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defective proventriculus is required for pattern formation along the proximodistal axis, cell proliferation and formation of veins in the *Drosophila* wing

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SUMMARY

Many genes have been identified that are required for the establishment of the dorsoventral (DV) and anteroposterior (AP) axes of the *Drosophila* wing. By contrast, little is known about the genes and mechanisms that pattern the proximodistal (PD) axis. Vestigial (Vg) is instrumental in patterning this axis, but the genes that mediate its effects and the mechanisms that operate during PD patterning are not known. We show that the gene *defective proventriculus* (*dve*) is required for a region of the PD axis encompassing the distal region of the proximal wing (PW) and a small part of the adjacent wing pouch. Loss-of-function of *dve* results in the deletion of this region and, consequently, shortening of the PD axis. *dve* expression is activated by Vg in a non-autonomous manner, and is repressed at the DV boundary through the combined activity of Nubbin and Wg. Besides its role in the establishment of the distal part of the PW, *dve* is also required for the formation of the wing veins 2 and 5, and the proliferation of wing pouch cells, especially in regions anterior to wing vein 3 and posterior to wing vein 4. The study of the regulation of *dve* expression provides information about the strategies employed to subdivide and pattern the PD axis, and reveals the importance of *vg* during this process.

Key words: Dve, Vestigial/Scalloped, Nubbin, *four-jointed*, Proximal wing, Pattern formation, Homeobox transcription factor

INTRODUCTION

The development of the *Drosophila* wing has become an important system to study the patterning and morphogenesis of an animal appendage (for a review, see Klein, 2001). The wing is formed by one of the imaginal discs, which are sheets of epithelial cells defined during embryogenesis that proliferate during larval development and form most of the adult fly.

Most work has concentrated on the patterning events that occur along the two existing axes, the anteroposterior (AP) and dorsoventral (DV) axes. Two patterning centres located at the DV and AP compartment boundaries provide positional information for the cells of the wing. At the AP boundary, a band of anterior cells along the boundary, defined by the Hedgehog (Hh) signal from posterior cells, express the secreted factor Decapentaplegic (Dpp). Dpp diffuses from these cells to both sides and generates a gradient, which supplies the wing cells with positional information along the AP axis (reviewed by Basler, 2000; Klein, 2001). Likewise, the Wg protein is produced in cells at the DV boundary under control of the *Notch* pathway and the nuclear factor Vestigial (Vg), and forms a bipartite gradient on each side of the boundary. This gradient is required to maintain the expression of *vg* in the cells of the wing pouch and to stabilize the expression of genes in the domain of Vg (Basler, 2000; Klein, 2001).

As the wing imaginal disc is a two-dimensional structure, the third axis, the PD axis, must be generated and patterned with help from the two existing axes. In the adult wing three regions of the PD axis are easily distinguishable. From proximal to distal, these are the hinge, the proximal wing (PW) and the wing blade (see Fig. 1A,C). Little is known about the genes and molecular strategies that establish and pattern this axis. It is known that the activity of the *vg* gene is required for the establishment of all distal wing fates (Klein and Martinez-Arias, 1998a; Klein and Martinez-Arias, 1999; Liu et al., 2000). Vg is a nuclear protein that associates with Scalloped (Sd) to form a bipartite transcription factor (Halder et al., 1998; Simmonds et al., 1998). The expression of *vg* is initiated at the DV boundary through the *Notch* signalling pathway (Kim et al., 1996). The descendants of the cells of the DV boundary will form the wing pouch (Klein and Martinez-Arias, 1999). Recent work has shown that Vg does not only determine the fate of cells within its domain of expression, but also in cells outside in the PW. Vg seems to activate an unidentified signal that induces the expression of *rotund* (*rn*) and *nubbin* (*nub*) in larger domains. Rn is a Zinc-finger containing transcription factor that is required, together with the POU domain

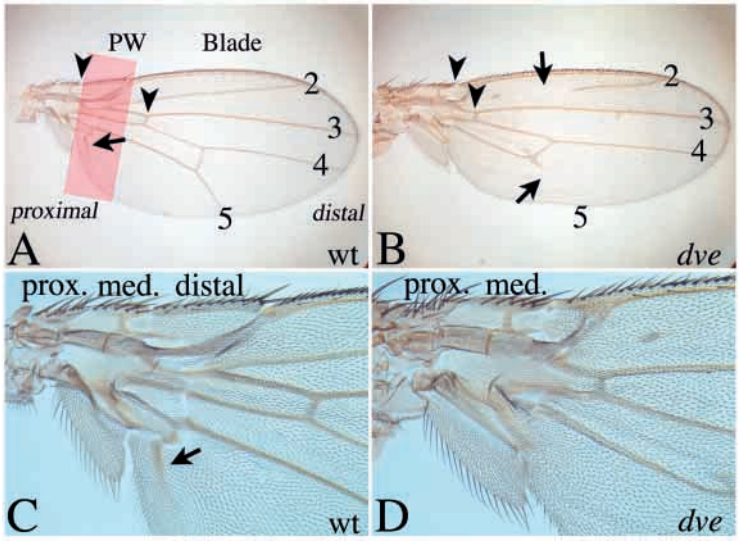

Fig. 1. The wing phenotype of *dve*^{P1738}-mutant flies. All wings are oriented proximal to the left and anterior to the top. (A) A wild-type wing. The arrowheads highlight the distance between the end of the medial costa and the anterior cross-vein. The longitudinal veins are numbered 2-5. The arrow indicates a small spot of vein material present in the proximal part of the wing blade. (B) A *dve*^{P1738}-mutant wing. The arrows indicate regions where the longitudinal veins 2 and 5 are interrupted. Note that the distance between the arrowhead in the PW and that at the anterior cross-vein is strongly reduced in comparison with the normal wing. Furthermore, the wing is smaller than the wild-type wing. (C) A magnification of the PW of a wild-type wing. The anterior margin of the PW is subdivided into three easily distinguishable regions: the proximal, medial and distal costa. (D) The PW of a *dve*^{P1738}-mutant wing. Most of the distal costa is deleted leaving only a very small remnant intact. Furthermore, the small spot of vein material, which can be observed in the adjacent wing blade (highlighted by the arrow in A and C), is deleted in the mutant wing, indicating that a small stripe of the adjacent wing pouch is also deleted. These observations indicate that Dve is required for the formation of a region encompassing most of the distal PW and a small part of the adjacent pouch. This region is highlighted by the pink box in A. The deletion causes the reduction of the distance between the anterior cross-vein and the PW.

transcription factor Nub (Nub), to induce the expression of Wg in a ring-like domain that frames the wing pouch (St Pierre et al., 2002; del Alamo Rodriguez et al., 2002). The induction of *wg* is a crucial event for the formation of the medial region of the PW (Neumann and Cohen, 1996). To get further insight in the patterning mechanisms that operate along the PD axis, it is important to identify new genes that are involved.

We report that the gene, *defective proventriculus* (*dve*) is required for the establishment of the region of the PD axis that comprises the distal part of the PW and a small part of the adjacent wing pouch. The function of *dve* is further required for other aspects of wing development, such as the formation of wing veins 2 and 5, and the proliferation of cells of the wing pouch. *dve* encodes a novel type of homeobox protein, which was originally shown to be required for the proper development of the larval proventriculus (Fuss and Hoch, 1998; Nakagoshi et al., 1998). The expression of *dve* in the wing imaginal disc occurs in a disc-like domain that is smaller than that of Nub and Rn but larger than that of Vg. It is initiated by Vg in a non-autonomous manner, and suppressed at the DV boundary by the combined activity of Nub and Wg. Our work reveals that Vg patterns the PW through the induction of expression of genes in disc-like domains of different sizes. The size difference of the expression domains creates concentric regions with differential gene activity, which directs the formation of the three regions of the PW. The regulation of *dve* expression by Vg is also the first obvious connection between Vg and the regulation of cell proliferation, which is clearly distinguishable from its role in pattern formation.

MATERIALS AND METHODS

Fly stocks

The *dve*^{P1738}-FRTG13 and UAS-*dve* lines, as well as *Df(2R) 58-5*, have been described previously (Fuss and Hoch, 1998; Nakagoshi et al., 1998). *dve*Gal4 (*P(GT1)dve*^{BG02382}) was obtained from the Bloomington Stock Centre. UAS-*vg* is described by Kim et al. (Kim et al., 1996). *nub*^{1}, *spd*^{flg} were provided by Steve Russell. *fj-lacZ* (Villano and Katz, 1995) was a gift of F. Katz. *rn-lacZ* was a gift of J.-P. Couso and is described by St. Pierre et al. (St. Pierre et al., 2002). *vg*^{83b27R} is a null mutation of *vg*, and together with the UAS-*vg*, *vg*-QE and *vg*-BE, was provided by S. Carroll (Kim et al., 1996). The *arr*^{2}-FRTG13 chromosome (Wehrli et al., 2000) was a gift of S. DiNardo. *dpp*Gal4, UAS-*Nintra* and UAS-*wg* stocks are described by Klein and Martinez-Arias (Klein and Martinez-Arias, 1998), and UAS-*Flp* (Duffy et al., 1998) was provided by N. Perrimon.

Clonal analysis

The *arr*^{2}-FRTG13 chromosome was used to induce *arr*-mutant clones with help of the FLP/FRT system. The mutant clones were induced using an UAS-FLP construct activated by *vg*GAL4. *dve*^{P1738}-FRTG13 clones were induced using an hsFlp construct. Wing imaginal discs were prepared at the late third larval instar stage, 48 hours after heat shock. Flip-out clones were induced with help of the AyGal4-UAS-GFP chromosome, kindly provided by K. Ito (Ito et al., 1997).

Histochemistry

The following antibodies were used: anti-Wg, anti-Dve, anti-β-Gal and anti-Nub. The anti-Wg antibody was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Anti-Nub was a gift of M. Averof and anti-Dve (Nakagoshi et al., 1998) was a gift of F. Matsuzaki.

Staining was performed according to standard protocols. The FITC- and Texas Red-conjugated secondary antibodies were purchased from Jackson Immuno Research.

RESULTS

The *dve*^{P1738} mutation (also called *dve*^{1}) is an insertion of a P-*lacZ* transposable element in the second intron of the *dve* gene. The insertion causes a severe truncation of the mRNA of the large transcript encoded by the *dve* gene (Fuss and Hoch, 1998). Homozygous *dve*^{P1738} animals are reported to die during the first larval instar stage as a result of a failure of the formation of the proventriculus (Fuss and Hoch, 1998; Nakagoshi et al., 1998). Previous work has shown that the mutant phenotype is caused by the P-element insertion, and can

be reverted by precise excision of the P-element (Fuss and Hoch, 1998; Nakagoshi et al., 1998).

We found that, although the majority of the mutant animals die as first instar larvae, a small percentage of the animals develop until adulthood, and some even hatch. However, these flies displayed defects in several adult structures, such as the wing, the haltere, the leg and the head. We have identified another insertion of a P-Gal4 construct: *P(GT1)dve*^{BG02382} (Gene Disruption Project members, 2001.1.29), inserted in the second intron of *dve*, which will now be referred to as *dve*-Gal4. *dve*^{P1738} is lethal in trans-heterozygousity to the deficiency *Df(2R) 58-5* or *dve*-Gal4, indicating that the *dve*^{P1738} is not a null allele in general. However, we could not detect any protein in wing imaginal discs of homozygous-mutant animals (see below), indicating that *dve*^{P1738} is a strong allele for wing development. The lethality of the *dve*-Gal4/*dve*^{P1738}-heterozygous animals can be rescued by the presence of a UAS-*dve* construct. These 'rescued' animals exhibit a slightly weaker wing phenotype than *dve*^{P1738}-homozygous mutants. We could not detect any activity of *dve*-Gal4 in any imaginal disc using a UAS-GFP reporter construct. Thus, it appears that *dve*-Gal4 expression is restricted to the time of embryogenesis and that this expression is sufficient to let the *dve*-Gal4/*dve*^{P1738} animals survive in the presence of UAS-*dve*. Nevertheless, the adult phenotype displayed by this allelic combination indicates that the loss of *dve* function is the cause of the observed wing defect. In this work, we have concentrated on the analysis of the function of *dve* during wing development.

Dve is required for the patterning of the proximal wing, the wing blade and the formation of wing veins

The adult *Drosophila* wing is subdivided into several domains along the proximodistal axis. Proximal-most is the hinge, which connects the proximal wing and the wing blade to the body wall (Fig. 1A). The proximal wing consists of several regions, among them the costa at the anterior margin. The costa can be further subdivided into three easily distinguishable parts: a proximal, a medial and a distal part (Fig. 1A,C). We found that wings of *dve*-mutant flies were smaller and shorter than wild-type flies (Fig. 1B). A detailed analysis showed that a region that encompasses most of the distal costa and a small part of the adjacent wing blade is missing in *dve*-mutant flies (Fig. 1C,D). As a result of the deletion, the distance between the end of the medial costa and the first cross-vein of the wing blade is reduced (compare distance between the arrowheads in Fig. 1A and B).

The comparison of wild-type and mutant wings further reveals that *dve*-mutant wings are also reduced in size along the anteroposterior (AP) axis (compare Fig. 1A and B; see Fig. 3A). Furthermore, wing vein 2 is interrupted in the proximal part, and the distal part of vein 5 is lost (arrows in Fig. 1A,B).

These phenotypes indicate that Dve is involved in the formation of the distal part of the PW, and in the correct development of wing veins 2 and 5, and that it is required for the regulation of the size of the wing.

dve mutants show recognizable abnormalities by the late third larval instar wing imaginal discs (Fig. 2). In mutant discs, the anlage of the dorsal part of the wing pouch is shorter, as revealed by the distance between the DV boundary and the inner ring-like expression domain of Wg expression in the proximal wing (Fig. 2A,C). The defects are more easily recognized during the early pupal phase, when the wing has evaginated (Fig. 2B,D). The wing pouch of *dve* mutants is smaller than in wild type and has a small indentation at the anterior wing margin (arrow in Fig. 2B,D). Furthermore, the fold adjacent to the wing blade (asterisk in Fig. 2B,D) appears to be reduced in the mutant wings (see arrowheads in Fig. 2B,D). These observations indicate that the defect in *dve*-mutant wing imaginal discs occurs before the late third larval instar stage. As we do not find any abnormal cell death in *dve*-mutant wing imaginal discs (data not shown), it is probable that the anlage of the proximal part of the PW, as well as the adjacent area of the blade, is not established in the absence of Dve function.

Nakagoshi et al. reported that *dve*^{P1738}-mutant cell clones, including the wing margin, cause the formation of ectopic bristles as well as nicks in the wing margin (Nakagoshi et al., 2002). We could not observe such perturbations in animals homozygous for this allele. (For an explanation of this discrepancy, see Discussion.)

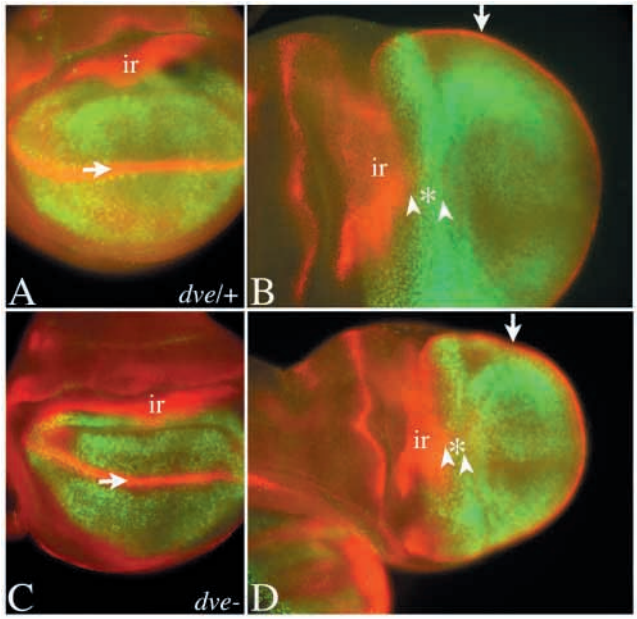

Fig. 2. Comparison of the wing region of a wild-type and *dve*^{P1738}-mutant wing imaginal disc, in animals of the late third larval instar stage and of the early pupal stage. The discs are double stained with anti-β-Gal (green) and anti-Wg (red) antibodies. (A) A wild-type wing imaginal disc of the late third larval instar stage. (C) A *dve*-mutant wing imaginal disc of the late third larval instar stage. Anterior is to the left; ventral to the bottom. The arrow (A,C) indicates the *wg*-expression domain along the DV boundary. ir, the inner ring-like expression domain of *wg* in the PW. The comparison of A and C reveals that the wing pouch of the *dve*-mutant wing disc is smaller. As a consequence the distance between the DV boundary and the inner ring-like domain of *wg* expression is reduced. (B,D) The defects become more obvious in wing imaginal discs of the early pupal stage. The *dve*-mutant wing (D) is smaller than its wild-type counterpart (B). The arrow highlights an indentation in the anterior margin of the mutant wing. Furthermore, the proximal fold, which is labelled by the asterisk (B,D) is smaller in the *dve* mutant (compare the distance between the two arrowheads in B and D).

Dve is required for the proliferation of cells in anterior and posterior regions of the wing

The reduction in size of the *dve*-mutant wings along the AP axis could simply be due to lack of cell growth. This conclusion is supported by the feeding defect reported for *dve* mutants (Fuss and Hoch, 1998), as the development of starved flies is slower and their cells are smaller. However, the size of the area outlined by the wing veins 3 and 4, and the anterior cross-vein and wing margin is of similar size in wild type and mutant (Fig. 3A). This suggests that at least in this area the cells are of similar size. The size reduction of the mutant wings could also be a result of increased cell death. However, we did not observe any enhanced cell death in *dve*-mutant wing imaginal discs of the early and late third larval instar stage (see above).

A further possibility is that the cells proliferate less in the mutant wings. To test this possibility, we compared distances and cell densities in several regions of the mutant and wild type wing (Fig. 3A). The cell density of both types of wings was very similar in the area between wing veins 3 and 4. Furthermore, the distance between wing vein 3 and wing vein 4, measured by the numbers of cells between them, is the same. Hence, no differences exist between mutant and wild-type wings in this region. These data are consistent with our observation that this region is of similar size in both genotypes.

However, we found differences between wild-type and *dve*-mutant wings in more anterior and posterior regions. Although the cell density in the region anterior to vein 3 was similar, the distance between vein 3 and the anterior wing margin was reduced in the *dve*-mutant wing, indicating that this area consists of fewer cells (Fig. 3A, measurements A and D). A similar difference was observed in the area between wing vein 4 and the posterior margin. We found that the distance from vein 4 to the posterior margin is again reduced. In addition, the cell density in this region is lower in the mutant (Fig. 3A, measurements C and F), indicating that the mutant cells are larger. Thus, there are fewer cells and the cell size is increased in the posterior area of *dve*-mutant wings. Enlargement of cells is a typical reaction of wing cells if their proliferation is inhibited and it is interpreted as a compensatory mechanism in order to achieve a normal organ size (Weigmann et al., 1997; Neufeld et al., 1998). The data suggest that the observed size reduction of *dve*-mutant wings is the result of a lower proliferation rate of cells located within the regions anterior of vein 3 and posterior of vein 4. Thus, Dve is required for the correct proliferation of wing pouch cells in these regions.

To further explore whether *dve*-mutant cells proliferate less than wild-type cells, we examined the behaviour of dve^{P1738}-

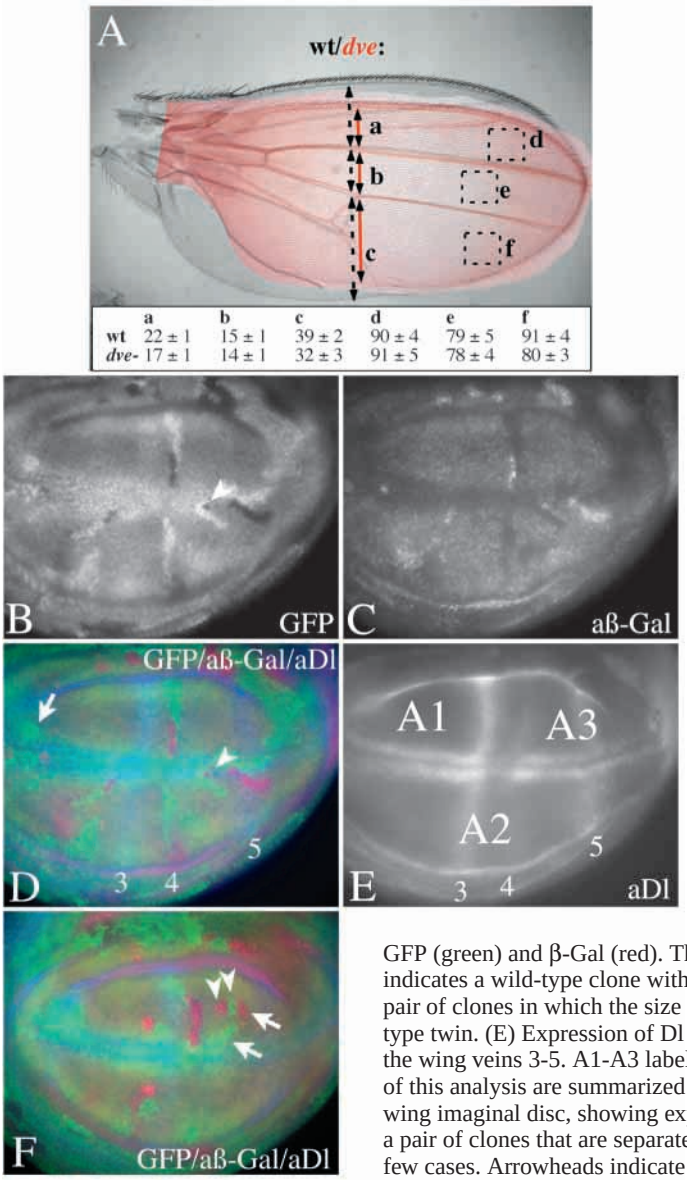

Fig. 3. Analysis of the proliferation defect of cells in dve^{P1738}-mutant wing pouches. (A) An overlay of a wild-type (grey) and mutant (red) wing. The double-ended arrows show the distances between wing vein 3 and the anterior wing margin (labelled a), between vein 3 and vein 4 (labelled b), and between vein 4 and the posterior margin (labelled c). Distances are counted in cell numbers. The cell density was measured in three different areas (labelled d-f). The results are summarized in the table below (d-f, *n*=14; a and c, *n*=4; b, *n*=14 wings counted for each genotype). In the region between vein 3 and vein 4, no differences in the distance (b) and the cell density (e) were observed. These results confirm the observation that both wings are of the same size in this region, as seen by the overlay of the wings. By contrast, anterior to vein 3 and posterior to vein 4 the distances in the mutant wings are shorter (a,c). Furthermore, the cell density is similar in the anterior area (see d) and even slightly lower in the posterior area (see e) in the mutant. This indicates that the observed reduction in size of these areas in the mutant is caused by having fewer cells. (B-F) Clonal analysis of dve^{P1738}. Clones were induced using hsFlp, and the wing imaginal discs were prepared 48 hours after heat shock. Discs are stained with anti-Dl and anti-β-Gal antibodies. (B) dve^{P1738}-mutant clones revealed by the absence of the GFP marker. (C) The same disc as in B showing the expression of β-Gal. The expression of β-Gal is complementary to that of GFP, showing loss of staining in the wild-type clones and stronger staining in the dve^{P1738}-homozygous clones. (D) Expression of Dl (blue), GFP (green) and β-Gal (red). The expression of Dl reveals the primordia of wing veins 3-5. The arrow indicates a wild-type clone with no obvious mutant counterpart. The arrowhead (B,D) points to a twin pair of clones in which the size of the mutant clone is dramatically reduced in comparison to its wild-type twin. (E) Expression of Dl in the disc also shown in B-D. The numbers highlight the primordia of the wing veins 3-5. A1-A3 labels the different areas in which the clones have been analysed. The results of this analysis are summarized in Table 1. (F) Another example of a dve^{P1738}-mutant clone bearing wing imaginal disc, showing expression of Dl (blue), β-Gal (red) and GFP (green). The arrows indicate a pair of clones that are separated by a band of heterozygous cells. This separation has been found in a few cases. Arrowheads indicate a pair of clones that are adjacent to each other.

mutant cell clones (Fig. 3B-F; Table 1). The clones were induced with help of the Flp/FRT system and were analysed 48 hours after clone induction by hsFlp. Because our analysis of the adult wing suggests that the behaviour of *dve*-mutant cells is dependent on the region of the wing, we subdivided the wing blade along the AP axis into three areas. These areas were defined by the expression pattern of Delta (Dl), which is expressed in the anlagen of wing veins 3, 4 and 5 (Fig. 3E). Area 1 (A1) extended from the anterior wing margin to vein 3, area 2 (A2) extended from vein 3 to vein 4, and area 3 (A3) extended from vein 4 to the posterior margin (Fig. 3E). Three effects were observed (Fig. 3B-F; Table 1). First, we found that mutant clones had fewer cells than their wild-type twin clones (see Table 1). However, the number of cells in mutant clones was dependent on its location: whereas the number of cells in mutant clones in A1 and A3 was roughly half that of their wild-type counterparts, the mutant clones in A2 had around two thirds of the cells that their wild-type twins did (see Table 1). The results indicate that after 48 hours, the mutant cells in A1 and A3 have gone through one cell cycle less than their wild-type counterparts.

Second, we found that in A1 and A3 (but not in A2), nearly half of the wild-type clones did not have a mutant counterpart (47% and 43%, respectively), which suggested that the mutant cells had died. As the mutant cells do not die in homozygous animals, we believe that cells of mutant clones undergo apoptosis because of a disadvantage in competing with wild-type neighbours. Cells defective in proliferation typically apoptose (Neufeld et al., 1998; Moreno et al., 2002). Altogether, these results further support our conclusion that *dve*-mutant cells are defective in cell proliferation.

A third interesting aspect is that, in some cases, a wild-type clone is separated from its mutant twin clone by a band of heterozygous cells (see arrows in Fig. 3F). This suggests that the two types of clones might have different adhesive properties. Alternatively, the heterozygous cells could have migrated into the area between the two clone types because some of the mutant cells died.

Table 1. Analysis of the *dve*-mutant clones.

Area	Number of orphan wild-type clones/total number of clones	Average number of cells in mutant clones	Average number of cells in the wild-type twin clones	% cell number (mutant versus wild-type clones)
A1	16/34 (47%)	6.7	13.8	49%
A2	0/13	10.9	16.6	66%
A3	6/14 (43%)	7.6	14.8	51%

Clones were induced with help of the Flp/FRT system and analysed 48 hours after clone induction by hsFlp. Areas A1-3 are described in Fig. 3E. A1 is the area anterior to the primordium of wing vein 3, A2 extends from vein 3 to vein 4, and A3 is the area posterior to vein 4. The table reveals that the size of the mutant clone in comparison to its wild-type twin is always smaller but varies depending on the area. In A1 and A3 the size of the clone is only around 50% of its wild-type twin, whereas in A2 it is 66%. This suggests that after 48 hours, the mutant cells have gone through one less cell cycle than their wild-type counterpart. Furthermore, although we found that 47% and 43% of the wild-type clones in A1 and A3, respectively, do not have a mutant counterpart, in A2 no orphan wild-type clone was found. These data indicate that Dve is required for the proliferation of all cells in the wing pouch, but the degree of its requirement is dependent on the region.

Expression of Dve during wing development

To gain further insight in the function of Dve, we monitored its expression pattern during wing development by use of an anti-Dve antibody. We compared the expression pattern of Dve with that of Wg, which is expressed throughout wing development in a pattern that reveals the organization of the developing wing (Fig. 4B,E,H,K). Wg is initially expressed in a ventral domain during the second larval instar stage and defines the wing area or wing field (Fig. 4B,C) (reviewed by Klein, 2001). At this time, Dve is not expressed in the wing imaginal disc (Fig. 4A,C). At the beginning of the third larval instar stage, Wg expression resolves into a stripe along the future DV compartment boundary and a proximal ring-like domain (Fig. 4E). In the middle of third larval instar stage a second ring-like domain in the proximal region of the anlage is added. The two ring-like domains of Wg expression highlight the anlagen of the proximal and medial regions of the proximal wing, as deduced from X-Gal staining of adults carrying a *wg-lacZ* construct (see Fig. 5A). Dve expression is initiated at the time when Wg resolves into a ring-like domain in the periphery and a domain along the DV boundary, and it becomes expressed in all cells inside the region framed by the ring-like domain of Wg (Fig. 4D,F). Dve continues to be expressed in a disc-like domain that fills the inside of the inner ring-like expression domain of Wg until the late third larval instar stage (Fig. 4G,I).

The anlage of the distal region of the PW, is located outside the wing pouch and inside the inner ring-like domain of Wg expression (Fig. 4I; see also Fig. 5A). Dve is expressed continuously in this region, and is present at the right place and time to control the development of this structure, which is absent in the mutants.

At the DV boundary, Dve is initially expressed (Fig. 4D,F), but it becomes downregulated soon after its initiation (arrowhead in Fig. 4G,I), with the exception of a short stretch at the anterior side (arrowhead in Fig. 4G). During the late third larval instar stage, it is also downregulated in the primordia of wing veins 3 and 4 (arrows in Fig. 4G).

We failed to detect any Dve protein in wing imaginal discs of homozygous-dve^{P1738} larvae (Fig. 4J,K), which suggests that this allele is probably a null allele for wing development.

Comparison of the expression domain of *dve* with that of other genes required for pattern formation along the PD axis

We have mapped the expression domain of *dve* in relation to that of other genes known to be involved in PD patterning of the wing, and in relation to the ring-like domains of *wg*. The ring-like domains label the region of the proximal and medial costa, as revealed by the X-Gal staining of adult wings bearing a *wg-lacZ* insertion (Fig. 5A).

vestigial (*vg*) is required for all distal fates from the medial costa distalwards. It is initially expressed in all pouch cells (Kim et al., 1996; Klein and Martinez-Arias, 1998a; Liu et al., 2000) and its expression is controlled through the *vg*-Quadrant enhancer (*vg*-QE) (Kim et al., 1996). We found that the expression domain of *dve* is larger earlier than that of the *vg*-QE. In addition, *dve* expression is initiated before the *vg*-QE is activated, which indicates that *dve* expression is initiated before the wing pouch forms (Fig. 5C-E; data not shown).

Nub is involved in patterning the wing from the medial costa distalwards (Ng et al., 1995). The *nub* gene is expressed in a disc-like domain that is slightly larger than that of *dve* (Fig. 5F-H) and that extends to the area between the two ring-like domains of *wg* expression (Fig. 5G,H). Examination of wing discs of early third instar larvae revealed that *nub* expression is initiated earlier than *dve*, and is always expressed in a larger domain than *dve* (data not shown).

The boundary of the expression domain of *rotund* (*rn*) falls between that of *dve* and *nub*. Its domain reaches the proximal boundary of the inner ring-like domain of *wg* expression (Fig. 5J).

By contrast, the expression domain of *dve* is larger than that of the *four-jointed* (*fj*) gene, which is expressed in a similar pattern to *vg* (Fig. 5I). The results of the comparison of the expression domains are schematically summarized in Fig. 5K. The cartoon reveals that the cells of the different regions of the proximal wing contain different combinations of gene activities. These specific combinations appear to trigger the region-specific differentiation in these cells.

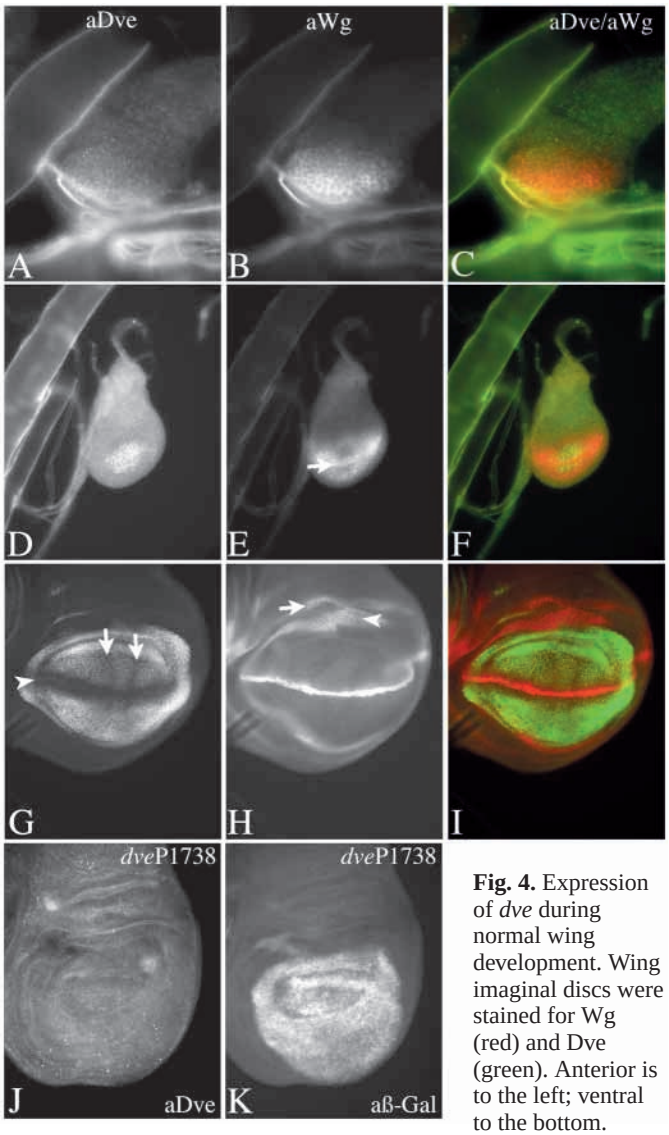

Fig. 4. Expression of *dve* during normal wing development. Wing imaginal discs were stained for Wg (red) and Dve (green). Anterior is to the left; ventral to the bottom. (A-C) A wing imaginal disc of the late second larval instar stage. Wg is expressed in a ventral area of the disc (B). At this time, Dve is not expressed (A,C). (D-F) A wing imaginal disc of the early third larval instar stage. Wg expression (E) resolves into a ring-like domain that is bisected by a stripe of Wg expression along the future DV compartment boundary (arrow). At this time, expression of Dve is initiated (D) and is observed in the region that is framed by the ring-like domain of Wg expression (F). (G-I) A wing imaginal disc of the late third larval instar stage. Wg is expressed in two ring-like domains and along the DV boundary (arrow and arrowhead in H). During this phase, Dve continues to be expressed in the area framed by the inner ring-like domain of *wg* expression (G,I). Note that Dve expression is suppressed at the DV boundary, with exception of a short stretch at the anterior side (arrowhead in G). Dve expression is further lowered in the region of the primordia of wing veins 3 and 4 (arrows in G). (J) No specific staining is observed in dve^{P1738}-mutant wing imaginal discs of the third larval instar stage following anti-Dve antibody staining. This suggests that in dve^{P1738} is a null allele of the locus for wing development. (K) Expression of β-Gal in the disc shown in J.

Regulation of the expression of *dve*

Vg activates the expression of *dve* in a non-autonomous manner

Vg is required for the establishment of distal wing fates, including the medial and distal areas of the proximal wing (Klein and Martinez-Arias, 1998a; Liu et al., 2000; del Alamo Rodriguez et al., 2002). This raises the possibility that Vg might activate the expression of *dve*. To test this possibility, we first monitored the expression of *dve* in *vg*-mutant wing imaginal discs. We found that in vg^{83b27R} mutants, the expression of *dve* is lost (Fig. 6A), which indicates that Vg activity is required for its expression. Note that the expression domain of *vg* is always smaller than that of *dve* (see above), which suggests that Vg regulates the expression of *dve* in a non-autonomous manner. We next investigated whether Vg is sufficient to activate *dve* expression. To address this question, we generated clones of *vg*-expressing cells in the wing imaginal disc with help of the Flip-out technique (Ito et al., 1997). Clones of *vg*-expressing cells were indeed able to induce ectopic expression of Dve (Fig. 6B,C). This result indicates that Vg is sufficient to activate expression of *dve*. The ectopic expression of *dve* was not restricted to the clones, but also occurred in cells surrounding the clones (arrows in Fig. 6B,C). The result confirms the conclusion that Vg induces *dve* expression in a non-autonomous manner. Hence, the induction of *dve* expression by Vg is indirect and probably mediated by a diffusible factor, the expression of which is controlled by Vg. Note, that the ability of Vg to ectopically induce *dve* expression is restricted to the wing and pleural regions, indicating that it requires the activity of other factors in other regions of the disc. As co-expression of *vg* and *wg* can induce wing fates in the notum (Klein and Martinez-Arias, 1998), we tested whether this combination is also sufficient to activate expression of *dve*. Indeed, we found that the combination of UAS-*vg* and UAS-*wg* activated by *dpp*-Gal4 was able to induce expression of *dve* in the notum (data not shown).

Wg and Nub suppress the expression of *dve* near the DV boundary

The study of *dve* expression during wing development revealed that it is downregulated at the DV boundary (see Fig. 4). The *Notch* pathway is active at the DV boundary and regulates the expression of genes such as *wg* and *vg*. We therefore wondered

whether it is the activity of *Notch* that suppresses the expression of *dve* at the DV boundary. The ectopic expression of the activated intracellular domain of Notch, UAS-Nintra, in the wing pouch with *dpp*-Gal4 results in the loss of *dve* expression (Fig. 6D), which indicates that activation of the pathway suppresses the expression of *dve* in pouch cells. However, the suppression of *dve* expression in normal wing imaginal discs occurs gradually and reaches several cell diameters from the DV boundary into the wing pouch, where the *Notch* pathway is not active. This suggests that the influence of Notch on the expression of *dve* is mediated by a diffusible factor that is controlled by *Notch* signalling at the DV boundary. Wg is such a factor and we tested whether it can suppress the expression of *dve*. We found that UAS-*wg*, expressed with *dpp*-Gal4, can suppress *dve* expression ectopically in pouch cells in the same manner as Nintra (Fig. 6E). Furthermore, pouch cells that lack the Wg co-receptor Arrow and cannot receive the Wg signal (Wehrli et al., 2000), express higher levels of Dve at the DV boundary, and even further away from the DV boundary (Fig. 6F-I). Both results suggest that Wg mediates the suppressive effect of the *Notch* pathway on *dve* expression in pouch cells near the DV boundary.

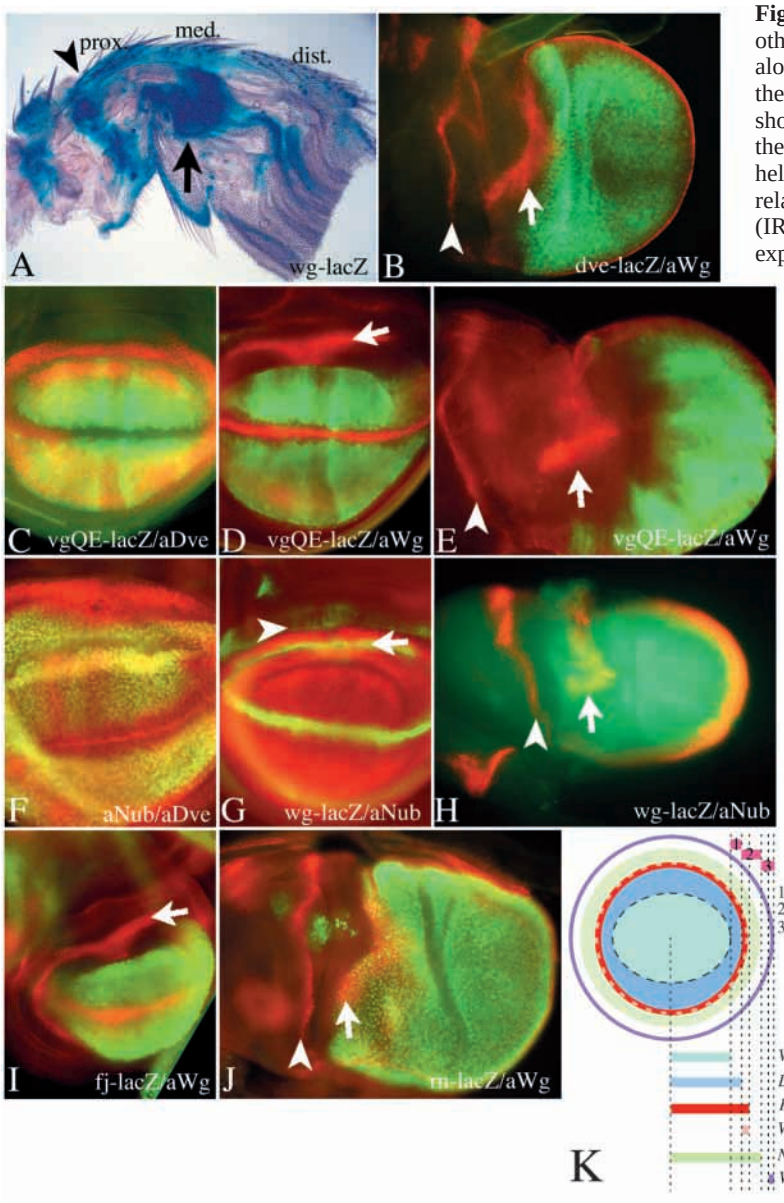

Fig. 5. Comparison of the expression domain of *dve* with that of other genes involved in the patterning of the *Drosophila* wing along the PD axis. In C,D,F,G,I, anterior is to the left; ventral to the bottom. In A,B,E,H,J, discs of the early pupal phase are shown where the wing has everted, revealing the PD axis. In these images anterior is up; distal to the right. The everted wing helps to clarify the limits of the examined expression domains in relation to the expression of Wg. The arrow highlights the inner (IR) and the arrowhead the outer (OR) ring-like domain of Wg expression in the PW. (A) Persistent β-galactosidase activity in adult flies carrying a P-*lacZ* insertion in the *wg* locus. The staining allows the determination of the structures that arise from the regions of Wg expression. It reveals that the ring-like domains of *wg* expression at the late third larval instar stage (arrow and arrowhead in Fig. 4H) label the anlagen of the proximal and medial regions of the PW (compare also with B). These observations suggest that the region between the IR and the actual wing pouch is the anlage of the distal region of the PW. (B) A $dve^{P1738/+}$ wing imaginal disc of the early pupal phase, stained with anti-β-Gal antibody, to reveal the expression of *dve* (green), and anti-Wg antibody, to reveal the expression of Wg (red). The expression domain of *dve* reaches close to the IR. (C) Expression of *vg*-QE (green), revealed by anti-β-Gal, and Dve (red), revealed by anti-Dve antibody staining. The expression domain of Dve is larger than that of the *vg*-QE. (D,E) Expression of the *vg*-QE (green) relative to that of Wg (red). The expression domain of *vg*-QE is restricted to the wing pouch and a broad band of non-expressing cells separates it from the IR (arrow). (F) Anti-Nub (red) anti-Dve (green) double-antibody staining of a wing imaginal disc of the late third larval instar stage. The double staining reveals that the disc-like expression domain of Nub is larger and includes that of Dve. (G,H) Anti-Nub anti-Wg double staining. (G) A wing imaginal disc of the late third larval instar stage, showing Wg (green) and Nub (red) expression. (H) A wing imaginal disc in the early pupal phase stained with anti-Wg (red) and anti-Nub (green) antibodies. G and H reveal that the border of the Nub expression domain lies between the two ring-like domains of Wg expression. (I) Expression of *fj*, revealed by anti-β-Gal staining (green), and Wg, revealed by anti-Wg antibody staining (red). *fj* is expressed in the wing pouch in a similar domain to Vg and is not expressed in the distal region of the PW. Thus, the expression domain is smaller than that of Dve. (J) A wing imaginal disc of the early pupal stage containing an *rn-lacZ* insertion to reveal the expression of *rn*. Anti-β-Gal (green) anti-Wg (red) double staining reveals that the boundary of the *rn* expression domain is identical to that of the IR. Thus, the expression domain of *rn* is larger than that of Dve. (K) Summary of the comparison. The proximodistal extent of the expression domains are depicted as follows: Vg/Fj, turquoise; Dve, blue; Rn, red; the IR, pink; Nub, green; and the outer ring-like domain of Wg, mauve. The cartoon highlights the fact that the tested genes are expressed in ring-like (Wg) or disc-like (Dve, Nub, Fj and Vg) domains of different sizes. The size of the domain increases from Vg/Fj to Dve to Rn, and from the inner ring-like domain of Wg to Nub to the outer ring-like domain of Wg. The result of these different expression domains is the definition of concentric regions with different combinations of gene activities that probably define the different regions of the PW.

The *spade*^{flag} (*spd*^{flg}) mutation of *wg* is lacking the regulatory region that directs expression of *wg* in the inner ring-like domain and causes the loss of the medial block of the proximal wing. We found that in *spd*^{flg} mutants, the expression of *dve* is not affected (Fig. 6M). In agreement with this is that the distal part of the PW forms normal in *spade*^{flag} mutants.

We also found that *dve* is still expressed in *nub* mutants (Fig. 6J-L), indicating that Nub is not required for the expression of *dve*. However, *dve* expression was not suppressed at the DV boundary (Fig. 6K,L). Hence, in addition to Wg, Nub seems to be required to suppress *dve* expression at the DV boundary. As expected, Nub expression is not altered in *dve*-mutant wing discs, indicating that Dve is not required for its expression in the wing region (Fig. 6N).

Ectopic expression of Dve

To further explore the function of Dve during the development of the wing, we studied the effects of ectopic expression of Dve in the wing imaginal disc. We used the Flip-out technique to ectopically express UAS-*dve* in clones of cells.

We observed three effects caused by clones of *dve*-expressing cells (Fig. 7A-G). *dve*-expressing clones induced the formation of folds around the clone if they were located in the region of the anlage of the distal part of the PW (arrowhead in Fig. 7A-C); this is a region where it is normally expressed.

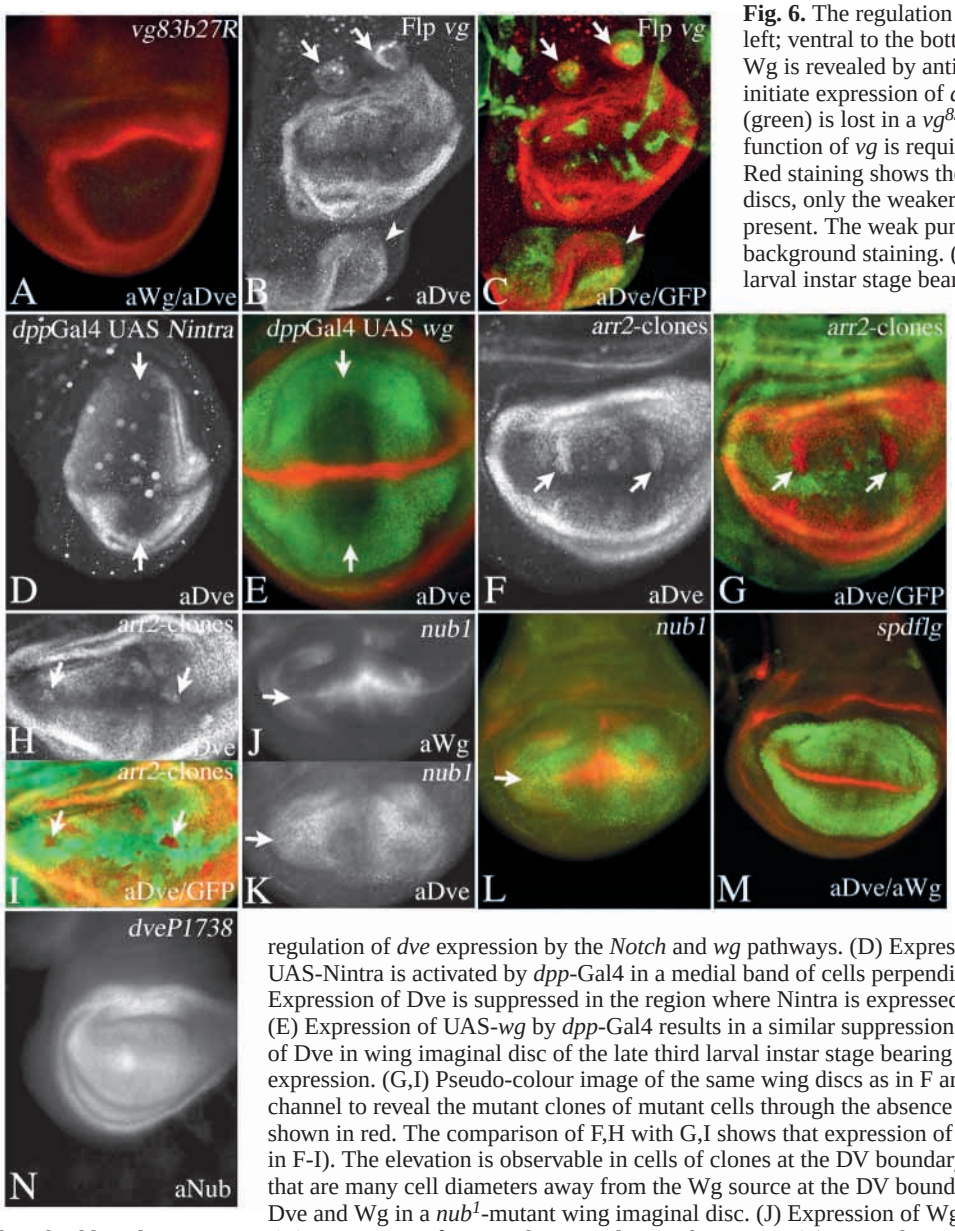

Fig. 6. The regulation of the expression of *dve*. Anterior is to the left; ventral to the bottom. In all images, expression of Dve and Wg is revealed by antibody staining. (A-C) Vg is sufficient to initiate expression of *dve* in the wing area. (A) Expression of Dve (green) is lost in a *vg*^{83b27R}-mutant wing disc, indicating that the function of *vg* is required for the induction of expression of *dve*. Red staining shows the expression of *wg*. In *vg*^{83b27R}-mutant wing discs, only the weaker outer ring-like expression domain of Wg is present. The weak punctuate green staining is unspecific background staining. (B,C) A wing imaginal disc of the late third larval instar stage bearing Vg-expressing cell clones. The clones of UAS-*vg* expressing cells were induced with the help of the AyGal4-UAS-GFP chromosome during the second larval instar and are labelled by the green GFP marker in C. (B) Expression of Dve. The arrows indicate Vg-expressing clones located outside the normal Dve expression domain. (C) Pseudo-colour image of the same disc as in B, revealing the Vg-expressing cell clones in green and expression of Dve in red. The double staining reveals that Vg-expressing clones can induce ectopic expression of Dve in the PW (see arrows) and in the pleura (arrowhead). Note that Vg can induce expression of Dve in adjacent non-expressing cells (see clones highlighted by the arrows), indicating that the induction of Dve expression occurs in a non-autonomous manner. The ability of Vg to induce expression of Dve is restricted to certain regions of the wing, indicating that additional factors are required in other regions. (D-I) Negative regulation of *dve* expression by the *Notch* and *wg* pathways. (D) Expression of Dve in a wing imaginal disc where UAS-Nintra is activated by *dpp*-Gal4 in a medial band of cells perpendicular to the DV boundary (arrows). Expression of Dve is suppressed in the region where Nintra is expressed (highlighted by the arrows). (E) Expression of UAS-*wg* by *dpp*-Gal4 results in a similar suppression of the expression of Dve. (F-I) Expression of Dve in wing imaginal disc of the late third larval instar stage bearing *arr*^{2}-mutant cell clones. (F,H) Dve expression. (G,I) Pseudo-colour image of the same wing discs as in F and H, respectively, including the green channel to reveal the mutant clones of mutant cells through the absence of GFP fluorescence. Expression of Dve is shown in red. The comparison of F,H with G,I shows that expression of Dve is elevated in *arr*^{2}-mutant cells (arrows in F-I). The elevation is observable in cells of clones at the DV boundary (arrows in H,I) and also in mutant cells that are many cell diameters away from the Wg source at the DV boundary (arrows in F,G). (J-L) Expression of Dve and Wg in a *nub*^{1}-mutant wing imaginal disc. (J) Expression of Wg in a *nub*-mutant wing imaginal disc of the late third larval instar stage. (K) Expression of Dve in the same disc as shown in J. (L) Merged view of both channels shown in J and K, showing Wg expression in red and Dve expression in green. The double staining reveals that expression of *dve* at the DV boundary is not suppressed in most of the regions (arrow in J-L). This suggests that Nub is required to suppress the expression of Dve at the DV boundary. (M) Expression of Dve (green) is not affected in a *spade*^{flg}-mutant wing imaginal disc. Red shows the expression of Wg and reveals that the inner ring-like domain of expression is lost. (N) Expression of Nub is unaffected in *dve*^{P1738}-mutant wing imaginal discs.

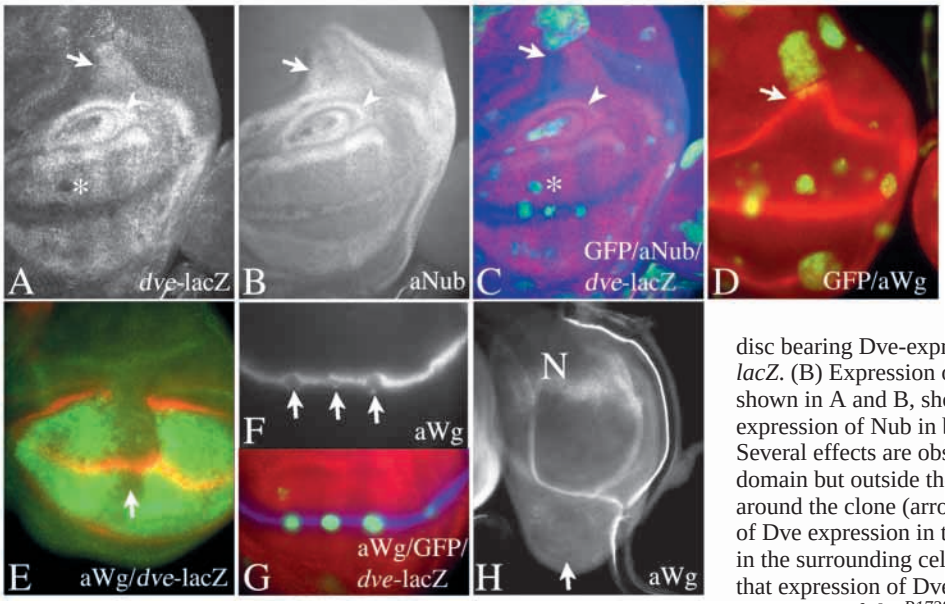

Fig. 7. The consequences of ectopic expression of *dve*. (A-D,F,G) Wing imaginal discs bearing clones of UAS-*dve* expressing cells in the wing area. The Dve-expressing clones were induced with help of the Flip-out method, using hsFlp and the AyGal4-UAS-GFP constructs, during the second larval instar stage and labelled by the green fluorescence of the GFP. Expression of all genes is detected by antibody staining. (A-C) Expression of dve^{P1738}-*lacZ* and Nub in a wing imaginal disc bearing Dve-expressing clones. (A) Expression of dve^{P1738}-*lacZ*. (B) Expression of Nub. (C) The same wing imaginal disc as shown in A and B, showing the Dve-expressing clones in green, expression of Nub in blue and expression of dve^{P1738}-*lacZ* in red. Several effects are observed. First, clones in the *dve* expression domain but outside the wing blade cause the formation of folds around the clone (arrowheads in A-C). This suggests that elevation of Dve expression in the PW can locally enhance the proliferation in the surrounding cells. Secondly, with a low frequency, we find that expression of Dve can non-autonomously induce ectopic expression of dve^{P1738}-*lacZ* and *nub* (see arrow in A-C). The ectopic Dve-expressing clone (above the arrow in C) is located at the hinge/notum boundary and expression of both genes is extended towards the clone. The pseudo-colour image shown in C reveals that the ectopic expression of Nub occurs in a larger domain than that of Dve, as is the case during normal development. (D) In a similar manner, we find that the expression of the inner ring-like domain of *wg* is correspondingly expanded. Expression of Wg is shown in red, the clones of Dve expressing cells are shown in green. The arrow indicates a clone near the inner ring-like expression domain of Wg. The clone induces an expansion of the inner ring-like expression domain of Wg in a similar manner to that shown for Nub and dve^{P1738}-*lacZ* (see A-C). The third effect caused by the Dve-expressing clones is highlighted by the asterisks in A and C. Elevation of Dve expression suppresses the expression of β-Gal, suggesting the existence of a negative feedback of Dve on its promoter. This negative influence of Dve on the activity of its promotor can also be observed when Dve is expressed with *dpp*-Gal, as shown in E. (E) Expression of UAS-*dve* with *dpp*-Gal4. Ectopic expression of dve^{P1738}-*lacZ* and of Wg is shown in green and red, respectively. Expression of Dve suppresses the expression of dve^{P1738}-*lacZ* (arrow). In addition expression of *wg* in the PW is interrupted in the domain of *dpp*-GAL4. (F,G) Forced expression of Dve at the DV boundary abolishes the expression of *wg*. (F) Expression of Wg. (G) Expression of Wg (blue) and dve^{P1738}-lacZ (red) in a disc bearing Dve-expressing clones (green). A comparison of F with G reveals that *wg* expression is interrupted (arrows in F) in the Dve-expressing clones. (H) Expression of UAS-*dve* with *sd*-Gal4 abolishes expression of Wg in the wing. Arrow indicates the wing area, which is poorly developed and does not exhibit any expression of Wg.

Thus the formation of these ectopic folds suggests that elevation of Dve levels can induce the proliferation in cells of the distal part of the PW. This is probably mediated by a diffusible factor.

The second effect was that clones at the DV boundary suppressed the expression of Wg (Fig. 7F,G). In accordance with this result, we found that expression of UAS-*dve* with *sd*-Gal4 or *vg*-BE-Gal4, which are strongly expressed at the DV boundary throughout wing development, results in a loss of wing structures as indicated by the absence of *wg* expression in the wing region (Fig. 7H; data not shown). These results suggest that expression of Dve at the DV boundary is deleterious for wing development, probably because it suppresses the expression of *wg*.

In addition, we found a third, low frequency phenotype. Clones of *dve*-expressing cells located outside the normal expression domain were able to non-autonomously expand the expression of *nub*, *wg* and *dve* itself (Fig. 7A-E). The ability to expand the expression of these genes was restricted to clones located in the region of the PW and the hinge. As in the wild-type discs, the ectopic expression domain of *nub* was larger than, and included, that of *dve* (Fig. 7A-C; highlighted by the arrows). *wg* expression was correspondingly expanded (Fig. 7D). As the functions of Nub, Wg and Dve are necessary to specify the medial and distal hinge, the results suggest that Dve can induce these more distal fates in the proximal region of the PW, albeit with a low frequency.

However, the low frequency of this effect and the observation that *dve*-expressing clones located in the notum had no detectable effects, suggests that during normal development Dve is probably not sufficient to establish the distal part of the PW. In agreement with this conclusion is the observation that ectopic expression of UAS-*dve* with *dpp*-Gal4 deletes most of the PW. This can be seen in the wing imaginal disc depicted in Fig. 7E, where both ring-like domains of Wg are interrupted in the *dpp* domain. This interruption is not caused by an extension of the anlage of the distal area of the PW, because in the corresponding adult wings this part, as well as the other regions of the PW, is severely reduced or deleted (data not shown). These data suggest that in the majority of cases the ectopic and overexpression of Dve is deleterious for wing development. Thus expression of Dve has to be tightly coordinated with that of other factors.

DISCUSSION

Dve is required for pattern formation along the PD axis of the *Drosophila* wing

Relatively little is known about the genes and mechanisms that

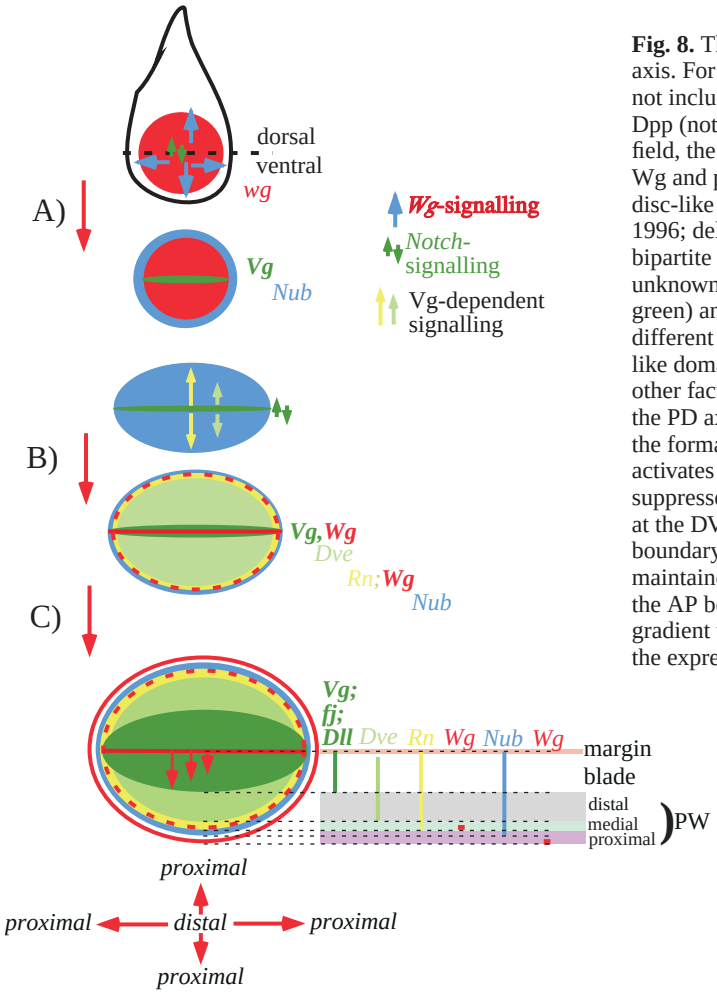

Fig. 8. The genetic hierarchy that controls pattern formation along the PD axis. For the sake of simplicity, the contribution of the AP patterning system is not included in the cartoon. (A) In the first step, the activity of Wg (red) and Dpp (not shown) define the position and extent of the wing field. Within this field, the *Notch* pathway induces the expression of Vg along the DV boundary. Wg and possibly Vg are required to induce the expression of Nub (blue) in a disc-like domain slightly larger than that of the early Wg domain (Ng et al., 1996; del Alamo Rodriguez et al., 2002). (B) Vg associates with Sd to form a bipartite transcription factor (Vg/Sd) that controls the expression of an unknown diffusible factor. This factor activates the expression of *dve* (light green) and *rn* (yellow) independently from each other and in domains of different sizes. The combined activity of Nub and Rn activate the inner ring-like domain of *wg* expression (dashed red circle). Dve, together with Nub and other factors, establishes the distal region of the PW, which is defined along the PD axis by the length of the medial and distal costal regions. Wg organizes the formation of the medial part of the PW. At the DV boundary, Vg/Sd activates the expression of Wg together with the *Notch* pathway. Wg suppresses the expression of Dve in cells near the DV boundary. (C) The cells at the DV boundary divide and the daughter cells that are displaced from the boundary form the wing pouch. Expression of Vg in the pouch cells is maintained by Wg, secreted from the DV boundary, and Dpp, secreted from the AP boundary (not shown). As a consequence, Vg is expressed in a gradient with the peak at the DV boundary. In the nascent pouch, Vg activates the expression of several patterning genes, such as *Dll*, and together with Wg, the genes of the *achaete-scute complex*, adjacent to the cells at the DV boundary (Neumann and Cohen, 1997; Zecca et al., 1996; Klein and Martinez-Arias, 1999), and *fj*. The activation of these targets is indicated by the three red arrows in the lower drawing of Step C. Fj is required for the establishment of a region within the pouch, and for the generation of planar polarity of the wing. The cartoon highlights the fact that all of the genes controlled by Vg/Sd are expressed in disc-like domains of different sizes. Their expression leads to concentric areas with different combinations of gene activities. These combinations are likely to establish different parts of the PW (see Step C).

pattern the PD axis. We have identified *dve* as a gene that is involved in pattern formation along this axis, i.e. for the specification of most of the distal region of the proximal wing and the adjacent proximal region of the pouch. In *dve*-mutant flies this region is deleted, whereas all other pattern elements of the PD axis are not affected. Expression of *dve* is initiated at the beginning of wing development, and it is expressed in the region from which the distal part of the PW and a small stripe of the adjacent wing blade form. We could observe defects in the morphology of mutant-wing discs by the late third larval instar stage. Because abnormal cell death was not observed in *dve*-mutant wings at earlier stages, the lack of the distal part of the PW in *dve* mutants could be caused by a failure in establishment of this region. However, we found that overexpression of Dve achieved through the Flp-out technique results in excessive proliferation of cells in the region of the distal part of the PW. This suggests that Dve might be required for the correct proliferation of the cells in this region. Hence, the loss of the distal part of the PW in *dve* mutants could also be explained by a failure in proliferation of the cells in the anlage of the distal region of the PW.

We found that ectopic expression of Dve does not cause the more proximal regions of the PW to become more distal, which indicates that other factors are required in addition to Dve to establish the distal part of the PW. One of these factors is Nub, which is involved in the establishment of the medial as well as the distal area of the PW (Ng et al., 1995; Rodriguez et al., 2002). However, neither ectopic expression of Nub (Neumann and Cohen, 1998) (T.K., unpublished), nor a combination of Nub and Dve (T.K., unpublished), consistently induces ectopic structures characteristic of the PW. Therefore, it is likely that a combination of Dve, Nub and other factors is required for the establishment of the distal area of the PW and the adjacent blade region.

Recent work has revealed that Nub seems to act in combination with Rn to establish the medial part of the PW. Both factors cooperate to establish the inner ring-like domain of *wg* expression (del Alamo Rodriguez et al., 2002). Thus, it appears that separate regions of the PW are established independently through different combinations of transcription factors.

Nakagoshi et al. reported that dve^{P1738}-mutant cell clones near the DV boundary of the wing lead to the formation of ectopic bristles characteristic for the wing margin (Nakagoshi et al., 2002). Concomitant with these pattern disturbances, the authors found ectopic expression of *wg* in the mutant cell clones. Based on these observations, they proposed that Dve is required for the refinement of *wg* expression. However, we do not find any defects in the bristle pattern of flies, homozygous for the same allele, or in other *dve*-mutant situations.

Therefore, we believe that the disturbances in the bristle pattern caused by the mutant clones are a result of the artificial apposition of Dve-expressing and non-expressing cells near the DV boundary, created by the induction of clones. We think that these disturbances do not reveal the biological function of Dve. In accordance with this conclusion is the observation that expression of Dve is suppressed along the DV boundary.

Dve is required for the proliferation of wing pouch cells

In addition to its function in pattern formation along the PD axis, our work showed that Dve is required for the proper proliferation of the wing pouch cells. Interestingly, the requirement for Dve differs along the PD axis. In the area anterior to wing vein 3 or posterior to wing vein 4 (areas A1 and A3 in Fig. 3E), *dve*-mutant cell clones contained only half as many cells as their wild-type counterpart. Hence, the mutant cells trailed their wild-type counterpart by one cell cycle after 48 hours. In addition, in many cases orphan wild-type clones without a mutant twin were found, which suggested that the mutant cells had died. Cell death is a typical reaction for cells that are impaired in cell proliferation (Weigmann et al., 1997; Neufeld et al., 1998). Both observations indicate that *dve*-mutant cells have a slower proliferation rate than wild-type cells. It is likely it is the slower rate of proliferation that causes the size reduction we observed in regions A1 and A3 of the *dve*-mutant wings. Proliferation of *dve*-mutant cells in the area A2 is also reduced, albeit to a lesser degree. The mutant clones contained 66% of the number of cells that their wild-type counterparts did. More importantly, we did not observe orphan wild-type clones, which indicates that the mutant cells do not undergo apoptosis in this region. Furthermore, the A2 area is of the same size in *dve*-mutant and wild-type wings. Hence, it appears that proliferation of *dve*-mutant cells is not as severely affected in A2 as it is in the other regions. This milder defect in proliferation of mutant cells in A2 seems to be compensated during later development. Altogether, our data suggest that Dve is required for the proliferation of all wing pouch cells, but the requirement for its activity varies along the AP axis.

Why do *dve*-mutant cells proliferate less? The observed cell death of mutant cells in A1 and A3 gives a hint to the answer. Cell death is probably not caused by a defect in the cell cycle machinery itself, as no increased cell death was found in homozygous *dve*-mutant animals. Furthermore, overexpression of Dve using the Flp-out technique does not lead to an over-proliferation of pouch cells. Hence, it is probable that the mutant cells die as a result of being disadvantaged when in competition with normal cells for survival factors, as has been recently shown for cells heterozygous for *Minute* mutations (Moreno et al., 2002). In the case of the *Minute* mutations, the survival factor is Dpp, which is also responsible for pattern formation along the AP axis (Moreno et al., 2002). The differential requirement of Dve along the AP axis suggests that it might be required for the reception of Dpp in pouch cells. However, one result argues against this possibility: Dve is required most in cells that are far away from the source of Dpp (which is at the AP boundary). However, these cells are not, or are only weakly, dependent on Dpp for their survival. Hence, it is unlikely that *dve*-mutant cells cannot properly receive Dpp.

Regulation of the expression of *dve*

We found that *dve* expression is initiated shortly after the start of wing development, during the early phase of the third larval instar stage. It is expressed in a disc-like domain that fills the region inside the inner ring-like domain of *wg* expression. We found that Vg is required, and is sufficient, for *dve* expression in the wing region. Importantly, our data show that Vg activates the expression of *dve* non-autonomously, which indicates that it must be mediated by a secreted factor that is regulated by Vg.

Nakagoshi et al. presented results that show that the expression of *dve* is dependent on Dpp and Wg signals (Nakagoshi et al., 2002). As *vg* is itself regulated by these signals (Williams et al., 1994; Kim et al., 1996; Kim et al., 1997), we think that Vg mediates the effect of these signals on the expression of *dve*.

We also found that expression of *dve* at the DV boundary is suppressed shortly after its initiation. We confirm the findings of Nakagoshi et al. (Nakagoshi et al., 2002) that Wg is required for this repression. In addition, we identify Nub as another factor required for the repression of *dve* expression. Our data suggest that this suppression is important, because we show that forced expression of *dve* along the DV boundary is deleterious for wing development. One gene affected by the forced expression of *dve* is *wg*, which is required for the development of the wing through maintenance of the expression of Vg in pouch cells (Klein and Martinez-Arias, 1999). Although the expression of other genes might be also affected, the loss of the expression of Wg is already sufficient to explain the loss of wing development upon forced expression of *dve*.

Pattern formation along the proximodistal axis

The wing imaginal disc is a single-cell layered epithelium and, thus, is a two-dimensional structure. Therefore, establishment and patterning of the PD axis must occur with the help of the existing AP and DV axes. The *vg* gene is an important translator of the positional values of these axes in corresponding PD values. Previous work showed that *vg* is required for the establishment of distal wing fates (reviewed by Klein, 2001). This work, together with that previously reported, gives insight into how Vg organizes the PD axis.

Previously, it has been shown that Vg is required for the establishment of the medial part of the PW (Liu et al., 2000; del Alamo Rodriguez et al., 2002). During this process Vg induces the expression of *rn*. Expression of *rn* is in turn required to set up the inner ring-like expression domain of Wg, which subsequently organizes the formation of the medial part of the PW (del Alamo Rodriguez et al., 2002; Neumann and Cohen, 1996). Our work shows that Vg is further required for the establishment of the distal part of the PW. It shows that one crucial event during this process is the establishment of the expression of *dve* by Vg. Importantly, Vg induces both parts of the PW in a non-autonomous manner. This indicates that Vg controls the expression of a diffusible factor that induces the expression of genes, such as *dve* and *rn*, in cells inside and outside of its expression domain, in order to establish the corresponding regions of the PW. Furthermore, we find that the induction of expression of *rn* and *dve* occurs independently from each other. The expression domain of *rn* is larger than that of *dve*. Taking for granted that expression of both genes is

induced by the same diffusible factor, this observation suggests that it might act in a concentration dependent manner. In this scenario the induction of *rn* expression would require less activity than the induction of *dve*.

del Alamo Rodriguez et al. reported evidence that expression of *nub* is lost in *vg*-mutant wing imaginal discs (del Alamo Rodriguez et al., 2002), suggesting that Vg is also required non-autonomously for the activation of *nub*, in a yet larger domain than *dve* and *rn*. However, these results are in conflict with earlier work that reports that *nub* expression is not dependent on Vg function (Klein and Martinez-Arias, 1998; Ng et al., 1996). This showed that Wg, but not Vg, is able to induce ectopic expression of *nub* in the notum of the wing imaginal disc. Furthermore, expression of *nub* RNA was observed in *vg*-null mutant wing imaginal discs. These data strongly suggest that Wg is required to activate expression of *nub*. Hence, further work is necessary to resolve the contradictions, and to determine whether Vg also plays a role during activation of the expression of *nub*. Despite this uncertainty, all of the mentioned genes are expressed in disc-like domains of different sizes. Their expression leads to concentric areas with different combinations of gene activities. It seems likely that a particular combination of these genes establishes a specific part of the PW (see Fig. 5K).

Our data provide evidence that Vg controls the expression of *fj*, within an expression domain that corresponds to the wing pouch. Fj is required for the establishment of a proximal region of the wing pouch and also for planar polarity of the wing (Villano and Katz, 1995; Zeidler et al., 2000). Furthermore, Vg regulates the expression of *Distal-less* (*Dll*), which is required to pattern the wing margin (Klein and Martinez-Arias, 1999). Thus, Vg is involved in the patterning of the PD axis inside as well as outside its expression domain.

It is widely accepted that pattern formation and cell proliferation are closely connected during wing development. However, it has not been clear how these processes are connected. The fact that expression of *dve* is initiated by one of the central patterning factors, Vg, provides a possible link.

Wing development in *Drosophila*

The data presented here, together with recently published work, reveal how patterning along the PD axis might occur with help of the two other existing axes (Fig. 8). Previous work has established that wing development starts at the cross-point of the expression domains of Dpp and Wg in the ventral part of the wing disc (Fig. 8, Step 1) (Klein and Martinez-Arias, 1999; Wu and Cohen, 2002) (for a review, see Klein, 2002). It appears that the combined activity of the two signals define the wing field. Although the activity of Wg is sufficient to establish the proximal-most pattern elements, the hinge and the proximal region, of the PW (Ng et al., 1996; Klein and Martinez-Arias, 1998a or b?), the establishment of all distal regions requires the additional activity of *vg* (Klein and Martinez-Arias, 1998; Klein and Martinez-Arias, 1999). In the wing field, the *Notch* signalling pathway activates the expression of *vg* in cells at the future compartment boundary (Fig. 8A). In addition, Wg, perhaps in collaboration with Vg/Sd, activates the expression of *nub.*

In the next step Vg induces the expression of *wg* in cells at the DV boundary, in collaboration with the *Notch* pathway (Fig. 8B). In addition, it activates an unknown diffusible factor that induces the expression of *dve* and *rn* in disc-like domains of different sizes (Fig. 8B). All these domains are larger than that of Vg, and expression of the three genes is established independently from each other. This fact suggests that the diffusible factor might act in a concentration-dependent manner, as is typical for morphogens. Dve and Rn act in collaboration with Nub to establish the medial and distal parts of the PW.

When the expression of *nub*, *rn* and *dve* is initiated, Vg is expressed in cells at the DV boundary (Fig. 8B). These cells will later form the distal-most structure, the wing margin. The wing pouch is formed by the progenies of cells at the DV boundary, and is therefore intercalated between the margin and the anlagen of the PW (Fig. 8C) (Klein and Martinez-Arias, 1999). During its formation, the pouch will be further subdivided through the combined activity of Vg and Wg. Both proteins generate gradients that further subdivide the pouch along the DV axis.

In summary, the data suggest that pattern formation along the PD axis occurs in several steps and uses a similar strategy to that observed during leg development (Galindo et al., 2002; Campbell, 2002; Goto and Hayashi, 1999). It is initiated by the definition of the proximal (hinge and the distal part of the PW) and the distal-most point (wing margin), with help of the existing AP and DV axes. During development, the intermediate pattern elements (first the anlagen of the medial and distal part of the PW, then the wing blade) are intercalated stepwise with respect to these reference points.

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