

# Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing

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## SUMMARY

The mammalian growth factor TGF $\beta$  negatively regulates cell proliferation in various systems. Here we provide evidence that another TGF $\beta$  superfamily member, *Drosophila* Decapentaplegic (Dpp), stimulates cell proliferation. In the developing wing blade, somatic clones lacking the Dpp receptors Punt or Thick veins (Tkv), or lacking Schnurri, a transcription factor involved in Dpp signal interpretation, fail to grow when induced early in larval development. Furthermore the spatial requirement for

these signaling components indicates that Dpp has to travel several cell diameters from its source in order to reach all cells that require its signal. The requirement for Tkv also depends on the distance of cells from the source of the Dpp signal. We propose that Dpp can act at a distance to positively control cell proliferation.

Key words: *Drosophila*, Decapentaplegic, imaginal disk, patterning, signaling molecule, cell proliferation, Dpp receptors, TGF $\beta$

## INTRODUCTION

A key process in development is the controlled growth of tissues by cell division and proliferation. Cells unable to respond to external regulatory signals may either under-proliferate, or proliferate in the random and uncontrolled manner typical of tumor outgrowths. Inductive signals are also required to convey positional information to cells and specify their developmental fates. Often these two processes, the control of cell proliferation, and specification of cell fates, are tightly linked, and are organized by the same regulatory signals. Identification of these signals and elucidation of their mode of action is a vital step in the understanding of general developmental mechanisms.

The imaginal discs of *Drosophila* grow from small originator populations of 20-40 cells, into large patterned epithelial sheets, several thousand cells in size, from which the limbs, eyes, and genitals of the adult fly are formed (reviewed by Cohen, 1993). This rapid expansion takes place during the four days of larval development. Although a large number of genes are thought to play a role in this disc growth, those encoding secreted signaling proteins, and with restricted expression domains within the discs, have attracted special interest. The major compartment boundaries along the anteroposterior and dorsoventral axes have long been proposed to act as organizing centers of proliferation and patterning in the imaginal discs (Crick and Lawrence, 1975; Meinhardt, 1982), and signals expressed at these boundaries are natural candidates to carry out such developmental control.

One such gene, which is expressed in a stripe on the anterior side of the anteroposterior (A/P) compartment boundary of wing and leg imaginal discs, is *decapentaplegic* (*dpp*; Posakony,

1991). A member of the TGF $\beta$  family of signaling molecules (Padgett et al., 1987), Dpp has been implicated as a positional signal for cells along the anteroposterior axis of the adult wing (Gelbart, 1989; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Zecca et al., 1995). Various combinations of mutations in the 'disc' regulatory region of the *dpp* locus cause patterning defects in virtually all imaginal disc derived adult tissues (Spencer et al., 1982; Masucci et al., 1990; St Johnston et al., 1990), and in the wing such phenotypes are typified by the loss of central structures. Dpp also plays a critical role in the specification of cell fates in early embryonic development, where it acts in a concentration dependent manner to define dorsal cell fates (Ferguson and Anderson, 1992; Wharton et al., 1993).

The question arises as to whether in addition to specifying cell fate, Dpp might also control the growth of the discs. Clonal analysis in the wing has revealed that the production of Dpp is required only in those cells that normally express *dpp*, in a stripe along the A/P boundary (Posakony et al., 1991). Clones lacking *dpp* activity in this region cause a general reduction in wing size, in addition to loss of pattern, suggesting that *dpp* expression at the center of the disc is required for the growth of the entire wing primordium.

The non-autonomous effect of *dpp* on disc growth prompts a further question. If *dpp* can influence cell proliferation over the entire wing blade, how does it mediate this effect when its expression is restricted to a small subset of cells in the imaginal discs? One possibility is that Dpp protein itself can diffuse from its source to physically interact with distant cells. Alternatively, Dpp could act purely locally, by inducing and maintaining the production of other regulatory signals that control proliferation at a distance.

The issues raised above can be addressed by examining the

requirement for Dpp signal transduction components within the developing wing blade. *tkv* and *punt*, which encode type I and type II serine/threonine kinase receptors respectively, are both necessary for Dpp signaling in the early embryo, indicating that they are the main receptors of the Dpp signal (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994; Ruberte et al., 1995; Letsou et al., 1995). A gene encoding a putative transcription factor, *schnurri* (*shn*), is also required for interpreting Dpp signaling in the patterning of the embryonic endoderm (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). By eliminating these components individually in clones, we can see whether cells require the Dpp signal to proliferate, and whether the Dpp signal is required in cells at a distance from the *dpp*-expressing cells, or only in cells within the *dpp* expression domain.

Our results demonstrate that all three components are required cell-autonomously early in wing blade development, in order for cells to proliferate. This indicates that all wing cells must receive the Dpp signal during early larval development, although we cannot rule out the possibility that cells require the activity of another TGF $\beta$ -like ligand that also acts via Punt, Tkv and Shn. Ectopic expression of *dpp* induces non-autonomous over-growth supporting a direct role for Dpp in stimulating cell proliferation. In addition, our analysis of *dpp* expression in wing imaginal discs at the time when *tkv* mutant clones are unable to grow suggests that the Dpp molecules have to move several cell diameters in order to reach all the cells that apparently require this signal. We have also revealed an additional, later role for Dpp signaling in wing vein formation; wing cells require *tkv* and *shn* to be competent to differentiate into vein material. Taken together, these data indicate that Dpp plays roles in the growth, patterning and differentiation of the wing imaginal discs.

## MATERIALS AND METHODS

### Induction of somatic recombination

#### Adult wings

Marked clones of cells homozygous mutant for *tkv*, *shn* and *punt* were generated by Flp-mediated recombination (Xu and Rubin, 1993). To induce clones in a *Minute* background, first instar larvae of the genotypes  $f^{36a} hsp70-flp, tkv^{a12} FRT40 / M(2)25A P[f^+] FRT40; f^{36a} hsp70-flp, FRT42 shn^{1B} / FRT42 P[f^+] P[f^+] M(2)58F; f^{36a} hsp70-flp, FRT82 punt^{135} / FRT82 M(3)67C P[f^+]$  were subjected to heat shock (60 minutes at 35°C) to trigger Flp-mediated mitotic recombination. Mutant clones of all three genes produced similar adult phenotypes although only results for *tkv* mutant clones are shown.

To induce such clones in a wild-type background, larvae of the genotypes  $f^{36a} hsp70-flp, tkv^{a12} P[f^+] ck FRT40 / FRT40$  (for twispot analysis);  $f^{36a} hsp70-flp, FRT42 shn^{1B} / FRT42 P[f^+]; f^{36a} hsp70-flp, FRT82 punt^{135} / FRT82 P[f^+]$  were subjected to heat shock (30 minutes at 33-35°C) at precisely timed developmental stages.

#### Wing imaginal discs

To generate *tkv*, *punt* and *shn* homozygous mutant clones marked in the imaginal discs by loss of the *IMyc* epitope, larvae of the following genotypes.  $hsp70-flp, tkv^{a12} FRT40 / 2IMyc FRT40; hsp70-flp, FRT42 shn^{1B} / FRT42 IMyc; hsp70-flp; FRT82 punt^P / FRT82 2IMyc$  were subjected to heat shock (30 minutes at 33-35°C) at precisely timed developmental stages. Resulting third instar larvae were subjected to a second, severe heat shock (60 minutes at 37°C) to induce *IMyc* expression. Imaginal discs were fixed after a 1 hour

recovery period and stained for *IMyc* expression. *tkv* mutant clones in a *Minute* background were generated in larvae of the genotype:  $hsp70-flp / +; tkv^{a12} FRT40 / M(2)25A IMyc FRT40$ , again by heat shocks in first instar.

In contrast to *tkv* and *shn* mutant clones, *punt* mutant clones were present in the wing blade region of the imaginal discs even when induced 60 hours AEL, but not when induced 48 hours AEL. Thus requirement for *punt* seems to be lower than that for *tkv*. However, neither of the two *punt* alleles tested (*punt<sup>P</sup>* and *punt<sup>135</sup>*) have been confirmed as null alleles (Ruberte et al., 1995; Letsou et al., 1995). It is possible that this difference in requirement for the type I and type II receptors is due to weak residual activity of the *punt* mutant allele, and not to a lesser requirement for the type II receptor Punt. The *shn* allele (*shn<sup>1B</sup>*) used here and previously (Grieder et al., 1995) for clones in the wing is a strong hypomorphic allele.

### Staging of larval developmental time points

When time points after egg laying (AEL) for clone induction and *dpp* expression are indicated, these larvae were accurately staged at the beginning of first instar. Larvae hatching over a 2 hour period were selected and grown at constant temperature (25°C) until clone induction by heat shock. Hatching time is defined as 24 hours AEL.

### Statistical comparison of *tkv* mutant and twispot clones

To compare the growth rate of *tkv* mutant clones versus that of their wild-type twispots, we counted the number of each type of clone in 20 wings from each time point of clone induction (60, 72, 84 and 96 hours AEL). To simplify this analysis, only clones consisting of more than 20 cells (60, 72 and 84 hours AEL) or 10 cells (96 hours AEL) were included. Thus the figures quoted do not reflect absolute numbers of clones but rather those able to grow to a certain size.

### Immunohistochemistry

Staining for *IMyc* and *lacZ* expression utilized the monoclonal antibody 9E10 (Evan et al., 1985) and a polyclonal antibody against  $\beta$ -galactosidase (Cappel) respectively. Standard FITC, Texas Red and HRP-conjugated secondary antibodies (Jackson) were used. Immunofluorescent signals were analyzed on a confocal laser scanning microscope (Molecular Dynamics).

### Ectopic *dpp* and *tkv<sup>Q253D</sup>* expression

Transformants bearing  $UAS > CD2, y^+ > dpp$  or  $UAS > CD2, y^+ > tkv^{Q253D} flp$ -out transgenes (Nellen et al., 1996) were crossed to females containing a *hsp70-flp* transgene and the Gal4 driver *C765* (Nellen et al., 1996). The resulting progeny were subjected to a mild heat shock (30 minutes at 34°C) during first or second larval instar. Wing discs were removed during late third instar, fixed, and stained for CD2 expression as described previously (Zecca et al., 1995).

To increase the levels of *dpp* expression in its endogenous domain, we crossed males bearing the *blk-Gal4 40C.6* transgene (Staehling-Hampton et al., 1994) to females containing zero or two *UAS-dpp* transgenes. Experimental larvae were identified with the aid of the dominant mutation *Tubby* on the *TM6b* balancer.

## RESULTS

### *tkv*, *punt* and *shn* are required for proliferation of wing blade cells

To investigate where, and for what function the Dpp signal is required in the developing wing, we sought to determine the need for the Dpp signal transduction components Tkv, Punt and Shn in the wing. To this end we generated, by Flp-induced somatic recombination (Xu and Rubin, 1993), marked clones homozygous for loss-of-function mutations in these genes, and looked for phenotypic effects. Clones generated in first instar

larvae were never observed in the adult wing blade proper, only in the proximal-most hinge regions. However, such clones were present if induced later in development, in third instar larvae. Hence there is a differential temporal requirement for *tkv*, *punt* and *shn* in the developing wing blade, and cells lacking these factors are unable to proliferate during early larval development.

In order to more accurately determine the temporal and spatial requirements of one of these components, *tkv*, we performed a twinspace analysis. This method utilizes different cell markers to label the two daughter cells produced after a somatic mitotic recombination event (Lawrence et al., 1986). Normally these two cells will divide at similar rates to form two marked clones (defined here as the 'experimental' and 'twinspace' clones) in close proximity to one another. If one of these daughter cells is made simultaneously homozygous for a *tkv* mutant allele, then the proliferation rate of cells lacking *tkv* can be compared to that of the wild-type twinspace clone cells. Thus we have a direct internal means of measuring *tkv* mutant clone growth. We used the chromosome depicted in Fig. 1, so that a single recombination event produces a *tkv* homozygous mutant clone marked with *crinkled*, and a twinspace clone marked by the loss of the *forked*<sup>+</sup> transgene (see legend to Fig. 1 for a more detailed description). These recombination events were induced at defined time points during development by heat shock-induced expression of the Flp enzyme.

*Tkv* mutant clones induced up to the beginning of third instar development (approximately 72 hours AEL) are dramatically underrepresented in comparison to their twinspace sister clones in the wing (see Fig. 2B for pictorial representation). In fact *tkv* mutant clones generated 60 hours AEL (38 clones compared to 245 wild-type twinspace clones) and 72 hours AEL (64 clones compared to 301 wild-type twinspace clones) are observed exclusively in the most peripheral regions of the blade, the costa (anteriorly) and the alula and nearby region (posteriorly, see Fig. 2B). These clones do not cause any phenotypes. No clones induced at these stages are observed in the wing blade proper, indicating that *tkv* activity is required for proliferation of wing blade cells at these time points.

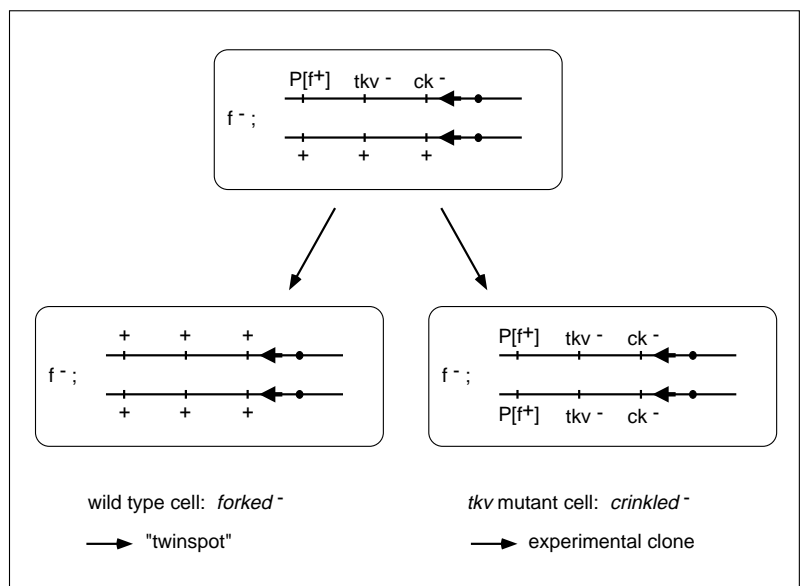
When induced 84 hours AEL, *tkv* mutant clones are still strongly underrepresented (77 clones compared to 289 wild-type twinspace clones), although *tkv* mutant clones are regularly seen in the alula and costa. At this stage *tkv* mutant clones are also often present in the region between vein 5 (L5) and the posterior margin, and occasionally between vein 4 (L4) and L5. Clones in the rest of the blade are still very scarce (4 from a total of 77 *tkv* mutant clones). This suggests that there is less requirement for *tkv* in regions most distant from the source of Dpp at the center of the wing blade. In these wings occasional small *tkv* mutant clones, not included in the statistical analysis due to their size (see Materials and Methods), are seen distributed throughout the entire wing blade.

Numerous small *tkv* mutant clones are present in the adult wing when induced 96 hours AEL (Fig. 2C). However when the analysis of these wings was simplified by excluding clones with less than ten cells, a strong requirement for *tkv* function was still apparent even at this late stage. Only 208 *tkv* clones were observed compared with 360 wild-type twinspace clones. This requirement was again lowest between V5 and the posterior margin where *tkv* mutant clones now survive equally well as their twinspace.

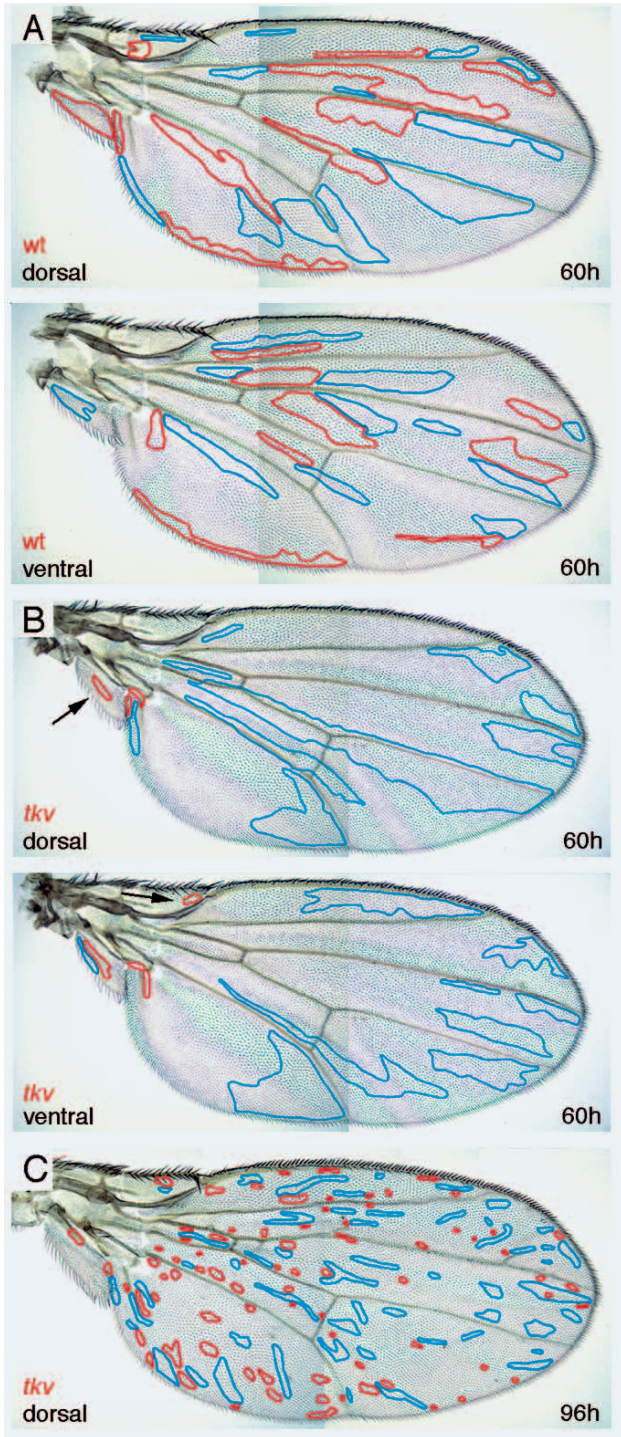
### Temporal requirement for *tkv*, *punt* and *shn* in the proliferation of wing imaginal disc cells

The twinspace analysis of early-induced *tkv* mutant clones in the adult wing revealed a basic absence of clones. This could be either because such clones are unable to grow, or because the cells in such clones do not differentiate into adult tissue. To distinguish between these two possibilities, we looked for clones in late third instar wing imaginal discs, this time using the *IMyc* epitope tag (Xu and Rubin, 1993) to visualize the experimental clones and their twinspace (see legend to Fig. 1). If clones are seen in the wing discs but not in the adult wings, then these clone cells must be lost during pupariation. Adult wing and imaginal disc clones were scored in animals from the same cross, thus eliminating any differences in genetic backgrounds.

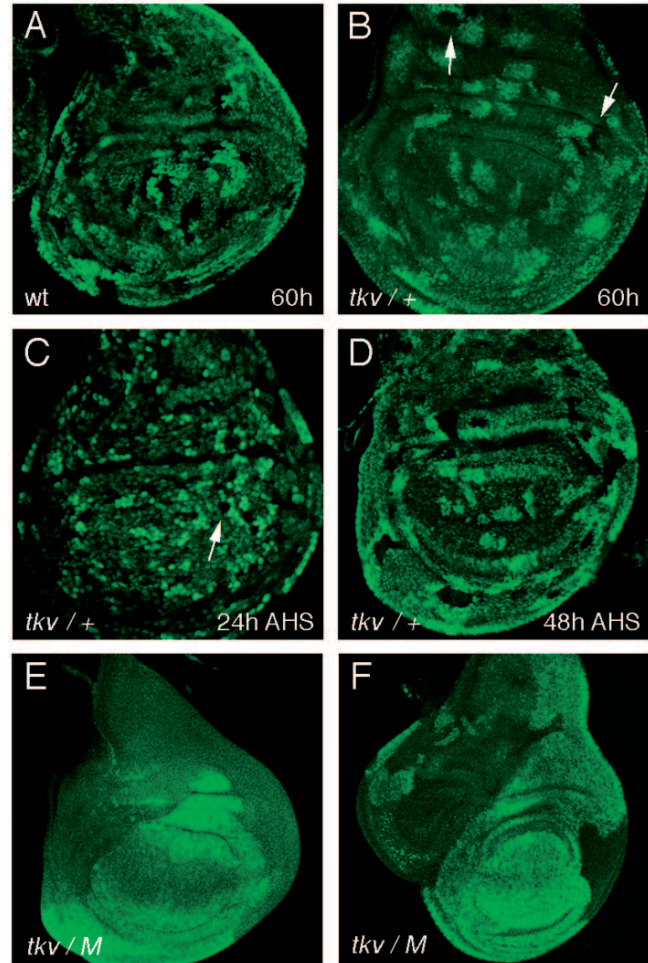
*Tkv* mutant clones induced 60 hours AEL were never



**Fig. 1.** The two products of a mitotic recombination event are differentially marked in the twinspace system. Schematic representation of the twinspace system used here to mark the homozygous experimental *tkv* mutant clones and the wild-type twinspace clones produced by somatic recombination. Flp-mediated recombination between FRT repeats (represented by short arrows) on two sister chromatid arms during mitosis results in two daughter cells of different genotypes. One cell is now homozygous for both *tkv* and *crinkled* alleles, and its descendants are detectable in the adult wing by the distinct *crinkled* wing hair phenotype. The other sister cell has lost the *forked* rescue construct (*P[f<sup>+</sup>]*), and 'twinspace' clones derived from such a cell appear *forked* mutant in the wings of adults that are in a *forked* mutant background. To visualize clones in the imaginal discs, a chromosome carrying the *IMyc* transgene is used in place of the wild-type chromosome depicted in the figure. Cells in *tkv* mutant clones will have lost the epitope, while cells of the twinspace gain an extra copy, thereby expressing twice the amount of *IMyc* epitope compared to the surrounding cells. When imaginal discs are stained with an anti-*IMyc* antibody, the experimental clone cells will show no staining, while the twinspace clone cells will stain brighter than the original heterozygous cells.



**Fig. 2.** *Tkv* mutant clones are greatly underrepresented compared to their wild-type twin-spot clones in the adult wing. Sample wings with multiple wild-type or *tkv* mutant experimental clones generated at precisely timed developmental stages. Dorsal and ventral wing surfaces are shown separately, except in C where only dorsal is shown. Anterior is up. Experimental and twin-spot clones are outlined in red and blue respectively. (A) Wild-type experimental clones survive as well as their twin-spots when induced 60 hours AEL (applies for 72, 84, and 96 hours AEL as well, not shown), indicating that the markers used do not affect cell survival or proliferation. (B) *tkv* mutant experimental clones induced 60 hours AEL survive only in the costa (arrow, ventral surface), alula (arrow, dorsal surface) and the region adjacent to the alula. Similar results for clones induced 72 hours AEL are not shown. (C) *tkv* mutant clones induced 96



**Fig. 3.** *tkv* mutant clone survival in wing imaginal discs. Anterior is to the left, dorsal is up. (A,B) Comparison of wild-type (A) and *tkv* mutant (B) experimental clones induced 60 hours AEL. Experimental clones are marked by loss of the green *lIMyc* staining. Twin-spot clones are a brighter green because they have two copies of the *lIMyc* epitope. Wild-type experimental clones survive over the entire disc (A). *Tkv* mutant experimental clones do not survive in the prospective wing blade but do survive in other regions of the discs (arrows). (C,D) Comparison of wing discs stained 24 (C) and 48 (D) hours after induction of *tkv* mutant clones, 69 hours AEL. 24 hours after clone induction (AHS – after heat shock) the *tkv* mutant clones are similar in size to their twin-spots, even in the prospective wing blade (arrow, C). A further 24 hours later, *tkv* mutant clones are generally seen only in non-blade regions (D). C was recorded at twice the magnification of the other discs. Clones in C are similar in size to those induced 84 hours AEL and observed at the end of third instar. (E,F) *Tkv* mutant clones in a *Minute* background. The clones were induced during the first larval instar, and are again marked by the loss of the green *lIMyc* staining, although no twin-spot clones are produced as the *Minute* allele is homozygous lethal. The dorsal presumptive notum is reduced in size when consisting entirely of *tkv* mutant tissue (E). *Tkv* mutant tissue bordering the prospective wing blade region can cause outgrowths and loss of pattern in the disc (F).

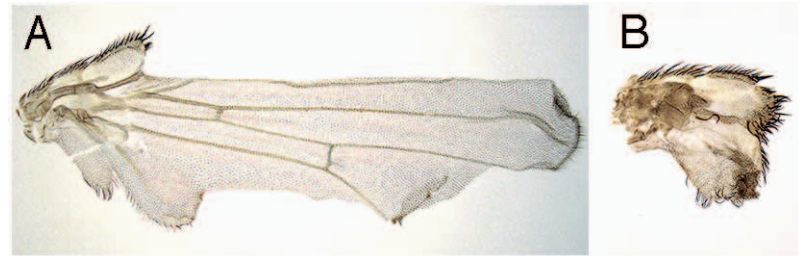
hours AEL survive over the entire wing blade, but predominantly in the costa and alula, and in the region between vein 5 and the posterior margin. Comparison of B and C illustrates the change in requirement for *tkv* as development progresses. Clones induced later are smaller and more numerous because they have less time to grow, and because larger discs have more cells in which mitotic recombination can take place.

observed in the wing blade region of the third instar larval wing disc, although large twinspace clones were often present (Fig. 3B). However such clones are often present in all other regions of the wing disc, including the hinge, notal and pleural precursor cells. Small and very rare *tkv* mutant clones are occasionally seen in the prospective wing blade when induced 72 hours AEL. When generated 84 hours AEL, *tkv* mutant clones are observed in the entire wing disc and are present in equal number and size to their twinspace clones (data not shown - clones similar in size to those in Fig. 3C). Similar results were obtained for *shn* and *punt* mutant clones (see Materials and Methods).

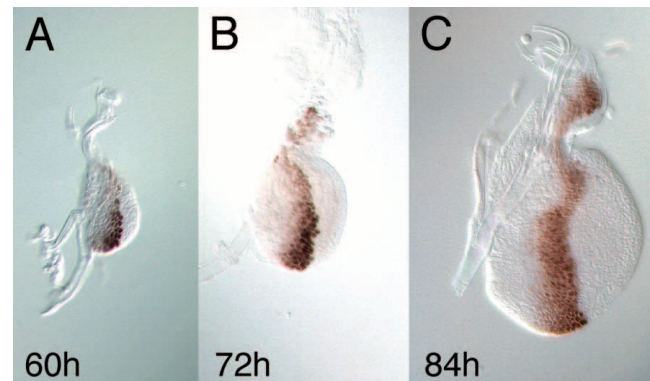
When induced 84 hours AEL, clones are observed in the wing blade region of the late third instar imaginal discs, but not in adult wings (see above). Possibly these clones are initially able to grow, but the cells are lost at a later point. To address this possibility, clones were induced 69 hours AEL and the discs analyzed 24 and 48 hours later. Small *tkv* mutant clones were observed in the wing blade region of the small discs dissected 24 hours after clone induction (Fig. 3C), but not in the large discs dissected a further 24 hours later (Fig. 3D). This indicates that early-induced *tkv* mutant clones are initially able to grow, possibly due to perdurance of *tkv* activity (see Discussion). However these cells must later be displaced because clones are not seen in the mature discs or the adult wings.

To determine whether *tkv* mutant clones could grow in the wing blade if given a growth advantage, we then generated such clones in a *Minute* background. In this system proliferation of disc cells is retarded by the dominant *Minute* allele present, putting clonal cells at an advantage because they have lost this allele during the mitotic recombination process (Lawrence et al., 1986). When induced in *Minute* mutant first instar larvae, *tkv* mutant clones appear in all regions of the wing disc except the central wing blade primordium. *tkv* mutant

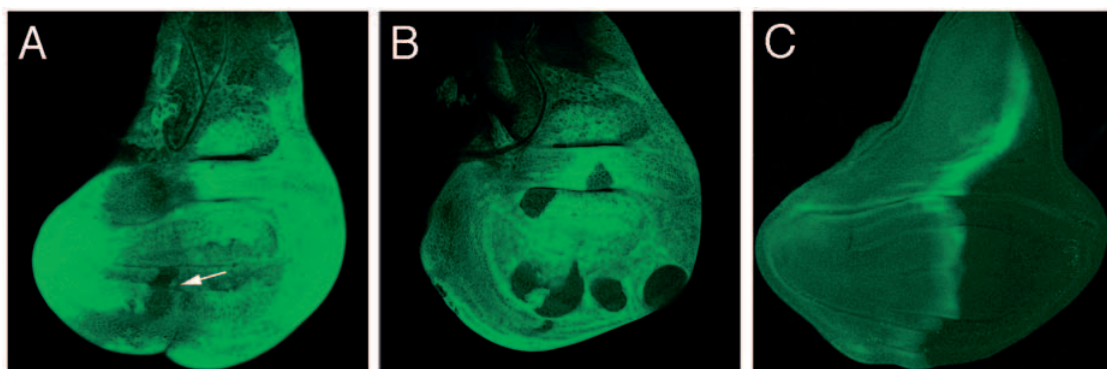
clones covering dorsal regions of the disc can cause a reduction in size of this area (Fig. 3E), and clones bordering the wing pouch occasionally result in outgrowths and pattern disruption (Fig. 3F). Only small patches of mutant tissue are observed in



**Fig. 4.** Adult phenotypes caused by *tkv* mutant clones induced in a *Minute* background. (A,B) Wing phenotypes of flies with *forked*-marked *tkv* mutant clones induced in a *Minute* background in first instar larvae. *tkv* mutant tissue is often seen in the proximal-most regions of such wings. Cells are wild-type at the borders of the wing notches illustrated in A. In B the main body of the wing is completely absent. Wild-type control clones induced in the same background result in normal wings with very large *forked*-marked clones (not shown). Orientation is the same as in Fig. 2.



**Fig. 5.** Expression of *dpp* in wing discs at different developmental stages. Wing imaginal discs dissected from larvae 60 (A), 72 (B), and 84 (C) hours AEL. These time points correspond to mid-second, end-of-second, and early-third larval instars, respectively. *dpp* expression is monitored by the *dpp lacZ* reporter *dpp<sup>P10638</sup>*. Orientation is the same as in Fig. 3.



**Fig. 6.** Ectopic or elevated *dpp* expression causes non-autonomous over-proliferation, unlike activation of the Dpp receptor Tkv. (A,B) Wing imaginal discs containing clones expressing either a *UAS>dpp* (A) or a *UAS>tkv<sup>Q253D</sup>* (B) transgene. Expression of both transgenes is under the control of Gal4 line *C765*. Clones have lost the CD2 epitope and therefore do not stain green. Ectopic *dpp*-expressing clones cause non-autonomous outgrowths (A, arrow indicates clone). *tkv<sup>Q253D</sup>*-expressing clones cause no such outgrowths but have an abnormal, smooth circular morphology, and appear to grow larger than *dpp*-expressing clones. (C) Wing disc from a larvae containing two copies of a *UAS-dpp* transgene under the control of the *dpp*-enhancer-Gal4 driver *blk-Gal4 40C.6* (Staebling-Hampton et al., 1994), causing over-expression of *dpp* in the normal *dpp* expression domain (Masucci et al., 1990). The wing blade region of such discs is significantly expanded along the anteroposterior axis, while the dorsal half of the disc remains unaffected. Antibody staining reveals expression of the *Cubitus interruptus* protein in this disc, which marks all cells of the anterior compartment. In all discs, anterior is to the right, dorsal is up.

adults, accompanied by dramatic loss of wing (Fig. 4A,B) and notal (not shown) structures, indicating that much of the mutant tissue seen in the imaginal discs is not able to undergo proper differentiation.

### ***tkv* is required several cells away from *dpp*-expressing cells**

Since the Dpp receptors, Tkv and Punt, are required early in development for prospective wing blade cells to proliferate, we infer that these cells must normally receive the Dpp signal. This would require movement of Dpp molecules from their source at the A/P compartment boundary to the more peripheral cells of the disc. 60 hours AEL, *dpp*-expressing cells occupy a relatively large portion (approximately one-third) of the tiny imaginal discs (for example see Fig. 5A), and thus the source is very close (2-3 cells) to the cells that require the signal. However, later in development the distance from the Dpp source to the outlying wing blade precursor cells increases as the disc grows.

This distance from the Dpp source to cells that receive the Dpp signal can be estimated by determining the number of cells in the prospective wing blade region at the time of clone induction. While the extent of *dpp* expression in the anterior compartment is somewhat ambiguous due to its graded nature, no Dpp is produced by cells of the posterior compartment, so any Dpp signal received by posterior cells must come from anterior cells. The clonal analysis data demonstrated that *tkv* is required for proliferation in all wing blade cells up until 72 hours AEL. At this stage the posterior compartment is on average 5 cells in width in the region of the future wing blade (Fig. 5B), although it is impossible to determine exactly which cells will eventually take part in wing formation. Therefore secreted Dpp molecules would have to travel at least 4 cell diameters in order to signal to all future wing blade cells in the posterior compartment. If *tkv* is still required 84 hours AEL, as suggested by the adult wing data, then the Dpp molecules would be required 7 to 8 cell diameters away from their site of synthesis (Fig. 5C), albeit *tkv* mutant clones do survive substantially better in the posterior-most region of the wing blade than in the rest.

### **Ectopic or elevated expression of *dpp* causes non-autonomous over-proliferation**

Although we have demonstrated that the Dpp receptors are required for cells to proliferate, it is not clear whether this indicates a direct role for Dpp in promoting growth, or whether loss of proliferation in receptor mutant clones is merely a secondary consequence of loss of Dpp input. We wanted to test whether ectopic expression of *dpp*, or constitutive activation of Dpp receptors would promote proliferation.

For this we have used the flip-out method and a combination of transgenes that allow us to induce marked clones of cells ectopically expressing either *dpp* or a constitutively active form of Tkv (Tk<sup>v</sup><sup>Q253D</sup>, see Materials and Methods and Nellen et al., 1996). We noted that even small *dpp*-expressing clones induced significant over-proliferation (Fig. 6A). The large majority of cells in an outgrowth caused by a *dpp*-expressing clone are wild-type and hence do not express *dpp* themselves. Conversely, Tk<sup>v</sup><sup>Q253D</sup>-expressing clones did not affect the overall size or general shape of the discs (see Fig. 3 for wild-type disc shape). However, compared to the *dpp*-expressing

clones, the Tk<sup>v</sup><sup>Q253D</sup>-expressing cells themselves appear to proliferate faster than surrounding tissue, resulting in round patches of cells that, if given enough time, bulge out from the epithelium (Fig. 6B). These results are consistent with and complement our observation that *tkv* and *punt* are required for cell proliferation in discs.

The difference in effect on proliferation between ectopic *dpp* expression and ectopic Tkv activity (non-autonomous versus autonomous action) indicates that the Dpp signal stimulates cell proliferation over a wide range of cells surrounding *dpp*-expressing cells. To investigate whether the ability of Dpp to promote proliferation depends on the levels of Dpp secreted by *dpp*-expressing cells we asked whether increased levels of *dpp* expression along the A/P compartment boundary would lead to correspondingly larger wing discs. To do this, we have combined in the same animals, a GAL4 transgene under the control of a *dpp* disc enhancer (Masucci et al., 1990; Staehling-Hampton et al., 1994) with a *dpp* transgene under the control of UAS sequences (Brand and Perrimon, 1993). Wing imaginal discs from larvae containing one copy of the *dpp-GAL4* and two copies of the *UAS-dpp* transgenes are greatly expanded along the anteroposterior axis of the wing blade region (Fig. 6C), although the dorsal, notal region of these discs remains unaffected. Thus the number of cells, and therefore the size of the wing, can be controlled, at least in part, by the amount of Dpp produced along the A/P compartment boundary.

### ***tkv* and *shn* are also required for vein cell differentiation**

A role for Dpp signal reception in allowing proliferation and/or conveying positional information during the first and second larval instars has been demonstrated. However, as also described above, clones homozygous mutant for *tkv*, *punt* and *shn* are often able to survive when induced late in development, especially in mid-to-late third instar larvae. These clones are of course small due to their late induction time, but they still cause visible mutant phenotypes, suggesting a later role for these genes in cell fate specification. Wings with such clones display small gaps, splits, and indentations in the veins, as well as additional vein material abutting the normal veins. *tkv* and *shn* mutant clones were examined to determine the role of Dpp signaling in vein differentiation.

*Drosophila* wing veins are dorsoventrally asymmetrical (Garcia-Bellido and de Celis, 1992). Some protrude on the dorsal wing surface (e.g. vein 3 and vein 5) while others protrude on the ventral surface (e.g. vein 2). *tkv* and *shn* clones cause loss of vein when they are present on the dominant (protruding) side of the vein (Fig. 7A,B, 33 from total 39 *tkv* mutant clones, 52 from total 57 *shn* mutant clones). No effect of such clones is seen when they are on the non-protruding surface (20 *tkv* mutant clones, 24 *shn* mutant clones). It is possible that loss of vein structures from the non-protruding side alone is obscured by the main body of the vein on the dominant side and is therefore not visible. Rare, large *tkv* and *shn* mutant clones on the dominant surface remove veins from this surface while a faint vein-like pigmentation remains on the opposite side (Fig. 7B), consistent with the knowledge that vein differentiation is surface autonomous and doesn't require induction from vein cells on the opposite surface (e.g. Diaz-Benjumea and Hafen, 1994). While the requirement for *tkv* and *shn* on the protruding side of the vein is basically cell autonomous, *tkv* or *shn*

mutant cells at the edges of clones are sometimes able to form vein material (Fig. 7A). This incomplete cell autonomy, and the general behavior of *tkv* and *shn* mutant clones is strongly reminiscent of the effect of various loss-of-vein genes, known collectively as the 'vein' (vn) genes (Garcia-Bellido, 1977; Diaz-Benjumea and Garcia-Bellido, 1990). Paradoxically, the *tkv* gene was originally classified as an excess-of-vein gene, together with *Notch*, due to the thickened vein phenotype caused by a homozygous viable *tkv* allele (*tkv<sup>l</sup>*; Diaz-Benjumea and Garcia-Bellido, 1990). This allele has not yet been molecularly characterized, but it is possible that it acts as a gain-of-function *tkv* allele (Penton et al., 1994).

This observed role for Dpp signal transduction components in the specification of wing vein cells is consistent with the existence of a class of *dpp* alleles, collectively known as the 'shortvein' alleles (Segal and Gelbart, 1985), that specifically affect vein formation. Also, *dpp* mutant clones in the posterior compartment of the wing can cause loss of vein while not affecting the main *dpp* expression domain (Posakony et al., 1991). Hence *dpp* may be expressed and required in vein precursor cells during pupal stages. This late role for Dpp in wing differentiation can be separated both genetically and temporally from its earlier role in the control of disc growth and patterning.

## DISCUSSION

Without tightly regulated control of cell proliferation, the development of correctly patterned multicellular tissues would not be possible. The rapidly growing imaginal discs of the *Drosophila* larvae provide a useful in vivo system to study how proliferation is regulated in developing animal tissues, and also how this proliferation is linked to patterning processes and eventual cell fate specification. Reduction in the activity of the signaling molecule Dpp has striking effects on the patterning of imaginal disc derived structures, and this has been closely studied in the wing where the veins and marginal bristles can be used as markers for positional information along the antero-posterior axis (Posakony et al., 1991; Spencer et al., 1982; Segal et al., 1985; Zecca et al., 1995). However, reducing the level of *dpp* expression also causes reduction in the size of the wings hinting that Dpp may also play a role in the growth of the wing primordium, possibly linked with its patterning function. The recent identification of molecules involved in Dpp signal transduction means that the requirement for Dpp signaling in target cells can now be studied. In particular we can address the key questions of what individual cells need the Dpp signal for, and where in the developing wing blade this signal is required.

### Dpp receptors are required cell autonomously for proliferation

The results presented here demonstrate that three components involved in Dpp signal interpretation, *tkv* and *punt* (the Dpp type I and type II receptors) and *shn* (a transcription factor), are all required by all prospective wing blade cells for proliferation early in larval development. When these components are genetically removed by mitotic recombination, mutant cells are no longer able to proliferate, and thus clones are not seen in the mature imaginal discs or in the adult wings. Conversely

ectopic expression of *dpp* results in over-proliferation of surrounding cells.

Taken together these results provide strong evidence that Dpp is not only required for growth of the future wing blade, but that it is also able to initiate proliferation of these cells. The requirement for components of the Dpp signaling pathway is cell autonomous over the entire presumptive wing blade during early larval development. Therefore the growth function of Dpp is not a consequence of a secondary signal triggered by Dpp, but is a direct response to Dpp itself.

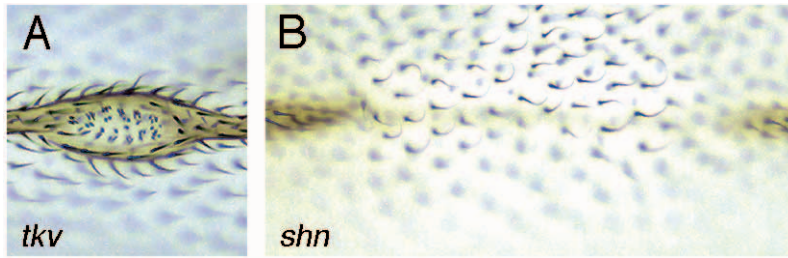
In contrast to the non-autonomous effect of *dpp*-expressing clones, expression of an activated form of *Tkv* has a purely autonomous effect on proliferation, with surrounding cells remaining unaffected. Such clones, expressing activated *Tkv*, appear to be larger than *dpp*-expressing clones, and have a striking appearance. Their tendency to form smooth, circular patches is reminiscent of clones ectopically expressing *engrailed* (Zecca et al., 1995), and is thought to be caused by mutant cells minimizing their interaction with surrounding wild-type cells.

While we propose a direct role for Dpp in promoting proliferation of wing blade cells, a second possibility is that Dpp is involved principally in patterning the wing blade and that without the Dpp signal, cells can no longer interpret their position within the disc. As a consequence these cells may be at a competitive disadvantage, possibly undergoing apoptosis, and loss of growth could merely be an indirect result of lack of positional identity.

Even though *dpp* is expressed along the entire A/P compartment boundary of the wing imaginal disc, our analysis reveals a strong requirement for Dpp receptors only in the wing blade primordium. Correspondingly, increased Dpp levels along the entire length of the compartment boundary have dramatic effects on growth only in the wing blade region of the disc. It is possible that Dpp can only promote cell proliferation in combination with other, wing blade-specific regulatory signals. Such cofactors might be secreted as a consequence of interactions between cells on opposing sides of the dorsoventral compartment boundary. One candidate signal that has been proposed to work in concert with Dpp to promote distal outgrowth is Wingless (Campbell et al., 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994; Diaz-Benjumea and Cohen, 1995). Wing cell proliferation may also be strongly dependent on Wg signal input (e.g. Peifer et al., 1991). In fact, a third possibility is that Dpp is not a growth promoting signal per se, but that Dpp exposure merely renders wing blade cells competent to respond to other organizing signals.

### Dpp signaling is required at a distance from the Dpp source

Although *dpp* is expressed only in a subset of cells in the anterior compartment of the developing wing disc, all cells of the early prospective wing blade require *tkv*, *punt* and *shn*. This implies that, especially in the posterior compartment, Dpp must diffuse away from its source to fulfill its function, although we cannot rule out the possible action of a second, as yet unknown, TGF $\beta$  ligand. Assuming that requirement for the Dpp receptors indicates that the Dpp signal is required in these cells, we have determined that Dpp must travel several cell diameters in order to reach all the cells that need its signal. In



**Fig. 7.** *Tkv* and *shn* mutant clones cause loss of wing vein. (A) *tkv* mutant clone (marked by *crinkled*) on the dorsal surface of vein 5, causing a split in the vein. *Tkv* mutant cells also appear on the vein at the border of the clone. (B) Rare, large *shn* mutant clone (marked by *forked*) on the dorsal surface of vein 3, causing a large gap in the vein within the boundaries of the clone. Note the faint vein-like pigmentation visible on the opposite ventral surface.

recent experiments involving the Dpp-responsive genes *optomotor blind* and *spalt*, we have shown that Dpp can induce gene expression many cells away from a source of ectopic Dpp (Nellen et al., 1996). The results here indicate that this is also the case for Dpp's ability to stimulate cell proliferation. Taken together, there is strong evidence that early in wing imaginal disc development, Dpp is secreted from its site of synthesis and travels to all prospective wing blade cells where it signals through its receptors Punt and Tkv. *shn* is also required in these cells indicating its product acts to propagate the Dpp signal, as it does in the endodermal cells of the developing embryonic midgut (Grieder et al., 1995), supporting a general role for Shn downstream of the Dpp receptors Punt and Tkv.

Although we have demonstrated a strong requirement for *tkv* in most wing blade cells up until 84 hours AEL, this requirement may extend even later in development. It is possible that *tkv* mRNA or protein perdures in cells after mitotic recombination has decreed them genetically null. Residual *tkv* activity may be sufficient to allow further rounds of replication. Alternatively, the proliferation process initiated by Tkv activation might not be immediately switched off by removal of Tkv production. If either of these theories is valid, then the Dpp diffusion distance would be even greater than that estimated in the Results section. Such a theory would also explain why some *tkv* mutant clones can initially survive, but later are not seen. Cells may be able to undergo a few more rounds of division before proliferation stops, and these cells are lost in the expanding disc.

*Tkv* mutant clones induced late in larval development also revealed a differential spatial requirement for *tkv* in the wing blade, surviving more readily in regions furthest away from the Dpp source at the A/P compartment boundary. This spatial requirement also changes as the disc grows, reflecting the increasing distance of peripheral wing blade cells from the basically static Dpp source. Therefore requirement for *tkv* depends strongly on the distance of the cells from the Dpp source. The variation in *tkv* requirement could be due to a lower requirement for the Dpp signal in more distant cells, so that perduring *tkv* activity is able to remain in effect for longer time periods after production is shut off. Alternatively, cells in these outlying regions may no longer require the Dpp signal for proliferation later in larval development, unlike cells in the more central region of the wing blade, implying that, even in the wing blade, Dpp is not a ubiquitous trigger for cell division. Both scenarios would support our proposal (Nellen et al. 1996) that the graded activity of Dpp can regionally subdivide the developing wing blade along the anteroposterior axis.

Apart from Dpp, at least one other member of the TGF $\beta$  superfamily, TGF $\beta$  itself, has been implicated in the control of

cell proliferation, although this regulation is often exerted in a negative manner, arresting cells in G1 phase (Massagué, 1990; Reynisdottir et al., 1995). Mutations in TGF $\beta$  receptors have furthermore been correlated with the loss of response of cells to TGF $\beta$ -mediated growth inhibition (Markowitz et al., 1995). Here we demonstrate an opposite, positive effect on cell proliferation by a TGF $\beta$ -like signal. However, the known role of Dpp in cell fate specification suggests that it is perhaps unlikely that Dpp signaling affects proliferation by acting directly on cell cycle regulation. Given the importance of understanding the mechanisms of growth control in animals, Dpp signaling should serve as a useful model system to elucidate the intimate relationship between pattern formation and proliferation control in a simple multicellular tissue.

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