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A re-evaluation of the contributions of Apterous and Notch to the dorsoventral lineage restriction boundary in the *Drosophila* wing

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SUMMARY

The *Drosophila* limb primordia are subdivided into compartments: cell populations that do not mix during development. The wing is subdivided into dorsal (D) and ventral (V) compartments by the activity of the selector gene *apterous* in D cells. Apterous causes segregation of D and V cell populations by at least two distinct mechanisms. The LRR transmembrane proteins Capricious and Tartan are transiently expressed in D cells and contribute to initial segregation of D and V cells. Signaling between D and V cells mediated by Notch and Fringe contributes to the maintenance of the DV affinity boundary. Given that Notch is activated symmetrically, in D and V cells adjacent to the boundary, its role in boundary formation remains somewhat unclear. We re-examine the roles of Apterous and Fringe activities in DV boundary formation and present evidence that Fringe cannot, by itself, generate an affinity difference between D and V cells. Although not sufficient, Fringe is required via Notch activation for expression of an Apterous-dependent affinity difference. We propose that Apterous controls expression of surface proteins that confer an affinity difference in conjunction with activated Notch. Thus, we view Apterous as instructive and Notch activity as essential, but permissive.

Key words: Compartment boundary, Cell affinities, Cell interactions, *fringe*

INTRODUCTION

The *Drosophila* wing primordium is subdivided into adjacent territories called compartments (García-Bellido et al., 1973). Anteroposterior (AP) and dorsoventral (DV) compartment subdivisions are primarily established by the activity of the selector genes *engrailed* in P cells and *apterous* in D cells (reviewed by Blair, 1995). Short-range interactions between adjacent compartments induce expression of the signaling molecules Wingless (Wg) or Decapentaplegic (Dpp) along the compartment boundaries. Wg and Dpp organize pattern and growth of the wing anlage (Diaz-Benjumea and Cohen, 1995; Nellen et al., 1996; Zecca et al., 1996; Lecuit et al., 1996; Neumann and Cohen, 1997). One of the central features of compartments is that the cell populations that comprise them do not mix during development. Intermingling of cells between adjacent compartments has disastrous consequences in patterning and growth of the wing primordium (Milán and Cohen, 1999a).

The onset of *apterous* expression in the early wing primordium induces expression of the Notch ligand Serrate in D cells and restricts expression of Delta, another Notch ligand, to V cells (Diaz-Benjumea and Cohen, 1995; Milán and Cohen, 2000). Dorsally expressed Serrate and ventrally expressed Delta activate Notch symmetrically in cells on both sides of the DV compartment boundary (Fig. 1A) (Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996; Doherty et al., 1996). Expression of the glycosyltransferase Fringe makes D cells more sensitive to Delta and less sensitive to Serrate (Fleming et al., 1997; Panin et al., 1997; Brückner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000). Notch activation induces Wg expression in cells along the DV boundary. Later in development, an increase in dLMO (BX – FlyBase) levels reduces Ap activity in the wing primordium (Milán and Cohen, 2000). At this stage, another set of cell interactions takes over to maintain Wg expression along the DV boundary (Fig. 1B) (de Celis and Bray, 1997; Micchelli et al., 1997). Wg induces expression of Serrate and Delta in nearby D and V cells. Serrate and Delta signal back to activate Notch and thereby maintain Cut and Wg expression along the DV boundary. The persistent low level of Fringe in D cells continues to make D cells more sensitive to Delta and less sensitive to Serrate at this later stage (Milán and Cohen, 2000).

At the time compartments were discovered, it was proposed that lineage restriction along the compartment boundaries depended on compartment specific expression of adhesion molecules that conferred differential cell affinities (García-Bellido et al., 1973). More recent studies have also indicated a role for cell communication at the compartment boundary. Engrailed induces expression of the secreted signaling protein Hedgehog in P cells. Hedgehog acts through Patched and Smoothened to control gene expression in A cells. Hedgehog signaling is needed to maintain segregation of A and P compartments (Blair and Ralston, 1997; Rodriguez and Basler, 1997) can fulfill this function because it is intrinsically asymmetric. Thus, it is easy to understand how Hh signaling

can induce a difference in cell behavior at the AP boundary. The situation at the DV boundary is more complex. The role of Notch signaling is less easy to reconcile with compartment-specific cell segregation, because Notch is activated symmetrically on both sides of the DV boundary. Fringe-dependent Notch signaling has been shown to play a role in segregation of D and V cells (Micchelli and Blair, 1999; Rauskolb et al., 1999). However, we have previously reported that restoring Notch activation along the DV compartment boundary is not sufficient to support the DV boundary under conditions of reduced Apterous activity (Milán and Cohen, 1999a). This indicates the need for an additional Apterous-dependent process that keeps D and V cells apart. The LRR transmembrane proteins Capricious and Tartan are transiently expressed in D cells under Apterous control, at which time they contribute to formation of the boundary between D and V cells (Fig. 1A) (Milán et al., 2001). Their function at the DV boundary is transient and they are subsequently redeployed to produce a difference in affinities between medial and lateral cells (Milán et al., 2002). Thus, maintenance of the DV affinity boundary is independent of Capricious and Tartan. Notch signaling may contribute to this process.

In this report we re-evaluate the roles of Apterous and Notch activation in the DV boundary. Two models have been proposed to explain the roles of Fringe and Notch. According to one view, dorsal expression of Fringe may have Notch-independent functions in the generation of an affinity difference between D and V cells (O'Keefe and Thomas, 2001; Rauskolb et al., 1999). Our findings do not support this view and indicate that the activities of Fringe are mediated through Notch. The second model proposes that Notch activity induces an adhesive state that is qualitatively modulated by Apterous to generate dorsal and ventral boundary states (Blair, 2001; Micchelli and Blair, 1999). This model is based on the proposal that there is a difference in affinity between boundary cells and cells within each compartment (wing blade cells). Our findings do not support this feature of the model. We show that there is no intrinsic affinity difference between boundary cells and wing blade cells within a compartment. Only interactions between D and V cells induce an affinity difference. Instead, we propose that Notch activity cooperates with Apterous to produce an affinity difference between D and V cells. We present a model in which the role of Apterous is instructive and the role of Notch is essential, but permissive.

MATERIALS AND METHODS

Drosophila strains and antibodies

Fly strains

UAS-ap and *UAS-dLMO* (Milán and Cohen, 1999b); *UAS-fng-myc* (Brückner et al., 2000); fng^{13} and *UAS-fng* (Irvine and Wieschaus, 1994); EP-fringe (EP(3)3082, FlyBase) and ap^{Gal4} (Milán and Cohen, 1999a); $Chip^{e55}$ (Morcillo et al., 1997); ap^{UGO35} and ap^{rk568} (referred to as *ap-lacZ*) (Cohen et al., 1992); *en-Gal4* (Fietz et al., 1995); *UAS-*N^{ecd} (Micchelli and Blair, 1999); *UAS-* N^{intra} (Rauskolb et al., 1999); *UAS-Hairless* (Go et al., 1998); *UAS-mastermind*^{DN} (Giraldez et al., 2002); and *actin>CD2>Gal4* (Pignoni and Zipursky, 1997).

Antibodies

Guinea-pig anti-Ap and rat anti-dLMO (Weihe et al., 2001) were used. Other antibodies are commercially available.

Genotypes of larvae used for genetic mosaic analysis

Actin>CD2>Gal4; ap^{rk568} females were crossed to the following males:

hs-FLP; UAS-dLMO;
hs-FLP; UAS-Ap;
hs-FLP; UAS-dLMO UAS-p35;
hs-FLP; UAS-Ap UAS-p35;
hs-FLP; UAS-fng-myc;
hs-FLP; UAS-fng-myc UAS-dLMO;
hs-FLP; $UAS\text{-}N^{ecd}$ *UAS-dLMO*;
hs-FLP; $UAS\text{-}N^{ecd}$ *UAS-Ap*;
hs-FLP; UAS-H UAS-dLMO;
hs-FLP; UAS-H UAS-Ap;
hs-FLP; $UAS\text{-}mam^{DN}$ *UAS-dLMO*;
hs-FLP; $UAS\text{-}mam^{DN}$ *UAS-Ap*;
hs-FLP; UAS-Nintra;
hs-FLP (I); ap^{rk568}*/+;* fng^{13} *FRT80/arm-lacZ FRT80*; and
hs-FLP (I); FRT42 $Chip^{e5.5}$*/FRT42 arm-lacZ.*

To generate clones lacking Ap activity, we made clones lacking the essential co-factor *Chip* or clones expressing the Ap inhibitor dLMO. Such clones are phenotypically equivalent to removing the *ap* gene, but can be generated in larger numbers by use of the FRT and flip-out systems. *ap* is located proximal to the FRT on 2R precluding use of the FRT system to generate large numbers of *ap* mutant clones.

Measurements of clone shapes

Using NIH Image version 1.60, the perimeter (L) and area (A) of the clones were measured. The ratio 4π A/L^2 was used as a measure of the shape of the clones. 4π $A/L^2=1.0$ for a perfect circle. Lower values indicate more irregular shapes. For presentation, 4π A/L^2 numbers were rounded off to one significant digit. *t*-test analysis was carried out to analyze if the shape of mutant or expressing clones differed significantly from control clones.

RESULTS

Relative levels of Fringe and Apterous at the DV affinity boundary

It has been previously reported that Fringe is not sufficient to support the DV boundary under conditions of reduced Apterous activity (Milán and Cohen, 1999a). However, this conclusion has been questioned on the basis of additional experiments (O'Keefe and Thomas, 2001). Both studies made use of a rescue assay in which the Gal4-UAS system was used to restore Fringe expression in D cells of *apterous* (*ap*) mutant wing discs. The fact that similar rescue assays appear to produce different results might be explained by the fact that the two groups used *ap* mutant backgrounds of different strength and produced different levels of Fringe expression in D cells. Three alleles of *ap* were used. ap^{Gal4} is a weak insertional mutant that expresses Gal4 in D cells (Calleja et al., 1996). ap^{rk568} is a stronger insertional mutant that expresses β-gal in D cells (Cohen et al., 1992). ap^{UG035} is a null allele generated by imprecise excision of the rk568 P element, which results in a deletion of the first exon of the *ap* gene (Cohen et al., 1992). Two different transgenes were used to drive *fringe* expression in D cells: *EP(3)3082* is an EP insertion in the *fringe* locus that allows Gal4-dependent induction of *fringe*. *UAS-fringe* is a transgene insertion believed to express higher levels of *fringe* than *EP(3)3082*. Milán and Cohen (Milán and Cohen, 1999a) reported that *fringe* expression using *EP(3)3082* in D cells of the ap^{Gal4}/ap^{UG035} heteroallelic combination was not able to

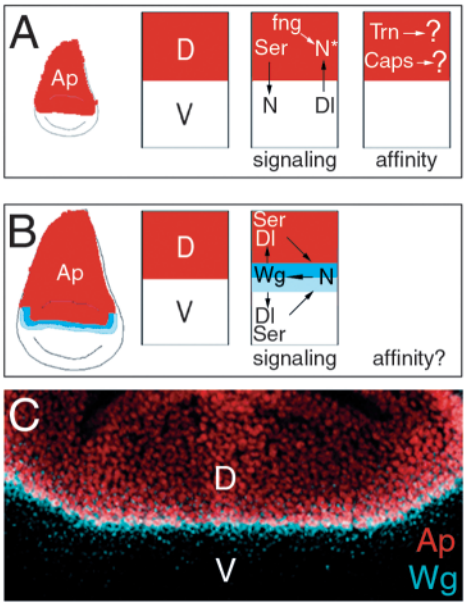

Fig. 1. Cell interactions at the DV boundary. (A) Establishment of the signaling center and the DV affinity boundary. Ap (in red) induces Serrate (Ser) and Fringe (fng) expression in dorsal (D) cells and restricts Delta (Dl) expression to ventral (V) cells. Ser signals to D cells and Dl to V cells to activate Notch along the DV boundary. Fng modifies Notch (N*) in D cells, thus making it sensitive to Dl but not to Serrate. Capricious (Caps) and Tartan (Trn) expression in D cells contribute to establishing the DV affinity boundary. Caps and Trn have been proposed to interact with an unknown partner (indicated by ?) expressed in D cells. (B) Maintenance of the signaling center and the affinity boundary. A positive-feedback loop between Wingless (Wg)-expressing cells along the DV boundary and Ser- and Dl-expressing cells in adjacent cells maintain the signaling center along the DV boundary. Caps and Tartan are not asymmetrically expressed at late stages; thus, maintenance of the DV affinity boundary is independent of Caps and Tartan activity. (C) Late third instar wild-type wing disc labeled to visualize expression of Apterous (red) and Wingless (blue).

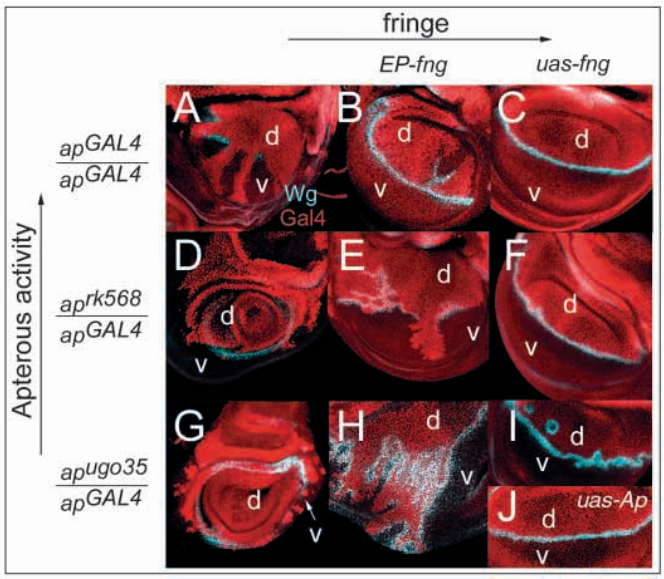

Fig. 2. Fringe and the DV affinity boundary. Wing discs of the following genotypes labeled to visualize Wg protein (blue) and Gal4 protein (red). (A) ap^{Gal4}/ap^{Gal4}. (B) ap^{Gal4}/ap^{Gal4}; *EP-fng*. (C) ap^{Gal4}/ap^{Gal4}; *UAS-fng*. (D) ap^{Gal4}/ap^{rk568}. (E) ap^{Gal4}/ap^{rk568}; *EP-fng*. (F) ap^{Gal4}/ap^{rk568}; *UAS-fng*. (G) ap^{Gal4}/ap^{UGO35}. (H) ap^{Gal4}/ap^{ugo35}; *EP-fng*. (I) ap^{Gal4}/ap^{ugo35}; *UAS-fng*. (J) Wing disc of ap^{Gal4}/ap^{rk568}; *UAS-Ap* labeled to visualize expression of Apterous (red) and Wingless (blue).

restore the DV affinity boundary (Fig. 2H). O'Keefe and Thomas (O'Keefe and Thomas, 2001) reported that expression of *fringe* at higher levels in D cells of the weaker heteroallelic combination ap^{Gal4}/ap^{rk568} was able to restore the DV affinity boundary (Fig. 2F).

To ask whether the differences between these experiments can be explained in terms of the amount of *fringe* activity relative to the degree of reduction of *ap* activity, we compared the ability of different amounts of Fringe expression to rescue three different *apterous* mutant combinations. ap^{Gal4} homozygous larvae show a relatively weak phenotype in wing discs (Fig. 2A). Providing a lower level of Fringe activity using *EP3082* did not fully rescue the ap^{Gal4} homozygous disc phenotype, whereas providing a higher level of Fringe activity using *UAS-fng* rescued well (Fig. 2B,C). In the ap^{Gal4}/ap^{rk568} combination, with intermediate levels of *Ap* activity, providing the lower level of Fringe activity using *EP3082* did not rescue the boundary phenotype. Providing the higher level of Fringe activity using *UAS-fng* rescued quite well, but not perfectly (Fig. 2D-F). The ap^{Gal4}/ap^{UG035} combination provided the strongest mutant phenotype. Neither of the Fringe-expressing genotypes rescued the boundary defects, though the *UAS-fng* was better than *EP3082* (Fig. 2G-I). We note that the level of Fringe in both cases was sufficient to generate robust Notch activation, as indicated by Wg expression. As a control, we verified that expression of *UAS-Apterous* under ap^{Gal4} control was sufficient to restore both Notch activation and the DV lineage restriction boundary in the strongest mutant combination (Fig. 2J). Thus, we reiterate our conclusion that Fringe activity in D cells does not appear to be sufficient to generate a DV affinity boundary under conditions of reduced Apterous activity.

Different effects of ubiquitous expression of Apterous or Fringe on the DV boundary

To further evaluate the contributions of Apterous and Fringe to maintenance of the DV affinity boundary, we expressed these proteins throughout the P compartment of the wing disc using engrailed-Gal4. Ectopic expression in P cells eliminates the difference between D and V cells in the P compartment and permits an assessment of their effects on the endogenous DV boundary (visualized by the expression of an *ap-lacZ* reporter gene). When *UAS-fng* was expressed in P cells, Wg expression was lost at the endogenous boundary between D and V cells in the P compartment, and an ectopic stripe of Wg expression was induced in cells along the edge of the engrailed-Gal4 domain (Fig. 3G-I). The interface between D and V compartment cells became irregular under these conditions. These observations confirm the report by Rauskolb et al. (Rauskolb et al., 1999) that a boundary between Fringe-expressing and non-expressing cells is needed to maintain the DV affinity boundary.

The effects of Apterous expression differed considerably

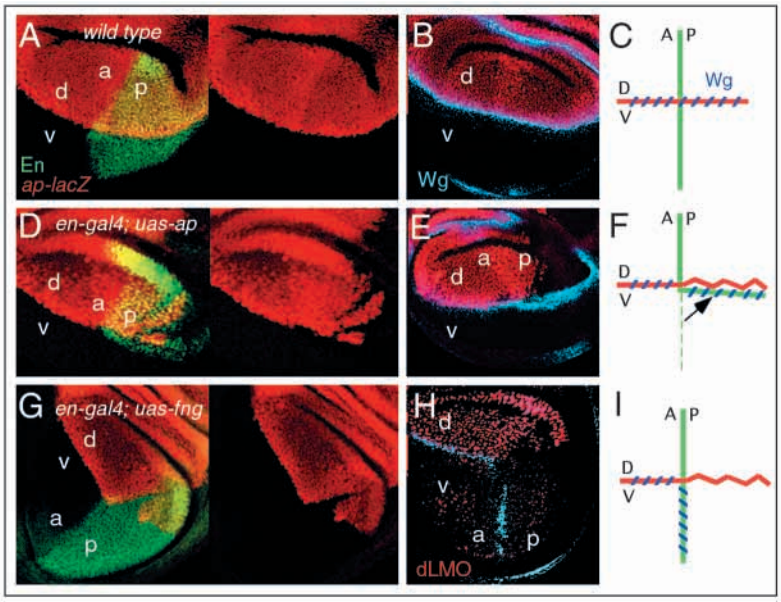

Fig. 3. Cell affinities and sorting at the DV boundary. (A,D,G) Late third instar wing discs labeled to visualize expression of Engrailed protein (green) and an *ap-lacZ* reporter gene (anti-β-gal, red). (B,E,H) Late third instar wing discs labeled to visualize expression of Wingless protein (blue) and *ap-lacZ* (red in B,E) or LMO (red in H). a, anterior; p, posterior; d, dorsal; v, ventral. (C,F,I) Drawing showing the relative locations of the AP compartment boundary (green) and the DV boundary (red). (A-C) Wild-type, (D-F) *en-gal4; UAS-ap* and (G-I) *en-gal4; UAS-fng*.

from Fringe in terms of the relative position of the AP and DV compartment boundaries. In wild-type wing discs, the AP and DV boundaries are perpendicular to each other (Fig. 3A-C). This was also the case when Fringe was expressed in P cells (Fig. 3G-I). The engrailed-Gal4 domain was perpendicular to the DV boundary (in the A compartment), despite expression of Fringe in all P cells. By contrast, expression of Apterous in all P cells caused P cells of ventral origin to relocate into the dorsoposterior quadrant of the disc (Fig. 3D-F). Although these cells were of V compartment origin, they appear to have sorted out into the D compartment by virtue of Ap expression. Under these conditions, the AP and DV compartment boundaries were no longer perpendicular (Fig. 3F). This suggests that Apterous and Fringe do not have comparable abilities to confer D compartment-specific cell behavior. Sorting out can be caused by differences in cell affinity.

Fringe is not comparable with Apterous in its ability to confer D cell affinity

In genetic mosaics, differences in cell affinity can be visualized by the shapes of mutant clones (Lawrence et al., 1999; Liu et al., 2000). To compare the effects of Apterous and Fringe on D compartment cell affinity we examined the shapes of mutant clones lacking these activities in the D compartment or clones expressing Apterous or Fringe in the V compartment. Wild-type clones were elongated along the proximodistal axis of the wing and their borders were irregular (Fig. 4A), except when they touched the DV or AP compartment boundaries (see Fig. 6D). For convenience, clones of cells lacking Apterous activity were produced either by removing *Chip*, a co-factor required for Apterous to function as a transcription factor (Fernandez-Funez et al., 1998; Morcillo et al., 1997), or by expressing the Apterous antagonist dLMO (Milán et al., 1998). In the D compartment, clones lacking Apterous activity were round in shape with smooth borders (Fig. 4A and see Fig. 5A). Clones mutant for the *apterous* gene behaved similarly in the D compartment (Blair et al., 1994), but for technical reasons were difficult to produce in large numbers (see Materials and Methods for details).

We measured the shape of wild-type clones and clones lacking Apterous activity using the formula $4\pi\ A/L^2$ (A=area and L=perimeter of the clone). $4\pi\ A/L^2$ equals 1.0 for a circle. The irregularly shaped wild-type clones had a longer perimeter relative to their area and generated a low value ($4\pi\ A/L^2$=0.36; Fig. 4A, quantitation in 4B). Dorsal clones lacking Apterous activity were significantly rounder than wild-type clones (Chip mutant clones: $4\pi\ A/L^2$=0.68 and $P<<0.001$; dLMO-expressing clones: $4\pi\ A/L^2$=0.81 and $P<<0.001$). Dorsal clones mutant for *fringe* were on average rounder in shape than wild-type clones (Fig. 4A; $4\pi\ A/L^2$=0.49 and $P<0.001$), but not as round as clones lacking Apterous activity ($P<0.001$). This difference is reflected in the observation that the borders of *fringe* mutant clones were irregular and highly indented compared with the relatively smooth borders of clones lacking Ap activity. These results agree with an earlier report that loss of *fringe* activity has an effect on clone shape (Rauskolb et al., 1999). However, our findings indicate that loss of *fringe* is not comparable with loss of Apterous activity in the severity of its effect on the local cell interactions that lead to sorting out of cells and smooth clone borders.

Comparable results were obtained when Apterous or Fringe was ectopically expressed in the V compartment. Ventral Apterous-expressing clones were significantly rounder than wild-type clones (Fig. 4A and B; $4\pi\ A/L^2$=0.83 and $P<<0.001$). Ventral clones expressing Fringe are on average rounder in shape than wild-type clones (Fig. 4B; $4\pi\ A/L^2$=0.53 and $P<0.001$), but not as round as clones expressing Apterous ($P<0.001$). These results indicate manipulation of Apterous activity confers a larger difference in cell affinities than modulation of Fringe alone can do, despite induction of Notch signaling. Consistent with this conclusion, we find that expression of the constitutively activated form of Notch in clones of cells is not sufficient to induce an affinity difference in the wing pouch, reflected by failure of the clones to adopt a round shape (Fig. 4C).

Apterous cannot confer a sustained affinity difference without Notch activation

Dorsal clones lacking Apterous activity lose Fringe expression.

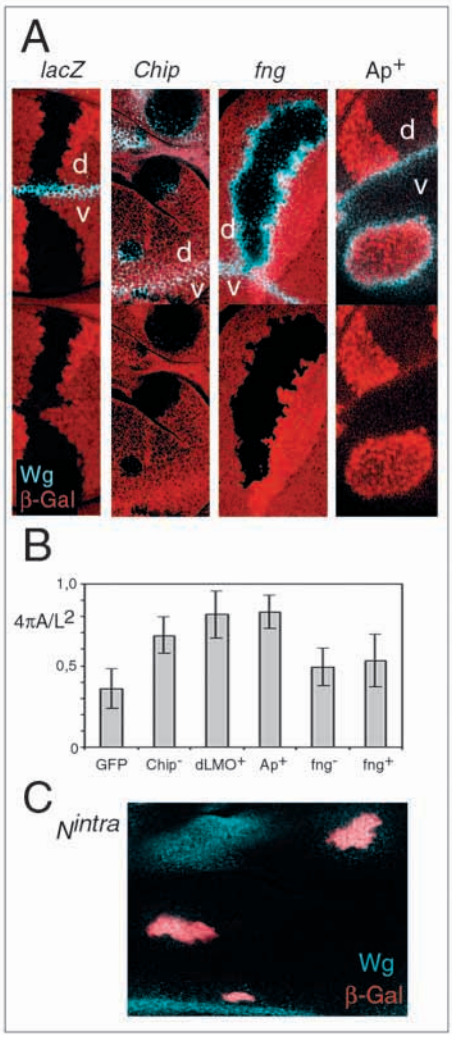

Fig. 4. Cell affinities at the DV boundary. (A) Wild-type (*lacZ*), *Chip* or *fringe* (*fng*) mutant clones located in the D compartment and marked by the absence of the *lacZ* marker (red). Apterous (Ap^+, red) expressing clone located in the V compartment. Wingless expression (blue). d, dorsal; v, ventral compartments. Note that wild-type clones were elongated with irregular borders, *Chip* clones and Apterous expressing clones were round with smooth borders, and *fringe* clones were intermediate in shape with irregular borders. (B) The $4\pi A/L^2$ ratio of D clones lacking GFP, *Chip* or *fringe*, or expressing LMO, and V clones expressing Apterous or Fringe. GFP, 0.4±0.1, n=35; dorsal *Chip* mutant, 0.7±0.1, n=15; dorsal LMO expressing, 0.8±0.1, n=16; ventral Apterous expressing, 0.8±0.1, n=19; dorsal *fringe* mutant, 0.5±0.1, n=25; ventral Fringe expressing, 0.5±0.1, n=14. Error bars indicate standard deviation. (C) Clones expressing $Notch^{intra}$ and β-gal (red) in the D compartment. Wingless is shown in blue. Clones did not 'round up' and had irregular borders.

Thus, it is possible that the effects of clones lacking Apterous activity on cell affinity may be due to loss of Fringe. The observation that removing Fringe activity did not produce as robust an effect on clone shape as removing Apterous activity suggested that Apterous would have multiple targets through which it affects cell affinity, Fringe being one of these. In this case we would expect that restoring Fringe activity in an *apterous* mutant clone would only partially compensate for the cell affinity defects. To test this possibility, we produced clones that lacked Apterous activity due to expression of the inhibitor dLMO (Milán et al., 1998). Use of Gal4 to remove Apterous activity allowed us to evaluate the effects of restoring Fringe expression on the shape of clones lacking expression of other Apterous target genes. Dorsal clones expressing dLMO were round and had smooth borders (Fig. 5A,E; $4\pi A/L^2$=0.81). When Fringe was co-expressed with dLMO, clones were elongated in shape and had irregular borders (Fig. 5B,E; $4\pi A/L^2$=0.34). As expected, Notch was not activated at the borders of these clones and Wg expression was not induced. However, contrary to our expectations, these clones were not significantly different in shape from wild-type clones (P=0.7). This indicates that Apterous-dependent alterations in cell affinity require the activity of Fringe, and therefore presumably Notch activation.

To test the dependence of dLMO-expressing clone shape on Notch activity more directly, we co-expressed a dominant-negative version of the Notch receptor. N^{ecd} encodes a truncated form of Notch that lacks the intracellular domain of the receptor, which blocks Notch activation in a cell-autonomous manner (Micchelli and Blair, 1999). When dLMO was used to remove Apterous activity, Notch signaling was induced in cells on both sides of the border of the clone, as revealed by Wg or Cut expression (Fig. 5A and data not shown, see drawing). This resembles the wild-type DV border, in that feedback signaling leads to activation of Notch on both sides of the interface between the two cell types (de Celis and Bray, 1997; Micchelli et al., 1997). Under these conditions, Delta and Serrate would be induced in cells adjacent to the Wg-expressing cells (i.e. offset from the borders of the clone by one or two rows of cells). When N^{ecd} was co-expressed, we observed two distinct outcomes. Notch activity and Wg and Cut expression were always lost in the mutant cells (Fig. 5C,D; see drawing). In some clones Notch was not activated in the adjacent wild-type cells, and Wg and Cut failed to be induced. These clones were irregular in shape (Fig. 5D,E; $4\pi A/L^2$=0.35). In other clones, Notch was activated and Wg and Cut were expressed in surrounding cells. Clones of this type were round in shape (Fig. 5C,E; $4\pi A/L^2$=0.77). When Notch activity was blocked in dLMO-expressing clones by co-expression of Hairless or a dominant-negative form of Mastermind, Wg and Cut failed to be induced inside the clone, but they were expressed in surrounding cells (not shown). Clones of this type were round in shape, as when N^{ecd} was co-expressed. The correlation between clone shape and Notch signaling in adjacent cells allows the possibility that recruitment of Serrate and Delta by Wg signaling may contribute to smoothing the clone border. It is interesting to note that in clones with a round shape, only some surrounding cells expressed Cut and Wg (Fig. 5C). This observation suggests that a lower level of Notch signaling activity is required to induce the genes that confer the affinity difference than is required to induce *wg* and *cut* expression.

Taken together, the data presented indicate that clones round up when Notch is activated in cells outside the clone, but fail to do so when Notch is not activated. This raises a question of why N^{ecd} is sometimes able to block Notch activation in cells outside the clone. One possibility is that, being a transmembrane protein, N^{ecd} may influence the ability of Serrate, Delta or Notch to function in the neighboring cells (we cannot exclude the possibility that N^{ecd} might also interact with

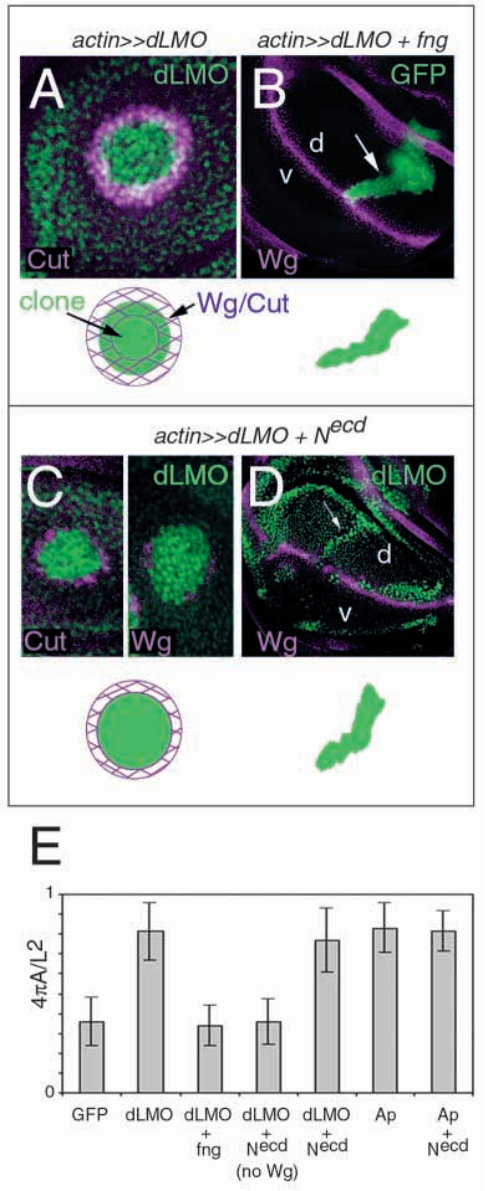

Fig. 5. Cell affinities at the DV boundary require Notch activation. (A) Clones expressing LMO (green). Cut is shown in purple. Dorsal clones were round and induced Cut expression in the clone (white) and in neighboring cells (purple). (B) Clones of cells expressing LMO and Fringe marked by the expression of GFP (green). Clones located in the dorsal compartment did not induce Wg expression (purple), were elongated and had irregular borders (arrow). (C,D) Clones of cells expressing LMO (green) and N^{ecd}. (C) Notch activation was blocked in the clone, as shown by absence of Cut expression. (D) When Notch was also blocked in the neighboring cells the shape of the clones became irregular and elongated. (E) Histogram indicating the 4π A/L^2 ratio of clones lacking *GFP*; dorsal clones expressing LMO, LMO and fng, or LMO and N^{ecd}; ventral clones expressing Ap or Ap and N^{ecd}. GFP, 0.4±0.1, *n*=35; dorsal LMO expressing, 0.8±0.1, *n*=16; dorsal LMO and Fng expressing, 0.3±0.1, *n*=12; dorsal LMO and N^{ecd} expressing, 0.4±0.1, *n*=16 clones without Wg expression in neighboring cells; dorsal LMO and N^{ecd} expressing, 0.8±0.1, *n*=14 clones with Wg expression in neighboring cells; ventral Apterous expressing, 0.8±0.1, *n*=19; ventral Ap and N^{ecd} expressing, 0.8±0.1, *n*=12. Error bars indicate standard deviation. d, dorsal; v, ventral compartment.

other as yet unidentified proteins to contribute to this effect). We note that similarly variable effects were observed in Serrate-expressing clones (Rauskolb et al., 1999). These results suggest that the effects of Fringe can be fully accounted for in terms of Notch activity. This contrasts with the previous proposal that Fringe acts independently of Notch in DV cell affinity (Rauskolb et al., 1999; O'Keefe and Thomas, 2001).

Sorting out at the DV boundary

The DV compartment boundary behaves as a lineage restriction boundary. Clones of cells born in one compartment do not give rise to progeny located in the adjacent one (Fig. 6A,D). This may be due to differences in affinity between D and V cells. However, the DV compartment boundary behaves also as a signaling center. Interactions between D and V cells induce Notch activation and Wg expression along the boundary (Fig. 1A). As illustrated in Fig. 6B, cells that change affinity and also acquire the signaling properties of the opposite compartment are expected to cross the boundary to intermingle freely with cells in the new compartment. By contrast, acquisition of the signaling properties of the opposite compartment might only be expected to cause displacement of the Wg stripe without allowing the clone to cross completely into the opposite compartment (Fig. 6C).

Apterous confers both the signaling and affinity properties of D cells. When Ap-expressing clones are produced in the V compartment, they cross into the D compartment and displace the Wg stripe (around the clone; Fig. 6E). The vast majority of these clones are topologically located in the D compartment, where they mix perfectly with D cells (Table 1). Likewise, clones lacking Ap activity in the D compartment because of dLMO expression can cross completely into the V

Table 1. Effects of clones on the DV boundary

	Frequency of clones displacing the Wg stripe	Frequency of clones crossing the DV boundary
Apterous-expressing clones*	13/13 (9/9)‡	12/13 (9/9)‡
dLMO-expressing clones†	24/24 (16/16)‡	20/24 (14/16)‡
Fng-expressing clones*	10/10	6/10
fng⁻ mutant clones†	23/23	9/23
dLMO- and Fng-expressing clones†	0/12	0/12
dLMO- and N^{ecd}-expressing clones†,§	0/14	2/14

*Clones of ventral origin abutting the DV boundary.
†Clones of dorsal origin abutting the DV boundary.
‡In parenthesis, clones of mixed dorsal and ventral origin.
§Only clones where N^{ecd} eliminated ectopic activity of Wg were scored.

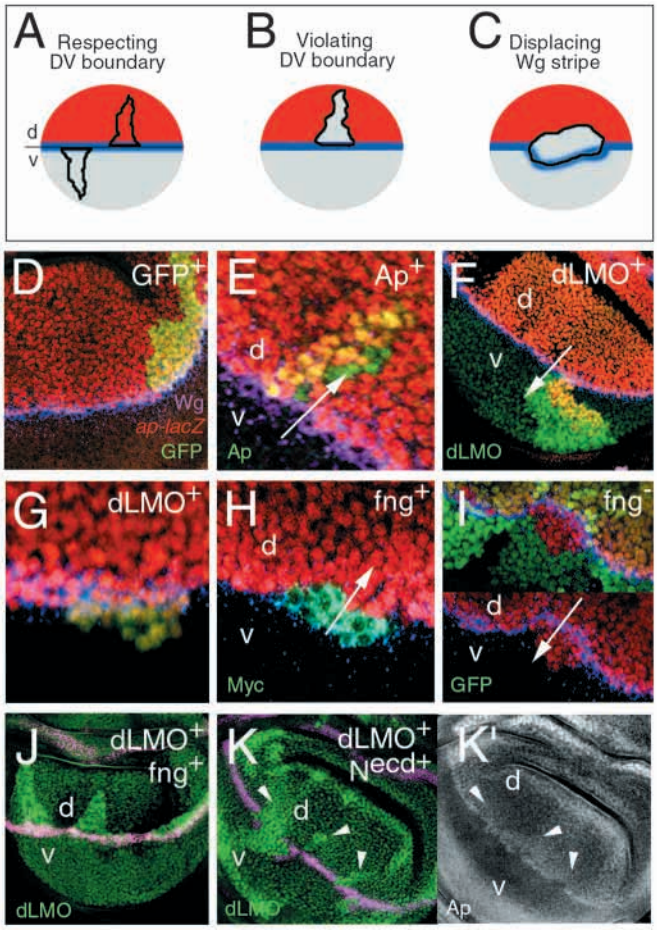

Fig. 6. Sorting out across the DV boundary. (A-C) Schematic representation of three types of clone behavior with respect to the DV boundary. Clones are outlined in black. Dorsal (d) cells are depicted in red; ventral (v) cells are depicted in gray. Notch activation and Wg expression is depicted in blue. (A) Clones respecting the DV boundary are elongated in shape with irregular borders, except when they abut the DV boundary. The endogenous DV boundary (between red and gray cells) corresponds to the stripe of Wg expression. (B) A clone of ventral cells that crossed the DV boundary into the D compartment. Under these conditions, the endogenous DV boundary does not correspond to the stripe of Wg expression. (C) A clone of ventral cells with D signaling properties that displaced the Wg stripe but did not cross completely into the D compartment. (D-I) Late third instar wing discs labeled to visualize expression of the *ap-lacZ* reporter gene (antibody to β-gal, red) and Wingless (blue). *ap-lacZ* labels the D compartment. Dorsal (d) and ventral (v) compartments are indicated. (D) Clone of cells expressing GFP and born in the D compartment. (E) Clone of cells expressing Ap (green) that was induced before the onset of *ap-lacZ* expression. Cells born in the dorsal compartment (red+green) or born in the ventral compartment (green) were located on the dorsal side of the Wg stripe and mixed with D cells. Thus, the clone crossed from the V into the D compartment (arrow). (F) Clone of cells expressing LMO (green) induced before the onset of *ap-lacZ* expression. Cells born in the dorsal (red+green) or the ventral (green) compartment were located on the ventral side of the Wg stripe and mixed with V cells. Thus, the clone crossed from the D into the V compartment (arrow). (G) Clone of D cells expressing LMO (green) induced after the onset of *ap-lacZ* expression (red+green) and located on the ventral side of the Wg stripe. (H) Ventral clone expressing Fng-myc labeled by the expression of Myc protein (green). The clone was born in the V compartment and displaced the Wg stripe toward ventral. Arrow in H indicates direction of crossing. (I) Dorsal clone mutant for *fng* labeled by the absence of the GFP marker (green). The clone was born in the dorsal compartment (red) and displaced the Wg stripe toward dorsal. (J) Dorsal clones expressing Fng-myc and LMO labeled by expression of LMO (green). (K,K′) Dorsal clones expressing LMO and N^{ecd} (LMO in green). Note clones born in the dorsal compartment (arrowheads) remain at the dorsal side of the Wg stripe (purple)

compartment if they contact the DV boundary. All clones analyzed displace the Wg stripe and intermingle freely with V cells (Fig. 6F,G; Table 1).

Fringe expression confers the signaling behavior of D cells, but as indicated above, appears not to confer all the affinity properties of D cells. We compared the behavior of Fringe-expressing clones born in the V compartment, with those of Ap. Although Fringe expressing clones displaced the Wg stripe with respect to the endogenous DV boundary in the same way as Ap-expressing clones (Fig. 6H), 6/10 clones crossed completely into the D compartment (Table 1). Co-expression of Serrate gave similar results; only 4/11 clones crossed completely into the D compartment. Likewise, *fringe* mutant clones born in the D compartment displaced the Wg stripe (Fig. 6I). However, only 9/23 clones were topologically located in the V compartment (Fig. 6I; Table 1). These findings provide another indication that Fringe is not comparable with Ap in establishing the cell affinities that control boundary formation.

As noted in the preceding section, Ap requires Notch activity to cause rounding up of clones. We therefore asked whether Notch activation is also required for sorting across the compartment boundary. We co-expressed Fringe to prevent activation of Notch in dLMO-expressing clones. In contrast to clones expressing dLMO alone, these clones did not sort into the V compartment (0/12 clones examined; Fig. 6J, compare with 6F,G). Comparable results were obtained when the dominant-negative form of the Notch receptor N^{ecd} was co-expressed with dLMO (Fig. 6K and K′). Clones that did not induce Notch activation in the adjacent wild-type cells did not sort out into the V compartment (Table 1).

Taken together, the results of the experiments with Fringe and N^{ecd} indicate that activation of Notch signaling is required for a sustained affinity difference between cells that express Apterous and those that do not. However, *fringe* cannot fully account for the effects of Apterous on D cell affinity. This comparison indicates that it is not the presence or absence of Fringe or of Notch activity per se that produces the affinity difference. Rather, Notch signaling appears to be required in conjunction with another Apterous-dependent process for maintenance of the affinity border.

DISCUSSION

Comparison of three models for maintenance of the DV affinity difference

The LRR transmembrane proteins Capricious and Tartan contribute to DV boundary formation, but their role is transient (Milán et al., 2001). Maintenance of the boundary requires an additional mechanism. Notch activity has been implicated in

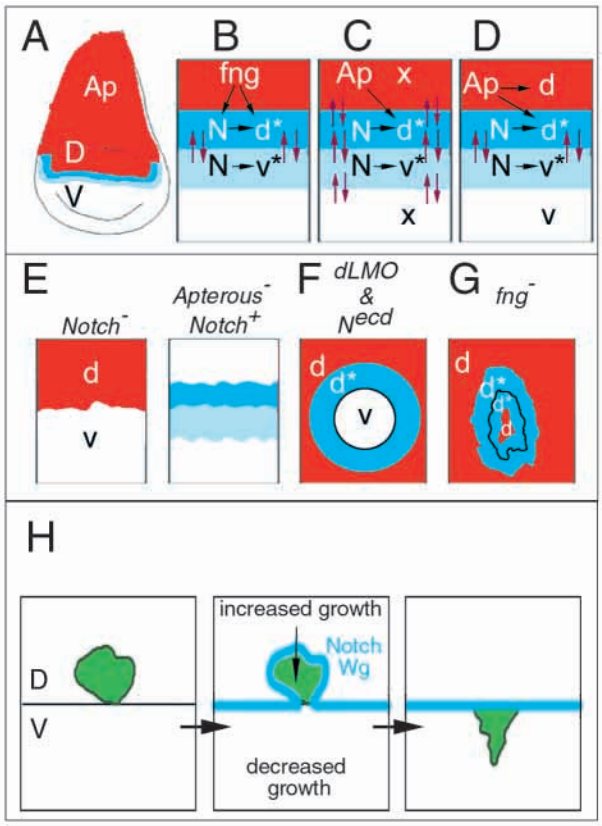

Fig. 7. Maintenance of the DV affinity boundary. (A-G) Dorsal cells are depicted in red, ventral in white. Notch activation is depicted in blue. Notch is activated in D and V cells. Apterous (Ap) activity in dorsal cells provides the underlying asymmetric cue. (B-D) Three models proposed to explain the activity of Notch and Ap in inducing a DV affinity difference. d* indicates a dorsal affinity state at the boundary; v* indicates a corresponding ventral affinity state. The model shown in B proposes that Fng acts on Notch and independently of Notch on another gene to induce the d* state (Rauskolb et al., 1999; O'Keefe and Thomas, 2001). According to this view, Fringe should be sufficient to induce a DV affinity difference. (C) Micchelli and Blair's model proposes that dorsal and ventral cells have the same cell affinity (x) in the absence of Notch activation. Thus, confrontation of boundary cells and cells in the wing blade should induce an affinity boundary. (D-G) We propose that Ap controls d and v affinity molecules, but that these require Notch activity to produce a sustained d* or v* affinity state. According to this model, Notch is required but cannot generate an affinity state on its own. Only interactions between cells of opposite compartments are able to induce an affinity boundary. (E) In the absence of Notch or Ap activity cell states d* and v* are not defined and the affinity boundary is not properly maintained. (F) Interaction between cell states d* or v* with cells of the opposite compartment is sufficient to induce an affinity difference. (G) *fng* mutant clones in the D compartment are not expected to confront d* and v* states and therefore cannot induce an affinity difference comparable with that produced by removing Ap activity (as in F). (H) A *fng* mutant clone in the D compartment (depicted in green) is expected to displace the Notch activity stripe (blue) around the clone. Notch induces symmetric growth, pushing the clone into the V compartment. Notch is not induced at the interface between the clone and the V compartment.

this process (Micchelli and Blair, 1999; Rauskolb et al., 1999; Blair, 2001), but its role has been questioned (Milán and Cohen, 1999a). Models for maintenance of the DV boundary must take into account the fact that Notch is activated symmetrically in cells on either side of the DV boundary. Therefore, an Ap-dependent process must be invoked to confer a DV difference. One proposal is that Fringe mediates the required Ap-dependent activity by acting in a Notch-independent manner, in addition to its role in Notch signaling (O'Keefe and Thomas, 2001; Rauskolb et al., 1999) (Fig. 7B). According to this view, confrontation of Fringe-expressing and non-expressing cells should induce a cell affinity difference. We have shown that increasing or decreasing Fringe activity has some effect, but does not produce affinity differences comparable with those produced by manipulating Apterous activity (Figs 3-6). Furthermore, we have shown that the effects of restoring Fringe in D cells that lack Apterous activity can be reproduced independently by blocking Notch activation using N^{ecd}. Thus, it is unlikely that Fringe has a Notch-independent role in DV cell interactions.

A second, very different, model proposes that Notch activation confers a boundary-specific affinity state and that this is modulated into D and V states by Apterous expression (Fig. 7C) (Micchelli and Blair, 1999; Blair, 2001). According to this model, there should be an affinity difference between boundary cells and internal cells within a compartment but not between D and V cells in the absence of Notch activity. This model proposes that Notch activity is sufficient to produce an affinity difference and hence smooth clone borders. However, as we have shown, clones of cells expressing the activated Notch receptor do not exhibit this property (Fig. 4C). This model is also difficult to reconcile with our observation that the borders of *fringe* mutant clones in the D compartment are highly irregular (Fig. 4A; illustrated in Fig. 7G). It is also incompatible with our finding that restoring Notch activity in the absence of Apterous function is not sufficient to generate a smooth DV boundary and prevent mixing of D and V cells (Fig. 2; illustrated in 7E).

The results reported here support the view that Notch activity is needed for cell affinity differences between D and V cells, but indicate that Notch activation is not sufficient to cause these differences. We therefore propose the model in Fig. 7D, which differs in one crucial respect from the model discussed above (Fig. 7C). We consider the role of Notch activation to be permissive rather than instructive, and suggest that Apterous controls expression of surface proteins in D and V cells. We envisage that Notch activity is an essential co-factor in allowing cells to convert this into an affinity state. In molecular terms, one possibility is that D and V surface proteins form complexes with activated Notch (N*). In this scenario D+N* and V+N* are the active components, D and V are needed and instructive but have no activity alone. Interestingly, it has been observed that loss of Notch activation only in one compartment does not alter the DV affinity boundary (Micchelli and Blair, 1999; Milán and Cohen, 1999a; Rauskolb et al., 1999). Thus, production of either the dorsal (D+N*) or the ventral (V+N*) boundary-specific cell state is sufficient to induce an affinity difference with cells of the opposite compartment (Fig. 7F). Another plausible molecular scenario is that Notch activity might control the subcellular localization of the predicted D and V proteins.

We present these examples to illustrate how Notch activity can be seen as a permissive co-factor rather than as an instructive principle defining cell affinity. Many other molecular explanations are possible. This model provides a satisfactory explanation for how Notch can be required, but not sufficient for boundary maintenance. The essential difference between the permissive and instructive models for Notch function lies in the observation that Notch activation leads to an affinity difference only in the context of juxtaposition of cells with opposite DV identity. Notch activation per se does not induce a robust affinity boundary, whereas clones expressing dLMO and N^{ecd} did so only when Notch was not blocked in the cells outside the clone (Fig. 7F). Comparable results were obtained with clones expressing Apterous and N^{ecd}.

Are the transmembrane proteins Serrate and Delta the D and V proteins, respectively? Early in development, Serrate is expressed in D cells and Delta in V cells (Diaz-Benjumea and Cohen, 1995; Milán and Cohen, 2000) (Fig. 1A). Late in development, both genes are regulated by Wg and are expressed in cells adjacent to the Wg-expressing cells at the DV boundary (de Celis and Bray, 1997; Micchelli et al., 1997) (Fig. 1B). Given that the Serrate- and Delta-expressing cells are offset from the DV boundary, we consider it unlikely that they confer the D* and V* activities. However, we do not exclude the possibility that they might contribute to the establishment of the DV affinity boundary in collaboration with Caps and Tartan.

Cell behavior at the DV boundary: sorting out by crossing versus pushing

The interface between D and V cells behaves as an affinity boundary and as a signaling center where Notch activation is required for the growth of the wing disc. Clones of cells can be induced to sort into the opposite compartment by manipulating Apterous or Fringe activities. As discussed previously by Blair and Ralston in the context of the AP boundary (Blair and Ralston, 1997), we would like to distinguish between crossing and pushing the DV boundary as possible mechanisms. Cells with altered Apterous activity also have altered Fringe activity. We suggest that these clones can cross the boundary and mix freely with cells in the opposite compartment because they change both their affinity state and signaling properties. Clones in which only Fringe activity is altered adopt signaling properties of the opposite compartment and displace the signaling center relative to the endogenous compartment boundary (Fig. 7H) (Rauskolb et al., 1999). In wild-type discs, symmetric activation of Notch and its targets leads to symmetric growth of D and V compartments. If growth is symmetric with respect to the displaced signaling center, the clone could be pushed into the opposite compartment by growth of the surrounding tissue (Blair and Ralston, 1997) (see also Fig. 7H).

At first glance, differential growth might explain how cells could be pushed to the interface between compartments. Can the model presented in the preceding section explain why some dorsal *fringe* mutant clones become able to mix with cells of the opposite compartment? As shown by Rauskolb et al. (Rauskolb et al., 1999) Notch is not activated in V cells adjacent to *fringe* mutant clones abutting the boundary (Fig. 7H). Our model suggests that these cells would become V instead of V+N*; hence, there would not be a sustained affinity difference between *fringe* mutant D cell and the adjacent V cells. This may explain why *fringe* mutant D cells can sometimes mix with V cells when they are pushed into the V compartment. A similar case can be made to explain how V cells expressing Fringe can be pushed into the D compartment and mix with D cells. In both situations, we note that these clones form smooth borders with the cells of the compartment of origin, suggesting symmetric growth induced by Notch may contribute to the smoothness of the affinity boundary. This type of 'pushing' mechanism provides a useful explanation for the behavior of clones of cells that contact the DV boundary. We note that the behavior of cells expressing Apterous and Fringe was not the same when the entire P compartment was involved. P cells of ventral origin expressing Apterous were able to sort into to dorsal posterior quadrant, but cells expressing Fringe were not. We suggest that this reflects an underlying difference between cells that have acquired a fully dorsal affinity state from those in which only the signaling properties have been altered. Fringe activity clearly plays an important role in the maintaining the segregation of D and V cells, but it is not the sole mediator of Apterous activity in this process.

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