Influence of Notch on dorsoventral compartmentalization and actin organization in the *Drosophila* wing

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Summary

Compartment boundaries play key roles in tissue organization by separating cell populations. Activation of the Notch receptor is required for dorsoventral (DV) compartmentalization of the *Drosophila* wing, but the nature of its requirement has been controversial. Here, we provide additional evidence that a stripe of Notch activation is sufficient to establish a sharp separation between cell populations, irrespective of their dorsal or ventral identities. We further find that cells at the DV compartment boundary are characterized by a distinct shape, a smooth interface, and an accumulation of F-actin at the adherens junction. Genetic manipulation establishes that a stripe of Notch activation is both necessary and sufficient for this DV boundary cell phenotype, and supports the existence of a non-transcriptional branch of the Notch pathway that influences F-actin. Finally, we identify a distinct requirement for a regulator of actin polymerization, *capulet*, in DV compartmentalization. These observations imply that Notch effects compartmentalization through a novel mechanism, which we refer to as a fence, that does not depend on the establishment of compartment-specific cell affinities, but does depend on the organization of the actin cytoskeleton.

Key words: Compartment, Boundary, Fringe, Capulet, *Drosophila*

Introduction

Many tissues become subdivided during their development by compartment boundaries – smooth lines of lineage restriction that prevent cells on one side from intermingling with cells on the other (reviewed by Blair, 2003b; Irvine and Rauskolb, 2001). These boundaries play crucial roles in tissue organization by separating distinct regions, and by serving as sites of morphogen synthesis. The *Drosophila* wing is subdivided by both anteroposterior (AP) and dorsoventral (DV) compartment boundaries. Although a number of genes that regulate the establishment of these boundaries have been identified, the cellular mechanisms that effect compartmentalization remain poorly understood.

The specification of AP and DV compartments is initiated by the restricted expression of transcription factors Engrailed/Invected (En), which is expressed by posterior cells, and Apterous (Ap), which is expressed by dorsal cells (Blair et al., 1994; Diaz-Benjumea and Cohen, 1993; Lawrence and Struhl, 1982; Morata and Lawrence, 1975; Simmonds et al., 1995; Tabata et al., 1995; Zecca et al., 1995). AP compartmentalization also requires Hedgehog (Hh)-mediated signaling from posterior cells to anterior cells (Blair and Ralston, 1997; Dahmann and Basler, 2000; Rodriguez and Basler, 1997). Although the molecular targets remain unknown, genetic manipulation that results in Hh pathway activation can cause cells to sort from posterior to anterior, whereas manipulation that results in a loss of Hh signaling can cause cells to sort from anterior to posterior. These observations imply that Hh signaling influences compartmentalization by promoting an anterior-type cell affinity.

Ap influences DV compartmentalization through at least two distinct mechanisms, which appear to act sequentially. First, Ap promotes the dorsal expression of Tartan (Trn) and Capricious (Caps). A role for Trn and Caps has been inferred from the observation that ectopic expression of these proteins can cause ventral cells to sort to the DV compartment border (Milan et al., 2001). Loss-of-function mutations in these genes do not affect compartmentalization, but it is thought that they might act redundantly with other factors (Milan et al., 2001). However, Trn and Caps are expressed specifically by dorsal cells in the second larval instar, when the DV compartment boundary forms, but in the third instar they become expressed in ventral lateral cells and stop being expressed by dorsal medial cells. Thus, any role of Trn and Caps in DV compartmentalization must be transient.

A second mechanism by which Ap influences compartmentalization is through its influence on Notch signaling (Micchelli and Blair, 1999; O'Keefe and Thomas, 2001; Rauskolb et al., 1999). Ap promotes the dorsal-specific expression of a Notch ligand, Serrate (Ser), and a Notch glycosyltransferase, Fringe (Fng) (Couso et al., 1995; Irvine and Wieschaus, 1994). Modification of Notch by Fng both inhibits Ser-to-Notch signaling, restricting Ser to signaling from dorsal cells to ventral cells, and potentiates Delta-to-Notch signaling, enabling the activation of Notch by the Delta

ligand in dorsal cells (Fig. 1A) (Fleming et al., 1997; Haines and Irvine, 2003; Panin et al., 1997). The action of Fng thereby positions and restricts a stripe of Notch activation along the DV border. Notch signaling is required for DV compartmentalization (Micchelli and Blair, 1999; Rauskolb et al., 1999), but there are crucial differences between the action of Notch at the DV boundary and that of Hh at the AP boundary. Signaling between dorsal and ventral compartments is bidirectional, rather than unidirectional. Additionally, neither ectopic activation of Notch nor loss of Notch activation cause directed changes in cell location (Micchelli and Blair, 1999; Milan and Cohen, 2003; Rauskolb et al., 1999). Rather, when Notch activation is disrupted, cells can intermingle in either direction, irrespective of their genotype or DV identity.

The influence of Notch indicates that it cannot affect DV compartmentalization simply by promoting a compartment-specific cell affinity on its own, and different models have been proposed to explain its role (Irvine and Rauskolb, 2001; Micchelli and Blair, 1999; Milan and Cohen, 1999; Milan and Cohen, 2003; O'Keefe and Thomas, 2001; Rauskolb et al., 1999). Two models are based on the conventional view that compartmentalization requires the establishment of distinct, compartment-specific cell affinities. In one version (Micchelli and Blair, 1999), Notch activation confers a distinct boundary cell affinity, which is modified by the presence of Ap in dorsal cells into a dorsal-boundary affinity. This model was based on loss-of-function experiments, and would appear to be inconsistent with the results of gain-of-function experiments: if Notch activation conferred a boundary-type cell affinity, then clones of cells in which Notch was constituitively activated would be expected to sort to the DV boundary, but this is not the case (Milan and Cohen, 2003; Rauskolb et al., 1999). A related model (Milan and Cohen, 2003) gets around this problem by proposing that Notch be considered 'permissive' and Ap 'instructive' for the specification of a distinct cell affinity. Thus, it shares the proposal that Ap and Notch act combinatorially to specify a dorsal-boundary cell affinity, but differs in that Notch activation alone is proposed to be insufficient to specify a distinct cell affinity that can influence compartmentalization.

By contrast, we have proposed a completely different model, in which Notch activation does not influence compartmentalization by contributing to dorsal- or ventral-type cell affinities, but rather creates a fence (Irvine and Rauskolb, 2001; Rauskolb et al., 1999), which we define as a property or behavior of cells at the border that keeps them from intermingling. In support of this model, we note that an ectopic stripe of Notch activation – created, for example, by mutation or mis-expression of Fng in clones of cells – can be sufficient to separate cells, even though cells on both sides of the border are all dorsal or are all ventral, and even though cells on both sides of the border have similar levels of Notch activation (O'Keefe and Thomas, 2001; Rauskolb et al., 1999) (Fig. 1B). These observations are inconsistent with models that propose a requirement for a Notch-independent influence of Apterous on cell affinity, but they have been disputed (Milan and Cohen, 1999; Milan and Cohen, 2003).

Thus, in the first section of this paper, we revisit the issue of the sufficiency of Notch activation in separating cells, providing both additional data in support of the fence model, and an alternative explanation for the observations of Milan

and Cohen (Milan and Cohen, 1999; Milan and Cohen, 2003). We then show that cells at the DV boundary have a distinctive shape, and that F-actin accumulates at the adherens junctions at the DV interface. Genetic manipulation establishes that a stripe of Notch activation is both necessary and sufficient for these phenotypes. The observation of a distinct, Notch-dependent boundary morphology further supports the fence hypothesis, and also suggests that a non-transcriptional branch of the Notch pathway participates in DV compartmentalization. Finally, we show that a regulator of actin polymerization exhibits a distinct requirement at the DV compartment boundary, consistent with the possibility that Notch influences compartmentalization via its ability to modulate F-actin. Together, our observations emphasize that DV compartmentalization is mechanistically distinct from AP compartmentalization, and that the establishment of a separation fence rather than specific compartmental affinities provides the best explanation for DV compartmentalization.

Materials and methods

Drosophila stocks and crosses

Clones of cells ectopically expressing Fng, Delta, N-ΔEN or N-intra were generated as described previously, using *UAS-fng27, UAS-Dl[30b], UAS-NΔEN[42B2]* or *UAS-NΔ34a* transgenes (Larkin et al., 1996; Panin et al., 1997; Rauskolb et al., 1999). Clones were induced in larvae at 24 to 48 hours after egg laying (AEL), and larvae were staged relative to the L2 to L3 molt by the anterior spiracles. *capt* and *chic* mutant clones were generated at 36-60 hours AEL by both standard Flipase-mediated mitotic recombination, and by the MARCM method (Blair, 2003a). For temperature-shift experiments with N^{ts}, animals were cultured at 18°C through the mid-second instar, and then maintained at 18°C or shifted to 22°C, 25°C or 29°C. *UAS-Abl[6]* (D. van Vactor, Boston, MA) was used for ectopic *Abl* expression, and $abl^1/Df(3L)stJ7$ for *Abl* loss of function.

Depending on the availability and species of antibodies available, dorsal or anterior cells were marked in some experiments by enhancer trap lines; we used ap-$lacZ^{rQ107}$, ap-$lacZ^{rK568}$ and ci-$lacZ$. *UAS-α-Cat:GFP arm-Gal4* was used to detect α-catenin (Oda and Tsukita, 1999).

Stocks used for making clones included:

y w hs-Flp[122]; act>y⁺>gal-4 UAS-GFP (AyGal4);
y w hs-Flp[122]; Ubi-GFP FRT40A;
y w hs-Flp[122]; FRT42B[G13] GFP:nls;
y w hs-Flp[122] tub-Gal4 UAS-GFP/FM7; tub-Gal80 FRT40A/CyO;
capt^{E636} FRT40A/CyO;
capt^{E593} FRT40A/CyO;
chic^{221} FRT40A; dpp-lacZ/L14;
capt^{E636} FRT40A; UAS-p35/L14;
FRT42B[G13] ena^{23};
FRT42B[G13] ena^{GC1}/CyO act-GFP; and
fat^8 FRT40A/CyO-GFP.

Immunostaining and image analysis

Imaginal discs were fixed for 15 minutes in 2% formaldehyde (methanol-free) in Ringer's solution (120 mM NaCl, 1.88 mM KCl, 2.38 mM NaHCO₃, 0.06 mM NaH₂PO₄, 0.82 mM CaCl₂) and then stained immediately. Primary antibodies used were rat anti-dLMO (Beadex) (at 1:100, S. Cohen, Heidelberg, Germany), mouse anti-WG 4D4 [at 1:400, Developmental Studies Hybridoma Bank (DSHB)], rat anti-E-cadherin DCAD2 (at 1:20, DSHB), mouse anti-Armadillo N2 7A1 (at 1:100, DSHB), mouse anti-Delta C594.9B (at 1:50, DHSB), mouse anti-Ena 5G2 (1:50, DSHB), mouse anti-phosphotyrosine PY20 (1:300, Covance), rabbit anti-β-gal (at 1:20, ICN), mouse anti-

En 4D9 (at 1:200, DHSB), mouse anti-alpha tubulin-FITC (at 1:50, Sigma F2168). Secondary antibodies were from Molecular Probes and Jackson Immunoresearch Laboratories. F-actin labeling was performed after immunostaining, using 488- or 546-conjugated phalloidin (Molecular Probes) at a 1:10 dilution for 40 minutes at room temperature.

Discs were analyzed by the acquisition of serial optical sections on a Leica TCS SP confocal microscope. Adjacent sections, including the apical disc surface, were combined by maximum projection with Leica software. In those instances where the curvature of the disc did not allow good visualization of the entire apical surface through a single maximum projection, groups of 10-30 sections (representing 1-6 µm) were combined by maximum projection, and then a composite image of the apical surface was created by using the layers feature of Adobe Photoshop. Similarly, because the nuclei are basal to the apical actin cytoskeleton, for purposes of illustration, we prepared images that combine projections through basal regions to show nuclear markers with projections through apical regions to show apical F-actin.
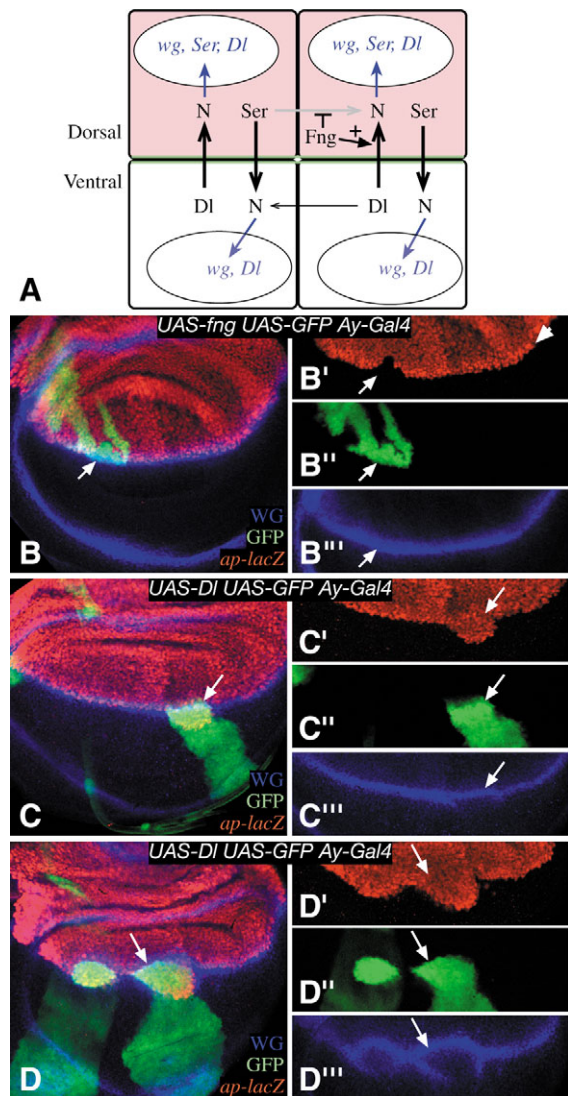
Results

A stripe of Notch activation is sufficient to separate cells

Because the normal DV compartment border occurs in the middle of a stripe of Notch activation, compartmental affinity

models require that Notch acts in conjunction with other, spatially restricted factors (Micchelli and Blair, 1999; Milan and Cohen, 2003). The sufficiency of Notch activation in separating cells is thus a crucial issue in evaluating potential models for compartmentalization. Normally, clones of cells in the wing tend to have irregular borders, except where they touch compartment boundaries. Clone edges with Notch activation can be created by the expression of Notch ligands, or, because of its influences on Notch-ligand interactions, by the expression or mutation of *fng*. Importantly, these clone edges can be as straight and smooth as the normal DV compartment boundary, and indeed can even be integrated into the normal DV boundary, leading us to infer that a stripe of Notch activation can be sufficient to effect DV compartmentalization (Fig. 1) (Rauskolb et al., 1999). By contrast, others have emphasized that stripes of Notch activation associated with altered expression of Fng can be irregular (Milan and Cohen, 1999; Milan and Cohen, 2003), and have inferred from this that Notch activation is insufficient for compartmentalization.

However, the ability of Fng to effect Notch activation varies across the wing (Irvine and Wieschaus, 1994; Milan and

Fig. 1. Relationship between Notch activation and DV compartmentalization. (A) Signaling interactions at the DV boundary. Delta (Dl) is expressed by both dorsal and ventral cells in response to Notch activation (blue arrow), but signals most effectively (thick black arrow) to dorsal cells owing to the presence of Fng, and only signals poorly (thin arrow) to ventral cells. Activated Notch in dorsal cells acts together with the dorsal-specific gene *ap* (not shown) to promote expression of Ser. Ser is blocked (black T) from signaling (gray arrow) to other dorsal cells by Fng, and thus is limited to signaling back across the compartment boundary (thick black arrow) to ventral cells, where it activates Notch. As shown here, Notch activation also results in the elevation of F-actin (green lines) along the cell interface where peak signaling occurs; the resolution of confocal microscopy is such that this usually appears as a single line (see also Fig. 4). (B-D) Third instar wing imaginal discs, stained for *ap-lacZ* expression (red) to mark dorsal cells, and Wg (blue), to mark Notch activation. Expression of *ap-lacZ* is a reliable marker of dorsal provenance, as it is not affected by Notch signaling or changes in cell location (Blair et al., 1994; Micchelli and Blair, 1999; Rauskolb et al., 1999). In this and subsequent figures, discs are oriented with ventral down and anterior to the left. (B'-D''') Individual stains of the discs shown in B-D. (B) *AyGal4 UAS-fng*, with a clone of Fng-expressing cells (arrow) marked by co-expression of GFP (green). Ectopic expression of Fng can effectively reposition the compartment boundary away from the normal DV interface by simultaneously creating an ectopic stripe of Notch activation within ventral cells, and eliminating normal Notch activation at the DV interface (Rauskolb et al., 1999). The normal DV interface (arrowhead) is relatively straight and smooth, and is disturbed by the Fng-expressing clone. (C,D) *AyGal4 UAS-Dl*, with clones of Dl-expressing cells (arrows) marked by co-expression of GFP (green). Ectopic expression of Dl can effectively reposition the compartment boundary away from the normal DV interface by simultaneously creating an ectopic stripe of Notch activation within dorsal cells, and eliminating normal Notch activation at the DV interface. In C, the clone edge is smoother apically, as evidenced by Wg expression, than basally, as evidenced by GFP expression. In D, the ectopic Notch activation stripes are smooth, but do not completely register with the wild-type DV boundary.

Cohen, 2000), presumably because Fng does not activate Notch directly, but rather influences its sensitivity to ligands, which are themselves distributed in a dynamic, spatial pattern. Thus, a trivial explanation for the observation that edges of Fng expression are more irregular than edges of Ap expression (Milan and Cohen, 2003) is simply that they effect different levels of Notch activation. Consistent with this, Fng clone edges near the DV boundary tend to be smoother than clone edges that are far from the DV boundary (data not shown). Indeed, the argument that Notch activation is not sufficient for compartmentalization (Milan and Cohen, 1999; Milan and Cohen, 2003) relies on the assumption that expression of Wingless (Wg), which has been widely used as a marker of Notch activation in the wing, is a reliable indicator of the quality of activation required for compartmentalization. To evaluate this assumption, we partially compromised Notch signaling using a temperature-sensitive allele of *Notch*, and then assayed both Wg expression, and the interface between dorsal and ventral cells (Fig. 2). At intermediate temperatures, expression of Wg remains detectable even as the DV interface becomes irregular (Fig. 2B). Thus, compartmentalization appears to require a level or quality of Notch activation that is distinct from that required for Wg expression.

In a complementary approach, we evaluated the consequences of driving high levels of ectopic Notch activation. Clones of cells expressing Delta under control of the *UAS-Dl[30b]* transgene have smooth edges, and often
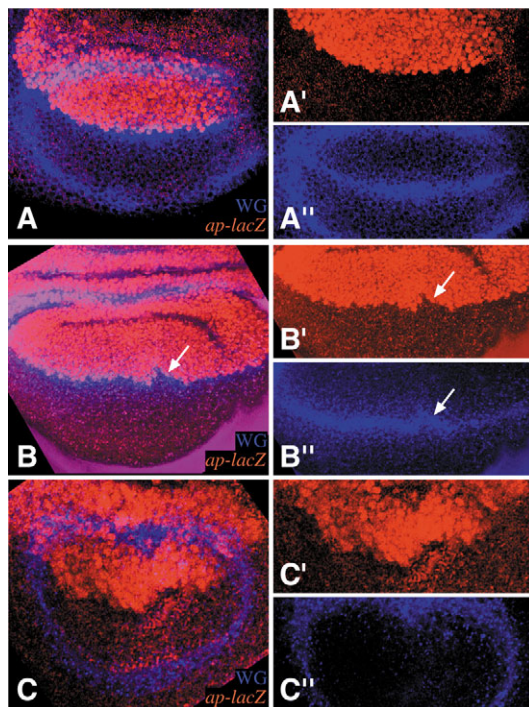
appear quite round (Fig. 1C,D, and see also below). As is the case for clones of cells expressing Fng or Ser (Milan and Cohen, 2003; Rauskolb et al., 1999), ectopic Notch activation stripes induced by Delta can also line up with the endogenous DV compartment boundary in flanking cells (Fig. 1C). Although these observations do not exclude the possibility of Notch-independent contributions of Ap to DV compartmentalization, they emphasize that there is no need to invoke such contributions, as Notch activation can be sufficient to effect a compartment-like cell separation.

Cell morphology and actin organization at compartment boundaries

To gain insight into potential cellular mechanisms by which Notch might effect DV compartmentalization, we investigated the actin and microtubule cytoskeletons. No special features of the microtubule cytoskeleton could be discerned in boundary cells, and the examination of mitotic spindles ruled out one potential model for fence construction, that of oriented mitoses (Fig. 3). However, staining with fluorescently labeled phalloidin, which binds to F-actin, revealed a distinct cellular
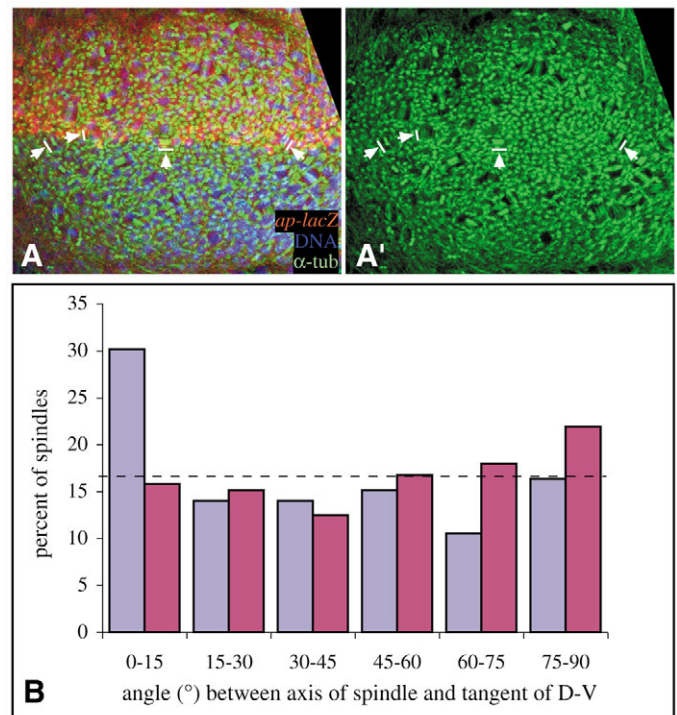

Fig. 3. Spindle orientations in the wing. (A) Third instar wing imaginal disc, stained for α-tubulin (green), *ap-lacZ* (red), and DNA (blue). Arrows indicate examples of dividing cells at the DV boundary, bars mark their axis of division. This can occur at different orientations relative to the DV boundary. (B) Histogram showing the percent of spindles at the DV boundary (blue bars, out of 86 total) or away from the DV boundary (red bars, out of 456 total) found in different orientations relative to the boundary. The angle was defined by the axis of the spindle and the tangent of the nearest DV interface. Angles were binned into 15° increments, thus a random distribution would predict 16.7% of spindle orientations (one sixth of the total) in each bin (dashed line). There is a slight bias for cells at the boundary to divide parallel to it, but not enough to account for compartmentalization.

Fig. 2. Differential sensitivity of compartmentalization and Wg expression to Notch. (A-C) N^{ts} wing imaginal discs, from animals cultured at 18°C during embryonic development, and then at (A) 18°C, (B) 22°C or (C) 29°C during larval development. At 22°C, in some instances (B, arrow), the DV interface is obviously disturbed despite continuous Wg expression. At 18°C, the DV interface is normal or subtly disturbed, and Wg expression is normal, whereas, at 29°C, the DV interface is grossly disturbed, and Wg is not expressed.

morphology and organization of F-actin at the DV border (Fig. 4).

Although F-actin is concentrated at adherens junctions throughout the epithelial cells of the wing imaginal disc, F-actin staining near the adherens junctions is noticeably thicker and more intense along the DV boundary than elsewhere in the disc (Fig. 4). Additionally, the interface between D and V cells is exceptionally straight and smooth at the level of the adherens junctions at the DV boundary. Both of these distinct features of the DV boundary are only observed at the level of the adherens junction – more basally the DV interface is not distinguishable from cell-cell contacts elsewhere in the disc (Fig. 4A″). The elevated F-actin staining is a particular property of F-actin organization, rather than cellular

Fig. 4. Actin and cell morphology at compartment boundaries. Wild-type mid-third instar wing discs, stained with phalloidin to reveal F-actin (green). To enable visualization across the curved surface of the disc in a single panel, the stains shown are projections through different focal planes. White arrows indicate the DV boundary. (A) Disc with elevated apical F-actin staining at the DV boundary clearly visible. A nuclear marker for dorsal cells (*ap-lacZ*, red) is not visible in apical focal planes, but is visible in more basal focal planes (A′). F-actin staining, however, is neither elevated nor smooth in basal-lateral sections (A′,A″). (B-E) Close up of a portion of the DV border, stained for F-actin and E-cadherin (B), F-actin and β-catenin (C), F-actin and anti-phospho-tyrosine (D), and Enabled and E-cadherin (E). (F) Vertical section through a disc stained for F-actin (green), E-cadherin (red) and *ap-lacZ* (blue). Arrow indicates F-actin at the DV boundary, which overlaps with E-cadherin.

morphology or adherens junctions, because other proteins that localize to the adherens junctions, including E-cadherin, α-catenin and β-catenin, are not elevated at the DV boundary (Fig. 4B′,C′ and data not shown). However, screening through actin-associated proteins and markers of adherens junctions identified Enabled (Ena), a member of the Ena/VASP family of actin regulators (Gertler et al., 1995), as a protein whose staining is elevated in conjunction with F-actin at the DV interface (Fig. 4E). Ena is a substrate for tyrosine kinases, including the actin regulator Abl (Gertler et al., 1995), and a general stain for phospho-tyrosine also reveals upregulation at the DV interface (Fig. 4D′).

Time-course experiments revealed that elevated F-actin staining is already visible at the beginning of the third instar and persists past the middle of the third instar (Fig. 5A-C; the DV F-actin stripe was visible in 61/61 discs at 0-12 hours of third instar, and 61/62 discs at 12-24 hours of third instar). After this, elevated F-actin staining is no longer consistently observed, although the interface between dorsal and ventral cells continues to be relatively straight and smooth at the adherens junctions (Fig. 5C-E, the DV F-actin stripe was visible in 16/78 discs at 24-36 hours of third instar, and 1/61 discs at 36-48 hours of third instar). Around 48 hours after the beginning of third instar, F-actin begins to accumulate in two stripes that flank the DV boundary, roughly 4 to 6 cells apart (Fig. 5E,F) (Blair, 1992). These late F-actin stripes appear around the time that Notch ligands become expressed in two stripes that flank the DV border, and peak F-actin staining at this stage is adjacent to peak ligand expression (Fig. 5F).

Interestingly, F-actin staining reveals that the AP boundary is not as straight as the DV boundary during early third instar (Fig. 5A,B), but does straighten out later in third instar (Fig. 5C-E) (Blair, 1992). Elevation of F-actin is also sometimes (18/120 discs) observed at the AP boundary, but this occurs preferentially at later stages, is consistently weaker than at the DV boundary, and in almost all cases only extends along part of the AP boundary, mostly in dorsal cells (Fig. 5 and data not shown).

Notch regulates the F-actin cytoskeleton in the wing

To determine whether the distinct features of the DV boundary require positional information at the juxtaposition of dorsal and ventral cells, or instead are regulated by Notch activation, we examined F-actin under conditions where the pattern of Notch activation was altered. Elimination of normal Notch activation by expression of Fng in ventral cells abolishes the distinctive features of cellular morphology and F-actin organization at the interface between dorsal and ventral cells (Fig. 6A). These features of the DV interface are also eliminated by downregulation of Notch function in N^{ts} animals (data not shown). Thus, Notch activation is required for F-actin organization and cellular morphology at the DV boundary. Importantly, the ectopic Notch activation stripe generated by high-level expression of Fng in ventral cells (i.e. in *UAS-fng ptc-Gal4* animals) is consistently associated with induction of a DV boundary-like cell morphology, F-actin organization and upregulation of Ena protein (Fig. 6A and data not shown). Because this now occurs entirely within ventral cells, Notch activation must also be sufficient for the regulation of these processes. Lower level Fng expression in clones of cells (i.e. in *UAS-fng AyGal4* animals) was less effective at influencing
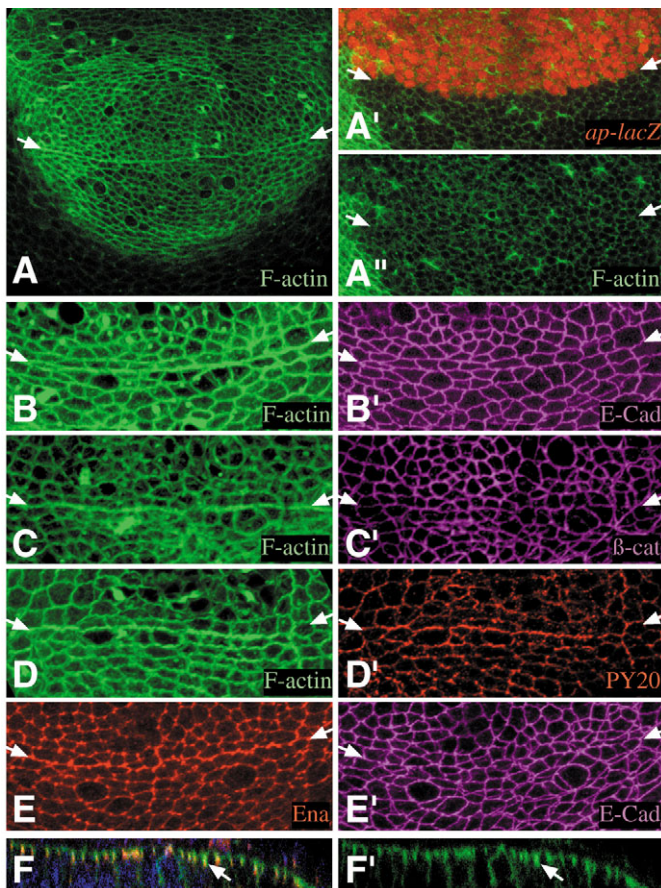
F-actin organization and cell morphology, but, as noted above, the edges of these clones are sometimes irregular. Importantly,

Delta-expressing clones, which are generally rounder than Fringe-expressing clones, are often associated with an upregulation of F-actin and a smooth interface at the adherens junctions (15/25 dorsal clones, and 27/51 total clones, exhibited upregulation of F-actin) (Fig. 6B,C). The change in F-actin staining does not appear to be a simple consequence of differences in cell affinity, because clones of cells mutant for the protocadherin gene *fat* (Mahoney et al., 1991) are not associated with upregulation of F-actin (Fig. 6E).

One striking feature of F-actin organization in DV boundary cells is its polarized nature: F-actin is specifically elevated along the interface between dorsal and ventral cells (Figs 4, 5). The resolution of confocal microscopy is not sufficient to separate F-actin staining in adjacent cells, but the accumulation does not appear to be biased with respect to staining for other markers (Fig. 4), suggesting that it occurs in both dorsal and ventral cells at the boundary. The polarization of F-actin accumulation does not require a DV interface, as it can also be observed along ectopic Notch activation stripes (Fig. 6A-C). Thus, it can be generated solely by Notch activation. Analysis of transcriptional targets in the wing suggests that levels of Notch activation are similar along both sides of the boundary, and thus it seems unlikely that the polarization of F-actin accumulation would be generated by quantitative differences in the transcriptional response. Moreover, no transcriptional upregulation of *ena* at the DV boundary could be detected by situ hybridization (not shown). The unique feature of the cell surface at which F-actin appears to be elevated is that it is the main surface at which Notch-ligand interactions occur (Fig. 1A). Thus, we take the polarization of F-actin accumulation as evidence for a non-transcriptional input from the Notch pathway to the actin cytoskeleton. Consistent with this hypothesis, expression of the intracellular domain of Notch (N-intra), which localizes to the nucleus and behaves as a constitutively activated receptor in regard to transcriptional outputs of the pathway (Schweisguth, 2004), is less effective at reorganizing F-actin and cell morphology than is Fringe or Delta (Fig. 6D; 6/40 N-intra clones had elevated F-actin at their edges). N-intra does exert some influence on F-actin, but as this occurs preferentially in older wing discs, and at the edges of N-intra expressing clones, it most likely results from its ability to induce the expression of Notch ligands (de Celis and Bray,
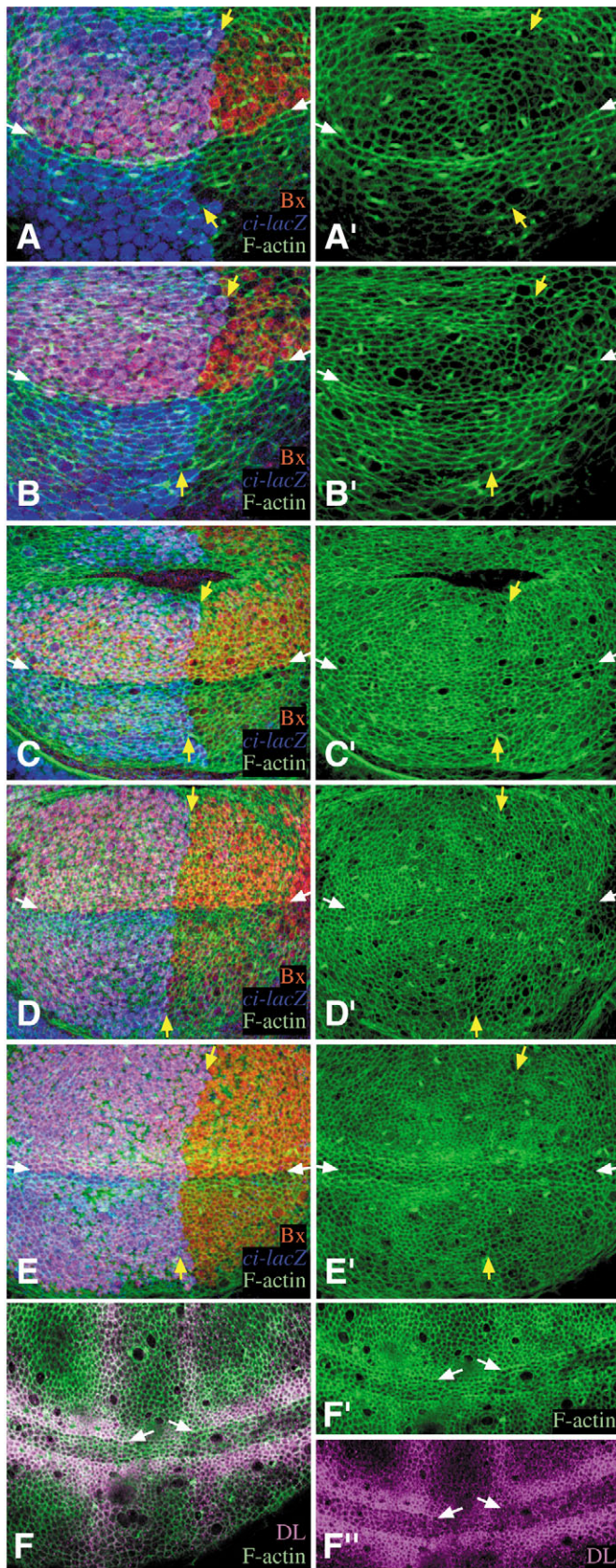
Fig. 5. Time course of F-actin organization. White arrows indicate F-actin at the DV boundary, yellow arrows point to F-actin at the AP boundary. (A-E) Discs of different ages stained for F-actin, an anterior marker (*ci-lacZ*, blue) and a dorsal marker (Bx, red). The apical DV F-actin line coincides precisely with the DV boundary during early-mid third instar, but because Bx and nuclear β-galactosidase are more basal, and cells are not perfectly vertical, the difference in focal planes gives a false impression in some cases of discordance between them. Larvae were staged from the beginning of the L2-L3 molt, and are shown at (A) 0-12 hours, (B) 12-24 hours, (C) 24-36 hours, (D) 36-48 hours and (E,F) 48-60 hours of third instar. The DV F-actin line is consistently observed from 0-24 hours of third instar (A,B). From 24-36 hours, F-actin is not consistently elevated, but cells still generally line up along the DV boundary (C). From 36-48 hours the DV boundary is no longer distinguishable by F-actin staining, but F-actin begins to appear elevated in flanking cells (D); this is even more obvious in older discs (E,F), and appears adjacent to late stripes of Dl expression (magenta, F).

1997; Panin et al., 1997), which could then activate both transcriptional and non-transcriptional branches of the Notch pathway. An activated form of Notch that also includes the transmembrane domain, Notch-ΔEN (Larkin et al., 1996), was similar to N-intra in terms of its influence on F-actin (not shown), from which we infer that the influence of Notch on F-actin probably requires Notch-ligand interactions.

Influence of Actin-regulatory proteins on DV compartmentalization

To evaluate the functional significance of F-actin structures in DV compartmentalization, we examined the consequences of mutation or ectopic expression of actin regulatory proteins. The *Abl* gene encodes a tyrosine kinase that interacts genetically with Notch in axon guidance (Crowner et al., 2003; Giniger, 1998), but neither mutation nor ectopic expression of *Abl* exerted detectable influences on DV compartmentalization. Ena is a substrate for Abl, and also interacts genetically with *Abl*, but clones of cells mutant for a hypomorphic allele did not affect DV compartmentalization, and clones of cells mutant for a null allele could not be recovered. Conversely, mutations in the Profilin homolog *chickadee*, or expression of dominant-negative forms of the actin-regulatory G proteins Rac, Rho or Cdc42, resulted in varying degrees of disturbance to the DV boundary, but only under conditions that also resulted in more gross defects like cell death, cells dropping out of the epithelium, invasive behavior, and/or disturbance of the AP compartment boundary (not shown). These defects made it difficult to assess the significance of affects of these mutations on DV compartmentalization.

However, mutations in *capulet* (*capt*, also known as *act up*) consistently and specifically disrupted the DV compartment boundary under partial loss-of-function conditions. *capt* is a *Drosophila* cyclase-associated protein (Baum et al., 2000; Benlali et al., 2000), which interacts genetically with *Abl* to influence axon guidance (Wills et al., 2002), and restricts apical actin polymerization in epithelial cells (Baum and Perrimon, 2001). When examined only two days after clone induction, no cell death or loss from the disc epithelium could be detected in *capt* mutant clones. Additionally, two-day-old *capt* clones failed to disturb the AP compartment boundary (Fig. 7C; 0/39 clones that touch the AP interface, as judged by the expression of Engrailed (En), were associated with an irregular boundary). Importantly then, two-day-old *capt* mutant clones exhibited

Fig. 6. Notch regulates F-actin organization in the wing. (A-E′) Third instar discs, stained for F-actin (green, shown from apical focal planes) and GFP (blue, marks Gal4-expressing cells), together with *ap-lacZ* (red, shown from basal focal planes) or Wg (red). (A) *UAS-fng UAS-GFP ptc-Gal4*. Normal Notch activation is observed at the DV boundary (arrowheads) and is disrupted near the AP boundary by ectopic Fng (asterisk), whereas ectopic Notch activation is induced along the AP boundary in ventral cells (arrow); corresponding changes occur in F-actin organization. In B-E arrowheads mark the normal DV F-actin line, arrows mark F-actin at clone edges. (B,C) A *UAS-Dl UAS-GFP AyGal4* disc, with clones of cells expressing Delta. (D) A *UAS-N-intra UAS-GFP AyGal4* disc, with clones of cells expressing N-intra. No autonomous influence on F-actin is observed, occasionally some upregulation is observed at clone edges, consistent with the ability of N-intra to induce Notch ligand expression. (E) Disc with a *fat⁸* mutant clone, marked by the absence of GFP (blue). No influence on F-actin is observed.
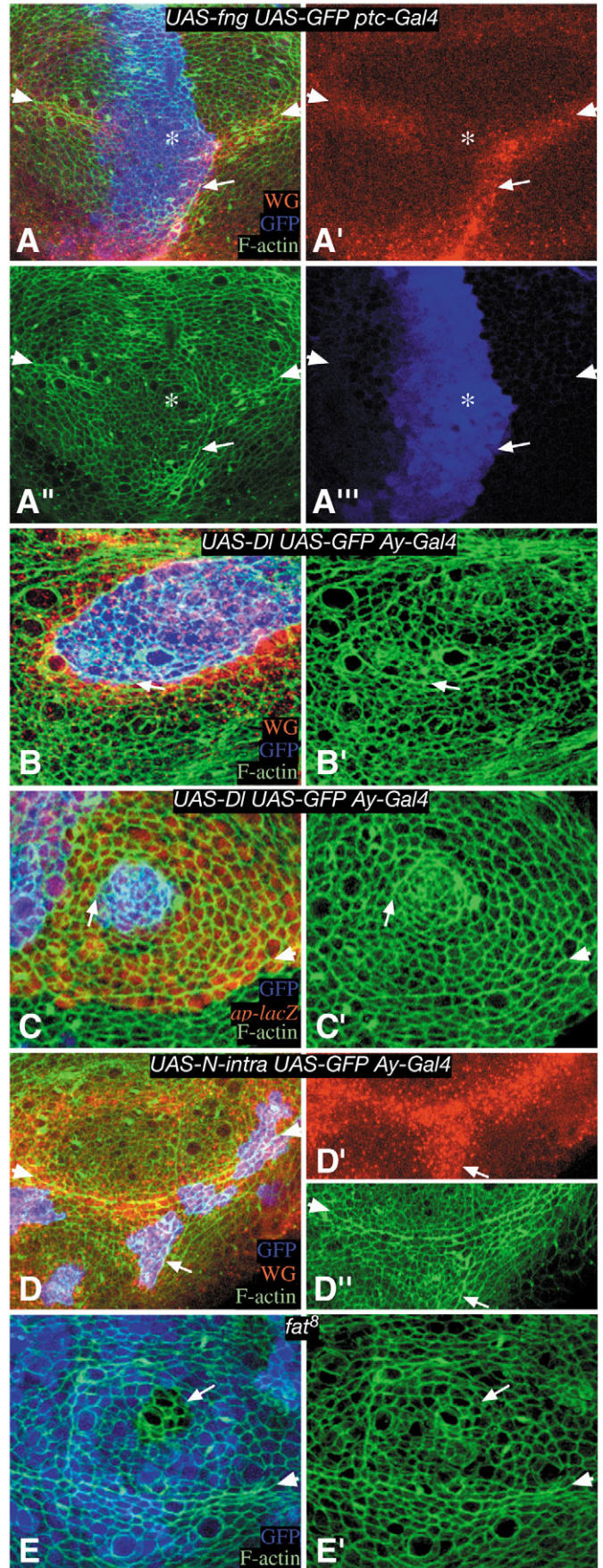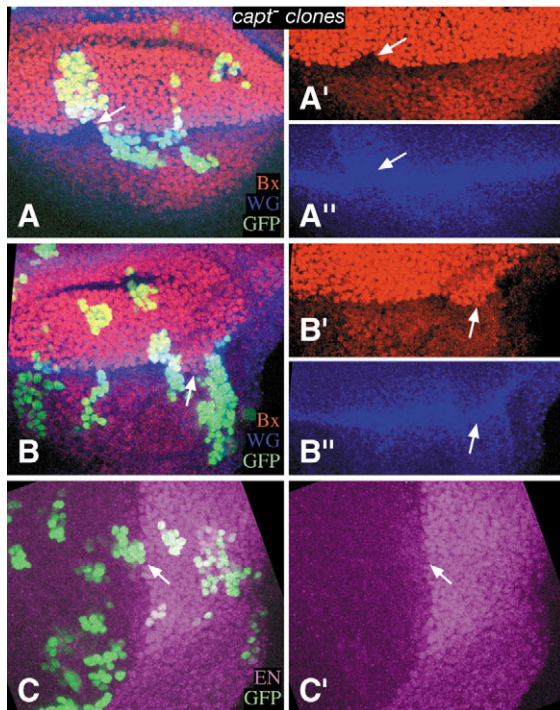
Fig. 7. Influence of *capt* on DV compartmentalization. Third instar wing imaginal discs, stained for Bx (red) and either Wg (blue) or En (magenta). Discs are shown two days after the induction of clones (marked by GFP expression, green) mutant for *capt*^E636^ and expressing p35. In A,B, arrows indicate disturbances in the DV boundary associated with mutant clones; in C, arrow points to a clone that is adjacent to the AP boundary but does not distort it.

strong and consistent disturbances of the DV compartment boundary [Fig. 7A,B; 27/82 clones that touched the DV interface, as judged by expression of the dorsal marker Beadex (Bx, also known as dLMO) (Weihe et al., 2001), were associated with an irregular boundary]. This presumably reflects a hypomorphic situation due to perdurance of *capt* gene product, as three days after clone induction the DV compartment boundary was still disturbed, but a fraction of mutant cells also appeared to be undergoing apoptosis or dropping out of the disc epithelium. Thus, although *capt* has a general role in epithelial integrity, reduction-of-function conditions reveal a particular requirement for *capt* at the DV compartment boundary.

The influence of *capt* mutant clones on DV compartmentalization is similar to that of *Notch* mutations in the bidirectional and non-autonomous nature of the disruptions (Fig. 7) (Micchelli and Blair, 1999; Rauskolb et al., 1999). That is, both can be associated with the appearance of ventral cells in the dorsal part of the disc, or of dorsal cells in the ventral part of the disc, and these often include wild-type cells that neighbor a mutant clone. Importantly, *capt* is not associated with loss of Notch signaling, at least as assayed at the level of transcriptional targets. In fact, Wg staining is elevated within *capt* mutant clones (Fig. 7A,B). However, neither ectopic Notch activation nor Wg expression alone cause disturbances of the DV boundary (Micchelli and Blair, 1999; Milan and Cohen, 2003; Rauskolb et al., 1999).

Discussion

Our results have advanced the understanding of DV compartmentalization in two crucial ways. First, they demonstrate that a stripe of Notch activation is sufficient to separate cells, which, together with prior studies on the requirements and geometry of Notch activation, lends support to the idea that a separation fence rather than promotion of a compartment-specific cell affinity provides the best explanation for the role of Notch in DV compartmentalization. Second, they identify both a distinct morphology and distinct genetic requirements for F-actin organization at the DV compartment boundary. In addition to these new insights into DV compartmentalization, our results also suggest that Notch influences F-actin in the wing through an alternate, non-transcriptional pathway.

Modulation of the actin cytoskeleton by the Notch pathway

The polarization of F-actin accumulation effected by Notch activation at the DV boundary cannot easily be accounted for purely by the transcriptional regulation of target genes associated with Notch activation. In the context of the normal DV boundary, or an ectopic boundary associated with altered Fng expression, a rectangular cell at the boundary has three neighbors with similar levels of Notch activation, but F-actin is elevated along only one of these cell interfaces (Fig. 1). In the case of ectopic Delta expression, Notch activation is actually inhibited inside of Delta-expressing clones through autonomous inhibition (de Celis and Bray, 1997; Doherty et al., 1996; Micchelli et al., 1997), and so is now asymmetric with respect to the clone boundary, yet it still effects a similar modulation of F-actin. The common feature of the cellular interface at which F-actin is upregulated in all cases is that it is the cellular interface at which most Notch-ligand interaction is actually occurring. This leads us to suggest that F-actin accumulation might be polarized through an alternate Notch pathway that impinges on the actin cytoskeleton. This inference is consistent with the observation that forms of Notch that are constituitively activated for transcriptional pathways do not result in a general, autonomous upregulation of F-actin.

The possibility of links from Notch or its ligands to the actin cytoskeleton that do not involve the canonical Notch transcriptional pathway has been suggested previously, based on studies of their influence on axon guidance, neurite or filopodial extension, and keratinocyte motility (Crowner et al., 2003; De Joussineau et al., 2003; Giniger, 1998; Lowell and Watt, 2001). The nature of this alternate pathway or pathways is not clear. In the context of the influence of Notch on axon guidance in the *Drosophila* embryo, this alternate pathway is characterized by genetic interactions with *Abl*, and a lack of genetic interactions with the Notch pathway transcription factor *Suppressor of Hairless* [*Su(H)*] (Crowner et al., 2003; Giniger, 1998). Although *Abl* mutations do not noticeably affect DV compartmentalization, *Abl* mutants in general have relatively mild effects, presumably because *Abl* is partially redundant (Gertler et al., 1989), and the elevation in phosho-tyrosine and Ena staining at the DV boundary is intriguing in light of potential links between Notch and Abl.

Sequential mechanisms in DV compartmentalization

Rather than a single mechanism for compartmentalization, studies of the DV boundary suggest that a series of distinct strategies affect what, by lineage analysis, appears to be a constant boundary. Trn and Caps, are expressed specifically in dorsal cells during the second larval instar, when the boundary first forms. Ectopic expression of these proteins can cause ventral cells to sort to and associate with dorsal cells (Milan et al., 2001), but their contribution to compartmentalization must be transient, as their dorsal-specific expression is lost during early third instar. Elevated F-actin staining at the DV boundary could not be consistently detected during second instar, was clearly visible from early through mid-third instar, but disappeared at late third instar. Thus, the role of the F-actin-dependent fence might also be transient. At late third instar, cells near the DV boundary stop proliferating (Blair, 1993; Johnston and Edgar, 1998; O'Brochta and Bryant, 1985). As the arrangement of cells in imaginal discs is largely a function of growth rather than movement, this cessation of proliferation presents a third potential mechanism for compartmentalization, which could be important at late stages.

Models for the role of Notch in DV compartmentalization

It has generally been assumed that compartmentalization is effected by the establishment of differential cell affinities, which result in cells sorting to their respective sides of a compartment boundary (Blair, 2003b; McNeill, 2000). Although this paradigm fits well with studies of AP compartmentalization, it is not easy to reconcile with studies of DV compartmentalization, given that Notch is activated and required on both sides of the compartment boundary, that neither mutation nor ectopic activation of Notch causes directed changes in cell location, that the requirement for Notch is non-autonomous, and that the requirement for Notch does not depend on the dorsal or ventral identity of a cell. Models that have proposed that Notch influences DV compartmentalization by affecting a compartment-specific cell affinity have required that it act in conjunction with Ap (Micchelli and Blair, 1999; Milan and Cohen, 2003). The crucial failing of such models, in our view, is that they cannot explain how a compartmental separation of cells is achieved by the ectopic Notch activation associated with a mutation of *fng*, or ectopic expression of Fng, Serrate or Delta, as in all of these cases cells on both sides of the boundary are identical with respect to the presence or absence of Ap.

An alternative hypothesis is that Notch activation influences a property or behavior of cells at the boundary in a way that prevents them from intermingling, which we refer to as a fence. The determination that Notch signaling effects a polarized elevation of F-actin and Ena supports this hypothesis, as it demonstrates that Notch can polarize the actin cytoskeleton in conjunction with its ability to separate cells, and that this influence of Notch is independent of the dorsal or ventral identity of the cell. Additionally, the bidirectional and non-autonomous disruptions of the compartment boundary effected by *capt* mutant clones are consistent with the inference that compartmentalization involves an F-actin-dependent fence. When the fence is broken, cells can intermix in either direction, irrespective of their DV identity. By contrast, it is not clear how the non-autonomous affects of *capt* mutant clones could be reconciled with models that postulate a compartment-specific cell affinity.

The possibility of a non-transcriptional influence of Notch on DV compartmentalization, as suggested above for its influence on F-actin, is appealing because it could explain the observation that Fng can influence compartmentalization even when co-expressed with N-intra (Rauskolb et al., 1999). Loss-of-function studies have provided mixed results as to the requirements for Notch transcriptional pathways in DV compartmentalization. Clones of cells mutant for a hypomorphic allele of *Su(H)*, *Su(H)*^{SF8}, respect the compartment boundary, even though transcriptional targets are affected (Micchelli and Blair, 1999). However, this is not a null situation for *Su(H)*, and we would predict that at a minimum, a Notch transcriptional pathway would be required at the DV boundary to maintain the expression of Notch ligands (Fig. 1A). Requirements for transcriptional mediator proteins confirm that transcription is required for DV compartmentalization (Janody et al., 2003), but a role for a transcriptional Notch pathway does not preclude a parallel role for a non-transcriptional pathway.

How might a compartmentalization fence be constructed?

The molecular nature of the compartmentalization fence is not yet clear, but some possibilities can be suggested. One model is based on the similarity of the F-actin stripe at the DV boundary to a prominent F-actin cable detected along the interface between leading edge cells and amnion-serosa cells during dorsal closure of the *Drosophila* embryo (Dequier et al., 2001). The F-actin cable and associated proteins are thought to help keep dorsal epidermal cells in register as they move, through actin-myosin-based contraction and/or influences on the protrusive behavior of filopodia (Jacinto et al., 2002; Kiehart et al., 2000). Similar processes could maintain a smooth separation between cells at the DV compartment boundary. Intriguingly, genetic studies have suggested a potential role for Notch in dorsal closure that does not involve *Su(H)* (Zecchini et al., 1999). The distinct requirement for a regulator of actin polymerization, *capt*, at the DV boundary is consistent with the hypothesis that the elevated F-actin detected at the DV boundary plays a crucial role in compartmentalization. In this view, the F-actin cable would be a physical manifestation of the Notch-dependent separation fence.

An alternative possibility is suggested by the observations Notch and its ligands can, at least in cultured cell assays, act as cell adhesion molecules (Fehon et al., 1990), and that association of Notch with its ligands can promote cleavage of both molecules (Bland et al., 2003; Kidd et al., 1998; Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998). Thus, while loss- and gain-of-function studies of Notch ligands do not support the possibility that they act as compartment-specific cell adhesion molecules (Rauskolb et al., 1999), we suggest that cleavage of Notch and/or its ligands might act as a boundary-specific de-adhesion mechanism. Boundary-specific de-adhesion, rather than compartment-specific adhesion, has been suggested as a possible mechanism for Eph-Ephrin-mediated cell separation (Cooke and Moens, 2002). In this model, the influence on F-actin might be a secondary consequence of the primary separation mechanism.

Development

Alternatively, because the cytoplasmic domains of Notch and its ligands have been reported to associate with proteins that can impinge on actin organization (Giniger, 1998; Six et al., 2004; Wright et al., 2004), Notch or ligand cleavage might be a direct mechanism for modulating F-actin.

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