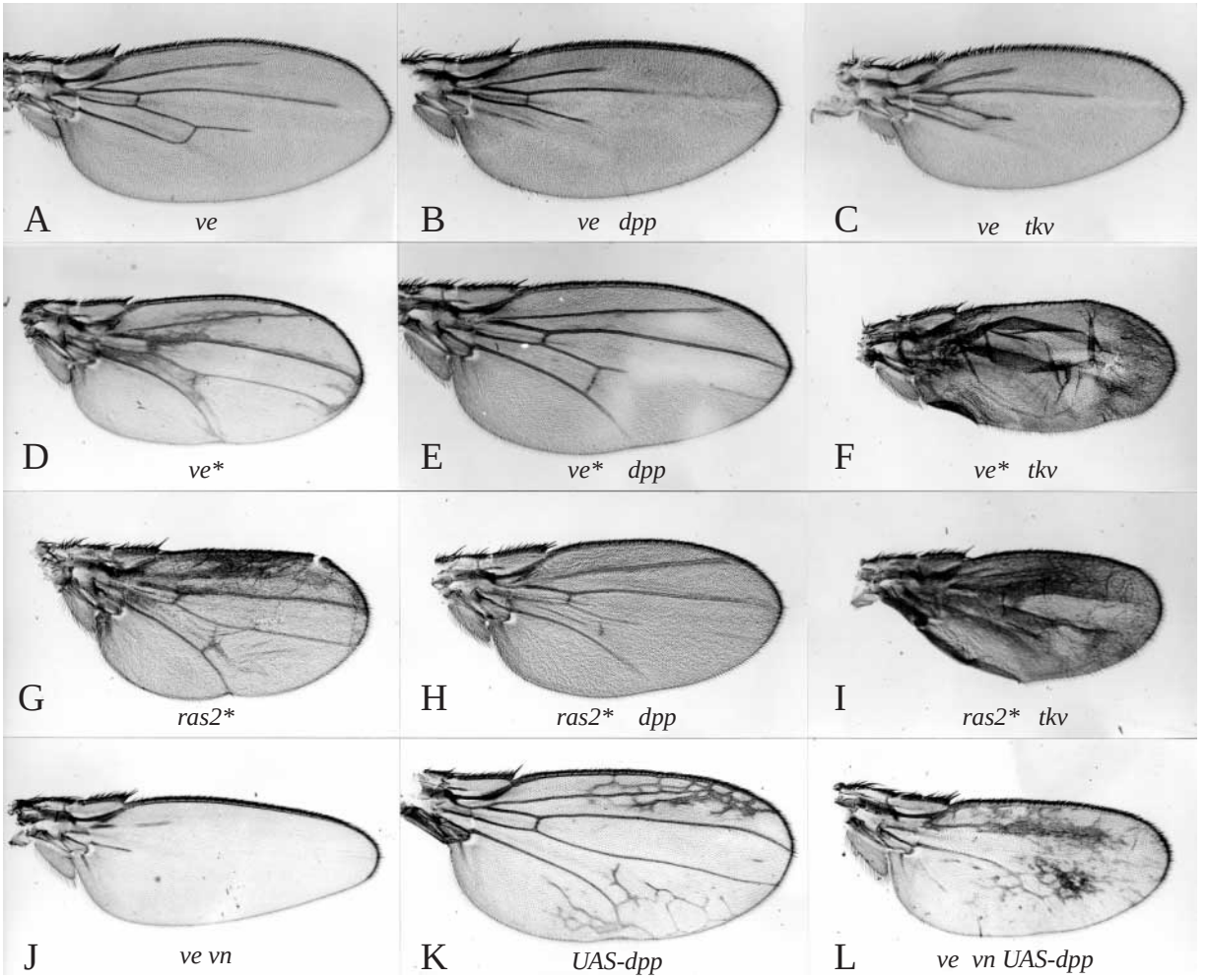

Fig. 7. Genetic interactions between dpp^s and *tkv* viable heteroallelic combinations with *ve* and *ras-2* variants. The genotype of the different mutant combinations are: (A) $ve^1/TM3$, ve^1 (*ve*). (B) dpp^{s4}/dpp^{s8}; $ve^1/TM3$, ve^1 (*ve dpp*). (C) tkv^1/tkv^{IIB}; $ve^1/TM3$, ve^1 (*ve tkv*). Control dpp^{s4}/dpp^{s8} and tkv^1/tkv^{IIB} phenotypes are shown in Fig. 3A and E respectively. (D) *rho30/+* (*ve**), (E) dpp^{s4}/dpp^{s8}; *rho30/+* (*ve* dpp*). (F) tkv^1/tkv^{IIB}; *rho30/+* (*ve* tkv*). (G) *ras-2*/Y* (*ras2**). (H) *ras-2*/Y*; dpp^{s4}/dpp^{s8}(*ras2* dpp*^{s}). (I) *ras-2*/Y*;tkv^1/tkv^{IIB}(*ras2* tkv*). (J) ve^1 vn^1 (*ve vn*), (K) $GAL4^{580}$/+; ve^1 vn^1 *UAS-dpp*/*TM2* (*UAS-dpp*), and (L) $GAL4^{580}$/+; ve^1 vn^1 *UAS-dpp*/ve^1 vn^1 (*ve vn UAS-dpp*).

that the expression of *Dl*, and consequently the maintenance of Notch signalling and *E(spl)mβ* expression, depends on Dpp function during pupal development.

DISCUSSION

The differentiation of the veins is a progressive process, in which vein territories are first specified in the imaginal disc, and then vein differentiation proceeds during the pupal stage through the combined activities of Top/Ve, Notch and Dpp signalling pathways. The function of *dpp* during the generation of the venation pattern is first required in the imaginal disc to establish the position of the veins, partly through the regulation of the transcription factors encoded by the genes *spalt* and *spalt-related* (de Celis et al., 1996b). Later, *dpp* expression is activated in the veins, where it is required for their differentiation. The restricted expression of *dpp* and its receptor Tkv to vein regions depends upon early acting genes (*top*/*ve* and *Notch*) that define the position of the veins in the disc. In addition, the presence of Dpp itself in the veins is required for the maintenance of at least *ve* and *Dl* expression in these places, since either reduced or ectopic Tkv activation is associated with similar changes in the expression of both *ve* and *Dl*. Thus it appears that the activity of early acting genes promotes the expression of late acting ones, and subsequently they become linked through feed-back mechanisms that keep their expression restricted to the developing veins (Fig. 10).

Vein differentiation requires localised expression of *dpp* to developing veins during pupal development

The expression of *dpp* in anterior cells abutting the anterior-posterior compartment boundary directs growth and patterning of the wing disc (Posakony et al., 1991). *dpp* mutations that reduce or eliminate this expression (dpp^d alleles) produce smaller wings in which the veins are absent or incorrectly positioned (Gelbart, 1989; Masucci et al., 1990). In addition, the differentiation of the veins is directly affected by *dpp* late during pupal development, when new expression of *dpp* appears in the developing veins. Strong dpp^s heteroallelic com-

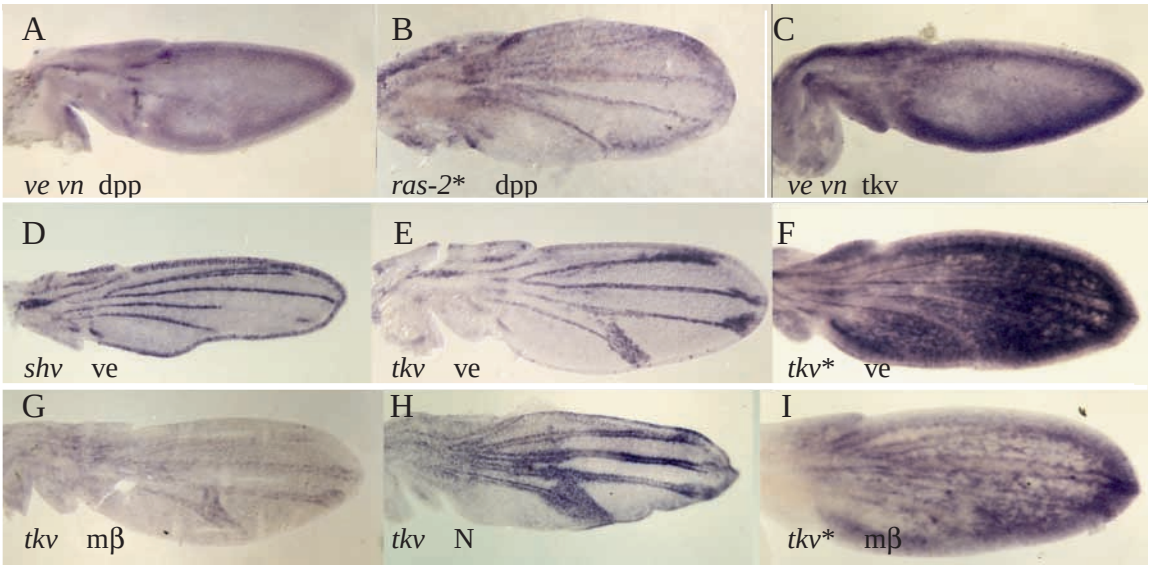

Fig. 8. Modifications in the expression of *dpp*, *tkv*, *ve*, *Notch* and *E(spl)mβ* in different mutant backgrounds. (A, B) Expression of *dpp* in ve^{1} vn^{1} (*ve vn* dpp; A) and in *ras2** (*ras2** dpp; B). (C) Expression of *tkv* in ve^{1} vn^{1} (*ve vn* tkv). (D-F) Expression of *ve* in dpp^{s4}/dpp^{s8} (*shv* ve; D) in tkv^{1}/tkv^{IIB} (*tkv* ve; E) and in GAL41348/+;UAS-tkv*/+ (*tkv** ve; F). (G,I) Expression of *E(spl)mβ* in tkv^{1}/tkv^{IIB} (*tkv* mβ; G) and GAL41348/+;UAS-tkv*/+ (*tkv** mβ; I). (H) Expression of *Notch* in tkv^{1}/tkv^{IIB} (*tkv* N). During pupal development the expression of both *Notch* and *E(spl)mβ* depends on Notch signalling (J. de Celis, S. Bray and A. Garcia-Bellido, unpublished data). All pictures are pupal wings 24-28 hours APF.

binations that remove most of the longitudinal veins do not affect the size of the wing or the expression of *dpp* in the imaginal disc. However they eliminate the expression of *dpp* in the affected presumptive vein territories, indicating a separation between the functions of *dpp* in the growth of the disc and in the differentiation of the veins. Mosaic analysis of dpp^{d} and dpp^{s} alleles confirm this separation, since posterior clones of dpp^{s22} affect the differentiation of the posterior veins, while posterior clones of a strong *dpp disc* allele (dpp^{d5}) differentiate wild-type veins (unpublished observations). dpp^{s} alleles map within a 10 kb region 5′ to the *dpp* transcription unit, whereas the dpp^{d} alleles map within a 20 kb 3′ region, and therefore the separate requirements of *dpp* can be attributed to distinct mechanisms involved in regulating its expression in the imaginal disc and in the pupal wing (St. Johnston et al., 1990).

thick veins participates in the *decapentaplegic* function in the differentiation of the veins

The activity of Dpp is mediated by receptor complexes, one of its elements being the Tkv protein (Affolter et al., 1994; Brummel et al., 1994), whose expression in the pupal wing is related to the differentiating veins. The effects on vein differentiation of complete removal of *tkv* (Burke and Basler, 1996), the extreme absence of veins seen in combina-

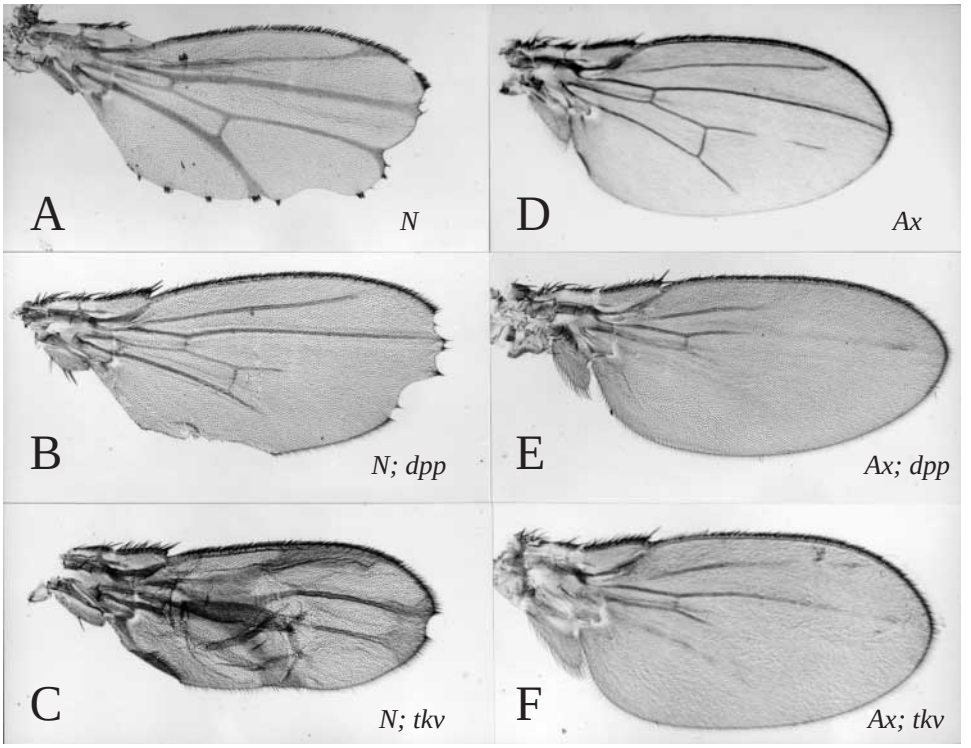

Fig. 9. Interactions between *dpp*, *tkv* and *Notch* alleles. (A-C) Genetic combinations involving the *Notch* loss-of-function allele fa^{nd} (*N*; A) with dpp^{s4}/dpp^{s8} (*N; dpp*; B) and tkv^{1}/tkv^{IIB} (*N; tkv*; C). (D-F) Genetic combinations involving the *Notch* gain-of-function allele Ax^{28} (*Ax*; D) with dpp^{s4}/dpp^{s8} (*Ax; dpp*; E) and tkv^{1}/tkv^{IIB} (*Ax; tkv*; F).

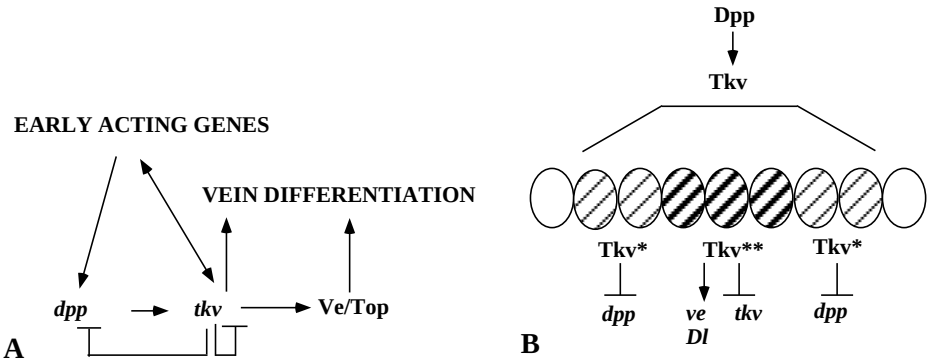

Fig. 10. Model of genetic and cellular interactions affecting vein differentiation during pupal development. (A) Antagonistic interactions between Top and Notch activities (early acting genes) determine the extent of vein competent territories during imaginal development, presumably by restricting the expression of specific transcription factors to these vein territories. One of the functions of these transcription factors would be the transcriptional activation of both *dpp* and *tkv* in the developing veins. Tkv activation by Dpp would produce different responses in different cells: within the vein Tkv downregulates its own transcription, activates and maintains the expression of early acting genes such as *ve*, and activates, probably in conjunction with Top, vein differentiation genes. In boundary cells that separate the vein from the intervein, Tkv would repress *dpp* transcription, maintaining its vein restricted expression and impeding the spread of Dpp. (B) Cellular interactions in vein cells (darkly hatched circles), and in boundary intervein cells (lightly hatched circles) that maintain the observed patterns of gene expression and activity. The different outcomes of Tkv activation in vein and boundary intervein cells (Tkv** and Tkv* respectively) could be determined by different levels of Tkv activation (above), by the presence or another Tkv ligand acting in the intervein, or by interactions between Tkv and another Dpp receptor in these cells.

tions between dpp^s and *tkv* mutations, and the effects of ectopic activation of Tkv, indicates that Dpp directs vein differentiation through activation of Tkv. The extra vein differentiation observed in viable *tkv* heteroallelic combinations correlates with ectopic *dpp* expression at the sites where ectopic vein tissue develops, and requires *dpp*, as illustrated by the suppression of this phenotype in combination with dpp^s alleles. Thus it appears that excess vein differentiation in hypomorphic loss-of-function *tkv* alleles is due to de-repression of *dpp* in cells close to the vein, the ectopic Dpp product acting on residual Tkv functional receptors being responsible for the differentiation of thicker veins. The repression of *dpp* transcription by Tkv could participate in restricting *dpp* expression to the veins, and implies that some mechanism must ensure that this repression is not operative in the vein cells that express *dpp*. It is possible that the observed downregulation of *tkv* expression in vein cells participates in generating the levels of Tkv activation necessary to activate vein differentiation, but insufficient to repress *dpp* expression. In addition, other elements such as additional Tkv ligands, or other Dpp receptors could participate in determining the two different outcomes of Tkv activation: vein differentiation in cells that express *dpp*, and *dpp* repression in adjacent cells (Fig. 10).

The correlation between sites of *dpp* expression and downregulation of *tkv* in the imaginal discs and in the developing veins is maintained when the distribution of *dpp* is altered. Thus when *dpp* is ectopically expressed or when its expression is reduced, the expression of *tkv* is reduced or increased respectively. It is possible that the downregulation of *tkv* is a direct consequence of Tkv activation, following Dpp binding and activation of downstream transcription factors that would regulate *tkv* expression. Interestingly, a similar repression of *torpedo* expression occurs following Top activation, both during embryonic development and during the differentiation of the veins (Sturtevant et al., 1994), suggesting that mechanisms which regulate the levels of receptor synthesis are linked with receptor activity, and could be significant in modulating the activities of signalling pathways.

The establishment of vein territories in the disc by *torpedo*/*veinlet* and *Notch* functions is implemented by *dpp* in the pupal wing

The establishment of vein territories in the imaginal disc requires the coordinate and antagonistic activities of Top and Notch pathways. In both cases, expression of at least some of their elements, namely *veinlet* and *Delta*, is restricted to the veins from the late third instar disc and throughout pupal development. The expression of *dpp* and *tkv* in vein territories depends, directly or indirectly on Top activity, because their transcription is not activated when Top activity is reduced (*ve vn* pupal wings). Once Dpp is established in the veins, local activation of Tkv in these cells is required both for the maintenance of *ve* and *Dl* expression and for the veins to differentiate. In experimental conditions in which *dpp* is expressed ectopically, vein differentiation proceeds in the absence of *ve* activity, suggesting that *dpp* is able to implement vein differentiation even when the levels of top activity are reduced. In addition, the extra vein differentiation produced as a consequence of ectopic Tkv activated receptor is corrected by the simultaneous presence of either a dominant negative DRaf protein, or the vein suppressing protein E(spl)mß, suggesting that vein differentiation genes are activated more efficiently by the coordinate activities of both Tkv and Top (Fig. 10).

Cross interactions between different signalling pathways in the pupal wing

The differentiation of the veins is a complex process that starts in the imaginal disc and continues during pupal development. It appears that signals that establish the position of the veins in the imaginal disc (*top*/*ve* and *Notch*), and late acting elements of the vein differentiation cascade (*dpp*) interact with each other during pupal development, as indicated by the existence of genetic interactions between mutant alleles affecting the activities of these signalling pathways. This work shows that an important outcome of the activity of early acting genes is the localised activation of *dpp* and *tkv* expression to the developing veins. *dpp* participates in the regulation of its own expression, presumably in a mechanism that involves up-

regulation of *tkv* transcription in the cells that separate the vein from the intervein. Tkv activation within the vein directs the expression of *ve* and *Dl*, offering a mechanism to link the activities of different signal transduction pathways in the differentiation of the veins.

I thank Michael Ashburner, in whose laboratory this work has been carried out, for continuous support; E. Martin-Blanco, J. Botas, S. Cohen, P. Martin, M. Baylies, K. Basler, M. Hoffmann, J. Roote and S. Gonzalez-Crespo for DNA clones and flies, and S. Bray for help in preparing the figures. Constructive criticism of the manuscript from S. Bray, D. Gubb, M. Ruiz-Gomez and S. Russell is also acknowledged. J. F.de C. is postdoctoral fellow of Spanish C. S. I. C.

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(Accepted 5 December 1996)