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The tumour suppressor gene *l(2)giant discs* is required to restrict the activity of Notch to the dorsoventral boundary during *Drosophila* wing development

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Abstract

During the development of the *Drosophila* wing, the activity of the *Notch* signalling pathway is required to establish and maintain the organizing activity at the dorsoventral boundary (D/V boundary). At early stages, the activity of the pathway is restricted to a small stripe straddling the D/V boundary, and the establishment of this activity domain requires the secreted molecule *fringe (fng).* The activity domain will be established symmetrically at each side of the boundary of Fng-expressing and non-expressing cells. Here, I present evidence that the *Drosophila* tumour-suppressor gene *lethal (2) gaint discs (lgd)* is required to restrict the activity of Notch to the D/V boundary. In the absence of *lgd* function, the activity of Notch expands from its initial domain at the D/V boundary. This expansion requires the presence of at least one of the Notch ligands, which can activate Notch more efficiently in the mutants. The results further suggest that Lgd appears to act as a general repressor of Notch activity, because it also affects vein, eye, and bristle development.

Keywords: Tumour suppressor genes; *l(2)giant discs*; Wing development; *Notch*-signalling; *Serrate*; *Abruptex*; *Delta*

Introduction

During the development of any multicellular organism, cell–cell communication is an essential mechanism in pattern formation and differentiation. One important pathway, which mediates short-range cell communication, is the *Notch* pathway. The *Notch (N)* gene was first characterized in *Drosophila* and encodes a *trans*-membrane receptor that is activated by ligands encoded by the genes *Serrate (Ser)* and *Delta (Dl).* The activation of Notch results in activation of the *Suppressor of Hairless [Su(H)]* protein, which regulates the expression of target genes in the nucleus (Fortini and Artavanis-Tsakonas, 1994). Recent work has shown that the activation of Notch by its ligands leads to the release of the intracellular domain by proteolytic cleavage, and this fragment is translocated to the nucleus. There, it acts in concert with Su(H) to direct gene expression (Brou et al., 2000; Lecourtois and Schweisguth, 1998; Mumm et al., 2000; Schroeter et al., 1998; Struhl and Adachi, 1998). So far, four vertebrate homologues of *N* have been isolated (Hunter, 1997). Three of them have been associated with cancer, indicating that *N* can act as a classical protooncogene (Hunter, 1997). This has been further confirmed by the observation that the intracellular form of Notch1 can collaborate with c-Myc in oncogenesis (Girard et al., 1996).

In the last few years, it has become clear, that the *N*-pathway is required in a great variety of developmental processes. The outcome of the activation of Notch is tissue-specific, but in several cases, its activity prevents cellular differentiation. During *Drosophila* wing development, the pathway acts as an inductive signal successively required for the establishment, patterning, and growth of the wing primordium in the wing imaginal disc (Klein, 2001). The activity of Notch is restricted to the dorsoventral boundary (D/V-boundary) during most stages of wing development and is required for the expression of several genes along this boundary. Among these genes are *wingless (wg), vestigial*

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(vg), genes of the *Enhancer of split complex [E(spl)-C]*, *Delta (Dl)*, *cut*, and *Serrate (Ser)*. The establishment of the Notch activity domain along the D/V-boundary requires, in addition, the activity of the gene *fringe (fng)* and is established in cells along both sides of the boundary between *fng*-expressing and nonexpressing cells (Irvine and Wieschaus, 1994; Kim et al., 1995; Klein and Martinez-Arias, 1998; Panin et al., 1997). This modification is thought to affect the ability of both ligands in opposite directions (Brückner et al., 2000; Fleming et al., 1997; Klein and Martinez-Arias, 1998; Panin et al., 1997). The activity of Notch along the D/V boundary leads to the activation of the expression of *Dl* and *Ser* through two different kinds of regulatory loops (de Celis and Bray, 1997; Klein and Martinez-Arias, 1998; Panin et al., 1997). In one, which is thought to operate during early stages of wing development, Notch activity results in the activation of the expression of *Dl* and *Ser* in the cells at the D/V boundary. In the other, later operating loop (from the middle of the third larval instar stage onwards), the expression of the ligands is mediated indirectly through the activation of *wg*-expression by Notch at the D/V boundary (de Celis and Bray, 1997; Micchelli et al., 1997). At the time when the later loop operates, Ser and Dl become expressed in cells adjacent to the cells at the boundary (de Celis and Bray, 1997; Micchelli et al., 1997). Hence, expression patterns of Dl and Ser during the third larval instar stage are dynamic, and expression at the D/V boundary is dependent on the Notch pathway. It has been demonstrated that this later operating loop is crucial for the maintenance of Notch activity (Micchelli et al., 1997). In contrast, the significance of the early operating loop is not clear.

Besides its role in pattern formation, the Notch pathway has an important function in the regulation of the cell proliferation during wing development. This is underlined by the observation that ectopic activation of the pathway causes an overproliferation of wing cells (Doherty et al., 1996; Jönsson F, 1996; Speicher et al., 1994).

In addition to the stimulatory effect of Fng, suppressive mechanisms also operate, which restrict the expression of target genes of the Notch pathway to the cells at the D/V boundary. The *nubbin* gene (*nub*) is involved in such a mechanism. In the absence of *nub* function, the expression of Wg as well as the activity of a Notch-dependent enhancer of the *vg* gene, the *vg* boundary enhancer (*vg*BE), is expanded (Neumann and Cohen, 1998). It has been shown that Nub can bind to the *vg*BE and suppress its activity in the wing pouch (Neumann and Cohen, 1998).

Imaginal disc development also depends on the *Drosophila* tumour suppressor genes (TSG). Fifty TSG have been identified and the loss-of-function of many of these genes results in overproliferation of the imaginal discs (Bryant et al., 1993; Watson et al., 1994). These genes can be divided into two groups based on the mutant phenotypes (Bryant et al., 1993; Watson et al., 1994). Deletion of genes belonging to the tumorous class causes cells to overproliferate and invade new regions so that eventually the epithelial and compartmental organization of the discs is lost. In contrast, the loss of genes of the hyperplastic group causes overproliferation, but does not disturb the epithelial and compartmental organization of the discs. One of the genes belonging to the second group is *l(2)giant discs (lgd)*. The loss of *lgd* causes massive overproliferation of imaginal disc cells and extended larval life (Bryant and Schubiger, 1971). It has also been observed that *wg* is expressed ectopically in the pouch of *lgd* mutants during wing development (Buratovich and Bryant, 1995). Similar phenotypes are observed, if the *Notch* pathway is ectopically activated during wing development (Couso et al., 1995; de Celis and Bray, 2000; Diaz-Benjumea and Cohen, 1995; Klein and Martinez-Arias, 1998; Speicher et al., 1994), raising the possibility that the *lgd* mutant phenotype could stem from the ectopic activation of the *Notch* pathway.

Here, I show that the *Notch* pathway is indeed ectopically active in *lgd* mutants and that hyperactivation as well as ectopic activation of the pathway accounts for the *lgd* phenotype during wing development. In *lgd* mutants, the expression of Notch target genes along the D/V boundary is expanded, indicating that Lgd is required for the restriction of Notch activity to the D/V boundary. Furthermore, the mutant phenotype of *lgd* is suppressed by concomitant loss of *Psn* or *Su(H)* function, indicating that it is caused by the activation of the Notch pathway. I provide evidence that the activity of *fng* and *Ser* seems to be dispensable in *lgd* mutant wing disc and that D1 can activate Notch efficiently enough to maintain its activity during wing development. The presented results indicate that the negative regulation of Notch by Lgd is not restricted to wing development and occurs during several other developmental processes, such as vein, eye, and bristle development, suggesting that Lgd suppresses the activity of the Notch pathway in a variety of developmental processes.

Materials and methods

Drosophila stocks

The following mutations were used in this work: Ser^{RX106} (Speicher et al., 1994), Ser^{94C} (Couso et al., 1995). The alleles of *l(2) giant discs (lgd)*, which are used in this study, are lgd^{d1}, lgd^{d4}, lgd^{d7}, and lgd^{d10}. In most of the experiments described, I used the lgd^{d7}-allele, but the results have been confirmed in many cases with other alleles. The Df(2L)FCK-20 (Barrio et al., 1999) is a gift of R. Barrio.

H^{E31} and the FRT40A $Su(H)^{SF8}$ chromosome are provided by F. Schweisguth. H^{E31} is a null mutant described in Schweisguth and Lecourtois (1998). Psn^{B3} and Psn^{I2} are null *Presinillin* mutants and were provided by Mark Fortini, and the Psn^{C1}-FRT2A chromosome (null allele) by G. Struhl (Lukinova et al., 1999; Struhl and Greenwald, 1999;

Ye et al., 1999). The *fng*^{13} FRT80 chromosome was provided by K. Irvine.

The *vg* boundary enhancer is described in Williams et al. (1993) and referred to here as *vgBE*. Sensory organ precursors were detected with a lacZ-insertion, A101, in the *neuralized* gene (Huang et al., 1991). *Delta* expression was detected with a lacZ insertion in *Dl* provided by J.F. de Celis or in anti-Dl antibody staining with the MAb 202 provided by M. Muskavitch. The *E(spl)m8 lacZ* is a gift of F. Schweisguth (Lecourtois and Schweisguth, 1995). The UAS *fng* construct was kindly provided by Sean Carroll (Kim et al., 1995, 1996); The UAS *Ser* (Speicher et al., 1994), UAS GFP (Yeh et al., 1995), the UAS *Notch*, and UAS ECN lines are described in Klein et al. (1997). The UAS FLN-CDC10 line is described in (Lawrence et al., 2000).

The *Dl*^{rev10} *Ser*^{VX82} FRT82B chromosome is a gift of S. Blair and is described in Miccheli et al. (1997). The FRT40A chromosome carrying two copies of a polyubiquitin-nls-GFP construct are kindly provided by Stefan Lüschnig. The FRT40A *lgd*^{d7} chromosome was generated in this work and was used together with the described FRT40A *Su(H)*^{SF8} chromosome (provided by F. Schweisguth) to generate the FRT40A *lgd*^{d7} *Su(H)*^{SF8} double mutant. The clones were generated by using various UASFlp constructs (Duffy et al., 1998) activated by *sd*Gal4 or *ptc*Gal4 or an hsFlp construct.

Ectopic expression of the different genes was achieved through the GAL4/UAS system (Brand and Perrimon, 1993). The expression of the different UAS constructs was driven in the imaginal discs with various GAL4 inserts. In the third instar, *decapentaplegic*Gal4 (*dpp*GAL4) and *patched*Gal4 (*ptc*Gal4) activate expression of UAS transgenes in a stripe along the AP boundary of the discs (Wilder and Perrimon, 1995). The *vg*Gal4 lines are described in Neumann and Cohen (1996). *scalloped* Gal4 (*sd* Gal4) is expressed in a pattern that is identical to that of *vestigial* and allows expression of the construct throughout the developing wing (Klein et al., 1997).

Stocks carrying different combinations of GAL4 and UAS chromosomes in wild type and mutant background were generated. All second- and third-chromosomal allelic combinations were balanced over the SM6a–TM6b compound balancer, which allowed the identification of larvae of the correct genotype because of the dominant larval marker Tb. Details of the stocks as well as the stocks themselves are available on request.

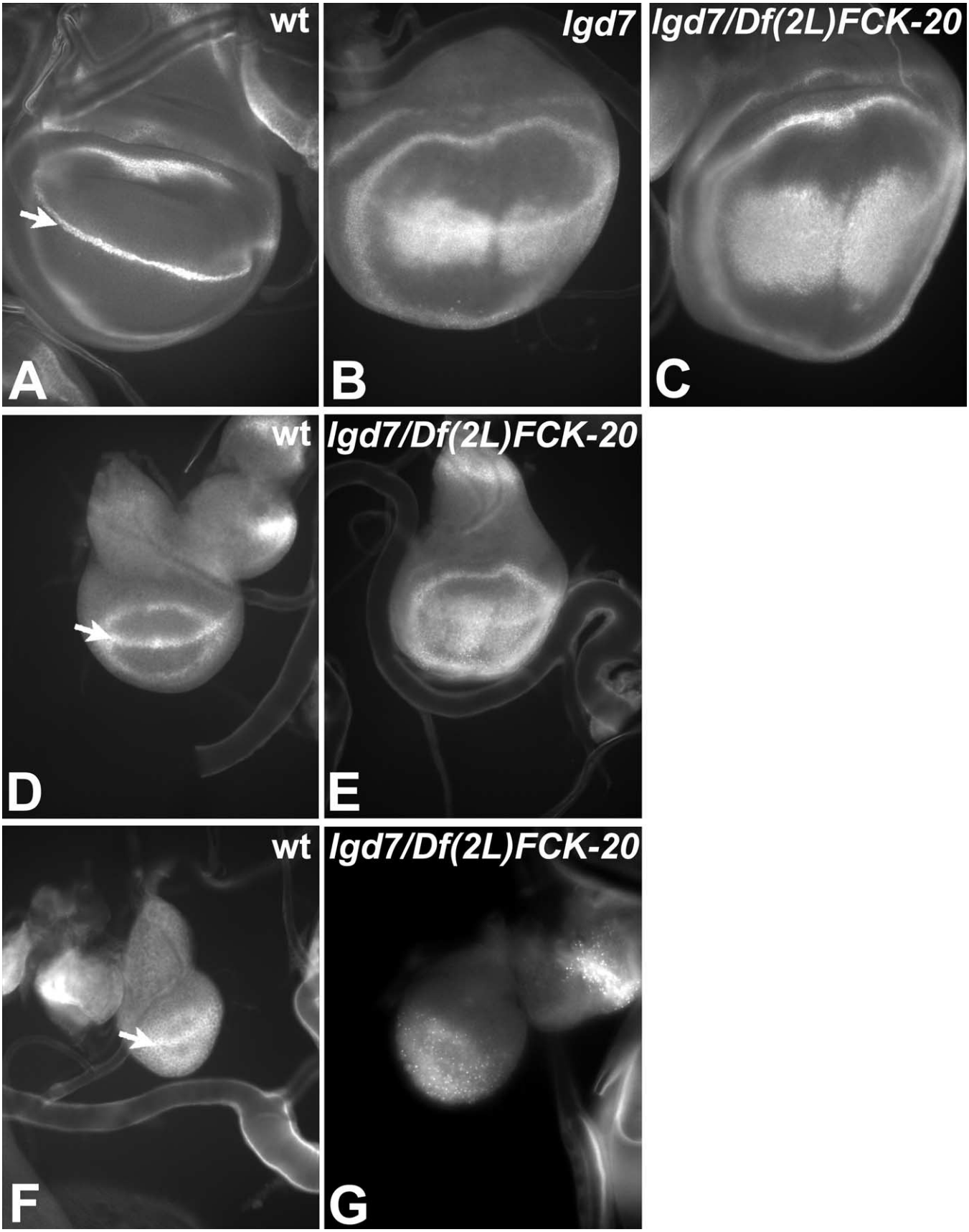
Immunohistochemistry and in situ hybridisation

The *vestigial* antibody is described in Williams et al. (1991) and, together with the anti-Ac antibody (Skeath and Carroll, 1991), was a gift of S. Carroll. The anti-Wingless antibody was kindly provided by S. Cohen (Neumann and Cohen, 1997). The *Ser* antibody (Speicher et al., 1994) was a gift from E. Knust. The anti-Dl MAb 202 was a gift of M. Muskavitch. The C17.9C6 anti-Notch antibody is a gift of S. Artavanis-Tsakonas. The *cut* antibody developed by G. Rubin 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). The anti-Sal antibody was kindly provided by R. Schuh. In situ hybridization was performed as described in Tautz and Pfeifle (1989). The fluorescence of the green fluorescent protein (GFP), Texas red-, and FITC-conjugated antibodies (purchased by Jackson Laboratories) were detected by using an appropriate filter set on a Zeiss Axiophot microscope.

Results

Loss of *lgd* function leads to an overgrowth of the imaginal discs, clearly noticeable in the wing region of the wing disc, which becomes enlarged and flat (Bryant and Schubiger, 1971). *wg* expression is normally restricted to the dorsoventral boundary (D/V boundary) of the wing pouch (Fig. 1A). In *lgd* mutants, *wg* is activated ectopically in a much broader domain that extends into the wing pouch (Buratovich and Bryant, 1995; Fig. 1B). In addition, *lgd* mutant wing discs often develop a second wing pouch in the region of the anlage of the scutellum (Buratovich and Bryant, 1995). Similar phenotypes are caused by gain-of-function alleles of *N*, for example, *Abruptex*, and are also observed upon expression of the activated intracellular form of *Notch*, *Nintra*, or expression of *Notch* ligands, such as *Dl*

Fig. 1. The wing imaginal disc phenotype caused by *lgd* mutations revealed by anti-Wg antibody staining. Anterior is to the left, dorsal to the top. (A) Wg expression in a wild type wing imaginal disc at the late third larval instar stage. Wg is expressed in two ring-like domains in the hinge region, framing the wing pouch. It is further expressed in a domain straddling the D/V boundary, highlighted by the arrow. (B) Wg expression in a *lgd*^{d7} mutant wing disc at the late third larval instar stage. The wing area is enlarged and flattened, and the expression of *wg* normally restricted to the D/V boundary (arrow in A) expands into the wing pouch. The *lgd* mutant phenotype is even more dramatic during the extended larval live, which is characteristic for these mutants (Buratovitch and Bryant, 1995). (C) The deficiency *Df(2L)FCK-20* deletes the *lgd* locus. The disc shown is of the genotype *lgd*^{d7}*/ Df(2L)FCK-20* and shows a phenotype comparable to that of homozygous *lgd*^{d7} mutant discs. This indicates that the *lgd*^{d7} is a strong allele of the locus. The deregulation of Wg expression is recognizable already in wing imaginal discs during the early third larval instar stage when no morphological differences to wild type discs are detectable. (D) A wild type wing imaginal disc of the mid third larval instar stage. At that stage, Wg is expressed in one ring-like domain and along the D/V boundary. (E) *lgd*^{d7}*/Df(2L)FCK-20* mutant wing imaginal discs of the same age as the wild type disc shown in (D). The expansion of Wg expression is clearly recognizable in the mutant disc. Wg expression is seen throughout the forming wing blade, which is framed by the ring-like expression domain of Wg. (F) A wild type disc of the early third larval instar stage. The expression of *wg* has just started to be expressed along the D/V boundary and the ring-like domain in the hinge. (G) In contrast, Wg expression has not resolved in a similar pattern in *lgd* mutant discs of the same age.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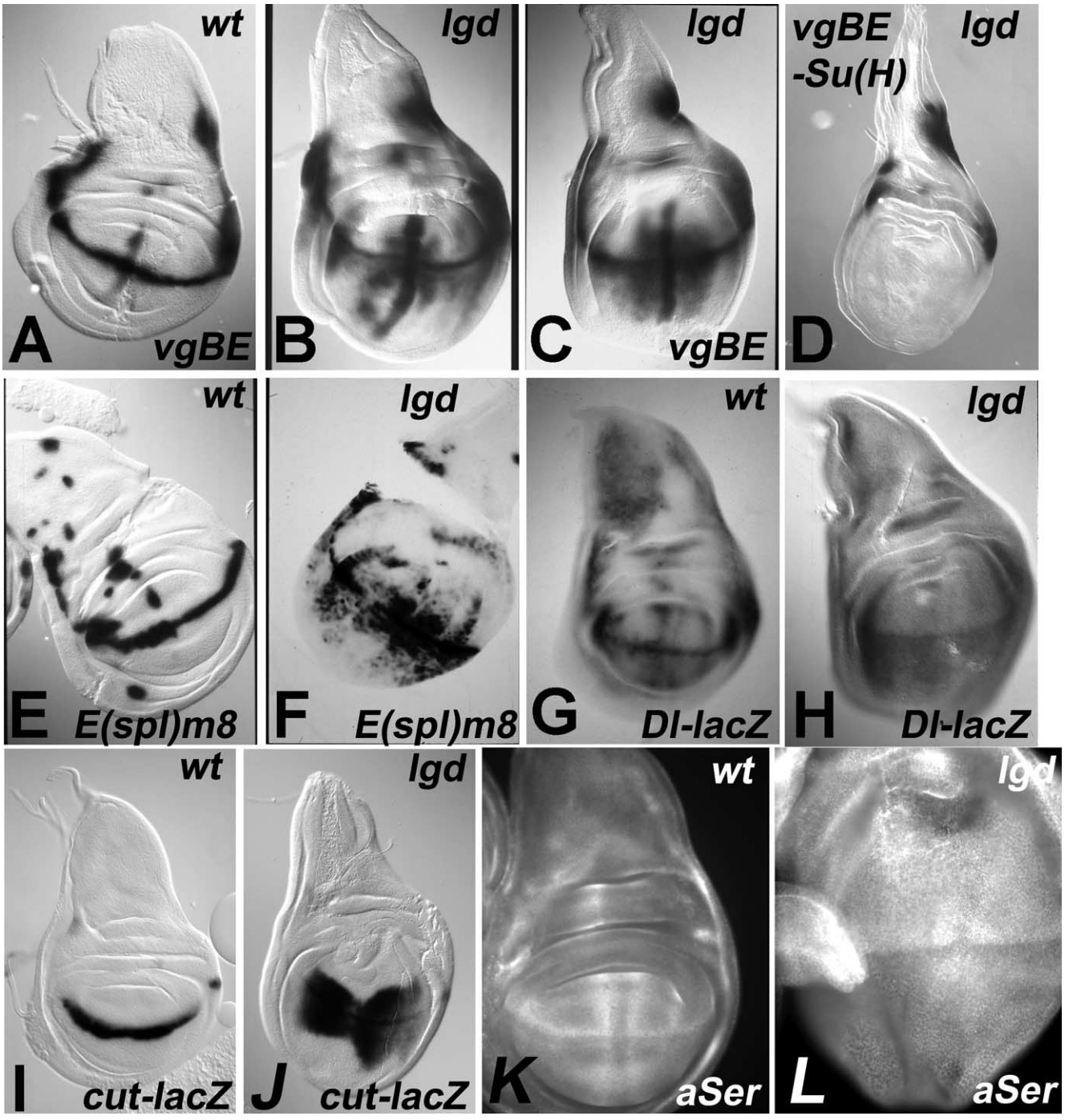


Fig. 2. Ectopic expression of *N*-regulated genes in *lgd* mutant wing discs. All discs are oriented with anterior to the left, dorsal to the top. (A) Expression of the *vg*BE in the wild type. The activity of the enhancer is expressed in cells along the dorsoventral boundary, which will later become the wing margin, and in a Stripe along the A/P compartment boundary. Both domains of expression are dependent on the activity of the Notch pathway (Klein et al., 2000; see also D). (B, C) Expression of this enhancer in a lgd^{d1} (B) and a lgd^{d7} mutant wing disc (C). In both cases, loss of *lgd* function causes the ectopic expression of the *vg*BE in the wing pouch. (D) Expression of a variant of the *vg*BE in which the *Su(H)* binding site is removed in a lgd^{d7} mutant wing disc. No expression is observed in the wing area, indicating that the *N/Su(H)* pathway is required for its ectopic activation in *lgd* mutant wing discs. (E) The expression of the *E(spl)m8* gene in late third instar revealed by a promoter lacZ-construct (*m8*-lacZ). The expression is restricted to the dorsoventral boundary and to the anlage of the third wing vein. (F) Expression of *m8*-lacZ in a third instar lgd^{d7} wing disc. β-Galactosidase activity is detectable in most regions of the pouch. (G) The expression of *Dl*-lacZ in a late third instar disc. Again, *Dl*-lacZ is ectopically expressed in the whole pouch area in late third instar lgd^{d7} discs (H). See also Fig. 6 for further evidence. (I) Expression of *cut*-lacZ (cut^{HZ1}) along the D/V boundary of a late third instar wing disc. (J) The expression of cut^{HZI} is strongly expanded in *lgd* mutant discs. (K, L) Expression of Ser in a wt and *lgd* mutant wing imaginal disc of the late third larval instar stage revealed by anti Ser antibody staining. (K) Expression of *Ser* in the wild type. Ser is expressed in two small stripes of cells adjacent to cells at the D/V boundary as well as in the anlagen of the wing veins. The dorsal stripe is stronger than the ventral one. Both stripes are dependent on the Wg signal produced by the cells at the D/V boundary. (L) In lgd^{d7} mutant discs, the expression of *Ser* has expanded and is detectable throughout the pouch. Again, expression in the dorsal side of the pouch is stronger than in the ventral side.

(de Celis and Bray, 2000; de Celis et al., 1996b; Jönsson F, 1996; Klein and Martinez-Arias, 1998). The ectopic activation of *wg* can already be observed in early third instar wing discs and precedes the visible morphological changes that occur at later stages (see Fig. 1D and E). The deficiency Df(2L) FCK-20 deletes the *lgd* locus, allowing the classification of the relative strength of the available alleles. The phenotype is always variable, but the overall phenotype of lgd^{d7} and lgd^{d10} in homozygotes and in *trans* over Df(2L)FCK-20 is very similar (Fig. 1B and C), indicating that these two alleles are strong, probably amorphic alleles. lgd^{d4} and lgd^{d1} are weaker alleles. All alleles display a qualitatively similar phenotype over the deficiency as in homozygotes, indicating that the observed phenotype is probably caused by the loss-of-function of the *lgd* gene.

Ectopic activation of Notch target genes in the absence of lgd function

The similarity between the loss of *lgd* function and ectopic *N* activation suggests that the phenotype of *lgd* could be caused by ectopic activation of the *Notch* pathway. To examine this possibility, I monitored the expression of *E(spl)m8*, *cut*, *Dl*, and *Ser* as well as the activity of the *vg*-boundary enhancer (*vg*BE) in mutant wing discs. The expression of all these markers is initiated in cells at the D/V boundary in a *Notch*-dependent manner (de Celis et al., 1996b; Doherty et al., 1996; Kim et al., 1996; Klein and Martinez-Arias, 1998; Micchelli et al., 1997; Panin et al., 1997). The *vg*BE is initially expressed along the D/V boundary of the wing, but late in the third instar, it is activated in an additional stripe along the anteroposterior compartment boundary (A/P boundary), which is also dependent on *Notch* activity (Klein et al., 2000). Both domains depend on the presence of a single *Su(H)* binding site in the enhancer (Kim et al., 1996; Klein et al., 2000). Similarly, the expression of *cut* and *E(spl)m8* is initiated in cells at the boundary by the *Notch*-pathway, and *E(spl)m8* is also dependent on the presence of Su(H) binding sites in its promoter (de Celis et al., 1996a; Lecourtois and Schweisguth, 1995). As described above, the expression of *Dl* and *Ser* is more complex but always dependent on the activity of Notch in cells at the D/V boundary. In *lgd* mutant wing discs, the *vg*BE as well as *cut*, *Dl*, *Ser*, and *E(spl)m8* are activated ectopically within the wing pouch (Fig. 2A–C and E–L; see also Fig. 6). The activation of the *vg*BE is dependent on the presence of the *Su(H)* binding site in the enhancer, since a version lacking it shows no ectopic activity in the mutants (Fig. 2D). As in the case of *wg*, the expression of the *vg*BE is already expanded in early third larval wing discs (data not shown). Altogether, these results show that the loss of *lgd* function leads to the ectopic expression of Notch target genes. This suggests that the *Notch* pathway is ectopically activated in *lgd* mutants.

Genetic interactions between lgd and genes of the Notch pathway

If the *lgd* phenotype is caused by the ectopic activation of *Notch*, inactivation of the *Notch* pathway should suppress the mutant phenotype of *lgd*. To test this prediction, I examined whether the *lgd* mutant phenotype is present in mutants, where Notch is not processed correctly, such as in *Presenilin (Psn)* (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). In *lgd; Psn* double mutant wing discs, the overproliferation of the disc cells, as well as the ectopic expression of *wg* is abolished (Fig. 3A–C). Furthermore, the formation of ectopic wings in the notum is missing. This suggests that the *Psn* mutant phenotype is epistatic over that of *lgd* mutants and that *lgd* acts through the Notch pathway. The slight rescue of the *Psn* phenotype (compare Fig. 3B with C) is probably due to a residual activity of the *Notch* pathway, since a similar rescue of the *Psn* mutant phenotype is observed if the *Hairless* gene is concomitantly removed (Klein et al., 2000). This residual activity seems to be enhanced in the absence of *lgd*.

The *Ax* mutations are gain-of-function alleles of the *Notch* locus, which lead to the overactivation of the pathway (de Celis et al., 1996b). I therefore looked for synergistic genetic interaction between *Ax* and *lgd* alleles. Homozygous or hemizygous Ax^{MI} mutant wing imaginal discs show a weak expansion of the expression of genes normally restricted to the D/V boundary (de Celis et al., 1996b). This phenotype is strongly enhanced by loss of one functional copy of the *lgd* (Fig. 3E). Double mutant wing discs show a very extreme phenotype, where the disc looses all visible organization and forms a rounded ball of cells (Fig. 3F). The synergistic genetic interaction between Ax^{M1} and lgd^{d7} further indicates a functional relationship between the two loci.

I further analysed the phenotype of *Ser; lgd* double mutant wing discs to examine the effect of loss of one Notch ligand in *lgd* mutants. Loss of *Ser* function leads to the loss of most of the wing blade and the margin (Jönsson F, 1996; Klein and Martinez-Arias, 1998; Speicher et al., 1994). The presence of a remnant of the wing pouch is due to the fact that the *Notch* pathway is active during early stages of wing development. This activation is achieved through a residual expression of *Dl* (Klein and Martinez-Arias, 1998). Animals of the *Ser; lgd* double mutant phenotype develop very slowly, and only few larva survive until the third instar. The wing imaginal discs of the larva have expanded wing pouches and, in contrast to *Ser*-mutant discs, they express *vg* and *Dl* (Fig. 3G and H, respectively) and *wg* (data not shown) in the wing blade. This shows, that in the absence of *lgd* function, the activity of *Ser* is not required to maintain Notch-dependent gene activity. In summary, the observed genetic interactions reveal a functional relationship between the *Notch* and *lgd* locus and support the conclusion that Lgd is a negative regulator of the *Notch* pathway.

The activation of Notch in lgd mutants is dependent on its ligands

The observation that loss of lgd function can compensate for the loss of Ser function raises the possibility that Notch could be activated in a ligand-independent manner in the absence of *lgd* function. To test this possibility, I generated *Ser/Dl* double mutant clones in *lgd*-mutant wing discs. The clones were induced through combining the Flp/FRT and the targeted Gal4-System. In the experiments described here, the expression of UASFlp was activated with *sd*Gal4. *sd*Gal4 is active throughout wing development and therefore activates UAS Flp expression at all stages of development. In the clones, the expression of the *Notch*-regulated genes *wg* and *cut* was interrupted in the centre of the clone area (asterisks, Fig. 4A–C), suggesting that the expression of these genes in *lgd* mutants depends on *Notch* ligands. However, several interesting additional effects were observed: First, *wg* and *cut* expression was surprisingly induced on both sides of the clone boundary, which can be clearly seen in clones located outside the expanded expression domain normally observed in *lgd* mutants (arrow in Fig. 4B and C). The effect is observed in the dorsal as well as the ventral half of the pouch (see arrows in Fig. 4B and C). This suggests that the removal of the ligands leads to the activation of Notch at the boundary of *Dl/Ser*-expressing and nonexpressing cells. Secondly, in several cases, the expression of *cut* and *wg* expands outside the clone, even far away from the clone boundary (Fig. 4B–D). This effect is biased, and the expansion toward the D/V boundary is stronger.

Thirdly, the expression of the *Notch* targets is activated up to three-cell diameter into the clone in a graded manner (Fig. 4D). Since the ligands are membrane anchored and thought to signal to adjacent cells, an activation of *Notch* target gene expression beyond one-cell diameter into the clone is not expected. One possibility is that the induction of Cut by Notch is indirect and mediated by a diffusible factor that is induced at the clone boundary.

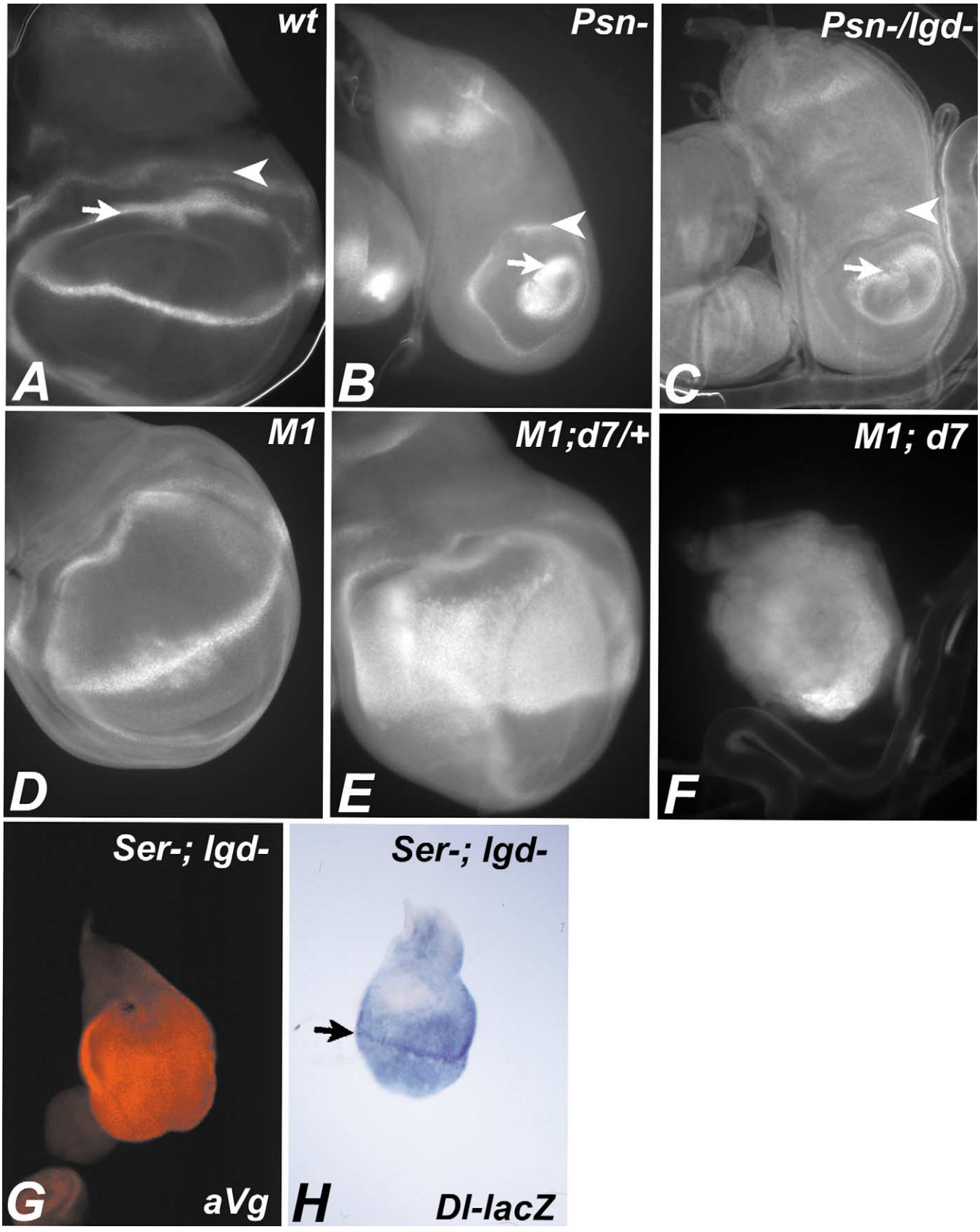
However, I found that clones of *Su(H)* mutant cells in *lgd* mutant discs loose expression of *Notch* target genes, such as Cut (Fig. 4E and F), indicating that the cells require a functional Notch pathway to activate expression of its target genes. Similar results were obtained with *Psn* mutant clones, using Wg expression as a read out of *Notch* activity (data not shown). These results rule out the possibility that the target genes of Notch are induced indirectly through a diffusible factor induced by the *Notch* pathway.

In summary, these results suggest that, in *lgd* mutant wing blades, all cells that express *Notch*-regulated genes require the activity of the signal cascade and receive a signal through Dl and/or Ser. In addition, they indicate that, in the *Ser; lgd* double mutant wing discs described above, Dl alone is sufficient not only to initiate, but also to maintain *N*-activity during wing development. Hence, it seems that Notch can be activated more efficiently by Dl in the absence of *lgd.*

Clonal analysis of lgd

To further characterize the function of *lgd*, I generated *lgd* mutant clones and monitored the expression of *Notch*-regulated genes, such as *cut*, *wg*, and *Dl*, as well as the activity of the Gbe+Su(H)m8 reporter construct. The Gbe+Su(H)m8 reporter construct consists of an ubiquitously expressing promoter of the *grainyhead* gene in which four copies of the Su(H) binding site derived from the E(spl) m8 promoter have been inserted (Furriols and Bray, 2001). This construct specifically detects Su(H)-dependent Notch activity in imaginal discs (Furriols and Bray, 2001). The clones were generated by using the FLP/FRT system. In a first experiment, the clones were induced with help of an hsFlp construct. I found that, if *lgd* mutant clones were induced during the first larval instar stage [24-48 h after egg laying (ael)], they were rarely found in wing pouches of the late third larval instar stage (Fig. 5A). In most cases, the twin clone, containing two copies of the GFP marker, was present but the mutant counterpart was missing (arrows in Fig. 5A), indicating that the mutant cells were not able to compete with their wild type neighbours in the wing pouch. In contrast, outside the pouch, e.g., in the hinge region, mutant clones could be frequently recovered (arrowhead in Fig. 5A), indicating that, in these regions, the mutant cells did not have any growth disadvantage. In addition, scars were often found in wing pouches where *lgd* mutant clones were induced (Fig. 5B–G; see arrowheads in Fig. 5E),

Fig. 3. Genetic interactions among alleles of genes of the *Notch* signalling pathway and *lgd.* (A–C) The *Psn* mutant phenotype is epistatic over that of *lgd* mutants. Wing imaginal discs are stained by anti-Wg antibody staining. (A) Expression of *wg* in a wild type disc at the late third larval instar stage. Wg is expressed in two rings in the proximal (arrowhead) and distal hinge (arrow) and along the D/V boundary. (B) The D/V boundary expression of Wg is lost in Psn^{C1} mutant wing imaginal discs. Furthermore, the diameter of the two ring-like domains in the hinge is dramatically reduced, indicating the loss of distal wing elements such as the pouch. (C) A lgd^{d7}; Psn^{C1} double mutant wing disc. The overproliferation of the wing imaginal disc of typical for *lgd* mutants is absent, no D/V boundary expression of Wg is detectable, and the diameter of the ring-like expression domains of Wg is reduced in a similar way as in *Psn* mutants. The phenotype is very similar to that of *Psn* mutants. (D–F) Genetic interactions between Ax^{M1} and lgd^{d7}. (D) In Ax^{M1} mutant wing discs, the expression of Wg along the D/V boundary is weakly expanded. (E) This expansion of Wg expression is strongly enhanced if only one copy of *lgd* is lost and is expanded over nearly the whole dorsal half of the wing blade. (F) In a Ax^{M1}; lgd^{d7} mutant, the organization of the wing imaginal disc is lost and only residual Wg expression is found. (G, H) Analysis of the *lgd; Ser* double mutant phenotype. (G) Anti-Vg antibody staining of a Ser^{94c}/Ser^{RX106}; lgd^{d7} mutant wing imaginal disc. Strong Vg expression is seen throughout most of the developing wing blade. This is not observed in *Ser* mutant wing discs, which have a phenotype very similar to that of *Psn* mutants, shown in (B). (H) Furthermore, D1 is expressed in these double mutant discs and is upregulated at the D/V boundary (arrow).


wt
A
Psn-
B
Psn-/lgd-
C
M1
D
M1;d7/+
E
M1; d7
F
Ser-; lgd-
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aVg
Ser-; lgd-
H
Dl-lacZ

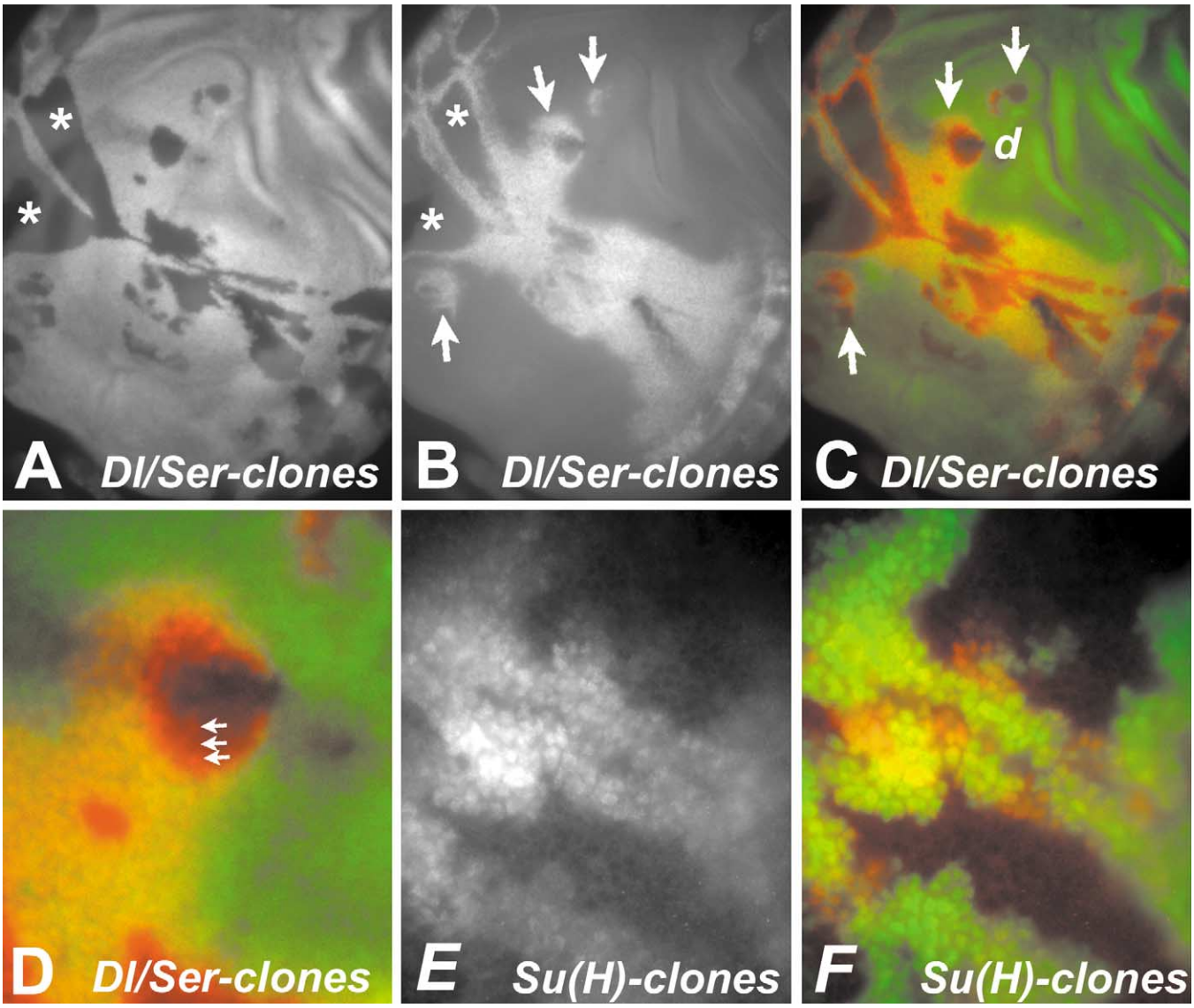


Fig. 4. Analysis of *Dl/Ser* mutant clones in lgd^{d7} mutant wing discs. Anterior is to the left; ventral is to the bottom. The expression of Cut is revealed by anti-Cut antibody staining, but similar results have been observed with anti-Wg antibody staining (not shown). The clones are induced as described in the text. (A) Clones of *lgd* mutant cells revealed by the absence of GFP marker. (B) Expression of Cut in the same disc as in (A). (C) Pseudocolour image of (A) and (B). Asterisks in (A) and (B) highlight large mutant clones. The centre of these areas is devoid of Cut expression (red), indicating that the Notch ligands are required for the induction its expression in *lgd* mutant cells. Interestingly, at the clone boundaries, *Dl/Ser* mutant cells express Cut up to several cell diameters within the clonal area. This becomes clear under higher magnifications of the region labelled in (C) with d and shown in (D). As Cut is a nuclear protein, it allows cellular resolution. Cut was expressed in *Dl/Ser* mutant cells up to three cells into the clone (see arrows). Cut was expressed in a graded manner with strongest expression in cells at the clone boundary. Another property of the clones is highlighted by the arrows in (B) and (C). They point to clones located outside the expanded expression domain of Cut, normally observed in *lgd* mutants. Note that the expression of Cut is initiated at the clone boundary (D) and expands in dorsal and ventral direction. Hence, in *lgd* mutant wing imaginal discs, a boundary of Ser- and Dl-expressing and nonexpressing cells initiates activation of the Notch pathway, and this activity spreads in each direction. Note that the expansion of the expression of Cut is greater toward the D/V-boundary, which is in the middle. (E, F) Behaviour of $Su(H)^{SF8}$ mutant clones induced in lgd^{d7} mutant discs by *sd*Gal4 UAS FLP. (E) Cut expression. (F) Pseudocolour image of the Cut expression and expression of the GFP marker revealing the mutant clones by absence of GFP. Cut expression is lost in all *Su(H)* mutant cells, suggesting that Su(H) is autonomously required for Cut expression in *lgd* mutants. Similar results have been observed with Psn^{C1} clones.

indicating that the mutant cells probably had undergone apoptosis. Even if the clones were induced during the second larval instar stage (48-72 h ael), many “orphan” wild type twin clones were found. However, in these cases, also some mutant clones were recovered (Fig. 5B–G). The mutant cells often expressed Notch target genes, such as *wg* and *cut* (see Fig. 5B–D and 5E–G respectively), even if they were located away from the D/V boundary and did not include the normal activity domain of Notch. Expression of Cut or Wg was not always activated in mutant clones (see, for example, the dorsal clone in Fig. 5B–D).

In this first set of experiments, expression of the genes

was always restricted to mutant territories, suggesting that *lgd* acts cell-autonomously. The mutant clones often had a round shape and seemed to try to minimize their contact to their normal neighbours. This suggests that the mutant cells have different adhesive properties than their normal neighbours. In a second set of experiments, *lgd* mutant clones were generated by using an UAS Flp construct, activated by *vg*BEGal4 or *sd*Gal4 (Fig. 5I–P and Fig. 6A–J). Using this method, I was able to induce large *lgd* mutant areas in wing pouches. This was surprising because of the difficulties of recovering mutant clones in the hsFlp experiment. The explanation of this difference might be the continuous expression of UAS FLP during all stages of wing development. Hence, clones are continuously induced, also beyond the phase of cell lethality of *lgd* mutant cells in early stages of wing development. In the large mutant territories, I often found an expansion of the expression of Wg within the clone area (data not shown). The use of the Gbe+Su(H)m8 construct in these experiments allowed for the detection of Notch activity outside the wing pouch, where the expression of genes like *wg* and *cut* is not controlled by Notch (Fig. 5I–P). The activity of this construct was often strongly upregulated in mutant territories in and also outside the wing pouch, such as the pleura (see arrows in Fig. 5I–K and L–O), in the notum, in regions of the leg disc (arrow in Fig. 5P) and the peripodial-membrane of the wing imaginal disc (Fig. 5L–O). This suggests that ectopic activation of Notch is a consequence of loss of *lgd* function in the wing imaginal disc outside the wing pouch and also in other imaginal discs. In the wing pouch, the activity of the Gbe+Su(H)m8 construct was often upregulated in mutant cells/regions that did not express Wg or Cut (see Fig. 6G–J; data not shown), indicating that Notch was activated in these cells but this activation was not sufficient for expression of Cut and Wg. Activation of the Gbe+Su(H)m8 construct can be observed already in early wing discs (Fig. 6G–J). At this stage, no morphological alteration of the wing disc is observed. This suggests that the activation of Notch is preceding the overproliferation of the disc.

In the set of experiments using UASFlp, expression of the Gbe+Su(H)m8 construct in some wild type cells was observed. This is especially clear if clones are located in the peripodial membrane. A good example of such a clone is shown in Fig. 5L–O. Although most of the normal cells at the clone boundary do not show activity of the Gbe+Su(H)m8 construct, a few cells that are highlighted by the arrow do so. This result shows that cell-autonomy of *lgd* is not complete.

As expected, *Dl* is strongly activated in *lgd* mutant clones (Fig. 6A and B). This observation raises the possibility that Lgd is a negative regulator of expression of *Dl*. Such a function of Lgd would explain the ectopic activation of the Notch pathway in *lgd* mutant imaginal discs and clones. Alternatively, *Dl* is also a target of the Notch pathway, and hence the strong ectopic expression of *Dl* in the mutant clones could be a consequence of the activation of the Notch pathway rather than its initial cause. Two experiments argue for the second alternative. Clones double mutant for *lgd* and *Su(H)* failed to express *Dl*, indicating that a functional *Notch* pathway is required for expression of *Dl* in *lgd* mutant cells. (Fig. 6C and D). Furthermore, *Dl* expression was strongly reduced in *Su(H)* mutant clones induced in *lgd* mutant wing imaginal discs (Fig. 6E and F). Both results indicate that the ectopic expression of *Dl* is not the cause but a consequence of the activation of the *Notch* pathway in the wing imaginal disc of *lgd* mutants. In agreement with this conclusion is the fact that *Dl* is not activated in *lgd* mutant clones located in the hinge region (e.g., see clone labelled by the arrowheads in Fig. 6A and B). This suggests that expression of Dl is not a consequence of loss of *lgd* function in all regions of the disc.

The negative effect of Ser on Notch signalling is suppressed in lgd mutant wing imaginal discs

Expression of *Ser* with *ptc*Gal4 during normal wing development results in interruption of the expression of Notch target genes, like *wg*, in the region where the *ptc* domain crosses the D/V boundary (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995). The reason for this interruption is that the activity of the Notch pathway is suppressed in cells expressing high levels of Ser (de Celis and Bray, 1997; Klein and Martinez-Arias, 1998; Miccheli and Blair, 1999; Thomas et al., 1995). In *lgd* mutants, this effect is not observed, and consequently, the expression of *wg* along the D/V-boundary is not interrupted (Fig. 7C and D). This observation suggests that the negative effect of strong *Ser* expression at the D/V boundary is absent in cells that lack *lgd*. To further support this conclusion, *Ser* was activated by *sd*Gal4 throughout the wing during normal development. Continuous expression of UAS*Ser* in the wild type leads to the loss of the wing margin and a dramatic reduction of the size of the wing pouch (Klein et al., 1997; Klein and Martinez-Arias, 1998). This negative effect is again absent in *lgd* mutants (data not shown). The results raise the possibility that *lgd* might be involved in the inhibition of the Notch pathway through high concentration of its ligands.

A similar effect of loss of *lgd* function on the ability to suppress *Notch* signalling cell-autonomously is observed if Fng is ectopically expressed (see Supplementary Material). Furthermore, clonal analysis of *fng* suggests that the loss of *lgd* seems to abolish the requirement of a boundary of Fng-expressing and nonexpressing cells for Notch activation (see Supplementary Material).

lgd is required for the regulation of the Notch pathway in other developmental processes

If ectopic activation of *Notch* signalling was a general consequence of loss of *lgd* function, one would expect other *Notch*-related processes besides that of wing development to be affected. To test this assumption, I analysed the effect

of loss of *lgd* function on other developmental processes that are dependent on *Notch* signalling. The selection of sensory organ precursors (SOP) out of the proneural clusters is one process regulated by the interactions between Notch and Dl. The function of Notch is to suppress neural development in the non-SOP cells of the proneural cluster by downregulating the activity of the proneural genes, such as *achaete (ac)* (Fig. 8A). In *lgd* mutant discs, some of the proneural clusters are formed (Fig. 8A, C and D), but in contrast to the wild type, the cells do not accumulate high levels of proneural activity, and as a consequence, most of the SOPs do not form. This is indicated by the absence of most of the expression of the SOP-specific marker A101 in *lgd* mutant wing imaginal discs (Fig. 8B, E, and G). A similar phenotype is also observed in *Abruptex* mutant wing imaginal discs (Couso and Martinez Arias, 1994; Heitzler and Simpson, 1993) and suggests that the *Notch*-pathway is hyperactive during SOP development in the absence of *lgd* function. The antineurogenic phenotype of *lgd* mutants is suppressed by concomitant loss of *Psn* function. *lgd; Psn* double mutant wing discs display a neurogenic phenotype similar to *Psn* mutant discs (Fig. 8I–K): clusters of large cells that strongly express Ac can be observed, and these cells express the neural differentiation marker Elav (Fig. 8K). The neurogenic phenotype of the double mutants indicates that the mutant phenotype of *Psn* is epistatic over that of *lgd* and that the antineurogenic phenotype of *lgd* mutants is mediated by the activation of the Notch pathway. Hence, *lgd* is involved in the regulation of *Notch* activity during this process. Notch plays an important role in the establishment of the equator and in cell proliferation within the eye disc (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopolous et al., 1998). Consequently, in *Psn* mutants, where the *Notch* pathway is inactivated, the eye disc remains small and poorly differentiated (Ye et al., 1999). In contrast to *lgd* mutants, the eye disc is enlarged (Bryant and Schubiger, 1971; data not shown). *lgd; Psn* double mutants resemble the *Psn* mutant, and the eye disc is small (Fig. 8L), suggesting that the *lgd* mutant phenotype in the eye is also caused by overactivity of the *Notch* pathway.

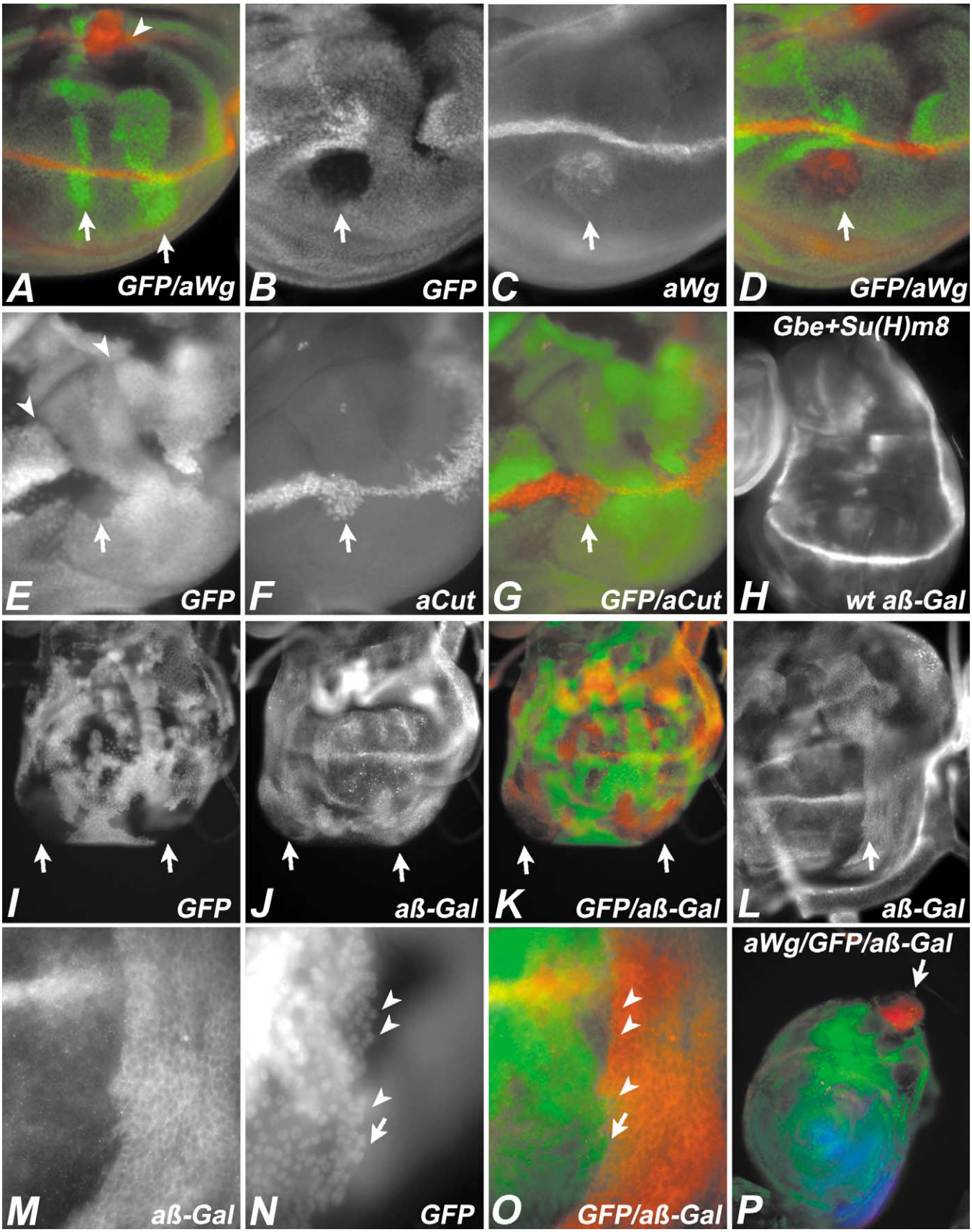
Another process affected by the overactivation of the *Notch* pathway is the development of the wing veins (Lindsley and Zimm, 1992). In flies, where *lgd* mutant clones have been generated, the veins are often interrupted (data not shown). Furthermore, vein formation is strongly affected in *lgd* mutant wing discs as assessed by the expression of *argos*-lacZ (Fig. 9F and G). Although it is not clear that this loss is due to the activation of the *Notch* pathway, the similarity of the phenotype to that of the Ax alleles makes it very likely that this phenotype is caused by overactivation of Notch.

The involvement of *lgd* in regulation of *Notch* activity in these developmental processes and the activation of the Gbe+Su(H)m8 construct in mutant clones outside the wing imply that loss of *lgd* function causes the activation of the *Notch* pathway in many developmental processes and suggest that Lgd might be a more general regulator of the *Notch* pathway during development of the adult fly.

Dpp signalling is not altered in lgd mutant wing imaginal discs

Ectopic expression of the *dpp* gene has been reported to contribute to the phenotype of *lgd* mutant wing discs (Buratovich and Bryant, 1995). In these experiments, expression of *dpp* was monitored with a lacZ-insertion in the *dpp* gene. I have examined the expression of *dpp* in *lgd* mutant discs by in situ hybridisation to see whether the lacZ-

Fig. 5. Clonal analysis of lgd^{d7}. Anterior is to the left; ventral to the bottom. Clones in (A–D) were induced with an hsFlp construct during the first (24–48 h ael) (A) or second larval instar stage (48–72 h ael) (B–G). In wing pouches of wing imaginal discs where *lgd* mutant clones were induced during the first larval instar, in most cases only the wild type twin clone survived. The wild type twin clone is recognizable because of its two copies of the GFP marker (see arrows). In many discs where clones were induced during later stages, scars were found (see arrows in E), suggesting that the mutant pouch cells undergo apoptosis. *lgd* mutant clones could be recovered outside the blade, e.g., in the hinge region (arrowhead), indicating that the cell death upon of loss of *lgd* function is not a general effect. (B–G) Mutant clones could be recovered if they were induced during second larval instar. (B) Clones of lgd^{d7} revealed by the absence of the GFP marker. Arrow highlights a clone in the wing pouch that does not include the D/V boundary. (C) Expression of Wg in the same disc indicating the activation of Wg expression in the clone highlighted by the arrow. (D) Pseudocolour image of the Wg and GFP channel revealing that activation of Wg expression is restricted to the mutant cells of the clone. (E–G) Cut expression in *lgd* mutant clones revealed by anti-Cut antibody staining. (E) Clones revealed by the absence of the GFP marker. (F) Channel showing the expression of Cut in this disc. (G) Pseudocolour image of the Cut and GFP channels shown in (E and F) revealing that Cut expression is autonomously activated in the cells of the mutant clone (arrows in E–G). (H) Expression of the Gbe+E(spl)m8-lacZ construct in a wild type wing imaginal disc of the late third larval instar stage. (I–O) Expression of the Gbe+E(spl)m8-lacZ construct in *lgd* mutant clones. *lgd* mutant areas were induced with *vg*Gal4 driving UASFlp. Discs contain the Gbe+E(spl)m8-lacZ reporter and are stained with anti β-Gal antibody staining. (I) *lgd* mutant territories recognizable by the absence of GFP. (J) Gbe+E(spl)m8-lacZ activity of the same disc. (G) Pseudocolour image of GFP (green) and Gbe+E(spl)m8-lacZ expression (red). Arrows in (I–K) highlight clones that are located outside the wing and strongly express the Gbe+E(spl)m8-lacZ construct. (L) Expression of Gbe+E(spl)m8-lacZ in another wing imaginal disc. Arrow points to a area in the peripodial membrane that ectopically express the Gbe+E(spl)m8-lacZ construct. (M) Higher magnification of the ectopic expression of the Gbe+E(spl)m8-lacZ construct in the peripodial membrane. (N) GFP expression of the disc shown in (L, M) revealing the *lgd* mutant territories by absence of the GFP marker. (O) Pseudocolour image of the channel showing the Gbe+E(spl)m8-lacZ (red) and GFP expression (green). Arrowheads in (N) and (O) highlight wildtypic cells that do express the Gbe+E(spl)m8-lacZ construct. The arrow points to the ventral clones boundary where the autonomy is complete. (P) *lgd* mutant clones in a leg imaginal disc. Disc is double stained for anti-Wg (blue) and anti-β-Gal. Clones are revealed by the absence of the GFP marker. Arrow points to a clone where the Gbe+E(spl)m8-lacZ reporter is strongly activated, indicating that, also in the leg, Notch activation is a consequence of loss of *lgd* function.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C aWg
D GFP/aWg
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F aCut
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Gbe+Su(H)m8
H wt aß-Gal
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L aß-Gal
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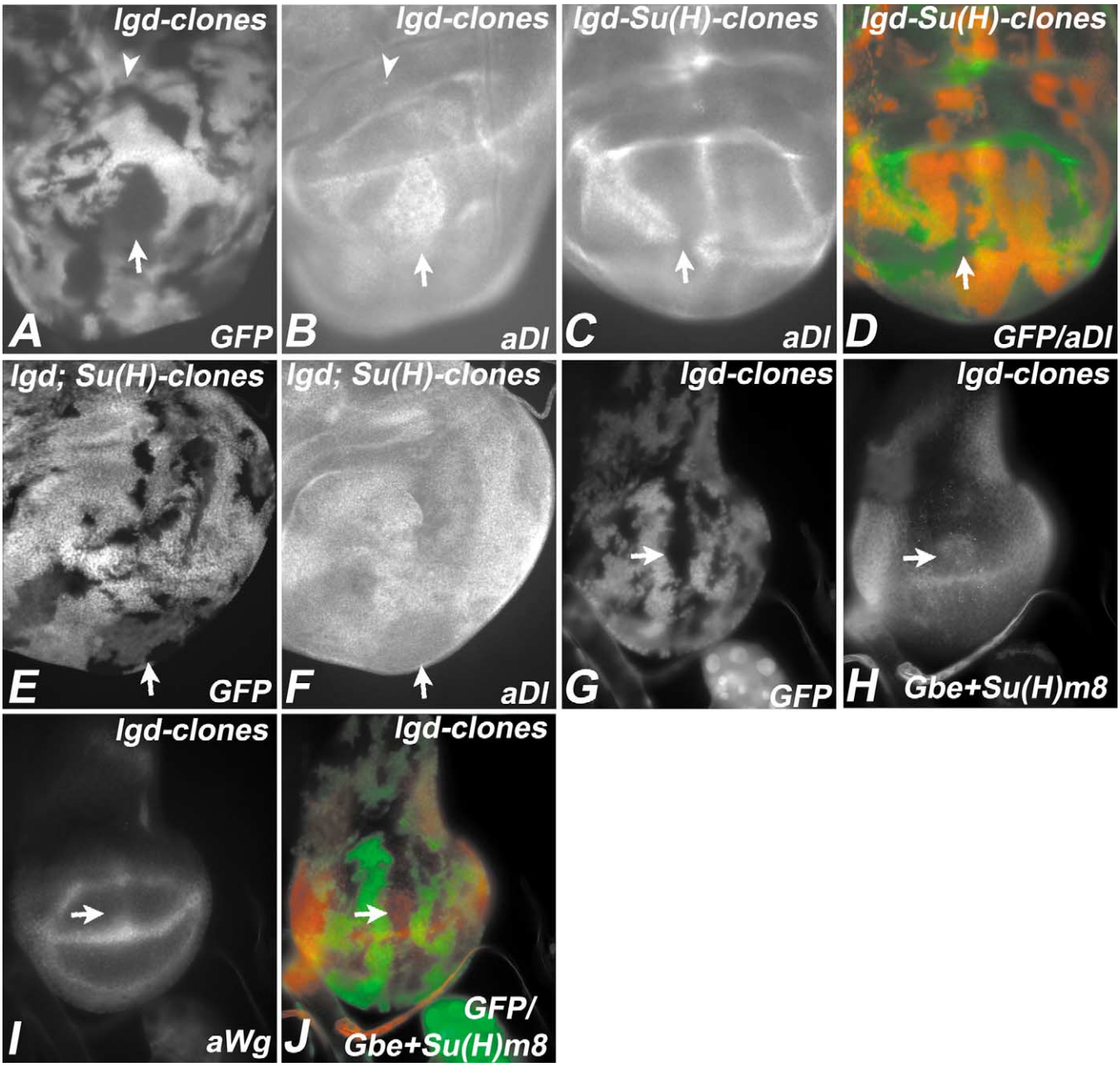

Fig. 6. Clonal analysis of lgd^{d7} mutants continued. Anterior is to the left, dorsal is to the top. (A) *lgd* mutant clones revealed by the absence of the GFP marker. (B) Expression of Dl in the same disc as in (A). Dl expression is detected by anti-Dl antibody staining. Arrows in (A) and (B) point to a large clone that expresses Dl ectopically. Arrowhead points to a part of a dorsal clone that is located in the hinge region and does not express Dl. This suggests that activation of Dl expression is not always a consequence of loss of *lgd* function. (C) Expression of Dl in a disc bearing $lgd^{d7}/Su(H)^{SF8}$ double mutant clones. (D) Pseudocolour image of the same disc as in (C) showing Dl expression in green and GFP expression in red. The image reveals that Dl expression is not expanded in the double mutant clones. Arrow in (C, D) highlights a clone that includes parts of the D/V boundary. (E, F) $Su(H)^{SF8}$ mutant clones in a *lgd* mutant wing disc. (E) Clones revealed by the absence of the GFP marker. (F) Expression of Dl in the same disc as in (E). The comparison of (E) and (F) reveals that the expression of Dl is reduced or abolished in many of the mutant areas of the disc. Dl expression is not abolished in all mutant regions, since it is also controlled by other inputs that are not affected by the loss of *lgd* function. (G, H) *lgd* mutant clones in a wing imaginal disc of the early third larval instar stage. (G) Clones marked by the absence of the GFP marker. (H) Expression of the Gbe+E(spl)m8-lacZ construct in the same disc as in (G). (I) Wg expression in the same the disc as shown in (G). Comparison with (H) reveals that the Gbe+E(spl)m8-lacZ construct is expressed in more mutant cells and further away from the D/V boundary than *wg*. (J) Pseudocolour image of the channel showing the expression of the Gbe+E(spl)m8-lacZ construct (red) and GFP (green). Arrow in (G–J) points to a large dorsal clone.

insertion might reflect the expression of *dpp* incorrectly (Fig. 9A–C). I observed a weak expression of *dpp* that seems to lie in the anterior compartment of the disc, similar to that which has been reported by Buratovich and Bryant using the P-lcaZ insertion line (Fig. 9A–C). However, closer examination revealed that this stripe is located in the peri-

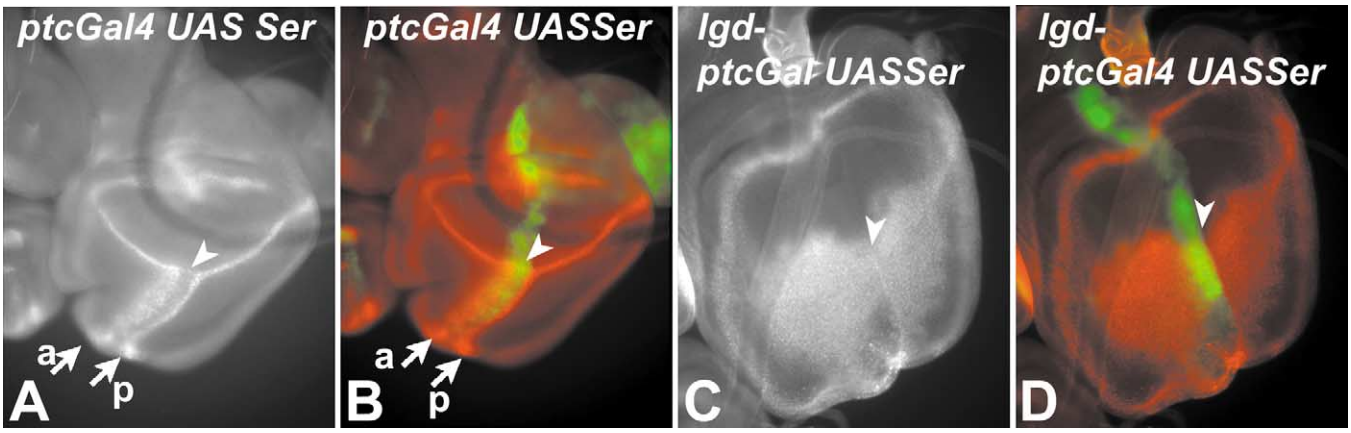

Fig. 7. Suppression of the negative effects of strong *Ser* expression in lgd^{d7} mutant wing discs. Anterior is to the left, dorsal to the top. UAS constructs in (A–D) are expressed with *ptc*Gal4. (A, B) Expression of UAS*Ser* during wild type development. (A) Expression of Wg revealed by antibody staining. The expression of UAS*Ser* induces ectopic expression of Wg in two stripes in the ventral side of the wing (arrows in A and B). The posterior stripe is one cell diameter in width. Between the two stripes, in the region at the D/V boundary where the *ptc* domain meets the D/V-boundary (arrowhead), the expression of Wg and all other Notch-regulated genes is interrupted due to negative effects associated with high *Ser* expression (Klein et al., 1997; de Celis and Bray, 1997; Miccheli et al., 1997). (B) Pseudocolour composite showing the expression domain of *ptc*Gal4 (green) in relation to Wg (red). It reveals that the ectopic posterior stripe of Wg expression is located outside the *ptc* domain in the posterior compartment (p in A and B). (C, D) The same experiment as described in (A, B), performed in a lgd^{d7} mutant disc. (C) Expression of Wg. (D) Pseudocolour composite revealing the *ptc*Gal4 expression domain (green) relative to the expression of Wg. The expression of Wg at the D/V-boundary is not interrupted (arrowheads in C and D), suggesting that the dominant negative effect caused by high *Ser* expression is suppressed in *lgd* mutant discs.

podial membrane (arrow in Fig. 9C), and it is likely that this "ectopic" domain is the normal expression domain of *dpp* in the peripodial membrane that is visible in the mutant due to a slightly stronger expression (compare Fig. 9A with 9B and C). In contrast, expression of *dpp* in the wing pouch seems weaker than in normal discs (Fig. 9B and C), and a weaker expression in the pouch is also observed with *dpp*-lacZ (Buratovitch and Bryant, 1995). I further found that the expression of the gene *spalt (sal)*, which is a target of the dpp signalling pathway, is not changed in *lgd* mutant discs (Fig. 9E). This suggests that *dpp* activity is normal in *lgd* mutant wing discs. Thus, ectopic *dpp* expression or overactivity of *dpp* does not appear to contribute to the phenotype caused by the loss of *lgd* function.

Discussion

The *Notch* pathway is an evolutionary conserved signalling pathway that is involved in a plethora of different developmental and pathological processes. Hence, it is important to gather more information about its different modes of regulation. So far, only little information is available about genes that are involved in its regulation. Here, I provide evidence that suggests that the tumour suppressor gene *lgd* is a negative regulator of the *Notch*-pathway. I show that all tested *Notch*-target genes are ectopically activated in *lgd* mutant wing discs or *lgd* mutant cell clones. The ectopic activation of Notch target genes as well as the observed overproliferation of *lgd* mutants is abolished in *lgd; Psn* double mutants. In addition, Notch target gene expression is also abolished in *Psn* or *Su(H)* mutant clones generated in *lgd* mutant wing imaginal discs. These data suggest that the Notch pathway becomes ectopically active in the absence of *lgd* function. Furthermore, the fact that Dl alone seems to provide sufficient *Notch* activity to sustain wing development in *lgd* mutants indicates that the pathway can be activated more efficiently in the mutant background. I show here that the activation of Notch is a consequence of loss of *lgd* function also in other developmental processes, such as bristle, leg, and wing vein development. Thus, the presented data make *lgd* a good candidate gene that regulates activity of the *Notch* pathway during adult development of *Drosophila.*

Although most aspects of the mutant phenotype of *lgd* mutants can be explained by the inappropriate activation of the *Notch* pathway, the cell death observed during induction of *lgd* mutant clones has not been observed if activated forms of Notch are expressed in the wing pouch or in gain-of-function mutants of *Notch*, such as *Ax*. These facts would suggest that *lgd* function might also have another function for cell viability that is separable from its role in the regulation of *Notch* activity. However, a recent paper by Milan et al. (2002) reports that inappropriate activation of the *Notch* pathway elicits apoptosis in wing pouch cells under certain circumstances. Hence, it is also possible that this aspect of the *lgd* mutant phenotype is a consequence of *Notch* activation.

The clonal analysis of *lgd* revealed several interesting effects. One effect is that Notch becomes activated at the boundary of *Dl Ser* double mutant cell clones. At the moment, it is not clear how this activation is achieved. A likely

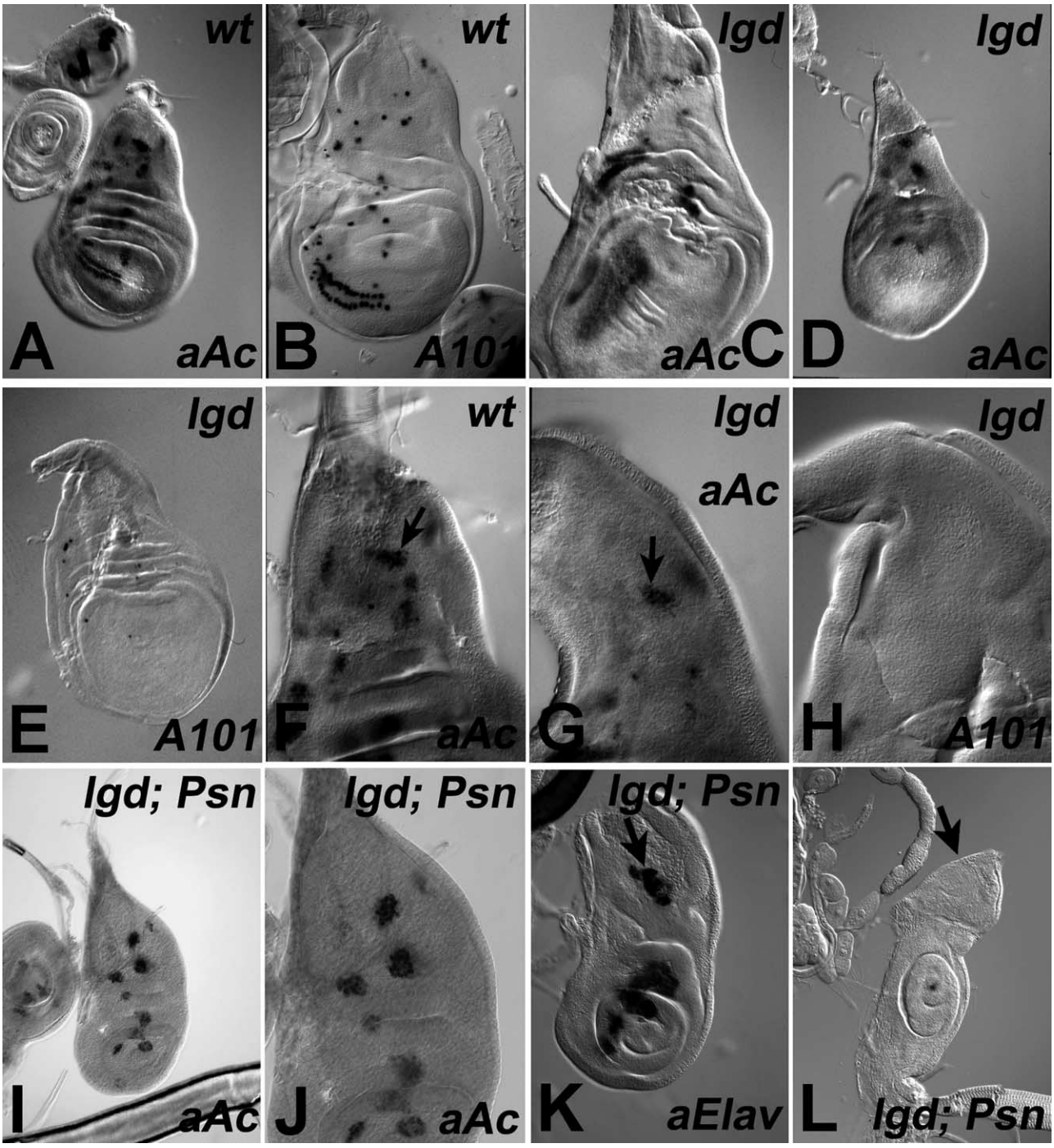

Fig. 8. Involvement of *lgd* in other Notch-related developmental processes. (A–L) Development of the adult PNS in lgd^{d7} mutants. Expression of the proneural gene *achaete* (*ac*) in *lgd* mutant and wild type wing discs is detected by anti Ac antibody staining. The A101-lacZ insertion reveals the sensory organ precursors (SOP). (A) Expression of Ac in a third larval instar stage wild type disc revealing the proneural clusters. Single cells in a cluster accumulate high amounts of *ac* protein and eventually develop as SOPs. (B) Expression of A101 labels the SOP at the end of the third instar stage. (C, D) Ac expression in a late (C) and early (D) third instar lgd^{d7} wing disc. Expression of Ac is still detectable in clusters and most of the clusters are present. (E) Expression of A101 in lgd^{d7} wing discs reveals that almost all SOPs fail to develop. (F, G) Expression of Ac in the notal area of wt (F) and lgd^{d7} (G) late third larval instar stage wing imaginal discs. The arrow points to the proneural cluster that gives rise to the dorsocentral machrochaete. The expression of Ac in *lgd* mutants seems to be weaker than the wild type (shown in F). As a consequence, most of the SMCs fail to develop in the *lgd* mutant (H). (I–K) Analysis of SOP development in lgd^{d7}, Psn^{B3}/Psn^{12} mutant wing discs. (I–K) Anti-Ac antibody staining. In contrast to lgd^{d7} mutant discs, the double mutant wing discs have big Ac-positive cells, and many of these cells express the Elav protein as revealed by anti-Elav-staining (K). The detection of a cluster of Elav-positive cells (arrow) shows that the cells of the proneural clusters have differentiated into neurons and therefore confirm the neurogenic phenotype of the double mutant discs. The data suggest that the antineurogenic phenotype observed in *lgd* mutants is mediated through the activity of the *Notch* pathway. (L) An eye-antennal disc of a *lgd; Psn* double mutant. The area of the eye disc is strongly reduced and is similar to *Psn* mutant discs (Ye et al., 1999). In contrast, the eye discs of *lgd* mutants are enlarged (data not shown; Bryant and Schubiger, 1971). The results suggest that *lgd* is required also during bristle and eye development to negatively regulate the activity of Notch.

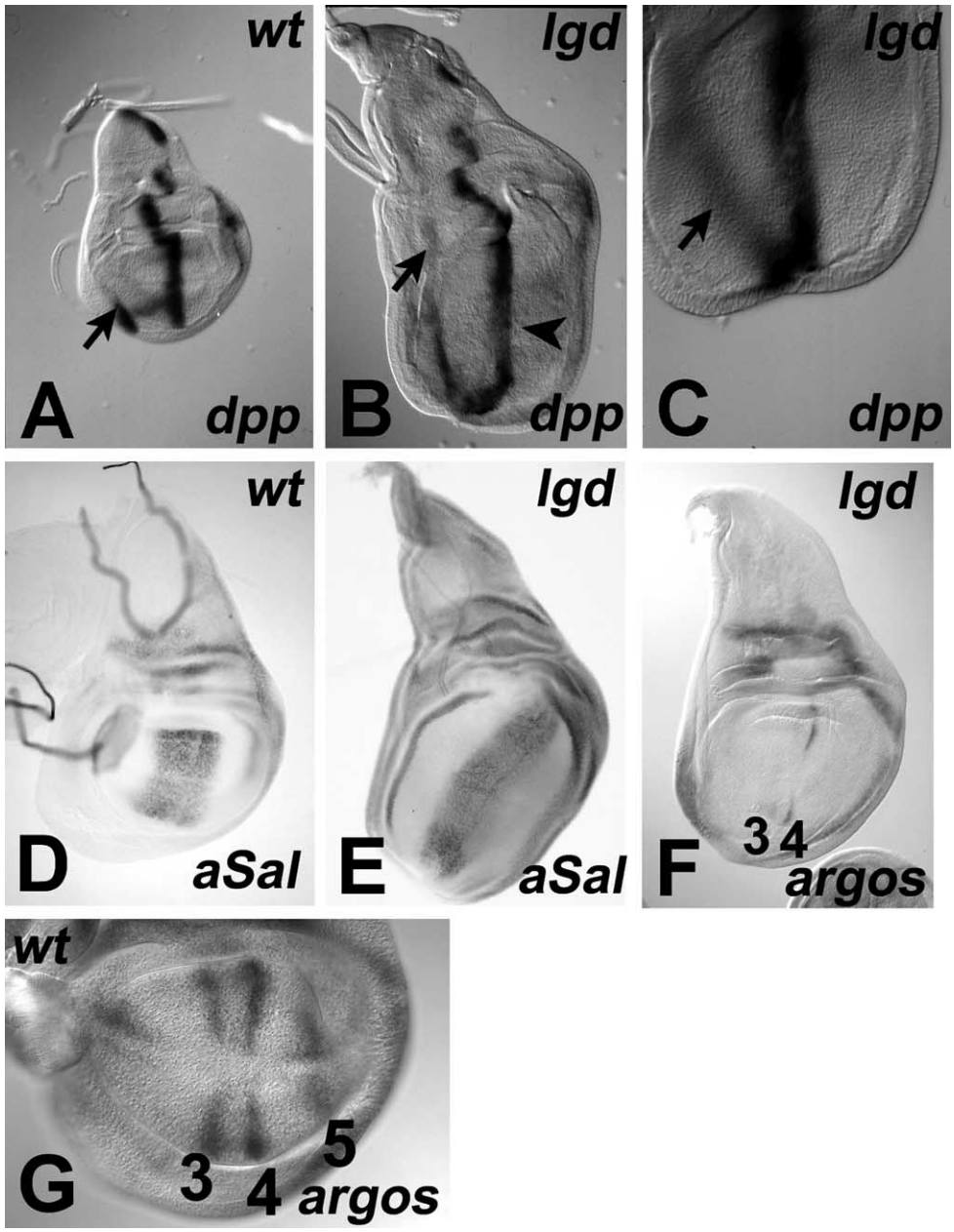

Fig. 9. Expression of *dpp* in *lgd* mutant wing discs detected by *in situ* hybridization. (A) Expression of *dpp* in a normal wing imaginal disc. It is expressed in a stripe along the anteroposterior compartment boundary. The arrow points to the region where the expression of *dpp* expands into the peripodial membrane. (B) Expression of *dpp* in a lgd^{d7} mutant disc. The expression in the pouch is weaker in some regions of the expanded wing pouch (arrowhead). The arrow points to the putative "ectopic" expression domain of *dpp*. As visible in (C), this expression domain is not in the same focal plane as the expression domain in the pouch, but is in the overlying peripodial membrane (arrow). (D, E) Expression of Spalt (Sal) in wt and *lgd* mutant wing discs detected by anti-Sal antibody staining. (D) During normal development, Sal is expressed in a block in the middle of the wing pouch. The extent of its expression is dependent on a gradient of Dpp activity, which has its source at the A/P boundary (see A, B). (E) The expression of Sal in a lgd^{d7} mutant wing disc. The comparison with (D) reveals that there is no change in the extent of Sal expression in the mutant, indicating that the activity of Dpp has not changed. (F) Expression of *argos*-lacZ in a lgd^{d7} mutant wing disc. See (G) for comparison with the expression in wild type. The expression in the anlage of veins 3 and 4 is strongly reduced and that of the fifth vein is missing, indicating the suppression of vein formation. (G) Expression of *argos*-lacZ in a wild type late third instar disc for comparison with (F). The numbers label the anlagen of wing veins 3–5. For further details, see text.

explanation is that activation of Notch at the clone boundaries is caused by the removal of the negative effects of strong Dl and Ser expression observed during late wing development (Micchelli et al., 1997). During normal development, Dl and Ser are expressed in a dorsal and ventral band of cells adjacent to the cells at the D/V boundary in later stages of the third larval instar. Both ligands signal from there to the cells at the boundary to maintain expres-

sion of Wg and other genes. It has been shown that activation of Notch is blocked in the cells expressing the ligands because of their autonomous inhibitory effect on Notch signalling at high concentrations (de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997). Loss of *Dl* and *Ser* expression leads to the loss of the suppressive effect, and the mutant cells at the clone boundary activate expression of *Notch* target genes (Micchelli et al., 1997). In *lgd* mutants, the expression domains of *Dl* and *Ser* are expanded and the pathway can be activated more efficiently. Thus, the effect of activation of Notch at the boundary of *Ser/Dl* double mutant clones should also be comparably enhanced.

The analysis of the *lgd* mutant clones suggests that *lgd* acts in a cell-autonomous way. However, this autonomy is not complete, and in some cases, *Notch* target genes are activated in wild type cells at the boundary of *lgd* mutant clones. An explanation for this observation is the fact that the activation of Notch results in the expression of the ligands Dl or Ser. Clones of wing pouch cells expressing the activated form of Notch, Nintra, also activate Notch target gene expression in cells outside the clone, indicating a nonautonomous behaviour of Nintra in this cases (de Celis and Bray, 1997). This nonautonomous behaviour is caused by the induction of the expression of the Notch ligands (de Celis and Bray, 1997). The nonautonomy of Nintra is not observed in all situations. For example, if UAS *Nintra* is expressed with *ptc*Gal4, activation of *Notch* target genes is cell-autonomous, although induction of ligand expression is observed (unpublished observation). Hence, the nonautonomous activation of *Notch* target genes by Nintra is dependent on other criteria, such as the level of expression or the time span of signalling. It is likely that the observed weak nonautonomy of *lgd* in clones is caused by the activation of expression of *Dl* and *Ser* close to threshold levels of activity that are required to activate Notch in some cells outside the clone.

Several explanations of how the *Notch* pathway is activated in *lgd* mutants are possible. A very simple one would be that the expansion of *Notch* target genes in *lgd* mutant clones or wing discs is caused by an overproliferation of the mutant cells that cause an expansion of the expression domains of the *Notch* target genes. Thus, the effects on *Notch* signalling would be a secondary effect. However, clones that are located in the wing pouch and do not have any contact with the normal domain of *Notch* activity at the D/V boundary are able to activate the expression of *Notch* target genes, indicating that the pathway is activated de novo. Furthermore, Notch is activated in mutant clones of wing discs of the early third instar (see Fig. 6). These discs do not show any visible overproliferation. Hence, it is very likely that the expansion of the target gene expression is not caused by a secondary effect, such as cell proliferation, but by the activation of the *Notch* pathway.

The expansion of *Notch* activity could also be caused by the loss of the suppressive effect on signalling of high concentrations of the ligands observed in the *lgd* mutants. Although this mode of regulation is important during the second half of the third larval instar stage, it cannot account for the ectopic activation of *Notch* targets in earlier wing discs observed here.

Lgd could act in a parallel pathway that is required to restrict the activation of the target genes by Notch. An example of this is the Nubbin transcription factor that seems to bind to the regulatory region of at least some Notch target genes and represses their expression away from the D/V boundary (Neumann and Cohen, 1998). Lgd could act in a similar way. However, there are important differences in the behaviour of *nub* and *lgd* mutants. *nub* mutants do not show the overproliferation of the imaginal discs seen in *lgd* mutants and, in contrast to *lgd*, the effects of Nub on *Notch* target gene expression are restricted to the wing. These differences make it unlikely that both genes act in the same pathway. In agreement with these conclusions, I found that *nub* expression is not affected in *lgd* mutant wing imaginal discs (data not shown).

A further possibility is that Lgd could modulate the effectiveness of the *Notch* signal, e.g., by creating a threshold for *Notch* activity required for activation of the target genes or influencing the activity of a selector gene such as Vg for the wing (see, e.g., Guss et al., 2001; Klein and Martinez-Arias, 1999). However, I show that the activity of one target gene of Vg/sd, *spalt*, is not affected in *lgd* mutants, suggesting that the activity of the selector is not affected.

The comparison of the *Ax* and *lgd* mutant phenotype reveals a striking similarity: In *Ax* mutant wing discs, as in those of *lgd* mutants, Notch activity expands into the wing pouch (de Celis and Bray, 2000; de Celis et al., 1996b; Ju et al., 2000). In addition, in *Ax* mutant wing discs, the dominant negative activity of the ligands is suppressed in a similar fashion to that observed in *lgd* mutants (de Celis and Bray, 2000; Ju et al., 2000). The phenotype of both of these mutants requires the activity of the *Notch* ligands (Heitzler et al., 1996); this work). Furthermore, in both mutants, the cell-autonomous suppressive effect of Fng on *Notch* signalling is strongly suppressed (see Supplementary Material). Finally, the development of the veins and SOPs is suppressed in both mutants (Heitzler et al., 1996; this work). The similarity of the phenotypes between *lgd* and *Ax* mutants raises the possibility that they are based by the interruption of the same process required to negatively regulate *Notch* activity. One argument against this conclusion is that the phenotype of the lgd^{d7}, Ax^{M1} double mutant wing discs described here is synergistic (see above). This suggests that the genes do not act in the same regulatory mechanism. The problem with this argument is that it is not clear whether any of the known *Ax* mutations are abolishing the affected function completely and thus does not rule out the possibility that *lgd* and *Ax* affect the same regulatory pathway.

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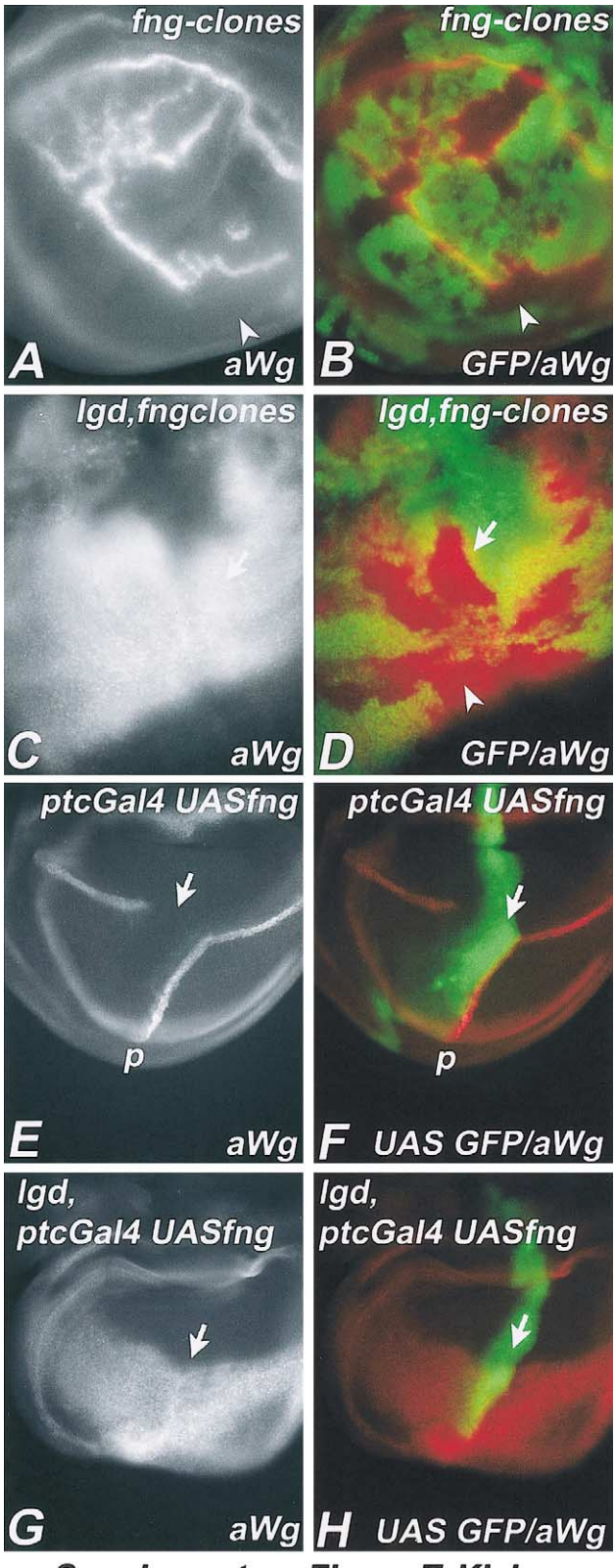
Appendix

Supplementary material:

A boundary of fng expressing and nonexpressing cells is not required for Notch activation in lgd mutant wing discs

The activation of the Notch pathway in the wing along the D/V boundary depends on the presence of a boundary between cells that express and cells that do not express the Fng protein (Irvine and Wieschaus, 1994; Kim et al., 1996; Klein and Martinez-Arias, 1998; Panin et al., 1997). Consistent with this model, expression of UAS*fng* with *ptc*Gal4 interrupts the expression of Notch-dependent genes along the D/V boundary and induces a new domain of expression along the posterior end of the *ptc* domain, where cells expressing high levels of Fng are juxtaposed to nonexpressing cells (Kim et al., 1995; see supplementary Fig. E and F). In contrast, performing the same experiment in *lgd* mutant discs, Fng does not interrupt the expression of *wg* at the D/V boundary (supplementary Fig. G and H). This raises the possibility that establishment of a distinct boundary of cells that express *fng* and those that do not is not necessary in *lgd* mutant wing discs. To further confirm this conclusion, I have expressed UAS *fng* throughout the wing blade with *sd*Gal4 to remove a sharp expression boundary of *fng* throughout wing development. Expression of UAS*fng* in this way during normal development results in the loss of the wing blade and distal hinge (Klein and Martinez-Arias, 1998). However, in *lgd* mutant discs, the expression of UAS *fng* by *sd*G4 has little effect on wing development, and the disc develops a wing blade similar to that of *lgd* mutants (data not shown). This result supports the conclusion that a sharp boundary between fng-expressing and nonexpressing cells is not required in *lgd* mutant wing discs for wing development. To find more evidence for this conclusion, fng^{13} mutant clones were induced in *lgd* mutant wing discs (supplementary Fig. A–D). Dorsal clones induced by *sd*Gal4 UAS FLP in wild type wing discs lead to the ectopic activation of the Notch pathway and the activation of *wg* expression at the clone boundaries (supplementary Fig. A and B; Kim et al., 1995). Mutant clones located in the ventral half of the pouch have no effect since *fng* is not expressed there during early development, and hence no ectopic boundary of *fng*-expressing and nonexpressing cells is



Supplementary Figure T. Klein

generated. In *lgd* mutant wing discs, *fng* mutant clones, which do not include the D/V boundary, behave like the clones in wild type discs (supplementary Fig. D) and *wg* expression is activated at the clonal boundaries in the dorsal half of the blade. However, unlike in the wild type, dorsal clones that are located within the expanded expression domain lead only to a weakening of *wg* expression in the centre of the clone but do not result in a loss of *wg* expression like in the wild type (see dorsal arrow in supplementary Fig. C and D). This result suggests that, in *lgd* mutant wing pouches, *wg* expression can be induced by Notch in the absence of Fng. Furthermore, clones that cross the D/V boundary do not lead to an interruption of *wg* expression at the D/V boundary within the mutant area (arrowhead in supplementary Fig. D), and clones that include parts of the ventral half of the expanded domain do not affect Wg expression at all, indicating that Fng has no function in the regulation of the ventral half of the expanded domain of Notch target genes. Altogether, the clonal analysis of fng^{13} confirms that, in the absence of Lgd, a boundary of *fng*-expressing and nonexpressing cells is not necessary for activation of Notch. Nevertheless, an ectopic boundary of Fng-expressing and nonexpressing cells can activate Notch.

Supplementary figure legend:

(A–D) Analysis of *fng* mutant clones in lgd^{d7} mutant wing discs. Clones were induced by using the amorphic fng^{13} allele (Irvine and Wieschaus, 1994) and activation of UAS FLP with *sd*Gal4. They are labelled by the absence of the GFP fluorescence. Discs are stained by anti-Wg antibody staining, shown in red (A, B) Induction of *fng* mutant clones in a wild type wing disc. (A) Wg expression. (B) Pseudocolour image revealing Wg (red) and mutant clones by the loss of the GFP marker (green). Expression of Wg is induced at the boundaries of dorsal clones. Arrowhead in (A, B) highlights a clone that crosses the D/V boundary. Expression of Wg along the D/V boundary is interrupted in the centre of these clones. (C, D) Induction of *fng* mutant clones in lgd^{d7} mutant wing imaginal discs. (C) Wg expression. (D) Pseudocolour image of the Wg staining (red) and clones revealed by loss of the GFP marker (green). As in the wild type, the dorsal clones induce Wg expression at their boundaries. However, clones that cross the D/V boundary (see arrowhead in D) do not interrupt expression of Wg along the D/V boundary. Furthermore, clones in the area of the expanded domain of *wg* expression do not loose expression completely (dorsal arrow in C, D). (E, F) Expression of UAS *fng* with *ptc*Gal4 during wild type development. (E) Wg expression. (F) Expression of *ptc*Gal4 relative to Wg. Wg expression (red) is interrupted where the *ptc* domain (green) crosses the D/V boundary (arrow in E, F). (p) highlights the ectopic stripe of *wg* expression at the posterior expression boundary of *fng*. (G, H) The same experiment as described in (E, F) now performed in lgd^{d7} mutant discs. (G) Wg expression. (H) Pseudocolour image showing Wg expression in red and *ptc*Gal4 expression revealed by the fluorescence of the UAS GFP construct in green. No loss of Wg expression (red) is observed in the region where *ptc* expression (green) overlaps with Wg expression (red) (arrow in G, H), indicating that the negative effects of strong *fng* expression observed in the wild type are suppressed if *lgd* is lost.

References

Barrio, R., deCelis, J.F., Bolshakov, S., Kafatos, F., 1999. Identification of regulatory regions driving the expression of the Drosophila spalt complex at different developmental stages. Dev. Biol. 215, 33–47.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R.A., Israel, A., 2000. A novel proteolytic cleavage involved in notch signaling: the role of the disintegrin–metalloprotease TACE. Mol. Cell 5, 207–216.

Brückner, K., Perez, L., Clausen, H., Cohen, S., 2000. Glycosyltransferase activity of Fringe modulates Notch–Delta interactions. Nature 406, 411–415.

Bryant, P.J., Schubiger, G., 1971. Giant and duplicated imaginal discs in a new lethal mutant of Drosophila melanogaster. Dev. Biol. 24, 233–263.

Bryant, P.J., Watson, K.L., Justice, R.W., Woods, D.F., 1993. Tumor suppressor genes encoding proteins required for cell interactions and signal transduction in Drosophila. Dev. Suppl., 239–249.

Buratovich, M.A., Bryant, P.J., 1995. Duplication of l(2)gd imaginal discs in Drosophila is mediated by ectopic expression of wg and dpp. Dev. Biol. 168, 452–463.

Cho, K.-O., Choi, K.-W., 1998. Fringe is essential for morror symmetry and morphogenesis in the Drosophila eye. Nature 396, 272–276.

Couso, J.P., Knust, E., Martinez Arias, A., 1995. Serrate and wingless cooperate to induce vestigial gene expression and wing formation in Drosophila. Curr. Biol. 5, 1437–1448.

Couso, J.P., Martinez Arias, A., 1994. Notch is required for wingless signaling in the epidermis of Drosophila. Cell 79, 259–272.

de Celis, J.F., Bray, S., 1997. Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. Development 124, 3241–3251.

de Celis, J.F., Bray, S.J., 2000. The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe. Development 127, 1291–1302.

de Celis, J.F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C., Bray, S., 1996a. Functional relationships between Notch, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal development. Development 122, 2719–2728.

de Celis, J.F., Garcia-Bellido, A., Bray, S.J., 1996b. Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. Development 122, 359–369.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A., Kopan, R., 1999. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain [see comments]. Nature 398, 518–522.

Diaz-Benjumea, F.J., Cohen, S.M., 1995. Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. Development 121, 4215–4225.

Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1996. Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in Drosophila wing formation. Genes Dev. 10, 421–434.

Domínguez, M., de Celis, J.F., 1998. A dorsal/ventral boundary established by Notch controls growth and polarity in the Drosophila eye. Nature 396, 276–278.

Duffy, J.B., Harrison, D.A., Perrimon, N., 1998. Identifying loci required for follicular patterning using directed mosaics. Development 125, 2263–2271.

Fleming, R.J., Gu, Y., Hukriede, N.A., 1997. Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. Development 124, 2973–2981.

Fortini, M.E., Artavanis-Tsakonas, S., 1994. The suppressor of hairless protein participates in notch receptor signaling. Cell 79, 273–282.

Furriols, M., Bray, S., 2001. A model response element detects Suppressor of Hairless-dependent molecular switch. Curr. Biol. 11, 60–64.

Girard, L., Hanna, Z., Beaulieu, N., Hoemann, C.D., Simard, C., Kozak, C.A., Jolicoeur, P., 1996. Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. Genes Dev. 10, 1930–1944.

Guss, K.A., Nelson, C.E., Hudson, A., Kraus, M.E., Carroll, S.B., 2001. Control of a genetic regulatory network by a selector gene. Science 292, 1164–1167.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C., Simpson, P., 1996. Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. Development 122, 161–171.

Heitzler, P., Simpson, P., 1993. Altered epidermal growth factor-like sequences provide evidence for a role of Notch as a receptor in cell fate decisions. Development 117, 1113–1123.

Huang, F., Dambly-Chaudière, C., Ghysen, A., 1991. The emergence of sense organs in the wing disc of Drosophila. Development 111, 1087–1095.

Hunter, T., 1997. Oncoprotein networks. Cell 88, 333–346.

Irvine, K.D., Wieschaus, E., 1994. fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. Cell 79, 595–606.

Jönsson, F.K.E., 1996. Distinct functions of the drosophila genes Serrate and Delta revealed by ectopic expression during wing development. Dev. Genes Evol. 206, 91–101.

Ju, B.-G., Jeong, S., Bae, E., Hyun, S., Carroll, S.B., Yim, J., Klm, J., 2000. Fringe forms a complex with Notch. Nature 405, 191–195.

Kim, J., Irvine, K.D., Carroll, S.B., 1995. Cell recognition, signal induction, and symmetrical gene activation at the dorsal–ventral boundary of the developing Drosophila wing. Cell 82, 795–802.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., Carroll, S. B., 1996. Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. Nature 382, 133–138.

Klein, T., 2001. Wing disc development in the fly: the early stages. Curr. Opin. Genet. Dev. 11, 470–475.

Klein, T., Brennan, K., Martinez-Arias, A.M., 1997. An intrinsic dominant negative activity of serrate that is modulated during wing development in Drosophila. Dev. Biol. 189, 123–34.

Klein, T., Martinez-Arias, A.M., 1998. Interactions among Delta, Serrate and Fringe modulate Notch activity during Drosophila wing development. Development 125, 2951–2962.

Klein, T., Martinez-Arias, A.M., 1999. The vestigial gene product provides a molecular context for the interpretation of signals during the development of the wing in Drosophila. Development 126, 913–925.

Klein, T., Seugnet, L., Haenlin, M., Martinez-Arias, A., 2000. Two different activities of Suppressor of Hairless during wing development in Drosophila. Development 127, 3553–3566.

Lawrence, N., Klein, T., Brennan, K., Martinez Arias, A., 2000. Structural requirements for notch signalling with delta and serrate during the development and patterning of the wing disc of Drosophila. Development 127, 3185–3195.

Lecourtois, M., Schweisguth, F., 1995. The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. Genes Dev. 9, 2598–2608.

Lecourtois, M., Schweisguth, F., 1998. Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. Curr. Biol. 8, 771–774.

Lindsley, P., Zimm, G.G., 1992. The Genome of *Drosophila melanogaster*. Academic Press, San Diego.

Lukinova, N.I., Roussakova, V.V., Fortini, M.E., 1999. Genetic characterization of cytological region 77A-D harboring the *Presinillin* gene of *Drosophila melanogaster*. Genetics 153, 1789–1797.

Miccheli, C.A., Blair, S.S., 1999. Dorsoventral restriction in wing imaginal discs requires Notch. Nature 401, 473–476.

Micchelli, C.A., Rulifson, E.J., Blair, S.S., 1997. The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. Development 124, 1485–1495.

Milan, M., Perez, L., Cohen, S.M., 2002. Short-range cell interactions and cell survival in the *Drosophila* wing. Dev. Cell, 797–805.

Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K.D., Haltiwanger, R.S., Vogt, T.F., 2000. Fringe is a glycosyltransferase that modifies Notch. Nature 406, 369–375.

Mumm, J.S., Schroeter, E.H., Saxena, M.S., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., Kopan, R., 2000. A ligand-induced extracellular cleavage regulates y-secretase-like proteolytic activation of Notch. Mol. Cell 5, 197–206.

Munro, S., Freeman, M., 2000. The Notch signalling regulator Fringe acts in the Golgi apparatus and requires the glykosyltransferase signature motif DxD. Curr. Biol. 10, 813–820.

Neumann, C.J., Cohen, S.M., 1997. Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. Development 124, 871–880.

Neumann, C.J., Cohen, S.M., 1998. Boundary formation in Drosophila wing: Notch activity attenuated by the POU protein Nubbin. Science 281, 409–413.

Panin, V.M., Papayannopoulos, V., Wilson, R., Irvine, K.D., 1997. Fringe modulates Notch-ligand interactions. Nature 387, 908–912.

Papayannopolous, V., Tomlinson, A., Panin, V.M., Rauskolb, C., Irvine, K.D., 1998. Dorsal–ventral signalling in the Drosophila eye. Science 281, 2031–2034.

Schroeter, E.H., Kisslinger, J.A., Kopan, R., 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain [see comments]. Nature 393, 382–386.

Skeath, J.B., Carroll, S.B., 1991. Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. Genes Dev. 5, 984–995.

Speicher, S.A., Thomas, U., Hinz, U., Knust, E., 1994. The Serrate locus of Drosophila and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. Development 120, 535–544.

Struhl, G., Adachi, A., 1998. Nuclear access and action of notch in vivo. Cell 93, 649–606.

Struhl, G., Greenwald, I., 1999. Presenilin is required for activity and nuclear access of Notch in Drosophila [see comments]. Nature 398, 522–525.

Tautz, D., Pfeifle, C., 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals

translational control of the segmentation gene hunchback. Chromosoma 98, 81–85.
Thomas, U., Jönsson, F., Speicher, S.A., Knust, E., 1995. Phenotypic and molecular characterization of SerD, a dominant allele of the Drosophila gene Serrate. Genetics 139, 203–213.
Watson, K.L., Justice, R.W., Bryant, P.J., 1994. Drosophila in cancer research: the first fifty tumor suppressor genes. J. Cell Sci. Suppl. 18, 19–33.
Wilder, E.L., Perrimon, N., 1995. Dual functions of wingless in the Drosophila leg imaginal disc. Development 121, 477–488.
Williams, J.A., Bell, J.B., Carroll, S.B., 1991. Control of Drosophila wing and haltere development by the nuclear vestigial gene product. Genes Dev. 5, 2481–2495.
Ye, Y., Lukinova, N., Fortini, M.E., 1999. Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants [see comments]. Nature 398, 525–529.
Yeh, E., Gustafson, K., Boulianne, G.L., 1995. Green fluorescent protein as a vital marker and reporter of gene expression in Drosophila. Proc. Natl. Acad. Sci. USA 92, 7036–7040.