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A permissive role of Notch in maintaining the DV affinity boundary of the *Drosophila* wing

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ABSTRACT

Cell affinities can contribute to organizing cells into tissues and organs. *Drosophila* limbs and the vertebrate central nervous system are subdivided into adjacent populations that do not mix. These cell populations are called compartments. Cell interactions mediated by receptor Notch have been implicated in the specification of compartment boundaries. However, the contribution of Notch in this process remains controversial. The instructive role of Notch and the transcriptional requirement of the pathway have been questioned in the last few years. Due to its central role in making developmental boundaries in vertebrates and invertebrates, we have reevaluated the contribution of Notch and its signaling pathway in the maintenance of an affinity difference between dorsal (D) and ventral (V) compartments in the *Drosophila* wing. Here we present evidence that unrestricted, low levels of Notch are sufficient for the formation of a stable DV affinity boundary. Cleavage of the Notch protein, release of the intracellular domain and a transcriptional function of Notch via the Suppressor of Hairless transcription factor are required and sufficient in this process. Our data support a permissive role of Notch in maintaining the DV affinity boundary. This contrasts with the instructive role of Notch in executing the organizing activity of the DV boundary.

Introduction

In multicellular organisms, stable subdivision into adjacent tissues and organs relies on the acquisition of different cell affinities. These are defined either by the differential expression of cell adhesion molecules or by interactions between adjacent cell populations that lead to cell repulsion. The imaginal discs of *Drosophila* and rhombomeres of the vertebrate hindbrain provide well-characterized experimental systems in which subdivision of the tissue depends on mechanisms that limit cell mixing to produce stable boundaries. These stable subdivisions are called compartments (García-Bellido et al., 1973). Compartment boundaries serve as signaling centers and organize the growth and pattern of the surrounding cellular territories. Intermingling of cells between adjacent compartments has disastrous consequences in patterning and growth (Milán and Cohen, 1999).

Notch signaling is involved in a variety of cell processes during development, including the establishment and maintenance of compartment boundaries in *Drosophila* imaginal discs and vertebrate hindbrain (Irvine, 1999; Irvine and Rauskolb, 2001; Takahashi, 2005). Notch is required at the compartment boundaries to define the affinity difference and execute the organizing activity of these boundaries. One key member in the process of Notch-dependent boundary formation is the glycosyltransferase Fringe, which modifies the extracellular domain of the receptor Notch, modulates the binding affinities to the Notch ligands Serrate/Jagged and Delta, and helps to restrict Notch signaling at the compartment boundary (Irvine, 1999; Irvine and Rauskolb, 2001; Takahashi, 2005). Fringe-dependent modulation of Notch signaling mediates boundary formation in a variety of tissues including *Drosophila* limbs, vertebrate somites, rhombomeres and forebrain (Bishop et al., 1999; Cheng et al., 2004; Cho and Choi, 1998; de Celis et al., 1998; Dominguez and de Celis, 1998; Micchelli and Blair, 1999; Papayannopoulos et al., 1998; Rauskolb et al., 1999; Rauskolb and Irvine, 1999; Zeltser et al., 2001).

The gene regulatory network involved in Notch activation at compartment boundaries is shared between the *Drosophila* wing imaginal disc and the vertebrate hindbrain. A positive feedback loop between boundary and non-boundary cells, mediated by Notch and the signaling molecule Wingless (Wg), in *Drosophila*, or Wnt-1, in vertebrates leads to restricted and high levels of Notch activation along the compartment boundary (Amoyel et al., 2005; de Celis and Bray, 1997; Diaz-Benjumea and Cohen, 1995; Micchelli et al., 1997). Restricted activity of Notch has a clear instructive role in the organizing activity of compartment boundaries. The contribution of Notch to the affinity difference between adjacent compartments remains controversial. Notch activity is required to maintain the lineage restriction boundary between dorsal (D) and ventral (V) cells in the fly wing and contributes to generating an affinity difference between boundary and non-boundary cells in the vertebrate hindbrain (Cheng et al., 2004; Micchelli and Blair, 1999; Rauskolb et al., 1999). However, the instructive role of Notch and the transcriptional requirement of the pathway have been challenged in the last few years (Cheng et al.,

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2004; Major and Irvine, 2005; Milan and Cohen, 2003; O'Keefe and Thomas, 2001). Due to the central role of Notch in making developmental boundaries both in vertebrates and invertebrates, we have reevaluated the contribution of Fringe, Notch and its signaling pathway in this process. Here, we present evidence in the *Drosophila* wing primordium that unrestricted, low levels of Notch activity are sufficient for the formation of a stable DV affinity boundary. Cleavage of the Notch protein at the membrane, release of the intracellular domain and a transcriptional function of Notch via the Suppressor of Hairless (Su(H)) transcription factor are required and sufficient in this process. Our data do not support any Notch-independent function of Fringe in DV boundary formation.

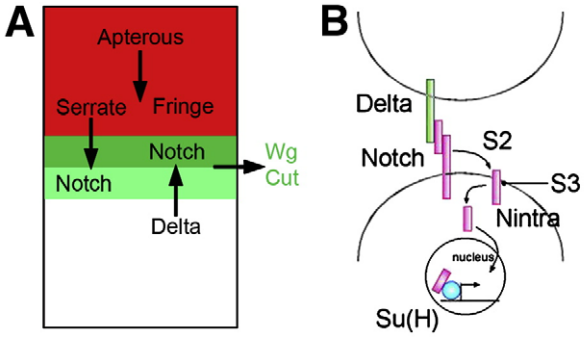

Fig. 1. Notch signaling and DV boundary formation in the *Drosophila* wing. (A) The onset of Apterous expression in dorsal cells is responsible for the complementary expression pattern of the Notch ligands Serrate and Delta in dorsal (D) and ventral (V) compartments, respectively. The polarized signaling of these two ligands towards the DV boundary is due to the activity of the Apterous-regulated gene *fringe*. *Fringe* encodes for a glycosyltransferase that modifies Notch. Notch modified by Fringe (in D cells) responds to Delta and unmodified Notch (in V cells) responds to Serrate. This results in high activation of the Notch pathway along the D/V boundary, which will induce the expression of Wingless (Wg) and Cut. (B) The Notch receptor in the signal receiving cells binds to its ligand Delta or Serrate in the signal-sending cells. Upon ligand binding, Notch undergoes two successive proteolytic cleavages. The first cleavage at the S2 site is ligand-induced, mediated by Kuzbanian, and generates a membrane-bound form of Notch (N^{EXT}) that is further processed by the Presinilin complex at the S3 site. This leads to the release of the Notch intracellular domain (N^{Intra}). N^{Intra} translocates to the nucleus where it interacts with Suppressor of Hairless transcription factor (Su(H)) to activate the transcription of Notch target genes.

Materials and methods

Fly strains

UAS-ap and *UAS-dLMO* (Milan et al., 1998); *UAS-fng-myc* (Bruckner et al., 2000); EP-fringe (EP(3)3082) and ap^{GAL4} (Milan and Cohen, 1999); ap^{UGO35} and ap^{rk568} (referred to as *ap-lacZ*) (Cohen et al., 1992); $msh^{lac\text{-}Z\ \Delta 69}$ (referred to as *msh-lacZ*) (Isshiki et al., 1997); *UAS-Su(H)-VP16* (Furriols and Bray, 2001), kuz^{1405} (Sotillos et al., 1997); aph^{1D35} (Hu and Fortini, 2003); *actin>CD2>GAL4* (Pignoni and Zipursky, 1997); *UAS-*N^{intra} (Struhl and Adachi, 1998); N^{ts2}, $scalloped^{GAL4}$, omb^{GAL4} and vg^{GAL4} (Flybase).

Antibodies

Antibodies against the following proteins are described in the Developmental Studies Hybridoma Bank: Notch extracellular domain (C458.2H), Notch intracellular domain (C17.9C6), Cut (2B10) and Wg (4D4). β-Galactosidase (Cappel). F-actin in early wing discs was labelled with FITC-Phalloidin as described previously (Major and Irvine, 2005).

Results

Subdivision of the developing wing primordium into D and V compartments relies on the restricted expression and activity of the LIM-homeodomain protein Apterous (Ap) in D cells (Diaz-Benjumea and Cohen, 1993). The interface between D and V cells behaves as a lineage restriction boundary as well as an organizing center, and both activities depend on the activity of Notch along the DV boundary. Ap induces the complementary expression of Serrate and Delta, two ligands of the receptor Notch, to D and V cells, respectively. Both ligands initiate a cascade of short-range cell interactions that lead to the activation of Notch at the DV boundary (Fig. 1A). Dorsally expressed Serrate and ventrally expressed Delta activate Notch symmetrically in cells on both sides of the DV compartment boundary (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1993; Doherty et al., 1996). Ap also induces the expression of the glycosyltransferase Fringe in D cells (Irvine and Wieschaus, 1994), which modifies the receptor Notch and makes D cells more sensitive to Delta and less sensitive to Serrate (Bruckner et al., 2000; Moloney, 2000; Munro and Freeman, 2000), thus helping to polarize Notch activation towards the DV boundary.

The organizing activity of the DV boundary clearly relies on the activity of Notch, since restricted activation of Notch in boundary cells is required and sufficient for this process (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Klein and Martinez-Arias, 1998; Milan and Cohen, 1999). In contrast, the contribution of Notch to the DV lineage restriction boundary is not well defined yet. Early in development, Ap induces the expression of the transmembrane proteins Capricious (Caps) and Tartan in D cells. Caps and Tartan contribute to the establishment of the DV affinity boundary, and they are able to do so in the absence of Notch activity (Milan et al., 2005; Milan et al., 2001a). Late in development, Caps and Tartan are re-deployed to generate differences in cell affinities between medial and lateral sides of the wing pouch (Milan et al., 2002), and Notch is required for the maintenance of the DV affinity boundary (Micchelli and Blair, 1999; Milan and Cohen, 1999; Rauskolb et al., 1999). In this study, we have performed a careful analysis of the contribution of the Notch signaling pathway to this process.

Low levels of Notch activity are required for maintenance of the DV affinity boundary

Soon after the onset of *apterous* expression in the early wing primordium, the positive feedback loop between Notch and its ligands leads to increased levels of Notch activity at the boundary. Above a particular threshold, activity of the Notch receptor causes the expression of the signaling molecule Wg first, and Cut later (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Herranz et al., 2006; Micchelli et al., 1997). We first analyzed the level of Notch activity required for the maintenance of a DV affinity difference. For this purpose, we monitored Cut and Wg expression as well as the DV affinity boundary in a thermo-sensitive Notch loss-of-function background (N^{ts2}) reared for several periods of time at permissive (18 °C) or restrictive (30 °C) temperatures. We used two reporter lines to monitor the DV boundary. The enhancer trap lines *ap-lacZ* and *msh-lacZ*, inserted in the *ap* and *msh* loci, respectively, are mutants for the corresponding genes and report the dorsal expression of *ap* and its target gene *msh* (Cohen et al., 1992; Lu et al., 2000; Milan et al., 2001b). *msh* is known to mediate the activity of Ap in D cell fate specification, and, most interestingly, it is not involved in setting the DV organizer, nor in placing the DV affinity boundary (Milan et al., 2001b).

At permissive temperature (18 °C), Wg and Cut were expressed along the DV boundary of N^{ts2} late third instar wing discs (Figs. 2B, C, E and F). At 30 °C during 24 h, Cut expression was almost completely lost and Wg expression levels were slightly reduced (Figs. 2B', C', E' and F'). Longer exposures (48 h) at restrictive temperature led to the loss of Cut and Wg expression (Figs. 2B" and E", and data not shown). Interestingly, loss of Wg expression was more evident in *ap-lacZ* heterozygous wing discs than in the *msh-lacZ*/+ background, most

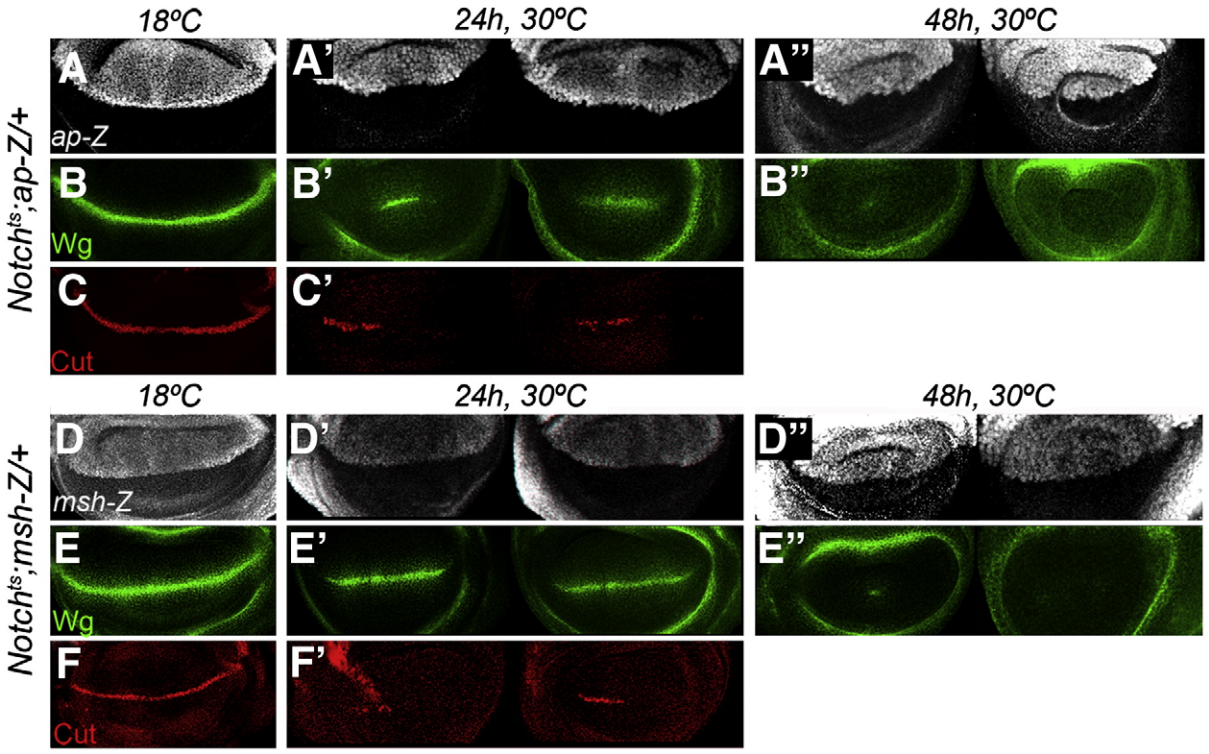

Fig. 2. Different levels of Notch activation required for target gene expression and DV affinity boundary formation. (A–C′) *Notch^{ts}/Y; ap-lacZ/+* late third instar wing discs labeled to visualize expression of *lacZ* (antibody to β-Gal, white, A–A″) and Wingless (Wg, green, B–B″) or Cut (red, C–C′) protein. Larvae were raised at 18 °C (A–C), or shifted during 24 h (A′–C′) or 48 h (A″, B″) at 30 °C before dissection. (D–F′) *Notch^{ts}/Y; msh-lacZ/+* late third instar wing discs labeled to visualize expression of *lacZ* (antibody to β-Gal, white, D–D″) and Wingless (Wg, green, E–E″) or Cut (red, F, F′) protein. Larvae were raised at 18 °C (D–F), or shifted during 24 h (D′–F′) or 48 h (D″, E″) at 30 °C before dissection. In this and all the figures, the discs are oriented with the ventral (V) compartment down.

probably because the former behaves as a sensitized background for Notch activity. We then compared the effects of compromised Notch activity on the DV affinity boundary in both genetic backgrounds. At permissive temperature, the interface between D and V cells visualized either by expression of *ap-lacZ* (Fig. 2A) or *msh-lacZ* (Fig. 2D) was straight and smooth. While the DV affinity boundary was already compromised in N^{ts2}; *ap-lacZ/+* late third instar wing discs reared only for 24 h at the restrictive temperature (Fig. 2A'), it was unaffected after 2 days at 30 °C in a N^{ts2}; *msh-lacZ/+* background (Figs. 2D' and D"). These results indicate that *msh-lacZ* is a more reliable DV reporter to analyze the role of Notch in the maintenance of the DV affinity boundary. Moreover, we show that the activity levels of Notch required for the maintenance of the DV affinity boundary are much lower than those required for Cut and Wg expression. Finally, we can also conclude that soon after the onset of *apterous* expression in the early wing primordium, the relatively low levels of Notch activity induced by the positive feedback loop between Notch and its ligands are already sufficient to generate a DV affinity difference.

A transcriptional role of Notch at the DV affinity boundary

Notch is a transmembrane protein that binds to its ligands Delta or Serrate on the signal-sending cells. Upon ligand binding, Notch undergoes two successive proteolytic cleavages (Fig. 1B, reviewed in Bray, 2006). The first cleavage at the S2 site is ligand-induced, mediated by Kuzbanian, a member of the ADAM family of metalloproteases, and generates a membrane-bound form of Notch (NEXT), which is further processed by the Presinilin/γ-Secretase complex at the S3 site. This leads to the release of the Notch intracellular domain (N^{intra}), which translocates to the nucleus where it interacts with the transcription factor *Su(H)* to activate the transcription of target genes (Fig. 1B).

Although Notch is required to maintain the DV affinity boundary, in the absence of Su(H) activity this boundary is not compromised (Micchelli and Blair, 1999; Rauskolb et al., 1999). These results may be explained by the dual activity of Su(H) as a transcriptional activator and repressor, as suggested by (Koelzer and Klein, 2006), in which case a critical role of Notch might be to relieve the Su(H)-mediated transcriptional repression of Notch target genes required to maintain the DV affinity boundary. Indeed, loss of Su(H) rescues the DV affinity defects caused by loss of Notch signaling (Koelzer and Klein, 2006). Since Koelzer and Klein used a viable null allele of Presenilin (Psn) to reduce Notch activity, Notch at the membrane might be still responsible for the generation of a DV affinity difference (Herranz and Milan, 2006). Consistent with this, a non-transcriptional role of Notch in modulating the actin cytoskeleton has been proposed to be involved in this process (Major and Irvine, 2005). In this case, Notch at the membrane might modulate the actin cytoskeleton since the cytoplasmic domain of Notch associates with proteins that can impinge on actin organization (Giniger, 1998). We then decided to reevaluate the contribution of Notch at the membrane in this process and the requirement of Su(H)-mediated transcription.

We first monitored the capacity of full-length Notch or processed Notch at the membrane to mediate DV affinity boundary formation. For this purpose, we made use of the FLP/FRT system (Xu and Rubin, 1993) to generate homozygous mutant clones for *kuzbanian* (*kuz*), and *aph*, a member of the Presinilin/γ-Secretase complex, which mediate S2 and S3 cleavages of Notch, respectively (Fig. 1B). In these mutant conditions, unprocessed full-length Notch and a truncated form of Notch lacking the extracellular domain (NEXT) are generated. The homozygous mutant clones were identified by the lack of the green fluorescent protein (GFP) and *msh-lacZ* was used as a marker of the dorsal cells. Clones of cells mutant for *kuz* or *aph* disturbed the DV affinity boundary (Figs. 3A and F, *n* clones (*kuz*)=13/26; *n* clones (*aph*)=9/14). The level and sub-cellular localization of Notch protein

detected with antibodies against the extracellular or intracellular domains were not affected in either case (Figs. 3B–E and G–J). A slight increase in the level of N^{intra} protein at the membrane was observed in *aph* mutant cells. We can therefore conclude that full-length Notch (in the absence of *kuz*) or NEXT (in the absence of *aph*) at the membrane is not sufficient to maintain the DV affinity boundary.

The requirement of S2 and S3 cleavages in the maintenance of the DV affinity boundary suggests that processing of Notch and release of N^{intra} are required for the transcription of genes involved in this process (Koelzer and Klein, 2006). We then analyzed whether a transcriptional output of Notch is sufficient to induce a DV affinity difference in the absence of detectable Notch protein at the membrane. Expression of a Notch RNA interference construct (*N-dsRNA*; Presente et al., 2002) at the DV boundary (in *vg-gal4*; *UAS-N-dsRNA* larvae, or in clones of cells; Fig. 4A, and Figs. 4B, C, respectively) gave rise to a reduction in Wg expression and a disturbed DV affinity boundary (*n* clones (*N-dsRNA*)=13/15). We noted that some *N-dsRNA* expressing clones did not loose Wg expression when abutting the DV boundary even though the DV affinity boundary was disturbed (e.g. Fig. 4C). Raising the larvae at a higher temperature (29 °C, which is known to increase Gal4 mediated gene expression) caused loss of Wg expression in all clones, as well as stronger disturbance of the DV affinity boundary (Figs. 4D–G; *n* clones (*N-dsRNA*)=14/14). Co-expression of the intracellular domain of Notch (N^{intra}, in *vg-gal4*; *UAS-N-dsRNA*, *UAS-*N^{intra} larvae, or in clones of cells, Fig. 4H, and Figs. 4I–L, respectively), which behaves as a S2- and S3-independent constitutively activated form of the receptor, restored Notch activation, as revealed by Wg expression, and the DV affinity difference at 25 °C (*n* clones (*N-dsRNA*, N^{intra})=30/30), and at 29 °C (*n* clones (*N-dsRNA*, N^{intra})=9/11). Interestingly, this rescue happened in the absence of detectable Notch protein levels at the membrane (Figs. 4J–L). Altogether, these results imply that a transcriptional output of Notch is required and sufficient for the maintenance of an affinity difference between D and V cells, and Notch at the membrane has no contribution in this process.

Restricted activation of Notch is not required at the DV affinity boundary

Activation of Notch at the DV boundary is required and sufficient to execute the organizing activity of the compartment boundary. Unrestricted activity of Notch has disastrous consequences on the growth and patterning of the wing primordium (Milan and Cohen, 1999). We then questioned whether restricted activation of Notch is also needed to mediate a DV affinity difference. To test this hypothesis, we took advantage of the Gal4/UAS system. Expression of *N-dsRNA* in a broad domain including boundary and non-boundary cells (in *scalloped-gal4*; *UAS-N-dsRNA*, larvae, Figs. 5A, B; or in *omb-gal4*; *UAS-N-dsRNA*, larvae, Figs. 5H, I) induced a reduction of Notch protein levels (Figs. 5B and H), loss of Wg expression, reduced size of the wing field and a disturbed DV affinity boundary, visualized by the expression of *msh-lacZ* (Figs. 5A and I). Co-expression of N^{intra} (in *scalloped-gal4*; *UAS-N-dsRNA*, *UAS-*N^{intra} larvae) restored Wg expression, wing growth and, most interestingly, the DV affinity boundary (Figs. 5C, D). As occurred with other Gal4 drivers (Fig. 4), this rescue happened in the absence of detectable Notch protein levels at the membrane (Fig. 5D, compare with Fig. 5G). The DV affinity boundary defects caused by expression of *N-dsRNA* were also rescued by co-expressing the constitutively activated form of Su(H) (Su(H)-VP16 (Furriols and Bray, 2000)), in *scalloped-gal4*; *UAS-N-dsRNA*, *UAS-Su(H)-VP16* larvae, compare Figs. 5E and F; and in *omb-gal4*; *UAS-N-dsRNA*, *UAS-Su(H)-VP16* larvae, compare Figs. 5I–K). First, these results demonstrate that a transcriptional output of Notch through Su(H) is required and sufficient for the maintenance of an affinity difference between D and V cells. Second, they indicate that uniform activation of Notch in all wing cells generates a DV affinity boundary and imply that restricted activation of Notch at the DV boundary has no instructive role in this process.

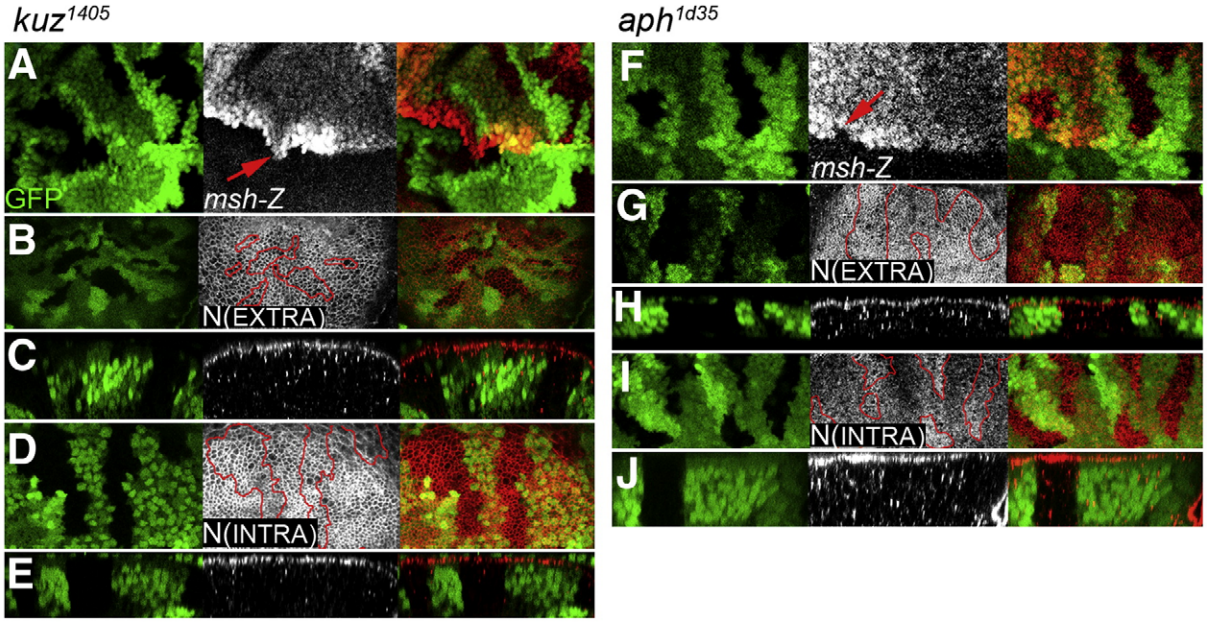

Fig. 3. S2 and S3 cleavages of Notch are required at the DV affinity boundary. (A–E) Wing discs with clones of cells mutant for *kuzbanian* (*kuz*^{1405}) marked by the absence of GFP, and labeled to visualize expression of *msh-lacZ* (antibody to β-Gal, red or white, A), the extracellular (N^{extra}) and intracellular (N^{intra}) domains of Notch (red or white, B–E). The level of Notch protein as well as its sub-cellular localization, visualized in the Z section (C, E), are not affected. Note in panel A that the *kuz*^{1405} clone of dorsal origin is crossing the DV lineage restriction boundary (arrow). (F–J) Wing discs with clones of cells mutant for *aph* (*aph*^{1D35}) marked by the absence of GFP, and labeled to visualize expression of *msh-lacZ* (antibody to β-Gal, red or white, F), the extracellular (N^{extra}) and intracellular (N^{intra}) domains of Notch (red or white, G–J). The level of N^{extra} protein and its subcellular localization, as visualized in the Z section (H), are not affected in *aph* mutant clones. The level of N^{intra} protein is increased due to a defect in S3 cleavage (I, J) but the sub-cellular localization of N^{intra}, as visualized in the Z section (J), is not affected in *aph* mutant clones. Note in panel F that the *aph*^{1D35} clone of dorsal origin induces wild-type cells in the ventral compartment to extend into the dorsal compartment (arrow).

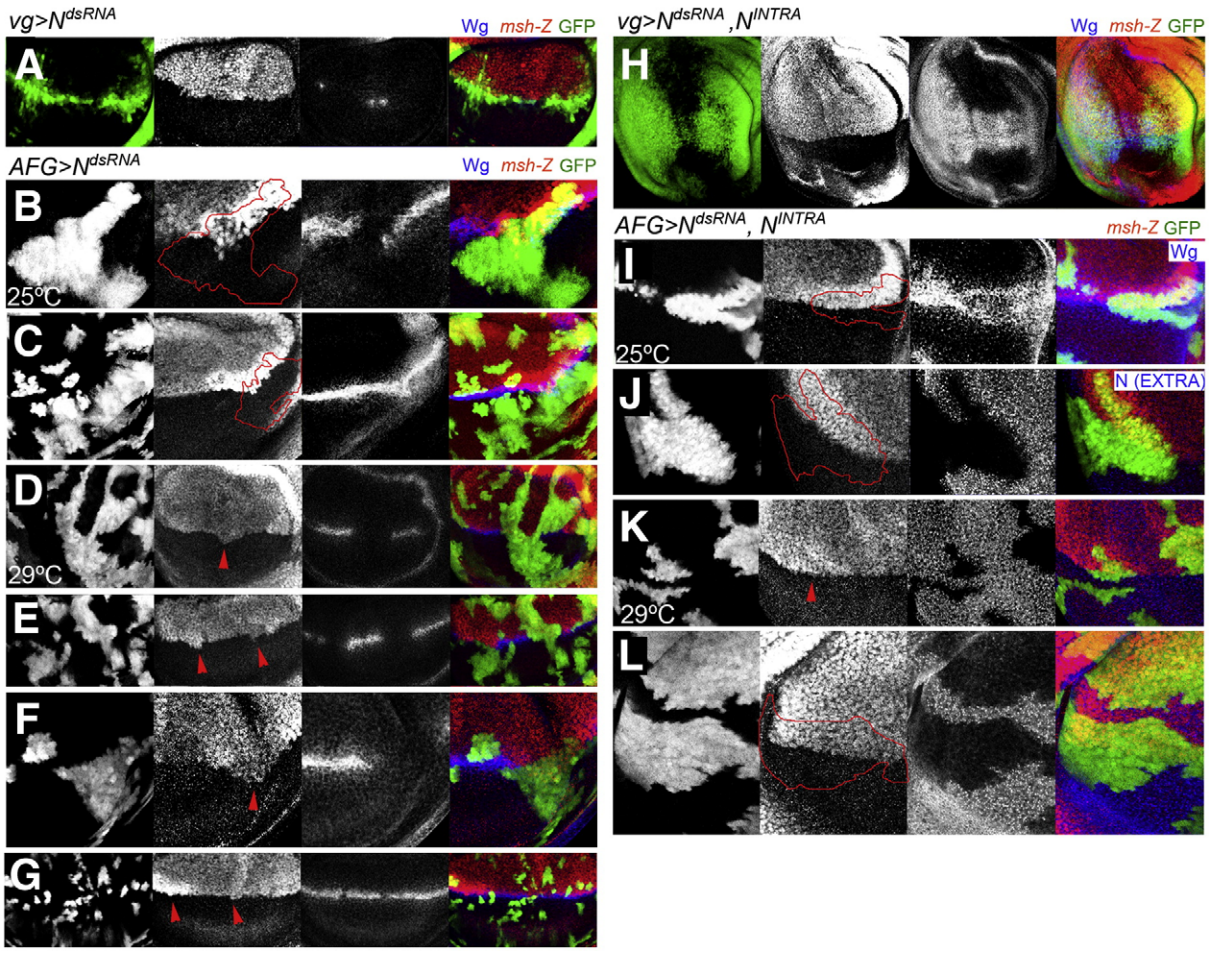

Fig. 4. A transcriptional function of Notch is required and sufficient to make a DV affinity boundary. (A) *vg-Gal4>UAS-GFP, UAS-N^{dsRNA}* wing discs labeled to visualize GFP (green), *msh-lacZ* (antibody to β-gal, red or white) and Wg protein (blue or white) expression. Note that Wg is not expressed and the DV boundary is irregular. (B–G) Wing discs with clones of cells expressing *N^{dsRNA}* and GFP (green), raised at 25 °C (B, C) or 29 °C (D–G) and labeled to visualize *msh-lacZ* (antibody to β-gal, red or white) and Wg protein (blue) expression. The contours of the clones of the cells are labeled by a red line or clones are labeled by a red arrowhead. Note altered Wg expression and disturbed DV boundary in the clones (H) *vg-Gal4>UAS-GFP, UAS-N^{dsRNA}; UAS-N^{intra}* wing discs labeled to visualize GFP (green), *msh-lacZ* (antibody to β-gal, red or white) and Wg protein (blue or white) expression. Note that expression of N^{intra} along the DV boundary, together with *N^{dsRNA}*, is sufficient to restore Wg expression and to generate a straight and smooth DV affinity boundary. (I–L) Wing discs with clones of cells expressing GFP (green) and *N^{dsRNA}*, *N^{intra}*, raised at 25 °C (I, J) or 29 °C (K, L) and labeled to visualize expression of *msh-lacZ* (antibody to β-gal, red or white) and Wg protein (blue, I) or Notch extracellular domain (N^{extra}, J–L). Expression of N^{intra} is sufficient to restore the formation of a straight and smooth boundary and the expression of Wg (I). Expression of N^{intra} restores the straight D/V boundary in the absence of Notch at the membrane (J–L). The contours of the clones of the cells are labeled by a red line or a red arrowhead.

A permissive and transcriptional role of Notch in locating the Actin fence along the DV boundary

The above results indicate that a transcriptional, unrestricted activity of Notch has a permissive role in the formation of the DV affinity boundary. However, a non-transcriptional activity of Notch in modulating the actin cytoskeleton has been proposed to have a role in the process of DV affinity formation (Major and Irvine, 2005). Major and Irvine showed that F-actin staining near the Zonula Adherens (ZA) is thicker at the DV boundary than elsewhere in the wing disc, and this accumulation is polarized within cells as it is stronger at the interface between D and V cells (Fig. 6A). In a situation of reduced Notch activity (in *omb-gal4*; *UAS-N-dsRNA* larvae), F-actin accumulation was abolished at the DV interface (Fig. 6B). We noted that Wg expression was reduced but not completely absent in these conditions, indicating that the activity levels of Notch required for the maintenance of the actin fence are much lower than those required for Wg expression. To address the proposed non-transcriptional activity of Notch in modulating the Actin cytoskeleton, we analyzed the ability of Su(H)-VP16 to rescue F-actin accumulation in the absence of Notch protein at the membrane. As shown in Figs. 6C and D co-expression of Su(H)-VP16 and *N-dsRNA* (in *omb-gal4*; *UAS-N-dsRNA*, *UAS-SU(H)-VP16* larvae) was able to rescue F-actin accumulation. These results rule out any possible transcriptional independent role of Notch in modulating the actin cytoskeleton.

The role of Fringe in the maintenance of the DV affinity boundary

Symmetric activation of Notch at both sides of the DV boundary defines an affinity difference between D and V cells. This observation implies that the role of Notch is permissive and translates the D specific activity of Apterous into a DV difference in cell affinities (Milan and Cohen, 2003). It has been postulated that the activity of the Glycosyltransferase Fringe in D cells is not only required to modify Notch and help to polarize Notch activity towards the boundary but also might be involved in modifying other transmembrane proteins required for the generation of a DV affinity difference (O'Keefe and Thomas, 2001; Rauskolb et al., 1999). We tested this hypothesis by analyzing the capacity of Notch activation to rescue the effects of uniform expression of Fringe and Ap in the maintenance of the DV affinity boundary. Uniform expression of *ap* or *fringe* in the entire wing pouch (in *scalloped-gal4*; *UAS-ap* or *scalloped-gal4*; *UAS-fringe*

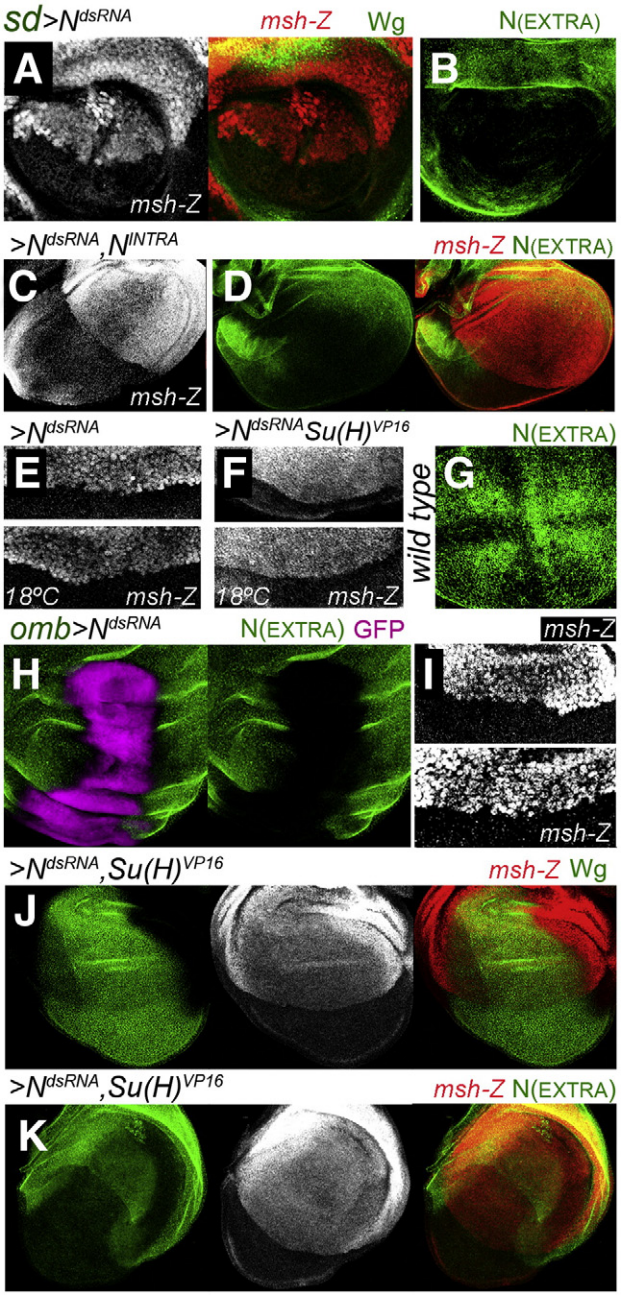

Fig. 5. Restricted Notch activation is not required for DV affinity boundary formation. (A–G) *sd-Gal4*, *UAS-N*^{dsRNA} (A, B, E), *sd-Gal4*, *UAS-N*^{dsRNA}, *UAS N*^{Intra} (C, D), *sd-Gal4*, *UAS-N*^{dsRNA} *UAS-Su(H)-VP16* (F) and *wild type* (G) wing discs raised at 25 °C (A–D and G) or 18 °C (E, F) and labeled to visualize the extracellular domain of Notch (N^{extra}, green, B, D, G), Wg protein (green A) and *msh-lacZ* (antibody to β-gal, red or white, A, C–F) expression. Expression of *N-dsRNA* in the domain of *sd*, which corresponds to the whole wing pouch (A), leads to an irregular DV boundary (A, E) and reduction in Notch protein levels (B). Ubiquitous co-expression of N^{intra} or Su(H)-VP16 in the entire wing pouch is sufficient to generate a straight and smooth DV affinity boundary (C, D and F) even in the absence of Notch at the membrane (D). (H–J) *omb-Gal4*, *UAS-N*^{dsRNA} (H, I) and *omb-Gal4*, *UAS-N*^{dsRNA} *UAS-Su(H)-VP16* (J, K) wing discs labeled to visualize the extracellular domain of Notch (N^{extra}, green, H, K), Wg protein (green, J) and *msh-lacZ* expression (antibody to β-gal, red or white, I–K). Expression of *N-dsRNA* in the domain of *omb* (H) leads to loss of Notch protein and a disturbed DV boundary. Co-expression of Su(H)-VP16 induced high level of Wg expression (J) and restored the DV affinity boundary (J, K) even in the absence of Notch protein (K).

larvae) induced a reduced wing size and disturbed the DV affinity boundary (Figs. 7A and B), visualized in this case by the expression of the *ap-lacZ* reporter. We noted that *ap* and *fringe* expression caused similar effects on wing growth, while the effects on the DV affinity boundary caused by *ap* were much stronger (compare Figs. 7A and B). In both cases, co-expression of N^{intra} restored the growth defects, but was able to restore the DV affinity boundary only in the case of Fringe (Figs. 7C and D). Similar results were obtained using the *vg-gal4* driver (data not shown). These results indicate that there is no Notch-independent contribution of Fringe in this process, and confirm the requirement of Ap together with Notch in the generation of an affinity boundary difference between D and V cells.

Discussion

The DV compartment boundary organizes the growth and pattern of the wing primordium. Restricted and high levels of Notch mediate, in a Su(H)-dependent manner, this organizing activity (Fig. 8). Unrestricted activation of Notch has disastrous consequences on the growth and patterning of the wing primordium (Milán and Cohen, 1999). The localization of the organizing center in the middle of the developing wing and at the interface between D and V cells is maintained by a lineage restriction boundary. This lineage restriction boundary is generated by stable and inheritable acquisition of distinct cell affinities between D and V cells. Here we have presented evidence that unrestricted and low levels of Notch activity is required, in a strictly Su(H)-dependent manner, to mediate the formation of the affinity boundary between D and V cells (Fig. 8). Full-length or processed Notch protein at the membrane does not contribute to this process. Thus, Notch has an instructive function in executing the organizing activity of the DV boundary, while its role in the formation of this boundary can be envisaged as permissive.

Compromised Notch disturbs the DV affinity boundary, while loss of Su(H) activity does not have any effect on this process. This observation has led to the proposal that Notch receptor has a Su(H)-independent role in this process, probably in modulating the actin

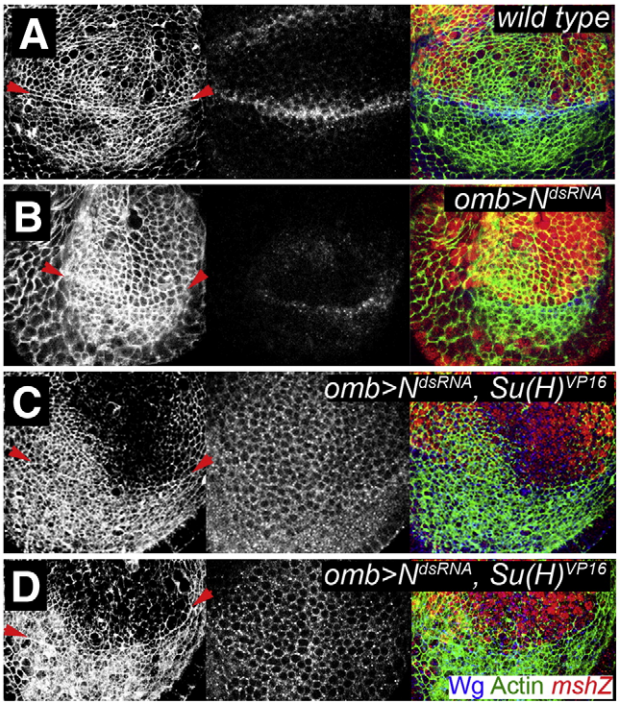

Fig. 6. F-actin accumulation at the DV affinity boundary. Early third instar *wild type* (A), *omb-Gal4*, *UAS-N*^{dsRNA} (B), and *omb-Gal4*, *UAS-N*^{dsRNA}, *UAS-Su(H)-VP16* (C, D) wing discs labeled to visualize the F-actin cable along the DV boundary (green or white), Wg protein (blue) and *msh-lacZ* (antibody to β-gal, red) expression. Expression of *N-dsRNA* in the domain of *omb*, abolishes the F-actin cable (B), while co-expression of *N-dsRNA* and Su(H)-VP16 rescues it (C, D).

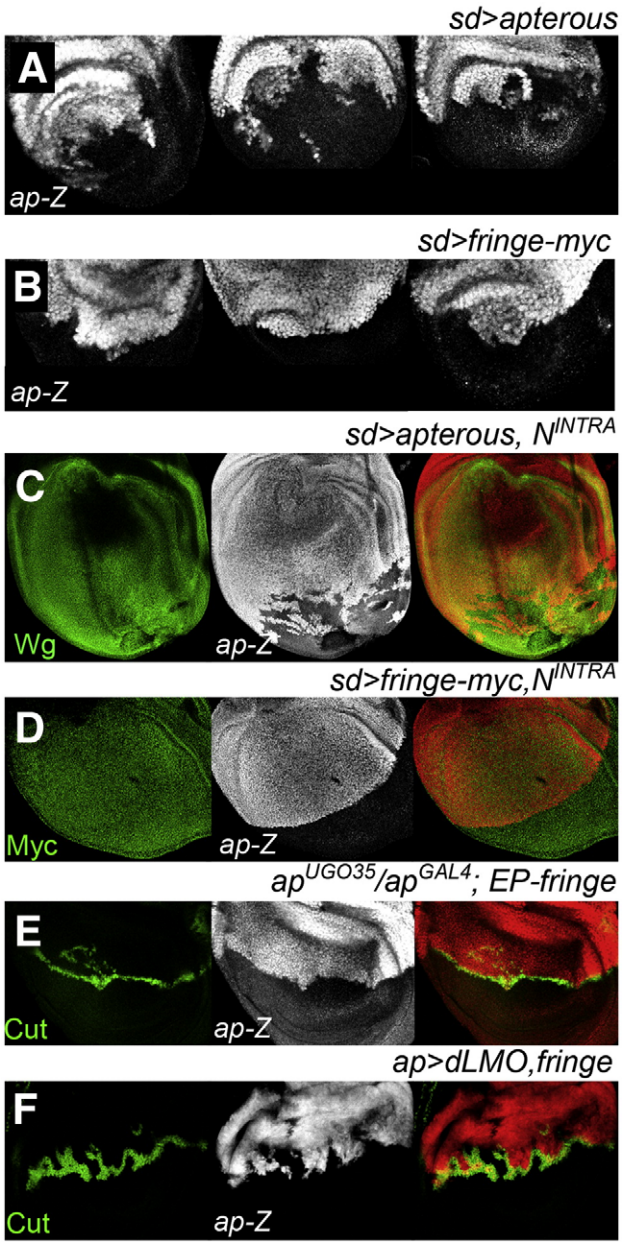

Fig. 7. The role of Fringe at the DV affinity boundary (A–D) *sd-Gal4; UAS-ap* (A), *sd-Gal4; UAS-fringe-myc* (B), *sd-Gal4; UAS-Ap; UAS* N^{intra} (C) and *sd-Gal4; UAS-fringe-myc UAS* N^{intra} (D) wing discs labeled to visualize *apterous-lacZ* expression (antibody to β-gal, red or white) and Wg (green, C) or Myc (green, D) protein expression. Expression of *apterous* or *fringe* in the entire wing pouch disturbed completely the formation of the DV affinity boundary (A, B). Note in panel C that co-expression of *apterous* and N^{intra} leads to a highly irregular DV boundary which is frequently violated. However, expression of N^{intra} in the entire wing pouch, together with *fringe*, is sufficient to generate a straight and smooth DV affinity boundary (D). (E, F) ap^{Gal4}/ap^{ugo35}*; EP-fng* (E) and *apGal4 UASGFP; UAS dLMO*, *UAS fng* (F) wing discs labeled to visualize Cut (green) and Gal4 (red) protein expression. Note in panels E and F that *fringe* expression in a situation of reduced levels (E) or absence of Ap activity (F) is sufficient to induce robust Notch activation at the DV boundary, as indicated by the expression of Cut (E, F), but the DV affinity boundary is still irregular.

cytoskeleton (Major and Irvine, 2005). Our results demonstrate that a transcriptional response to the Notch signal is required and sufficient for the segregation of D and V cells, that this response is mediated by Su(H), and that Notch at the membrane does not contribute to this process. The diverse effects of removing Notch or Su(H) at the DV affinity boundary can be explained by the dual activity of Su(H) as a transcriptional activator in the presence of Notch activity or a transcriptional repressor in its absence. Thus, a critical role of Notch might be to relieve Su(H)-mediated transcriptional repression of Notch target genes required in this process. Consistent with this, loss of Su(H) rescues the DV affinity defects caused by loss of Notch signaling (Koelzer and Klein, 2006). It is interesting to note that in vertebrates a transcriptional output of Notch via Su(H) defines an affinity difference between boundary and non-boundary cells at the rhombomere boundaries (Cheng et al., 2004). The possible conserved role of Notch in alleviating the transcriptional repression mediated by Su(H) remains to be elucidated.

Subdivision of the wing primordium into D and V compartments, the generation of an affinity difference between D and V cells and the establishment of a Notch-mediated organizing center at the DV boundary relies on the activity of the LIM-homeodomain Ap in D cells. Early in development, Ap induces the complementary expression of the Notch ligands Serrate and Delta to D and V cells, and the D specific expression of the glycosyltransferase Fringe, which restrict Notch activation to the DV boundary (Fig. 1). The DV affinity boundary is established by the Ap-dependent compartment specific expression of transmembrane proteins like Caps and Tartan, among others (Milan et al., 2001a). Later in development, maintenance of this affinity boundary also depends on the activity of Ap in D cells, but in this case Notch translates this activity into a difference in cell affinity. Moreover, our results indicate that the only role of Fringe in DV boundary formation is to polarize Notch signaling towards the boundary. Thus, the organizing activity of the DV boundary is executed by the activity of Notch, while Ap has a central role in the establishment and maintenance of the DV lineage restriction boundary (Fig. 8).

The permissive role of Notch and the instructive role of Ap in this process is consistent with previous reports addressing the requirements of Notch, Fringe and Ap in the maintenance of the DV affinity difference (Milan and Cohen, 1999; Milan and Cohen, 2003). These reports were based on two different functional assays. First, a change in Ap activity induces clones of cells to make smooth boundaries with the neighboring cells most probably due to a difference in cell affinities. When abutting the DV boundary, these clones completely sort towards the other compartment. In contrast, a change in Fringe activity or absence of Notch induces clones of cells to disturb the DV affinity boundary without a clear violation and sorting towards the opposite compartment. These clones do not

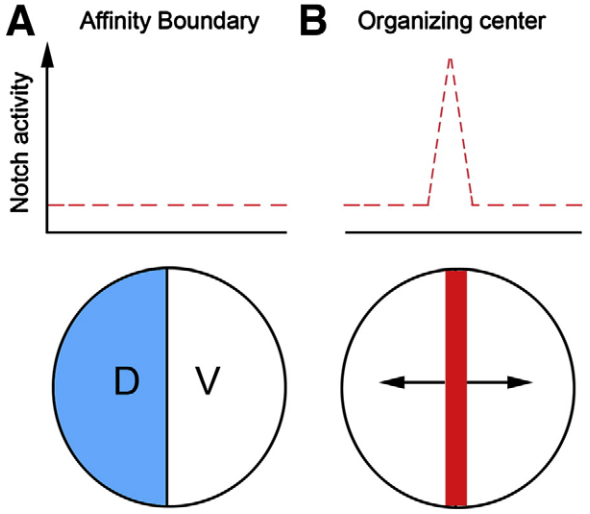

Fig. 8. Permissive and instructive roles of Notch at the DV boundary. (A) Low and unrestricted levels of Notch have a permissive role in mediating an affinity difference between D and V cells and conferred by the activity of Apterous in D cells. (B) High and restricted levels of Notch at the DV boundary have an instructive role in organizing in a symmetric manner growth and pattern at both sides of the boundary.

show smooth boundaries either (Fig. 4 and Milan and Cohen, 2003). Second, Fringe expression in D cells in a situation of absence of Ap activity (in *ap-Gal4*; *UAS-dLMO*/*UAS-fng* wing discs; note dLMO is a potent repressor of Ap protein activity, (Milan et al., 1998; Shoresh et al., 1998; Zeng et al., 1998)) is able to rescue Notch activation along the DV boundary but not the DV affinity difference (Fig. 7F, see also Milan et al., 2005). Even though it was argued that the activity levels of Notch in these latter assays were not sufficient to execute its role at the DV affinity boundary (Major and Irvine, 2005), this is not the case. The high threshold Notch regulated gene *cut* is expressed in these situations, while the DV affinity difference is completely abolished (Figs. 7E, F). Two different observations suggest, however, that high levels of Notch activity might confer, in an Ap independent manner, a boundary specific cell affinity that contributes to shape the DV affinity boundary. First, clones of cells expressing the activated form Su(H)-VP16 make smooth boundaries with wild type neighboring cells, most probably due to a difference in cell affinities (data not shown). Second, in a situation of reduced *apterous* activity (in ap^{Gal4}/ap^{ugo35}; *EP-fng* wing discs; note ap^{Gal4} is a hypomorphic allele of *apterous* (Milan and Cohen, 2003)), restored Notch activation is able to generate a relatively smooth DV affinity boundary (Fig. 7E). Thus, Notch has a permissive role in translating the difference between D and V cells conferred by Ap into an affinity difference, but Notch might also contribute to a small extent and in an Ap independent manner to shape the DV affinity boundary. It is interesting to note that the vertebrate orthologue of Ap, Lmx1b, is involved in defining a lineage restriction boundary in the limb bud mesenchyme (Arques et al., 2007; Pearse et al., 2007). We can therefore propose that the same genes, gene regulatory networks and signaling pathways are used in a reiterative manner to define similar tissue properties in developing vertebrates and invertebrates, and as such, the *Drosophila* wing primordium provides a very useful system to study the acquisition of these tissue properties at the genetic level.

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