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Distinct mitogenic and cell fate specification functions of *wingless* in different regions of the wing

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SUMMARY

Patterning and proliferation are coordinately controlled in the development of *Drosophila* imaginal discs. Localized expression of *decapentaplegic* (*dpp*) at the anterior-posterior and *wingless* (*wg*) at the dorsal-ventral compartment boundaries controls growth of the wing with respect to the A/P and D/V axes. The growth-promoting effects of these organizers are thought to be indirect, since growth is dispersed throughout the disc, and is not localized near the sources of *wg* or *dpp*. *wg* has also been implicated in proximal-distal patterning of the wing hinge. In this report, we present evidence that *wg* is principally required for local cell proliferation in the hinge. Loss of *wg* expression leads to a local reduction in cell division, resulting in the deletion of a distinct set of wing hinge structures. Ectopic activation of the *wg* pathway in cells of the wing hinge leads to overproliferation without repatterning, indicating that *wg* acts as a mitogen in this part of the disc. By contrast, overexpression of *wg* in the wing blade leads to repatterning and only secondarily to proliferation. These results suggest that the Wg signal elicits very different responses in different regions of the wing imaginal disc.

Key words: *wingless*, Wnt, *dishevelled*, mitogen, signal transduction, growth control, *Drosophila*, cell fate

INTRODUCTION

Among the more intriguing problems in pattern formation is how positional information is used to control cell proliferation. It is necessary to know how cell proliferation is integrated with patterning events and how final cell numbers are specified. Recent work has established the basic principles by which pattern is generated in the appendages of *Drosophila* (reviewed in Blair, 1995; Brook et al., 1996), making this developmental process a good starting point for examining the coordination of proliferation with patterning.

Drosophila appendages are subdivided into compartments (Garcia-Bellido, 1975). The nascent wing imaginal disc is divided into anterior-posterior compartments in the embryonic ectoderm (reviewed in Cohen, 1993) and becomes further subdivided into dorsal-ventral and proximal-distal compartments during the second larval instar (Garcia-Bellido et al., 1973). Crick and Lawrence (1975) suggested that compartment boundaries may serve as organizing centers responsible for generating spatial pattern in developing appendages, and recently a lot of evidence in favor of this hypothesis has accumulated (reviewed in Blair, 1995; Brook et al., 1996).

Interactions between the compartments have been shown to induce specialized cells near the compartment boundaries that control patterning and proliferation in the disc. Thus *engrailed* activates the secreted signaling molecule Hedgehog in posterior cells, which in turn induces the expression of *decapentaplegic* in nearby anterior cells (Basler and Struhl, 1994). Decapentaplegic is also a secreted signaling protein, and has been shown to be the mediator of the anterior-posterior organizer (Capdevila and Guererro, 1994; Diaz-Benjumea et al., 1994; Zecca et al., 1995). Ectopic expression of *decapentaplegic* in either compartment leads to a duplication of the anterior-posterior axis. Similarly, *apterous* specifies dorsal cell fate (Diaz-Benjumea and Cohen, 1993; Blair, 1993). *fringe* and *Serrate* signal from dorsal to ventral cells and induce expression of Wingless and Vestigial at the dorsal-ventral boundary (Irvine and Wieschaus, 1994; Kim et al., 1995; Diaz-Benjumea and Cohen, 1995; Couso et al., 1995). The D-V boundary corresponds to the wing margin in the adult wing and *wingless* function is required for the formation of wing margin structures (Couso et al., 1994; Phillips and Whittle, 1993). *wingless* (*wg*) function in the wing margin is also required for cell proliferation and/or cell survival in the wing blade, and ectopic expression of *wg* in the wing pouch not only locally respecifies cells to assume wing margin fate, but also stimulates overproliferation of surrounding cells (Diaz-Benjumea and Cohen, 1995). Thus *wg* mediates the organizing effects of the D-V boundary both on patterning and on proliferation. Analysis of the mutation *nubbin* has suggested the presence of a proximal-distal organizing center in the wing hinge which is responsible for growth of the wing (Ng et al., 1995). In the third instar wing imaginal disc, *wg* is expressed in two rings surrounding the wing pouch, one of which is missing in a *nubbin* mutant background. This observation has suggested that this domain of *wg* expression might be implicated in formation of the wing hinge and in growth of the wing (Ng et al., 1995).

While the molecular mechanisms by which organizers are established are starting to be understood, and key mediators of organizer function have been identified, downstream events are not well understood. Thus it is not at all clear how organizers regulate cell proliferation. Here we present evidence that the ring of *wingless* expression surrounding the wing pouch is required for local cell proliferation in the wing hinge, but not for long-range patterning. We also show that ectopic activation of the *wg* pathway can induce overproliferation without causing repatterning, indicating that *wg* is acting as a mitogen in this part of the wing disc. The mitogenic effect of *wg* in the hinge contrasts with its effects in the wing blade, where it directly specifies cell fates and apparently indirectly promotes proliferation (Diaz-Benjumea and Cohen, 1995). Region-specific differences in the mitogenic and fate specification responses to *Wnt-1*, the vertebrate orthologue of *wg*, have also been observed in the mouse central nervous system (Dickinson et al., 1994), raising the possibility that the pathways by which cells distinguish between growth and cell fate specification in response to Wnt signals may be conserved.

MATERIALS AND METHODS

Drosophila stocks

wg^{CX4} is described in Baker (1987) and van den Heuvel et al. (1993). spd^{fg} is described in Lindsley and Zimm (1992). wg^{r0727}, $Df(2L)spd^{j2}$ and $Df(2L)spd^{hL2}$ are described below. *neuralized-lacZ* is described in Ghysen and O'Kane (1989). UAS-Wg is described in Lawrence et al. (1995). The GAL4 driver MS1096 is decribed in Capdevila and Guerrero (1994).

P-element-generated alleles

wg^{r0727} is a pZ P-element inserted into the *wg* locus (a gift of Ulrike Gaul). It is embryonic lethal, although the segment polarity phenotype of wg^{r0727} homozygous embryos is weaker than that of wg^{CX4} homozygous embryos, arguing that it is a strong *wg* hypomorph (unpublished observation). By performing plasmid rescue, we mapped the insertion site of wg^{r0727} to about 100 bp 5′ of the *wg* transcription start site (see Fig. 4). We used this P-element to generate both $Df(2L)spd^{j2}$ and $Df(2L)spd^{hL2}$. $Df(2L)spd^{j2}$ was generated by imprecise excision of wg^{r0727} (scoring for loss of the $rosy^{+}$ marker). Hybridization of genomic DNA from flies heterozygous for $Df(2L)spd^{j2}$ with a probe spanning the insertion site of wg^{r0727} showed that the left flank is gone, while the right flank is still intact, thus leaving the *wg* transcript untouched (data not shown). Cytological analysis showed that the imprecise P-element excision has generated a large deficiency covering the 27C1-28A1 interval (Fig. 4). $Df(2L)spd^{hL2}$ was also generated by imprecise excision of the wg^{r0727} P-element. Southern blot analysis indicated that at least 30 kb are deleted in both the 5′ and the 3′ regions of *wg*. $Df(2L)spd^{hL2}$ is not visible cytologically and complements the lethal complementation groups ('G' and 'J') that Tiong and Nash (1990) identified on either side of the *wg* locus.

Histochemical methods

Whole-mount in situ hybridization was performed as described by Tautz and Pfeifle (1989) using a *wg* RNA probe. X-gal staining of pharate adults carrying *wg-lacZ* was done as in Hama et al. (1990). Anti-Dll staining was as in Diaz-Benjumea and Cohen (1995). Acridine orange staining was performed as in Masucci et al. (1990). Bromodeoxyuridine (BrdU) labeling was done as in Usui and Kimura (1992). BrdU incorporation was for 30 minutes for the experiment in Fig. 6 and for 10 minutes in Fig. 8.

Molecular methods

Southern blot analysis was performed following standard procedures (Ausubel et al. 1994). Enhancer activity of the 1.2 kb *Eco*RI fragment (indicated as probe c in Fig. 4D) was tested in the Casper-hs43-AUG-βGal vector (Thummel and Pirrotta, 1991). Several independent transformants inserted at different chromosomal locations produced the same expression pattern. UAS-Dsh was prepared by cloning a full-length *dsh* cDNA (Klingensmith et al., 1994) as an *Eco*RI fragment into pUAST (Brand and Perrimon, 1993).

RESULTS

The mutation *nubbin* identifies a class of genes required for patterning the hinge region of the *Drosophila* wing (Ng et al., 1995). In adult viable *nubbin* mutants, almost the whole wing is lost. However, removing *nubbin* function in clones of cells indicates that wild-type activity is not required in cells giving rise to most of the wing blade itself, but only in cells located in the hinge region. This indicates that there may be a patterning center in the hinge region which has both local and long-range influences on wing development. To learn more about this patterning center, we sought to identify other mutations with a similar phenotype to *nubbin*. One such mutation is $spade^{flag}$ (spd^{fg}). Here we show that spd^{fg} is a regulatory mutation of *wg* that specifically abolishes *wg* function in the hinge.

The $spade^{flag}$ mutant phenotype results from the loss of *wg* expression in the wing hinge

spd^{fg} was originally identified as a viable spontaneous mutation that causes an overall reduction of wing size, as well as a reduction of the alula and a variable loss of posterior wing margin structures (Lindsley and Zimm, 1992). A detailed characterization of the spd^{fg} phenotype indicates that the most penetrant aspect of this phenotype is the loss of specific hinge structures (Fig. 1). The hinge structures lost in spd^{fg} include the medial costa, the humeral cross-vein, septum 2 and the part of the dorsal radius which lies between the medial costa and the alula (Fig. 1B,D). The alula is sometimes completely absent and sometimes vestigial. The Sc1 campaniform sensillum is always absent, while the Sc12 group of campaniform sensillae is reduced, with more proximally located sensillae sometimes remaining. More proximal hinge structures, such as the proximal costa and the axillary cord are not affected by spd^{fg}.

The structures lost in the hinge of spd^{fg} mutants are centered on a domain of *wg* expression that runs through the hinge (Fig. 1B,D). In the third instar wing imaginal disc, *wg* is expressed in several domains including two rings surrounding the wing pouch (Fig. 2B). Examination of *wg-lacZ* expression in the adult wing shows that both of these rings of *wg* expression run through the hinge structures of the adult. The inner of the two rings in the imaginal disc corresponds to the more distal stripe running through the adult hinge, and we refer to this as the 'inner ring'. The inner ring runs from the medial costa through the humeral cross-vein and the dorsal radius and then curves around to the base of the alula. The outer ring runs from the base of the costa to the base of the axillary cord (see Phillips and Whittle, 1993; Fig. 1B). The inner ring of *wg* expression lies in the hinge domain, which it is deleted in spd^{fg} mutants.

The inner ring of *wg* expression is also absent in *nubbin* mutant wing discs which correlates well with the *nubbin* hinge phenotype (Ng et al., 1995). These observations suggest that spd^{fg} may reduce *wg* function associated with the inner ring of *wg* expression in the wing imaginal disc.

To test this hypothesis directly, we examined *wg* expression in spd^{fg} mutant imaginal discs by whole-mount *in situ* hybridization. The inner ring of *wg* expression is not detectable in the spd^{fg} mutant disc (Fig. 2). Careful examination also suggests that the intensity of staining in the wing margin is reduced relative to a wild-type control (compare Fig. 2A and B). The reduced levels of *wg* RNA in the wing margin of spd^{fg} mutants correlates well with the observation that spd^{fg} homozygotes show some loss of margin bristles, as noted by Couso et al. (1994).

$spade^{flag}$ is allelic to *wg*

The observation that spd^{fg} removes *wg* expression in the inner ring of the wing hinge and reduces it in the wing margin is consistent with the proposal that *spd* may be a regulatory allele of *wg* (Couso et al., 1994; Tiong and Nash, 1990). However, *spd* shows an ambiguous complementation behavior towards different *wg* alleles (Tiong and Nash, 1990). We sought to clarify this issue by crossing spd^{fg} to three well-characterized *wg* alleles: a *wg* null point mutant, a deficiency removing only the 5′ region of *wg*, and a deficiency removing both the 5′ and the 3′ regions of *wg*.

wg^{CX4} is a null point mutant caused by a small deletion removing the *wg* promoter (van den Heuvel et al., 1993). Flies of the genotype spd^{fg}/wg^{CX4} show a milder version of the hinge phenotype seen in spd^{fg} homozygotes (Fig. 3A,B) and there are no reductions of margin structures. If *spd* and *wg* are different loci that show a dominant genetic interaction, it is expected that a deficiency removing both would show the same phenotype as the spd^{fg}/wg^{CX4} heterozygous combination. However, deficiencies that uncover the whole region do not show any phenotype, suggesting that spd^{fg} is allelic to *wg*. This raises the question why the heteroallelic combination gives a weaker phenotype than either homozygote. This effect is probably due to transvection, i.e.. because the relevant enhancer on the wg^{CX4} chromosome is driving the expression of the *wg* gene on the spd^{fg} chromosome. There is further genetic evidence that the *wg* locus is subject to transvection (Neumann and Cohen, 1996). For example, the mutation wg^{1}, which has been shown to be a small deletion in the 3′ regulatory region of *wg* (Baker, 1987; van den Heuvel et al., 1993), is largely complemented by wg^{CX4}.

We also crossed spd^{fg} to two new alleles of *wg* generated by P-element mobilization. $Df(2L)spd^{j2}$ is a large deficiency that breaks about 100 bp 5′ of the *wg* transcription start site and removes the entire 5′ regulatory region of *wg* (Fig. 4). $Df(2L)spd^{j2}$ behaves as an embryonic lethal allele of *wg* in *trans* to wg^{CX4} (Neumann and Cohen, 1996). When heterozygous with spd^{fg}, $Df(2L)spd^{j2}$ produces a hinge phenotype indistinguishable from that of spd^{fg} homozygotes (Fig. 3C,D). This shows that removing the 5′ sequences of *wg* abolishes the ability to complement spd^{fg}. As the 5′ regulatory sequences of *wg* are present on the wg^{CX4} chromosome and wg^{CX4} can partially complement spd^{fg}, we conclude that the defect on the spd^{fg} chromosome must lie in the 5′ regulatory region of *wg*.

$Df(2L)spd^{hL2}$ is a small deficiency that removes the *wg* gene and flanking DNA extending at least 30 kb in both directions. $Df(2L)spd^{hL2}$ does not include the other known lethal complementation groups flanking the *wg* locus (Tiong and Nash, 1990). Flies of the genotype $spd^{fg}/Df(2L)spd^{hL2}$ have a hinge phenotype that is only slightly stronger than that of spd^{fg} homozygotes (Fig. 3E,F). The proximal costa is present, as is the proximal part of the distal costa, but the alula is absent. In addition, the anterior and posterior wing margins are absent, and adjacent wing blade tissue is scalloped. This wing margin phenotype is much stronger than that of spd^{fg} homozygotes and of flies of the genotype spd^{fg}/Df(2L)spd^{j2}.

Taken together with the effects of spd^{fg} on *wg* expression,

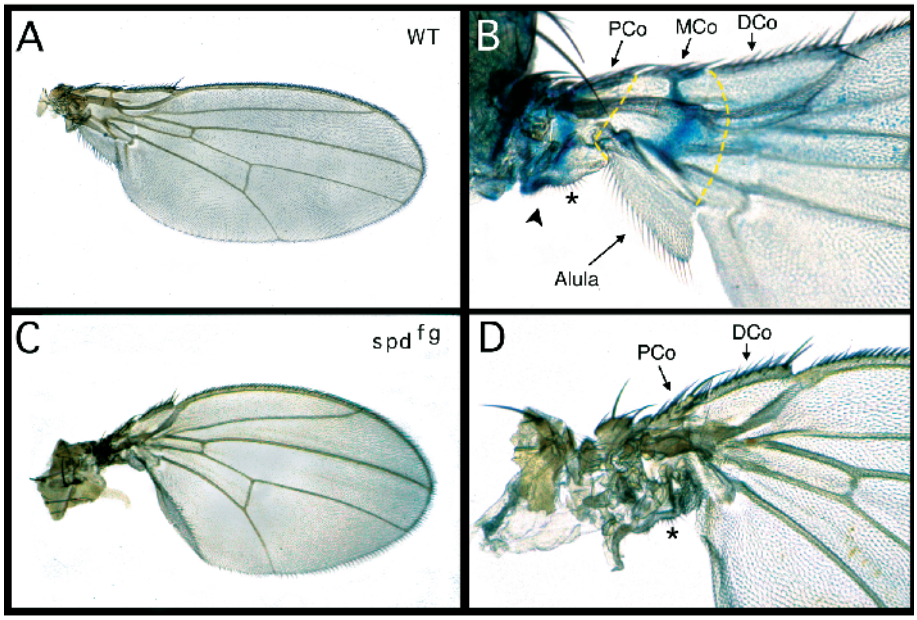

Fig. 1. The $spade^{flag}$ mutant phenotype. (A) Wild-type wing. (B) Close up view of the wild-type wing hinge from an individual carrying a *wg-lacZ* reporter. *wg*-expressing cells are stained blue. The proximal ring of *wg* expression (which corresponds to the outer ring in the wing imaginal disc) runs from the base of the proximal costa (PCo) to the proximal axillary cord (asterisk) adjacent to the body-wall (arrowhead). The more distal ring (which corresponds to the inner ring in the disc) runs from the center of the medial costa (MCo) to the base of the alula. (C) Wing from a fly homozygous for the mutation spd^{fg}. The wing hinge is strongly reduced and the wing shows a slight overall reduction in size. There is a variable loss of wing margin bristles, which is stronger in the posterior margin. (D) Close up view of the spd^{fg} hinge. Distal hinge structures including the medial costa (MCo), the humeral cross-vein, septum 2 and alula are missing. Proximal hinge structures, such as the proximal costa (PCo) and axillary cord (asterisk) are not affected. The hinge structures deleted in spd^{fg} mutants are contained in the region bounded by the yellow dashed lines on the wild-type wing in B. Note that the inner ring of *wg* expression lies in the center of this domain. DCo, distal costa

these observations suggest that *spd*^{fg} is a regulatory mutation that removes *wg* function in the hinge while only reducing it in the wing margin. Consistent with this suggestion, removal of *wg* function during third instar using the *wg*^{ts} mutation leads to a number of similar defects in wing development (Phillips and Whittle, 1993; Couso et al., 1994). Although not described in those reports, *wg*^{ts} produces a hinge defect indistinguishable from that of *spd*^{fg} homozygotes (see Fig. 5D in Couso et al., 1994). The hinge phenotype is partially masked by transvection when *spd*^{fg} is heterozygous over a point mutant of *wg*, but is uncovered by a deficiency that removes the 5′ regulatory region of *wg* The full margin phenotype is only uncovered by a deficiency that removes both the 5′ and the 3′ regulatory regions of *wg*.

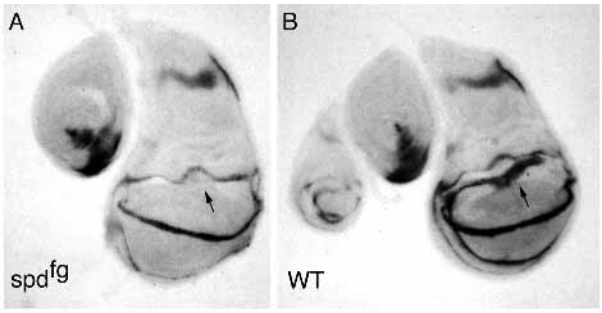

Fig. 2. The inner ring of *wg* expression is lost in *spade*^{flag} mutants. (A) Third instar wing imaginal disc from a *spd*^{fg} mutant stained for *wg* RNA by in situ hybridization. Note that all domains of *wg* expression are present, except the inner ring surrounding the wing pouch (arrow). This ring corresponds to the distal ring of the adult wing hinge. Careful comparison of A and B also indicates that there is a slight reduction in the level of *wg* expression in the wing margin. (B) Wild-type third instar wing imaginal disc stained as in A.

spade^{flag} deletes an enhancer located 5′ of *wg* that drives *wg* expression in the hinge and in the wing margin

The observation that *Df(2L)spd*^{j2} uncovers the *spd*^{fg} mutant phenotype suggested that *spd*^{fg} must affect sequences located 5′ of *wg*. We tested DNA in this region from *spd*^{fg} mutants for chromosome rearrangements and found a restriction fragment length polymorphism indicative of a small deletion located about 9 kb 5′ of the *wg* promoter (Fig. 4B-D). Comparison of the restriction enzyme digestion pattern of the mutant DNA with wild-type DNA shows that there is a deletion of about 1 kb that removes most of a 1.2 kb *Eco*RI fragment in the wild-type DNA (probe c in Fig. 4D). The 1.2 kb *Eco*RI fragment contains an enhancer element sufficient to direct reporter gene expression in a ring around the wing pouch and in a stripe along the wing margin (Fig. 5). *lacZ* expression driven by this DNA fragment is activated in second instar in a diffuse pattern (Fig. 5A) which resolves into a wing margin stripe and a ring around the wing pouch during early-mid third instar (Fig. 5B,C). The dynamics of this expression are similar to those of the endogenous *wg* gene in the margin and the inner ring.

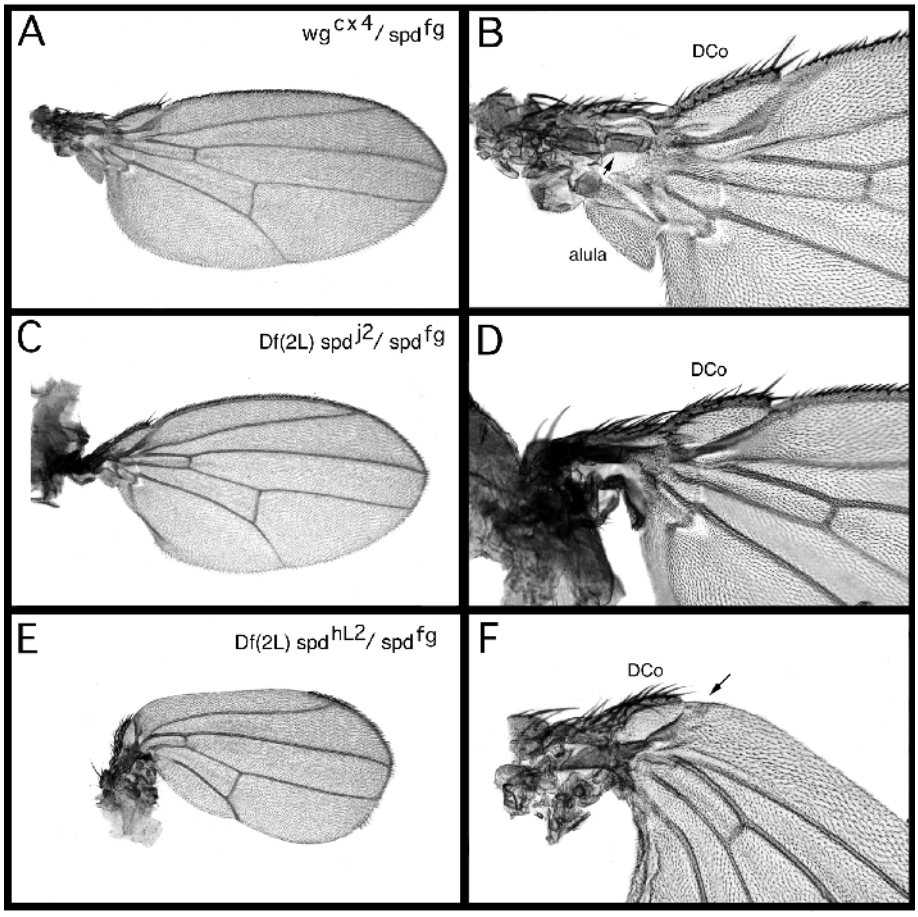

Fig. 3. *spade*^{flag} behaves genetically as a regulatory allele of *wg*. (A,B) Wing from a fly heterozygous for *spd*^{fg} and *wg*^{CX4}. The hinge shows a mutant phenotype milder than that observed in the *spd*^{fg} homozygous wing. The alula and septum 2 (arrow) are present, but the medial costa and humeral cross-vein are missing. (C) Wing from a fly heterozygous for *spd*^{fg} and Df(2L)*spd*^{j2}. (D) Detail of the hinge region of a comparable wing. This phenotype is indistinguishable from that of *spd*^{fg} homozygotes, suggesting that Df(2L)*spd*^{j2} completely removes *spd* function. (E,F) Wing from a fly heterozygous for *spd*^{fg} and Df(2L)*spd*^{hL2}. Note the extensive scalloping of the anterior and posterior wing margin. The wing is smaller than in *spd*^{fg} homozygotes (compare with Figs 1C and 3C, which is similar in size to the wing of a *spd*^{fg} homozygote). (F) The hinge phenotype is slightly stronger than in the *spd*^{fg} homozygote. Arrow indicates where vein 1 reaches the anterior margin. Loss of wing margin structures begins here and extends distally. PCo and DCo are unaffected.

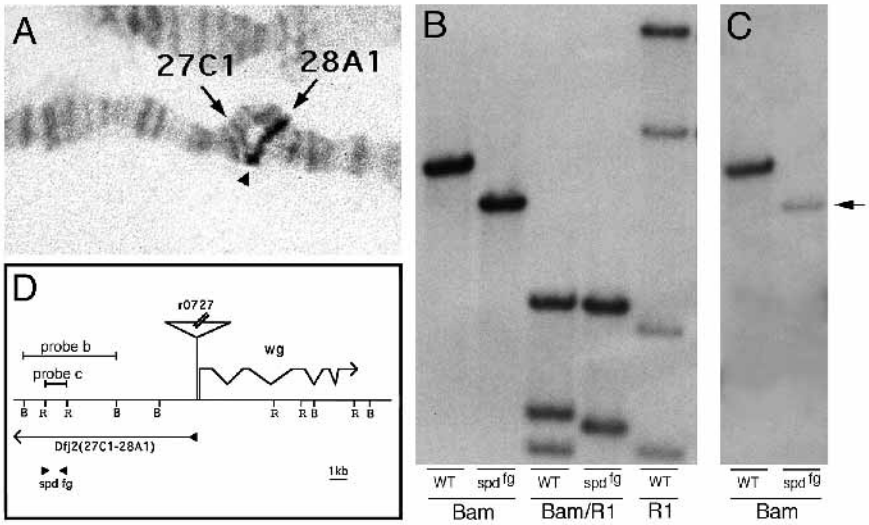

Fig. 4. The mutation *spade^{flag}* is due to a small deletion in the 5′ regulatory region of *wg*. (A) Polytene chromosome of an individual heterozygous for Df(2L)*spd^{j2}* hybridized with a *wg* cDNA probe (arrowhead). Although this deficiency leaves the *wg* coding region intact, it removes DNA 5′ of *wg* extending from 28A1 to 27C1. See also D. (B) Genomic Southern blot of wild-type DNA and DNA from *spd^{fg}* homozygotes as indicated underneath the lanes. DNA was digested with *Bam*HI or *Eco*RI (R1) as indicated. The blot was hybridized with a *Bam*HI fragment from the *wg* 5′ region that is indicated in D as 'probe b'. Note that the size of this *Bam* fragment is reduced in *spd^{fg}* mutant DNA (compare the first two lanes), indicating that some DNA has been deleted. Comparison with the wild-type *Eco*RI digest shows that the small *Eco*RI fragment is gone in *spd^{fg}* mutant DNA, and that a novel smaller fragment replaces the small *Bam*HI/*Eco*RI fragment seen in the double digest of wild-type DNA, suggesting that one *Eco*RI site is lost in the mutant. This digestion pattern can be explained by a deletion that removes most of the small *Eco*RI fragment as well as the left *Eco*RI site (see the diagram in D). (C) The first two lanes of the blot shown in B were stripped and reprobed with the small *Eco*RI fragment shown in D as 'probe c'. This fragment labels the wild-type DNA strongly, but the *spd^{fg}* DNA only very weakly (arrow), indicating that most of the DNA of this 1.2 kb *Eco*RI fragment is deleted in the mutant. (D) Schematic map of the *wg* gene, showing the location of the deletion in *spd^{fg}* (►◄), the proximal breakpoint of *Df(2L)spd^{j2}* (<), and the insertion site of the P-element *wg^{r0727}* (triangle). The DNA fragments used to probe the genomic Southern blots in B and C are indicated above the map.

Staining of adult flies shows that the ring of *lacZ* expression corresponds to the more distal ring of *wg* expression in the hinge (not shown). These results suggest that the *spd^{fg}* mutant phenotype is due to the deletion of an enhancer element that drives *wg* expression in the inner ring of the wing hinge and in the wing margin. Although the enhancer drives strong expression in the wing margin, removal of this fragment in *spd^{fg}* mutants does not cause a severe loss of wing margin structures, indicating that this aspect of *wg* regulation is partially redundant.

The *spade^{flag}* phenotype is due to decreased proliferation in the hinge region

To further investigate the *spd^{fg}* phenotype, we examined the expression pattern of the neuronal marker *neuralised/A101* (Ghysen and O'Kane, 1989; Huang et al., 1991) in the *spd^{fg}* mutant background (Fig. 6A-D). Specific groups of sense-organ precursors, including all or part of the Sc12 group of campaniform sensillae, are missing in late third instar wing imaginal discs of *spd^{fg}* mutants (Fig. 6C,D). These cells are inside, or close to the inner ring of *wg* expression (compare Figs 2B and 5D with 6A). Examination of the *spd^{fg}* mutant also shows that there is tissue missing in this area, bringing the Sc25 group of campaniform sensillae closer to the sensillae located on vein 3 (compare Fig. 6B with D). This indicates that the structures that are deleted in the adult hinge are already absent in late third instar wing imaginal discs of *spd^{fg}* mutants.

To determine whether the loss of distal hinge structures in *spd^{fg}* is due to cell death, we stained wing imaginal discs of *spd^{fg}* mutants with acridine orange, which labels apoptotic cells (Masucci et al., 1990). No abnormal cell death was detected in any part of the disc throughout third instar (data not shown). To further address this question, we crossed the *spd^{fg}* enhancer-*lacZ* reporter gene into the *spd^{fg}* mutant background. If the absence of *wg* activity in the inner ring leads to localized cell death, it is expected that, after correct initial activation, the expression of this reporter would fade away, as the cells in which it is expressed would die. However, this is not what we observe. During late third instar, when the loss of tissue in the hinge of *spd^{fg}* individuals is already apparent, the enhancer is

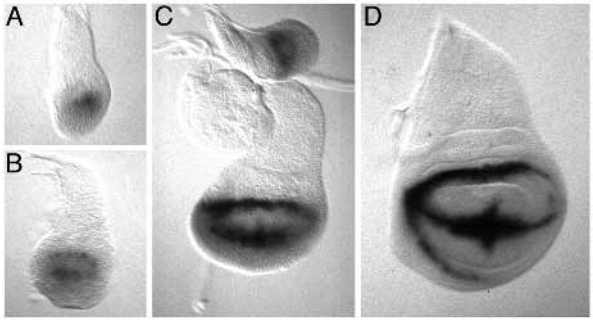

Fig. 5. The DNA fragment deleted in *spd^{fg}* contains an enhancer element. The 1.2 kb RI fragment (described as probe c in Fig. 4D) contains an enhancer element sufficient to drive *lacZ*-reporter gene expression in a pattern that corresponds to the inner ring and the wing margin expression domains of *wg*. All discs were stained with X-gal to reveal *lacZ* expression driven by the enhancer element. (A) A late second instar wing imaginal disc. Expression at this stage appears diffuse. (B) An early third instar wing disc showing an early stage in the formation of the wing margin stripe and a ring surrounding the wing pouch. (C) A mid third instar wing disc showing an essentially mature expression pattern. (D) A late third instar wing disc. Staining corresponds to the wing margin and the inner ring of wg expression (compare this disc to the one in Fig. 2B, which shows the *wg* expression at a comparable stage). Note that expression in the more distal regions of the wing margin is a bit broader than that of endogenous *wg* and that staining of the ventral part of the ring is not as strong as of *wg* in this domain (this is especially apparent in B and C).

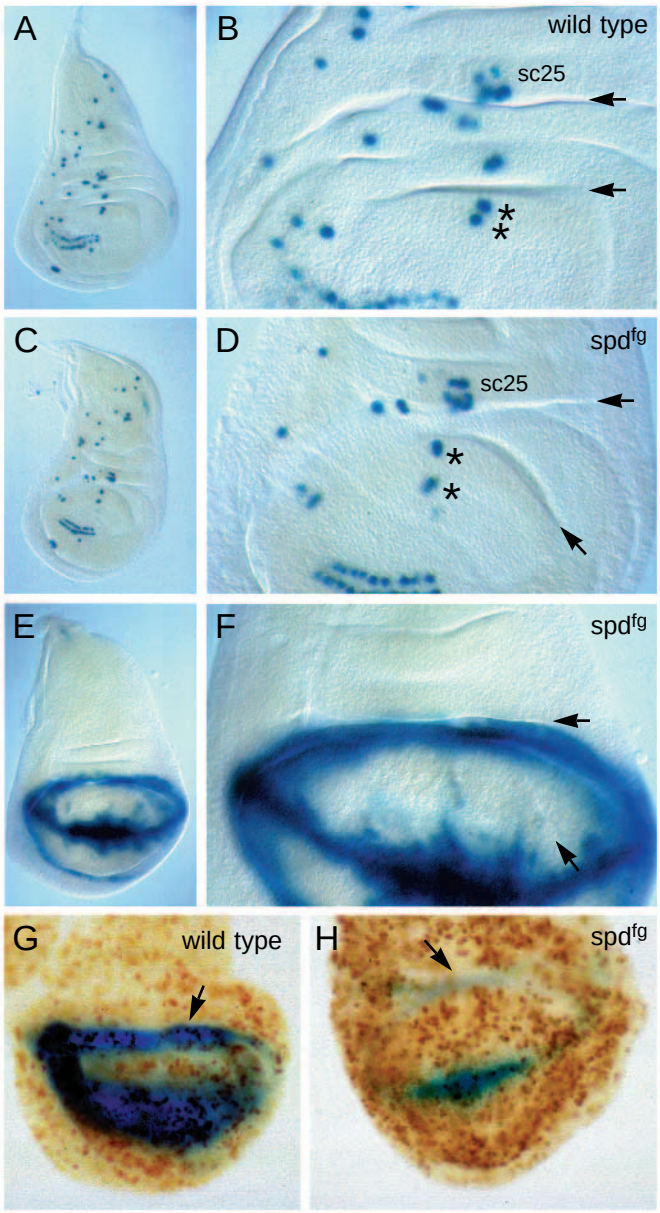

Fig. 6. Underproliferation of the wing hinge in *spd*^{fg} mutants. A-F were stained with X-gal, while G and H were stained for both BrdU incorporation (brown) and X-gal (blue). (A) *A101* expression in a wild-type late third instar wing imaginal disc. (B) A close up view of the dorsal hinge region of the disc shown in A. The SMCs of the Sc25 group of campaniform sensillae are labeled, and the SMCs of the sensillum of the anterior cross vein and the second sensillum of the third vein (L3-2) are marked with asterisks. The SMCs that lie between these two groups correspond to the giant sensillum of the dorsal radius and the Sc12 group of campaniform sensillae. Assignment of SMCs is according to Huang et al. (1991). The third instar wing imaginal disc has several characteristic folds, a pair of which are marked by arrows here and in D and F. (C) *A101* expression in a *spd*^{fg} late third instar wing imaginal disc. (D) Close up view of the dorsal hinge region of the disc shown in C. Note that tissue between the two folds marked by arrows appears to be missing in *spd*^{fg}, bringing the Sc25 SMCs closer to the ACV and L3-2 (asterisks). The GSR is absent, while the Sc12 group are partially absent. In this genetic background, there is an enhancement of the *spd*^{fg} phenotype that results in scalloping of the anterior and posterior wing margins. For this reason, SMCs of the proximal anterior wing margin are missing in the mutant. (E) Expression of *lacZ* driven by the *spd* enhancer in a *spd*^{fg} mutant wing disc. Note that the expression is identical to that in a wild-type background (compare with Fig. 5D). (F) A close up view of the dorsal hinge region of the disc shown in E. The inner ring of *wg* expression lies roughly midway between the two folds that are marked by arrows in B,D and F. Note that, even though the reduction in tissue between the two folds is clearly visible in the mutant, the *spd* enhancer still drives strong expression here, suggesting that loss of the folds cannot be due to cell death. (G) BrdU pulse-labeling of a wild-type mid-third instar disc showing the pattern of cell division in relation to the domain of expression of the *spd* enhancer. Note the presence of labelled nuclei (brown) in the cells expressing the enhancer (blue). (H) BrdU pulse-labeling of a mid-third instar *spd* mutant disc. Note the relative absence of cell division in the dorsal hinge region (arrow). Enhancer-driven *lacZ* expression was understained to emphasize the absence of incorporation of BrdU.

still strongly expressed (Fig. 6E,F). Furthermore, we labeled *spd*^{fg} mutant discs as well as wild-type discs carrying the *spd*^{fg} enhancer-*lacZ* gene for *lacZ* expression and simultaneously for BrdU incorporation, which marks dividing cells. In wild-type discs, many cells expressing the *lacZ* reporter in the hinge are dividing (Fig. 6G). In the *spd*^{fg} mutant background, however, only very few cells expressing the *lacZ* reporter in the hinge can be seen to divide (Fig. 6H). Taken together, these results indicate that the absence of distal hinge structures in *spd*^{fg} mutants is not due to cell death. Instead, they suggest that *wg* activity in the inner ring is required to promote local cell proliferation in the wing hinge.

Ectopic activation of the *wingless* pathway causes overproliferation in the hinge region of the wing

It has been shown that *wg* is able to induce wing margin cell fates in the wing pouch (Diaz-Benjumea and Cohen, 1995). To determine whether *wg* is also sufficient to specify pattern elements in the hinge region, we misexpressed *wg* using the GAL4:UAS system (Brand and Perrimon, 1993). As a GAL4 driver we used MS1096, which has been shown to express GAL4 in the dorsal wing pouch (Capdevila an Guerrero, 1994). However, we found that it is also expressed in the ventral wing pouch, as well as in the dorsal hinge, although at lower levels (Fig. 7A). When crossed to this driver, a UAS-*wg* line utilizing the wild-type *wg* cDNA (Lawrence et al., 1995) is able to activate Distal-less (Dll) expression throughout the wing pouch (Fig. 7C). Dll has been shown to be a target gene of *wg* in the wing pouch (Diaz-Benjumea and Cohen, 1995). Pharate adults recovered from this cross also have a high density of wing margin bristles on both wing surfaces, although sometimes with a lower density ventrally (Fig. 7D). We also observed that there is an increase in the size of the dorsal hinge in this combination (visualized by the area that expresses UAS-*lacZ* under the control of MS1096 in the dorsal hinge, Fig. 7C). This correlates with a broadening of the hinge region of the pharates (compare Fig. 7B with 7D), suggesting that there may be extra growth in the hinge induced by ectopic *wg* activity.

dishevelled (*dsh*) is required to transduce the *wg* signal (reviewed in Klingensmith and Nusse, 1994). Dsh is a phosphoprotein that becomes hyper-phosphorylated in response to the *wg* signal, and it has been shown that overexpression of

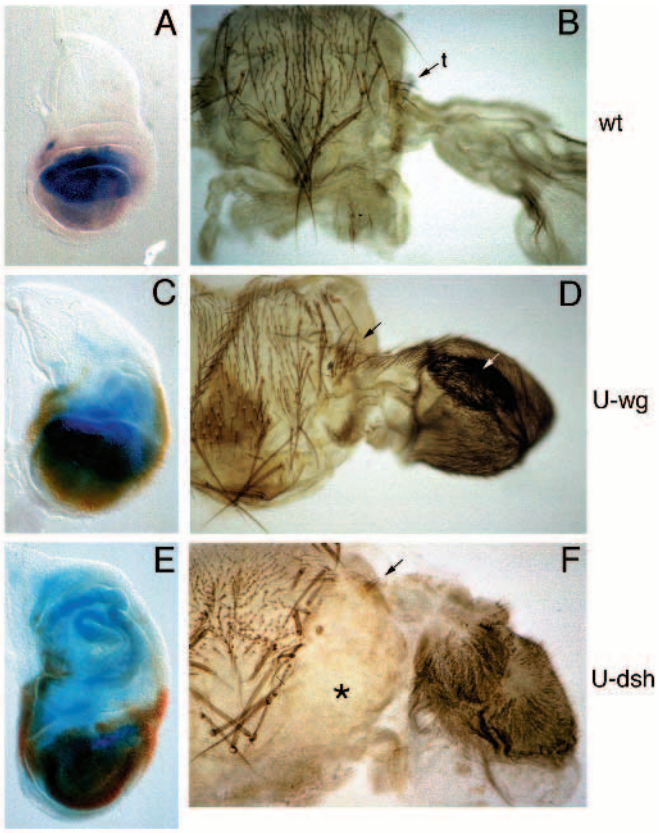

Fig. 7. Overgrowth of the wing hinge caused by ectopic expression of *wg* or *dsh*. A was stained with X-gal, while C and E were stained with anti-Dll antibody and X-gal. (A) Expression of the Gal4 driver MS1096 in the wing visualized by UAS-*lacZ*. Note the strong blue label in the dorsal wing pouch and the weaker (pink-blue) labeling in the ventral wing and dorsal wing hinge regions. (B) Cuticle from a wild-type pharate adult. t indicates the tegula. (C) MS1096:UAS-*wg*+UAS-*lacZ* wing disc double labeled for *lacZ* activity (blue) and Dll protein expression (brown). In wild-type discs, Dll is expressed in a narrow band of cells along the wing margin under control of *wg* (Diaz-Benjumea and Cohen, 1995). Note that Dll is expressed throughout the entire wing pouch, but not in the overgrown wing hinge region. Thus cells in the hinge respond differently to the Wg signal than cells in the wing blade. The lower level of *wg* expression in the ventral wing is still sufficient to respecify cell fates to direct Dll expression. The overlap of Dll and *lacZ* staining is more difficult to see in the dorsal wing pouch due to the higher level of *lacZ* activity. (D) Cuticle from a MS1096:UAS-*wg* pharate adult. Cells throughout the wing pouch differentiate structures characteristic of the wing margin. Note the dense cluster of anterior wing margin bristles (white arrow). The tegula shows an increase in bristle density (black arrow). The proximal wing hinge is expanded (compare with B), but does not show any pattern duplication. (E) MS1096:UAS-*dsh*+UAS-*lacZ* wing disc double labeled for *lacZ* activity and Dll protein expression. Note the vast expansion of the dorsal wing hinge, visible as a set of unusual folds in the disc epithelium. The cells contributing to the overgrowth express *lacZ* indicating that they come from the region where MS1096 is expressed. Dll is expressed throughout the wing pouch. (F) Cuticle from a MS1096:UAS-*dsh* pharate adult. Note the vast overgrowth of the proximal wing hinge (asterisk). This consists of apparently unpatterned tissue.

Dsh is sufficient to cause its hyper-phosphorylation and activation of the *wg* pathway, as assayed by the accumulation of Armadillo protein in cultured cells (Yanagawa et al., 1995).

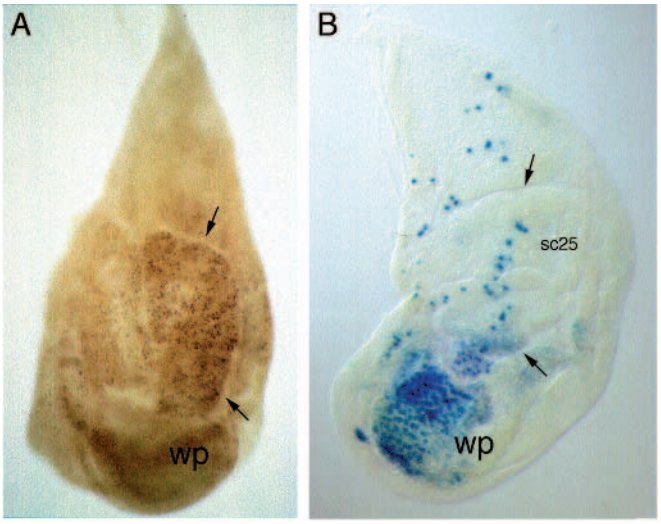

Fig. 8. Overproliferation of the wing hinge caused by UAS-*dsh*. (A) MS1096:UAS-*dsh* wing disc pulse labeled by BrdU incorporation shows an elevated level of cell division in the wing hinge region (arrows). The wing pouch (wp) and notum show a lower density of labeled cells. Control discs do not show the increased density of labeled cells (not shown). (B) MS1096:UAS-*dsh*; A101 wing disc stained with X-gal (compare with the wild-type pattern in Fig. 6A,B). The wing pouch is filled with sense organs, indicating that cell fates have been respecified toward margin. By contrast, the pattern of sense organ precursors is normal in the overgrown hinge region (arrows). For comparison, the fold indicated by the top arrow is the same as the fold at the top edge of Fig. 6B.

Sokol et al. (1995) have shown that injection of *Xdsh* mRNA into *Xenopus* oocytes can mimic the effect of injection of *Wnt* mRNA, even though *Xdsh* mRNA is present ubiquitously in the oocyte. These results suggested that overexpression of *dsh* in *Drosophila* could have a similar effect. Indeed, expression of UAS-*dsh* by several GAL4 drivers can phenocopy ectopic expression of *wg* in the wing and leg imaginal discs (Fig. 7E,F, and data not shown).

UAS-*dsh* crossed to MS1096 induces Dll expression throughout the wing pouch, as observed with UAS-*wg* (Fig. 7C,E). The dorsal hinge is greatly overgrown, as indicated by the abnormal folds of cells expressing UAS-*lacZ* and UAS-*dsh* (Fig. 7E). The pharates resulting from this cross also have densely packed wing margin bristles all over the wing blade surfaces, although in some cases the ventral surface is more sparsely covered (Figs 7F, 8B). The region occupied by the proximal dorsal hinge is greatly expanded. This effect of UAS-*dsh* is much stronger than that of UAS-*wg*.

To determine whether the expansion of the dorsal hinge region in MS1096:UAS-*dsh* individuals is due to increased proliferation, we pulse labeled imaginal discs with BrdU. When incubated with BrdU for 10 minutes, only few cells are labeled in a wild-type wing disc (not shown). By contrast, both the frequency and intensity of labeling is increased in the dorsal hinge of MS1096:UAS-*dsh* wing discs (compare the region between the arrows in Fig. 8A with the regions of the disc on either side, which resemble control discs). This result indicates that overexpression of *dsh* in the hinge region strongly stimulates cell division.

Examination of the extra tissue in the proximal hinge of

MS1096:UAS-*dsh* pharates indicates that it is not patterned. There does not appear to be a duplication of distal hinge structures more proximally. To further address this point, we crossed the neuronal marker A101 into this background (Fig. 8B). It is possible to locate most of the sensillar precursors in the hinge region in the correct location in these discs and in the correct numbers. This is consistent with the observation that sensory mother cells are selected from mitotically quiescent clusters of cells (Usui and Kimura, 1992). However, the spacing of cells within clusters of sensillae is abnormal. Thus the Sc25 group of sensillae appears as a compact cluster in wild-type discs, but is stretched into a long line in discs of the genotype MS1096:UAS-*dsh* (compare Fig. 6B with Fig. 8B). These results indicate that ectopic activation of the *wg* pathway in the hinge region of the wing does not respecify pattern, but instead stimulates cell division. However, the greatly increased cell numbers disrupt the organization of the hinge.

DISCUSSION

Distinct mitogenic and fate specification responses to the Wg signal

We have shown here that *wingless* function is required to promote proliferation of cells in the wing hinge. Reduction of *wg* gene activity in the wing hinge leads to underproliferation of this region and loss of distal hinge structures. Conversely, overexpression of *wg* or activation of the *wg* signal transduction pathway through *dsh* expression leads to local overgrowth of the proximal hinge. The effects of Wg in the proximal hinge suggest a rather direct mitogenic effect in this region of the disc. This contrasts with the effects of Wg in the wing pouch, where the primary effect of increasing Wg activity is to respecify cell fate toward wing margin. The difference in response to the Wg signal in the hinge and the wing blade is reminiscent of the effects observed when Wnt-1 is overexpressed in the mouse CNS using a HOXb-4 Region A enhancer (Dickinson et al., 1994). Although Wnt-1 is required for specification of fates at the mid-brain hind-brain junction, ectopic expression in the ventral CNS causes overproliferation without cell fate respecification. Skaer and Martinez-Arias (1992) have shown that *wg* stimulates cell division in the anlage of the Malpighian tubules, and several *Wnt* genes, including *Wnt-1*, the vertebrate orthologue of *wg*, have been found to have an oncogenic effect on mammary epithelial cells (reviewed in Nusse and Varmus, 1992).

Although *wg* is required for proliferation in the distal hinge region of the wild-type wing imaginal disc, this does not result in a zone of proliferation that stands out above that in the surrounding area. This suggests that other factors are stimulating proliferation in the rest of the disc at a similar rate. In this context it is noteworthy that, while removal of the inner ring of *wg* expression does not lead to a reduction of proliferation in the proximal hinge region, ectopic activation of the *wg* pathway strongly induces overproliferation in this region. This suggests that *wg* can synergize with the factor(s) that are regulating cell division in the proximal hinge.

wg locally mediates the effect of the P/D organizer

The reduction of the total size of the wing in spd^{fg} homozygotes is mostly due to the absence of hinge structures. This indicates that the *wg* expression domain in the inner ring surrounding the hinge does not have any long distance effects on wing development, unlike the *wg* expression domain in the wing margin (Diaz-Benjumea and Cohen, 1995). Therefore, *wg* cannot be the sole mediator of the putative hinge-organizing center identified by *nubbin* and only part of the *nubbin* phenotype can be due to removal of the inner ring of *wg* expression (Ng et al., 1995). However, the spd^{fg} phenotype suggests that *wg* locally mediates the stimulatory effect of the putative proximal-distal organizer on growth of the wing and suggests that one mechanism whereby organizers exert their influence on cell division may be to establish subdomains of cells in which proliferation is regulated independently from the rest of the disc.

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