The LRR Proteins Capricious and Tartan Mediate Cell Interactions during DV Boundary Formation in the *Drosophila* Wing

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Summary

Mechanisms to segregate cell populations play important roles in tissue patterning during animal development. Rhombomeres and compartments in the ectoderm and imaginal discs of *Drosophila* are examples in which initially homogenous populations of cells come to be separated by boundaries of lineage restriction. Boundary formation depends in part on signaling between the distinctly specified cell populations that comprise compartments and in part on formation of affinity boundaries that prevent intermingling of these cell populations. Here, we present evidence that two transmembrane proteins with leucine-rich repeats, known as Capricious and Tartan, contribute to formation of the affinity boundary between dorsal and ventral compartments during *Drosophila* wing development.

Introduction

During development of multicellular organisms, groups of cells assemble to form tissues that are initially homogenous. The elaboration of spatial pattern often begins by subdividing the field of cells into smaller territories. 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). In the imaginal discs, compartment boundaries serve as signaling centers. Short-range interactions between cells in adjacent compartments induce the expression of the signaling proteins Wingless (Wg) and Decapentaplegic (Dpp) in cells adjacent to the compartment boundaries. Wg and Dpp form long-range extracellular protein gradients centered on the compartment boundaries (Strigini and Cohen, 2000; Teleman and Cohen, 2000; Entchev et al., 2000). Stable boundaries between compartments result in tightly localized sources of these signaling proteins. Intermingling of cells at the compartment boundary causes disorganization of the signaling center, with disastrous consequences for patterning and growth control (Milán and Cohen, 1999a; Dahmann and Basler, 1999).

Compartments are formed by heritable expression of transcription factors. Engrailed/Invected expression con-

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fers posterior (P) identity and Apterous confers dorsal (D) identity in the wing disc. P compartment cells lacking *engrailed/invected* activity do not respect the anterior-posterior boundary (García-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Tabata et al., 1995; Zecca et al., 1995). Likewise, dorsal cells lacking *ap* activity fail to respect the dorsal-ventral (DV) boundary in the wing disc (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). One proposal to explain segregation of cells at compartment boundaries is that these selector genes confer compartment-specific differences in cell affinity, perhaps by differential expression of cell adhesion molecules (García-Bellido, 1972).

More recently, signaling between compartments has been implicated in producing the affinity differences that maintain the segregation of the distinctly specified cell populations. Hedgehog signaling is asymmetric. P cells produce Hedgehog, but are relatively insensitive to it, whereas A cells are Hedgehog responsive (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Dominguez et al., 1996; Ramirez-Weber et al., 2000). Anterior cells that are unable to transduce the Hedgehog signal are unable to recognize the affinity boundary between A and P compartments (Blair and Ralston, 1997; Rodriguez and Basler, 1997). However, these cells do not intermingle freely with P cells, suggesting that there may be an underlying affinity difference not accounted for by signaling (Blair and Ralston, 1997). A recent study proposes that differential expression of a single adhesion protein might be sufficient to explain the AP affinity border (Dahmann and Basler, 2000).

Modulation of Notch signaling has been implicated in DV boundary formation (Rauskolb et al., 1999; Micchelli and Blair, 1999). Fringe acts as a glycosyltransferase to modify the receptor protein Notch in the dorsal compartment (Moloney et al., 2000; Brückner et al., 2000). Fringe activity makes D cells more sensitive to Delta, a ligand expressed by V cells and less sensitive to Serrate, the ligand expressed by D cells (Panin et al., 1997; Fleming et al., 1997; Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996; Doherty et al., 1996). Consequently, signaling by each ligand is limited to nearby cells on the opposite side of the boundary, with the result that high levels of Notch activity are limited to a narrow band of cells along the DV boundary. Although altering the signaling properties of cells by modulation of Fringe activity has been shown to allow cells to cross the boundary (Rauskolb et al., 1999), Fringe activity has been shown to be insufficient to support boundary formation (Milán and Cohen, 1999a). This observation, together with the fact that Notch signaling is activated symmetrically has suggested that other Apterous-dependent cell interactions might be needed for formation of the DV affinity boundary. Here, we present evidence that *capricious (caps)* and *tartan* are targets of Apterous that contribute to DV boundary formation in the wing disc. *caps* and *tartan* encode transmembrane proteins with extracellular leucine-rich repeats (LRR) and are expressed in the D compartment during boundary formation. Caps and Tartan confer affinity for D cells, assessed by sorting-out
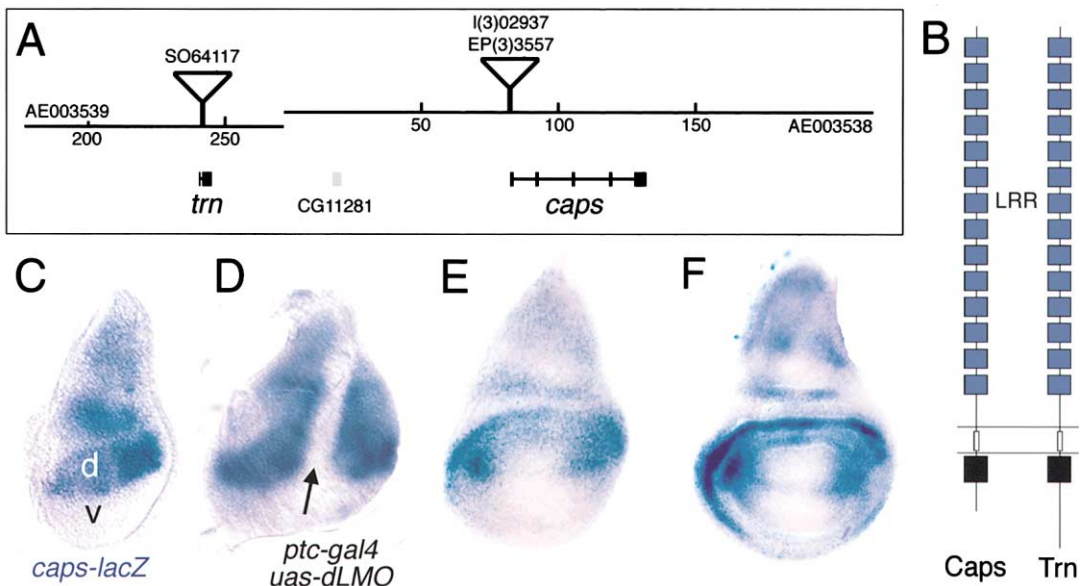
Figure 1. *caps* and *tartan* Expression

(A) Genomic organization of the *caps* and *tartan* genes. *caps* was identified by three transgene insertions. I(3)02937 serves as a lacZ reporter gene. The *caps* transcript consists of 5 exons spanning ~40 kb. The entire open reading frame is contained in exon 5. *tartan* is associated with the transgene insertion I(3)S064117, which serves as a lacZ-reporter gene.

(B) Comparison of the predicted Caps and Tartan proteins. Blue boxes indicate LRR domains. The two proteins are 65% identical and show 83% similarity in this region. Black boxes indicate highly conserved juxtamembrane domains.

(C) *caps-lacZ* expression in a 2nd instar wing disc visualized by histochemical staining for β-gal activity.

(D) *caps-lacZ* in a 2nd instar wing disc that expressed *dLMO* under *patched^Gal4* control. The arrow indicates repression of *caps-lacZ* in the *patched^Gal4* expression domain.

(E and F) *caps-lacZ* expression in mid third and mature third instar wing discs.

behavior. Caps supports boundary formation without conferring D signaling properties. Fringe, in contrast, confers dorsal signaling properties without affecting DV affinity. Thus, Caps, Tartan, and Fringe have complementary roles in boundary formation.

Results

caps and *tartan* Expression

The *tartan* and *capricious* genes encode closely related transmembrane proteins. The predicted Caps and Tartan proteins are 65% identical in the extracellular domain, which consists of 14 LRR domains. They also share a conserved domain adjacent to the membrane in the cytoplasmic tail, but differ at their C termini (Figure 1; Shishido et al., 1998). LRR domains are thought to mediate protein interaction, consistent with the possibility that these proteins might mediate cell interactions (Rothberg et al., 1990; Raghavan and White, 1997; Shishido et al., 1998). We became interested in the functions of Caps and Tartan on the basis of their expression patterns in the developing wing imaginal disc. In second and early third instar wing discs, *caps-lacZ* is expressed in the D compartment (Figure 1C). At this stage, expression of Caps protein coincides with that of Apterous, and *caps-lacZ* expression depends on Apterous activity. Expression of the Apterous inhibitor, dLMO, under control of *patched-Gal4* represses *caps-lacZ* expression (Figure 1D, arrow). During third instar, dorsal expression of *caps-lacZ* decreases and new lateral expression do-

mains arise. These domains are initially stronger in the D compartment but become symmetric in D and V compartments in mature third instar discs (Figures 1E and 1F). *tartan* expression was monitored using a *lacZ* reporter gene and antibody to Tartan protein, and was similar to *caps* expression at all stages (not shown). The dynamics of these expression patterns suggested that Caps and Tartan proteins might mediate cell interactions during early DV patterning and subsequently during medial-lateral patterning of the wing.

DV Boundary Formation

To assess the roles of Caps and Tartan in DV boundary formation, we made use of a rescue assay in which the Gal4-UAS system was used to restore Caps and Tartan expression in D cells of *apterous* (*ap*) mutant wing discs. *ap^Gal4*/*ap^-* mutant discs were not able to form a smooth DV boundary and failed to induce Wg expression uniformly along the interface between D and V cell populations (Figure 2A; see Milán and Cohen, 1999a). Expression of Caps in D cells under *ap^Gal4* control restored a smooth interface between D and V cells in the mutant discs, but did not restore Wg expression along the boundary (Figure 2B). Tartan was considerably less effective at producing a smooth interface between D and V cells (Figure 2C). We next tested Connectin, a GPI-anchored membrane protein that is related to Caps and Tartan in the LRR domains, because Connectin has been shown to mediate homophilic cell adhesion (Nose et al., 1992). Connectin was ineffective in the boundary rescue
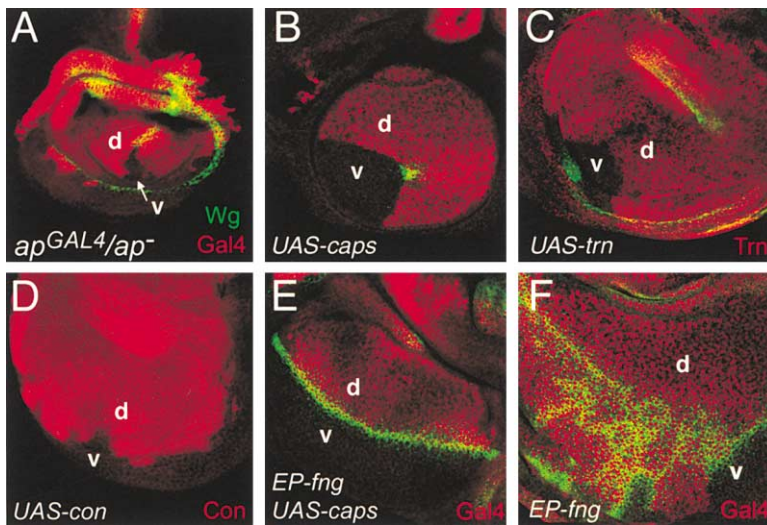
Figure 2. Caps Expression Restores the DV Affinity Boundary

(A) ap^{Gal4}/ap^{UGO35} wing disc labeled to visualize Wg protein (green) and Gal4 protein (red). The border between dorsal (d) and ventral (v) cells was irregular. The wing pouch was small (outlined by the ring of Wg expression). Note the small size of the v region (arrow) compared to the d region in the pouch.

(B) ap^{Gal4}/ap^{UGO35}; *UAS-caps* wing disc. A smooth border was restored between d and v cells. Note the increase in the size of the v territory relative to the disc in (A). A spot of Wg expression was observed in the center of these discs. This occurred in only 2/20 ap^{Gal4}/ap^{UGO35} discs (Milan and Cohen 1999b). The difference between *ap* mutant discs and those also expressing Caps may reflect improved signaling between d and v cells when the interface is stabilized.

(C) ap^{Gal4}/ap^{UGO35}; *UAS-trn* wing disc. Trn protein (red). The border between d and v cells was irregular and the V territory was small.

(D) ap^{Gal4}/ap^{UGO35}; *UAS-connectin* wing disc. Connectin protein (red). Connectin expression had no effect on the boundary or relative size of the v territory.

(E) ap^{Gal4}/ap^{UGO35}; *EP-fringe/UAS-caps* wing disc. Wg expression was restored. The interface between d and v cells was smooth and the compartments were of equal size in 10/10 discs examined. Similar results were obtained by coexpression of Caps with Serrate (10/10 discs, not shown).

(F) ap^{Gal4}/ap^{UGO35}; *EP-fringe* wing disc. Wg expression was restored. The interface between d and v cells was highly irregular in all discs and the compartment boundary was violated in 10/12 discs examined (Milan and Cohen 1999b). The v compartment remained small. Expression of Fringe at higher levels using UAS-Fringe reduced the intermingling of d and v cells, but the boundary was violated in 13/14 discs examined (not shown).

assay (Figure 2D). Fasciclin II, an unrelated adhesion protein, was also unable to restore the DV boundary (D.A. O'Keefe and J.B. Thomas, personal communication). These observations suggest that Caps expression produced an affinity boundary between D and V cells by a mechanism that is not simply due to increased cohesion among dorsal cells.

To test the requirement for Caps and Tartan in boundary formation, we produced clones of cells mutant for *caps* or *tartan*. Single mutant clones did not produce observable alterations in the wing disc. Clones simultaneously mutant for *caps* and *tartan* did not cause defects at the DV boundary, but did perturb medial-lateral cell interactions (not shown). In wild-type discs, loss of *caps* and *tartan* activity may be compensated for by other proteins. We therefore asked whether reduced levels of *caps* and *tartan* activity would cause defects when DV boundary formation was compromised by reduction of Ap activity. Two sensitized genetic backgrounds were examined. Bx^1 produces a wing scalloping phenotype that is very sensitive to the level of expression of other genes involved in DV patterning (Milán et al., 1998). Bx^1 is a dominant mutation that overexpresses the dLMO protein. dLMO competes with Ap for binding to its cofactor Chip and thereby reduces Ap activity (Fernandez-Funez et al., 1998; van Meyel et al., 1999; Milán and Cohen 1999b). The second sensitized genotype was provided by a mutant with reduced expression of the Ap cofactor *Chip*. $Chip^{e5.5}$ was selected because it is less sensitive to modification than Bx^1 but shows specific genetic interactions with *ap*, *dLMO*, *Serrate*, and *fringe* (Morcillo et al., 1997; data not shown). Interactions were scored on the basis of dominant wing scalloping phenotypes in flies heterozygous for $Chip^{e5.5}$ or Bx^1. Eighteen

deletions dominantly enhanced both phenotypes (Table 1). Nine of these uncover genes with known roles in DV patterning including *ap*, *vestigial*, *cut*, and *Serrate*. Df(3L)C190, a deletion that removes the *caps* and *tartan* genes, enhanced the Bx^1 and $Chip^{e5.5}$ phenotypes (Figure 3). The *caps tartan* double mutant chromosome used for the clonal analysis also enhanced both phenotypes (not shown). The contributions of *caps* and *tartan* were then tested individually. A *caps* lack-of-function mutant enhanced both phenotypes. A *tartan* mutant lack-of-function mutant enhanced Bx^1, but did not produce a

Table 1. Deficiencies Interacting with Bx and Chip

Deficiency	Candidate gene
Df(1)ct-J4	*cut*
Df(1)C246	*(sno)*
Df(1)N19	*dLMO*
Df(2L)al	
Df(2R)nap1	*ap*
Df(2R)M41A4	*ap*
Df(2R)Np5	
Df(2R)E3363	*(lola)*
Df(2R)vg-C	*vg*
Df(2R)CX1	*vg, mam*
Df(2R)X58-12	*(Minute)*
Df(2R)or-BR6	*Chip*
Df(3L)AC1	
Df(3L)C190	*caps, trn*
Df(3L)brm11	*(brm)*
Df(3R)M-Kx1	*(minute)*
Df(3R)DG2	*osa*
Df(3R)D605	*Ser*

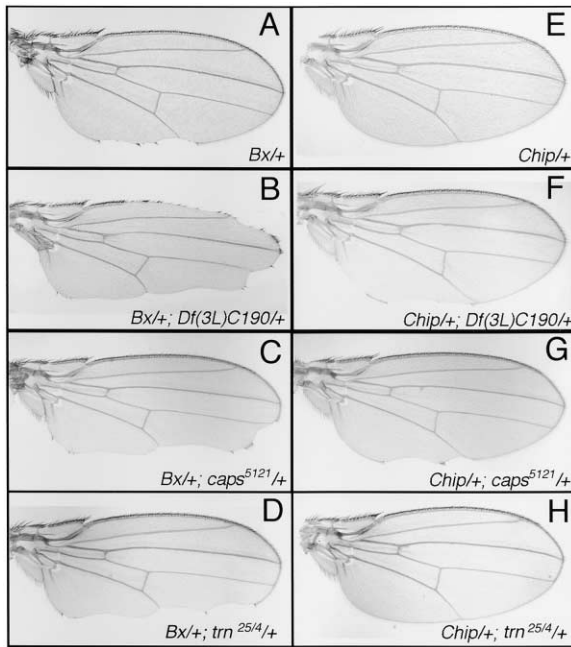Interaction with the candidate gene was confirmed for those not in parentheses.

Figure 3. Genetic Interactions

(A) Cuticle preparation of a *Bx[1]/+* wing showing mild scalloping in the P compartment.

(B) *Bx[1]/+*; Df(3L)C190/+ wing showing strong scalloping in A and P compartments.

(C) *Bx[1]/+*; *caps[l(3)05121]/+* wing showing strong scalloping in the P compartment.

(D) *Bx[1]/+*; *trn[25/4]/+* wing showing strong scalloping in the P compartment.

(E) *Chip[e5.5]/+* wing. Heterozygous *Chip* mutant wings are morphologically normal.

(F) *Chip[e5.5]/+*; Df(3L)C190/+ wing showing scalloping in the P compartment.

(G) *Chip[e5.5]/+*; *caps[l(3)05121]/+* wing showing scalloping in the P compartment.

(H) *Chip[e5.5]/+*; *trn[25/4]/+* wing. No scalloping was produced in this combination.

phenotype in the less sensitive *Chip[e5.5]* background. These observations indicate that reduced *caps* and *tartan* activity caused wing defects when the system was sensitized by reduction of Ap activity. Together with the observations presented in Figure 2, they suggest that Caps and Tartan contribute to DV boundary formation.

Sorting-out and Cell Survival

We next made use of the flip-out Gal4 system to produce clones of Gal4-expressing cells in the wing disc to examine effects of ectopic Caps and Tartan expression. In comparing the effects of clones expressing different transgenes, we assume that the initial distribution of Gal4-expressing cells is comparable prior to transgene expression. Consequently, differences in the distribution of clones at later stages must reflect transgene-dependent effects on cell behavior. To evaluate these effects, we plotted the ratio of ventral to dorsal clones (Figure 4D). Differences in clone survival are reflected in altered ratios of total clone recovery (T). Differences in clone location are reflected by altered ratios of clones

in contact with the DV boundary (B) and of clones located internally in the compartment (I).

Control GFP-expressing clones were evenly distributed in D and V compartments (Figure 4D). Of 178 clones examined, 23 contacted the boundary on the D side compared to 25 on the V side. Sixty-four clones were located internally in the D compartment, compared to 66 in the V compartment. Caps-expressing clones differ in two respects from control clones (Figure 4D). Fewer Caps-expressing clones were recovered in the V compartment (V/D ratio ∼0.7, n = 116). Nonetheless, twice as many V clones were recovered at the boundary as would be expected if Caps had no effect on their distribution. Comparable results were obtained for clones expressing Tartan or Caps and Tartan together (Figure 4D). These observations suggest that clones expressing Caps or Tartan survive poorly in the V compartment. To ask whether poor survival of V clones could be responsible for their accumulation at the DV boundary, we coexpressed Caps and Tartan with the viral apoptosis inhibitor p35. p35 expression suppressed the loss of V clones (V/D ratio =1). Yet, V clones were still overrepresented by ∼2-fold at the DV boundary (Figures 4B and 4D). Control clones expressing GFP and p35 were evenly distributed between D and V compartments (Figures 4A and 4D). These observations suggest that V clones expressing Caps or Tartan survive poorly if they fail to contact D cells, and that Caps or Tartan expression causes V cells to sort-out toward the D compartment.

Sorting-out Behavior

Caps- and Tartan-expressing clones of V compartment origin sorted-out toward the DV boundary but remained in the V compartment. Although these clones did not cross the boundary, many of them appeared to push the Wg stripe toward dorsal (Figure 4B). To examine this behavior more closely, we produced clones in early 2nd instar discs, before the DV boundary forms. Some of these clones were bisected by the nascent DV boundary so that they contributed to both compartments (referred to as D+V clones). Control D+V clones expressing GFP or lacZ reporter genes had no effect on the shape of the Wg stripe, and most were of similar size in both compartments (Figure 5A; Table 2). These clones were generally elongated in shape and had irregular borders where they contacted neighboring wild-type cells. In contrast, D+V clones expressing Caps or Tartan were more compact in shape, had smoother borders, and tended to be considerably smaller in the V compartment (Figure 5B, Table 2). Many of these clones distorted the Wg stripe where they crossed the boundary (arrows, Figure 5B). D+V clones expressing Caps and Tartan together had similar effects (Table 2).

We next examined the effects of smaller clones on the shape of the DV boundary, using expression of an *ap-lacZ* reporter gene to mark dorsal cells. GFP-expressing clones that contacted the DV boundary had no effect on the *ap-lacZ* border or on the Wg stripe (Figure 6A and Table 2). In contrast, ventral Caps- or Tartan-expressing clones often displaced both the *ap-lacZ* border and the Wg stripe toward dorsal (Figure 6B, arrow). In one case, we observed a ventral Caps-expressing clone that had separated a group of D cells from the rest of the D
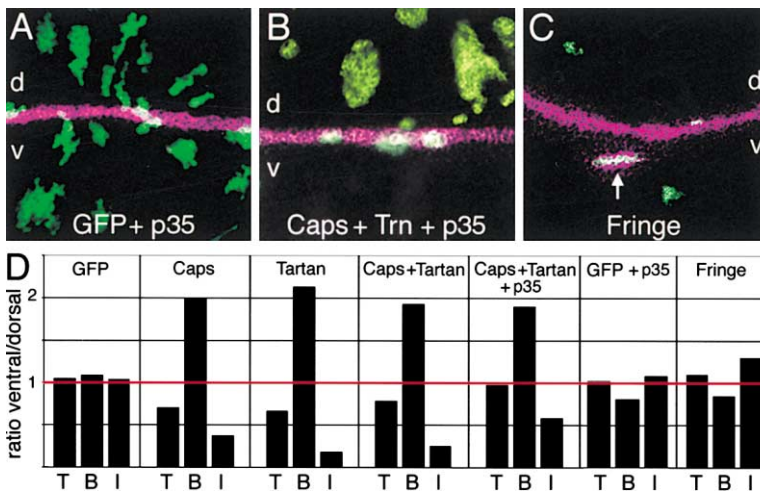
Figure 4. Caps and Tartan Expression Influences the Location of Clones in the V Compartment

(A) Wing disc with GFP+p35-expressing clones (green). The DV boundary is visualized by Wg expression (purple). Dorsal and ventral compartments are indicated (d, v).

(B) Caps + Tartan + p35-expressing clones (visualized by anti-Caps, green). Note the asymmetric distribution of clones in d and v compartments.

(C) Fringe-myc-expressing clones (visualized by anti-Myc, green) induce Wg expression where they abut ventral cells (arrow). At early stages, all ventral clones exhibit this behavior. At later stages, the zone in which this occurs narrows toward the DV boundary (see Milan and Cohen, 2000, for explanation).

(D) Histogram plotting the ratio of V/D clones. T = all clones counted. B = clones touching the DV boundary. I = clones not touching the DV boundary. Clones of D origin were identified by *ap-lacZ* expression. The red line highlights V/D ratio = 1. GFP: n = 178. Caps: n = 116. Tartan: n = 125. Caps + Tartan: n = 45. Caps + Tartan + p35: n = 67. GFP + p35: n = 220. Fringe: n = 90.

compartment (Figure 6C). The effects of clones expressing Tartan or Caps and Tartan together were similar to those of Caps-expressing clones (Table 2). D compartment clones had no effect.

Although it is not possible to observe how these distortions of the DV boundary arise, it is tempting to speculate that they result from V cells attempting to sort-out into the D compartment. Sorting out could be caused by increased affinity for D compartment cells or by repulsion by V compartment cells. Either mechanism could provide a force to push the clones into the D compartment and displace D cells and the Wg stripe. As Caps and Tartan are expressed by D cells, we asked whether V clones are attracted to the D compartment by homophilic cell adhesion mediated by Caps and Tartan. To measure homophilic adhesion, we made use of a cell aggregation assay described by Nose et al. (1992), in which Connectin expression caused aggregation of S2 cells. S2 cells expressing Caps and Tartan did not aggregate more than control cells. Likewise, we were unable to detect binding of a secreted Caps-Alkaline Phosphatase fusion protein to cells expressing Caps, Tartan, or both (not shown). Thus, the sorting-out behavior of Caps- and Tartan-expressing clones is unlikely to depend on homophilic cell adhesion mediated by these

proteins. We propose that Caps and Tartan interact with other surface proteins expressed in the D compartment.

Cellular Projections and Sorting-out

To examine how Caps and Tartan induce sorting behavior, clones that had sorted toward the DV boundary were studied using confocal microscopy. We observed membrane-bound cellular processes extending from Caps-expressing cells toward cells in the D compartment. Figure 7A shows four optical sections through a ventral Caps-expressing clone (labeled with anti-Caps, green). Caps protein outlined the cell surface and appeared in bright spots that may be intracellular vesicles (2 left panels). In more apical sections, Caps protein was located on thin structures that extended into the dorsal compartment (2 right panels). In cross-section, these structures can be seen to project from V cells over the apical surface of nearby D cells (arrow, Figure 7B). As Caps is a membrane protein, we infer that these are membranous cellular processes, perhaps filopodia. Processes were also observed projecting toward the D compartment from V clones that were not in contact with the boundary (Figure 7C, section S1). We did not observe similar structures extending between closely spaced clones within the V compartment (Figure 7D). All
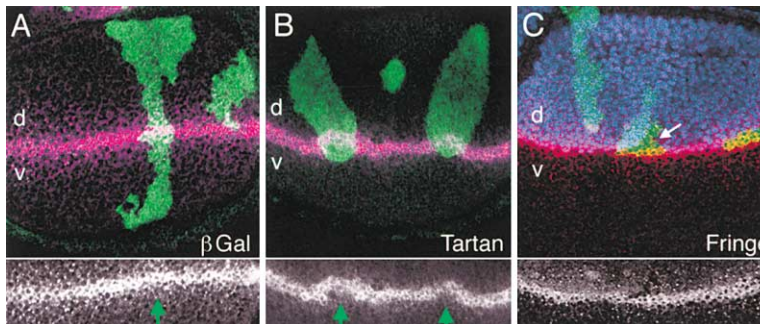

Figure 5. Effects of Large Clones on DV Boundary Shape

(A) Optical section of a wing disc with large lacZ-expressing clones. Clones were visualized by antibody to β-Gal protein (green). Dorsal and ventral compartments are indicated (d, v). Wg expression marks the DV boundary (purple). Lower panel: Wg expression alone. The green arrow indicates the position of the clone.

(B) Optical section of a wing disc with large clones expressing Tartan and β-Gal (green). The Wg stripe is displaced where the clones cross (green arrows).

(C) Optical section of a wing disc with large clones expressing Fringe-myc (green). D cells are marked by *ap-lacZ* expression (blue). The arrow indicates a large clone of combined d and v origin. Cells that were in the v compartment when the boundary was formed are labeled with anti-Myc (green) but not with *ap-lacZ* (not blue). Both types of cells from the clone were located on the dorsal side of the Wg stripe (red).

Table 2. Effects of Clones on the DV Boundary

	GFP	Caps	Trn	Caps+Trn
D clones[1]	0/23	0/14	0/24	0/16
V clones[1]	0/25	14/30	10/41	14/31
D+V clones[1]	0/15	15/25	22/34	11/17
D+V clones[2]	0/15	16/25	23/34	9/17

[1] Number of clones displacing the DV boundary/total.
[2] Number of clones larger in the dorsal compartment/total.

projections were oriented toward the D compartment. These observations support the idea that Caps may interact with another cell surface protein in the D compartment.

Tartan-expressing clones also sorted-out toward the D compartment. We examined clones of Tartan-expressing cells, but were unable to visualize cellular projections with anti-Tartan antibody. As an alternative, we made use of a transgene expressing cytoplasmic β-Gal to mark cellular processes when coexpressed with Tartan. Ventral Tartan-expressing clones also extend cytoplasmic processes toward D cells (Figure 7E). Projections produced by cells expressing Caps were similar in appearance when visualized using cytoplasmic β-Gal (not shown). Cellular processes expressing Caps and Tartan may help ventral cells to sort toward the D compartment. These observations suggest that the behavior of Caps- and Tartan-expressing V clones is guided by increased affinity for D cells.

Distinct Roles for Caps/Tartan and Fringe
We next considered why V clones expressing Caps and Tartan were unable to cross the Wg stripe. As shown in Figures 2B and 2C, Caps and Tartan were not able to restore Notch signaling and Wg expression when expressed in the D compartment of *ap* mutant discs. Likewise, clones of cells expressing Caps or Tartan did not induce Wg expression in adjacent V cells (Figures 5B and 6B). Instead, Wg was expressed normally where Tartan or Caps-expressing V cells contacted D cells. Thus, ventral Caps- and Tartan-expressing clones retain ventral signaling properties. In this respect, they differ from ventral Fringe-expressing clones, which acquire

the signaling properties of D cells (Panin et al., 1997; Fleming et al., 1997).

Fringe acts as a glycosyltransferase enzyme to modify Notch and make it differentially sensitive to its ligands (Moloney et al., 2000; Brückner et al., 2000). Consequently, ventral clones of Fringe-expressing cells induced ectopic expression of Wg where they contacted other V cells (arrow, Figure 4C). In cases where ventral Fringe-expressing clones contacted the DV boundary, Wg was induced at the interface with other V cells, but not at the interface with D cells. The change in signaling properties of these cells resulted in relocation of the stripe of Wg expression to the interface between the clone and other V cells (arrow, Figure 6C). Consequently, Fringe-expressing clones cross the boundary defined by the Wg stripe (Rauskolb et al., 1999). Caps- and Tartan-expressing clones retain ventral signaling properties and so cannot reposition the Wg stripe.

The behavior of Fringe-expressing clones differs in a second respect. Fringe-expressing clones were not lost from the V compartment and did not accumulate at the DV boundary (Figure 4D). Thus, Fringe-expressing clones did not acquire the ability to sort-out toward D cells that is conferred by Caps or Tartan expression. These properties are reflected in the different abilities of Fringe and Caps to rescue the DV affinity boundary in *ap* mutant discs. Expression of Fringe restored Notch signaling and induced Wg expression, but was unable to produce a smooth DV affinity boundary (Figure 2F, see Experimental Procedures). In contrast, Caps produced a smooth boundary but did not restore Notch signaling. Coexpression of Caps and Fringe restored Wg expression and produced a normal DV affinity boundary (Figure 2E). Likewise, clones expressing Caps, Tartan, and Fringe sorted-out toward the DV boundary and crossed into the D compartment (not shown). These observations suggest that Fringe and Caps/Tartan play distinct but complementary roles in boundary formation.

Discussion

Two Apterous-Dependent Processes in Boundary Formation
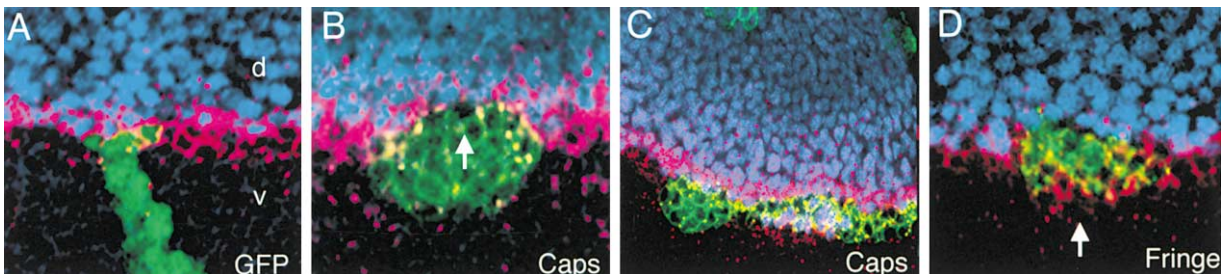Apterous controls formation of the DV compartment boundary in the wing disc. Apterous is needed to initiate

Figure 6. Effects of Small Clones on DV Boundary Shape
Dorsal cells (d) are marked by *ap-lacZ* (nuclear β-Gal shown in blue). Wg protein is shown in purple. Transgene expression is shown in green.
(A) Ventral GFP-expressing clone. The clone was elongated and irregular in outline and had no effect on the DV boundary.
(B and C) Caps-expressing clones. (B) The clone rounded up and cells were close to the DV border. Dorsal cells were displaced by the clone.
(C) Ventral Caps-expressing cells separated a group of d cells from the d compartment (visible as green cells lacking nuclear β-Gal between 2 groups of blue cells). (C) is at ~1/2 magnification of (A), (B), and (D).
(D) Ventral Fringe-myc expressing clone. Note that Wg was expressed at the interface between the clone and normal v cells (arrow), but not at the interface between the clone and d cells.
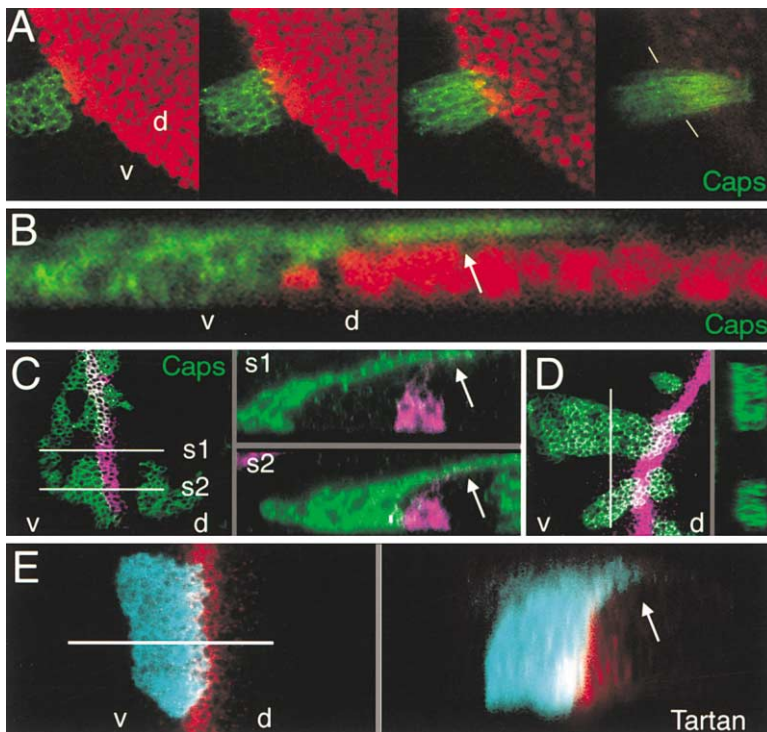
Figure 7. Projections Mediating Cell Contact

(A) Series of four horizontal optical sections through a ventral Caps-expressing clone. Caps protein shown in green. Dorsal cells (d) are marked by *ap-lacZ* (nuclear β-Gal shown in red). White bars indicate the position of the DV boundary in the apical-most section.

(B) Optical cross-section though the clone in (A). Arrow indicates Caps-containing processes extending into the d compartment. Apical is up.

(C) Optical section of a disc with several Caps-expressing clones. Wg (purple) marks the DV boundary. S1 and S2 indicate the positions of the optical cross-sections shown at right.

(D) Optical section of a disc with Caps-expressing clones. The white line shows the position of the cross-section at right. Caps-containing processes were not observed between v clones.

(E) Optical section of a ventral Tartan-expressing clone labeled by cytoplasmic β-Gal expression (blue). Wg (red) marks the DV boundary. The white line shows the position of the cross-section at right. The thicker base of cellular projections can be visualized by β-Gal.

signaling between compartments and to prevent intermingling of D and V cells. In this report, we have presented evidence that Apterous controls the signaling properties and affinity properties of dorsal cells via distinct sets of target genes. Apterous regulates expression of Fringe and the Notch ligands Serrate and Delta in D cells. Fringe ensures proper signaling between D and V cells, but is insufficient to support boundary formation (Milan and Cohen, 1999a; Figure 2). In contrast, the LRR protein Caps supports boundary formation but does not induce Notch signaling. When expressed in V cells, Caps and Tartan cause cells to extend processes toward D cells and to attempt to sort-out into the D compartment. However, Caps- and Tartan-expressing cells cannot cross the boundary because they retain ventral signaling properties. Fringe-expressing clones have the opposite properties: they do not sort-out toward the dorsal compartment (Figure 4D), but cross the boundary when they happen to contact it (Micchelli and Blair, 1999; Rauskolb et al., 1999) (Figure 5C). Our findings suggest that Fringe influences cell behavior predominantly through its effects on Notch signaling, whereas Caps and Tartan primarily influence cell behavior by increasing affinity for D cells.

We propose that the activities of Caps and Tartan, as well as those of the Notch ligands and Fringe, are required for DV boundary formation. Apterous controls expression of Serrate and Fringe as well as Caps and Tartan in dorsal cells during boundary formation. The ligands for Notch are transmembrane proteins. Therefore, ligand-receptor binding may contribute to adhesion between D and V cells at the boundary while inducing signaling. By increasing the affinity for Delta, Fringe may promote binding between Notch on D cells and Delta on V cells. Likewise, by reducing the affinity of D cells for Serrate, Fringe may promote binding between

Serrate on D cells and Notch on V cells. Increased binding between oppositely specified cells is likely to help to stabilize the interface between the two cell populations, but seems unlikely to help drive the initial segregation of the populations needed to establish a smooth boundary. Indeed, we have reported that restoring Fringe and Serrate expression in *apterous* mutant discs is not sufficient to restore a normal DV boundary (Milan and Cohen, 1999a) (Figure 2). We propose that the transient expression of Caps and Tartan in D cells initiates the segregation of the two cell populations. Once they are separated, Fringe-dependent cell interactions may stabilize the boundary (Micchelli and Blair, 1999; Rauskolb et al., 1999). Fringe has also been implicated in boundary formation in vertebrate limbs (Rodriguez-Esteban et al., 1997; Laufer et al., 1997). It will be of interest to learn whether Caps and Tartan homologs play comparable roles in DV boundary formation vertebrates.

The Cellular Basis for Caps- and Tartan-Mediated Cell Interaction

Caps and Tartan expression induced the formation of cellular processes that projected from V cells toward D cells. Cytonemes and similar structures have been proposed to mediate long-range cell interactions in imaginal discs (Ramirez-Weber and Kornberg, 1999; Cho et al., 2000). The structures we observed appear to differ from cytonemes in that they project across the signaling center into the opposite compartment, rather than projecting toward the signaling center. Filopodia have been implicated in guiding morphogenetic movements in epithelial sheets (Raich et al., 1999; Martin-Blanco et al., 2000). Filopodia expressing E-Cadherin have been implicated in the formation of adhesive zippers between epithelial cells which serve as nucleation centers for reorganization of the cytoskeleton (Vasiouk-

hin et al., 2000). We propose that imaginal disc cells use filopodia that express cell-surface proteins, including Caps and Tartan, to assess the identity of nearby cells and to control cell behavior. Caps and Tartan do not appear to mediate homophilic adhesion. This suggests that dorsal cells express another cell surface protein able to bind the LRR domains of Caps and Tartan. Expression screening and systematic searches for membrane proteins expressed on D cells may help to identify the Caps/Tartan binding partner.

Mechanisms of Cell Segregation and Boundary Formation

The mechanisms by which tissue boundaries form are not well understood. Differences in cell adhesion can contribute to tissue boundary formation. Sorting-out of cell populations can be guided by both the amount and types of adhesion proteins that cells express (Steinberg and Takeichi, 1994, and references therein). A different view comes from studies on Ephrins and Eph receptors, which suggest that repulsion or deadhesion can promote segregation of cell populations (Xu et al., 1999; Mellitzer et al., 1999). Many adhesion proteins form regulated connections with the cytoskeleton and participate in contact-mediated signaling (reviewed in Vleminckx and Kemler, 1999; Hynes, 1999). A useful distinction can be made between initial cell-cell contacts, which may be transient, and formation of stable contacts that may involve substantial reorganization of the cytoskeleton (Vasioukhin and Fuchs, 2001). If signaling promotes reorganization of the cytoskeleton, cell interactions might be destabilized (Brückner and Klein, 1998). Repeated cycles of deadhesion and readhesion could lead to sorting out behavior. It is possible therefore that adhesive differences and differences in cell behavior both contribute to forming tissue boundaries. At present, it is not clear which type of explanation best describes formation of the compartment boundaries in imaginal discs. Caps and Tartan are cell surface proteins that mediate cell interactions. Our findings suggest that contacts with appropriately specified cells mediated by Caps and Tartan might be stabilized, whereas contacts with inappropriate cells might be destabilized. A deeper understanding of these processes awaits identification of the cell surface proteins with which Caps and Tartan interact.

Experimental Procedures

Drosophila Strains
Previously described strains: Bx^1 and UAS-dLMO (Milan et al., 1998). $Chip^{e5.5}$ (Morcillo et al., 1997). UAS-fringe (Irvine and Wieschaus, 1994). EP-fringe (Milán and Cohen, 1999a). UAS-p35 (Hay et al., 1994). UAS-Connectin (Meadows et al., 1994). ap^{rk568} (Cohen et al., 1992). ap^{Gal4} (Calleja et al., 1996). $caps^{65.2}$ and UAS-caps (Shishido et al., 1998). $caps^{65.2}$ is a null allele generated by imprecise excision of a P element. l(3)05121 and l(3)02937 are P element insertion alleles of caps. $trn^{SO64117}$ is a hypomorphic trn allele (Salzberg et al., 1997). $trn^{25/4}$ is a null allele generated by P element excision (Chang et al., 1993). UAS-trn was prepared by cloning 5′ and 3′ fragments from two cDNA clones described in Chang et al. (1993). To prepare the tartan caps double mutant chromosome, $trn^{SO64117}$ was first recombined onto the $caps^{65.2}$ chromosome. New tartan alleles were then generated by imprecise excision of the $trn^{SO64117}$ P element. $trn^{\Delta2.9}$ was identified by lethality in trans to other trn alleles. Tartan protein was not detected in clones of cells homozygous for the $trn^{\Delta2.9}$ $caps^{65.2}$ double mutant chromosome.

Antibodies
The following antibodies came from the listed sources: mouse anti-Wg (Brook and Cohen, 1996); rabbit anti-Gal4 DBD (Santa Cruz); rabbit anti-Caps (Shishido et al., 1998); rabbit anti-Trn (Chang et al., 1993); and mouse anti-Connectin (Meadows et al., 1994). Guinea pig anti-Ap was provided by J. Botas.

Rescue of *apterous* Mutant Wing Discs
Rescue assays were performed using two combinations of ap alleles that reduced Ap activity to different extents. ap^{ugo35} is a deletion that lacks ap activity. ap^{rk568} and ap^{Gal4} are P element insertions that reduce ap activity. ap^{Gal4}/ap^{rk568} discs retain more ap activity than ap^{Gal4}/ap^{ugo35} discs. In ap^{Gal4}/ap^{rk568} discs, Wg expression along the DV boundary was not completely lost, indicating residual Ap activity (not shown). Expression of Fringe at high levels using a UAS fringe construct (ap^{Gal4}/ap^{rk568}; UAS-fng) fully restored Wg expression along the DV boundary and restored a normal DV boundary in 80% of discs (not shown), in agreement with a recent report by O'Keefe et al. (2001). Expression of Fringe at moderate levels in ap^{Gal4}/ap^{rk568} using EP-fng did not rescue the DV boundary. In the more severe mutant combination ap^{Gal4}/ap^{ugo35}, the wing pouch was smaller and Wg expression was lost from the DV boundary in most discs (Milán and Cohen, 1999). Expression of Serrate or Fringe at moderate or at high levels was not able to rescue boundary formation in this genotype.

Genotypes of Larvae Used for Genetic Mosaic Analysis
hs-FLP (I)/Actin>CD2>Gal4; ap^{rk568}/+; UAS-GFP/+. *hs-FLP (I)/Actin>CD2>Gal4; ap^{rk568}/+; UAS-GFP/UAS-p35. hs-FLP (I)/Actin>CD2>Gal4; UAS-caps/ap^{rk568} or uas-lacZ. hs-FLP (I)/Actin>CD2>Gal4; UAS-trn/ap^{rk568} or uas-lacZ. hs-FLP (I)/Actin>CD2>Gal4; UAS-caps UAS-trn/ap^{rk568}. hs-FLP (I)/Actin>CD2>Gal4; UAS-fng-myc/ap^{rk568}. hs-FLP (I)/Actin>CD2>Gal4; UAS-fng-myc/ap^{rk568}; UAS-caps UAS-trn/+. hs-FLP (I)/Actin>CD2>Gal4; UAS-p35/ap^{rk568};UAS-caps UAS-trn/+. hs-FLP (I); $caps^{65.2}$ FRT80/Ubi-GFP FRT80. hs-FLP (I); $trn^{25/4}$ FRT80/Ubi-GFP FRT80. hs-FLP (I); $trn^{\Delta2.9}$ $caps^{65.2}$ FRT80/Ubi-GFP FRT80.

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