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# Border of Notch activity establishes a boundary between the two dorsal appendage tube cell types

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**Abstract**

Boundaries establish and maintain separate populations of cells critical for organ formation. We show that Notch signaling establishes the boundary between two types of post-mitotic epithelial cells, the Rhomboid- and the Broad-positive cells. These cells will undergo morphogenetic movements to generate the two sides of a simple organ, the dorsal appendage tube of the *Drosophila* egg chamber. The boundary forms due to a difference in Notch levels in adjacent cells. The Notch expression pattern mimics the boundary; Notch levels are high in Rhomboid cells and low in Broad cells. *Notch*$^{-}$ mutant clones generate an ectopic boundary: ectopic Rhomboid cells arise in *Notch*$^{+}$ cells adjacent to the *Notch*$^{-}$ mutant cells but not further away from the clonal border. Pangolin, a component of the Wingless pathway, is required for Broad expression and for *rhomboid* repression. We further show that Broad represses *rhomboid* cell autonomously. Our data provide a foundation for understanding how a single row of Rhomboid cells arises adjacent to the Broad cells in the dorsal appendage primordia. Generating a boundary by the Notch pathway might constitute an evolutionarily conserved first step during organ formation in many tissues.


*Keywords:* Organ formation; Boundary formation; Patterning; Notch; Wingless

## Introduction

Animal organs contain a wide diversity of cell types arranged precisely to create a functional structure. Boundaries play a critical role during organ formation by generating distinct cell types and by preventing mixing between these specialized cells (Irvine and Rauskolb, 2001). In many tissues, such as the vertebrate hindbrain (Cheng et al., 2004), the *Drosophila* hindgut (Fusse and Hoch, 2002; Iwaki and Lengyel, 2002; Takashima et al., 2002) and the wing disc (Irvine and Rauskolb, 2001), Notch signaling establishes a boundary necessary for organ formation.

Notch signaling is an evolutionarily conserved mechanism used in a wide variety of cell fate decisions during animal development. Notch encodes a transmembrane receptor that is activated by the ligands Delta and Serrate (Jagged in vertebrates). Upon ligand binding, Notch is cleaved by Presenillin, producing the Notch Intra-Cellular Domain (NICD), which is the active form of the receptor. NICD translocates into the nucleus and acts as a transcriptional co-activator to promote expression of Notch target genes (Schweisguth, 2004).

Notch promotes cell fate decisions by multiple methods: lateral inhibition, asymmetric cell divisions, and establishment of boundaries. During development, boundaries function to keep different cell populations separated from one another and, in some cases, act as signaling centers that pattern cells on either

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side of the boundary during organ formation (Irvine and Rauskolb, 2001; Contakos et al., 2005). These boundaries might use the cytoskeleton to set up a physical separation between cells (Major and Irvine, 2005). Tubes, which come in a wide variety of shapes and sizes, are important simple organs or parts of organs and can be produced by several distinct mechanisms (Lubarsky and Krasnow, 2003). While the morphological changes necessary to make tubes are well characterized in many systems, little is known about the molecular pathways that coordinate distinct cell types during tube formation.

As a model for understanding the molecular and cellular interactions necessary to create tubes from simple epithelia, we study dorsal appendage formation during egg development in *Drosophila melanogaster*. The egg chamber consists of the germ line surrounded by an epithelial layer of somatic follicle cells. The dorsal appendage primordia are located on either side of the dorsal midline at the anterior of the egg chamber. Midway through egg development, the cells in each primordium reorganize from a flat epithelial sheet into a tube with two distinct surfaces distinguished by specific markers, the transcription factor Broad and the serine protease Rhomboid (Fig. 1; Dorman et al., 2004; Deng and Bownes, 1997; Ruohola-Baker et al., 1993). The distinct border between these markers is already present in the flat epithelium, prior to the morphogenesis of the tube (Fig. 1; Dorman et al., 2004; Ward and Berg, 2005). Each cell type occupies a stereotypic position within the tube. The Rhomboid cells form the ventral midline of the tube, whereas the Broad cells form a dorsal 'roof' of apically constricted cells over the Rhomboid cells. Both cell types are essential for tube formation, since in all dorsal appendage mutants, both cell types form and occupy the appropriate locations within the otherwise defective tubes. These and other observations suggest that a boundary separates the Rhomboid and Broad cells (Ward and Berg, 2005). Since cell division ceases prior to specification of the dorsal appendage primordia, this boundary is not determined by cell lineage. Rather, the boundary is established via cellular interactions. The molecular pathways that produce this boundary, however, are unknown.

Initially, Broad is expressed in all follicle cells over the oocyte while *rhomboid* appears in a triangle pattern in response to EGF and TGF-β signaling (Deng and Bownes, 1997; Ruohola-Baker et al., 1993). Here, we show that high levels of Notch arise in a T-shaped region on the dorsal midline, repressing Broad (Figs. 1C, E, G). In more posterior

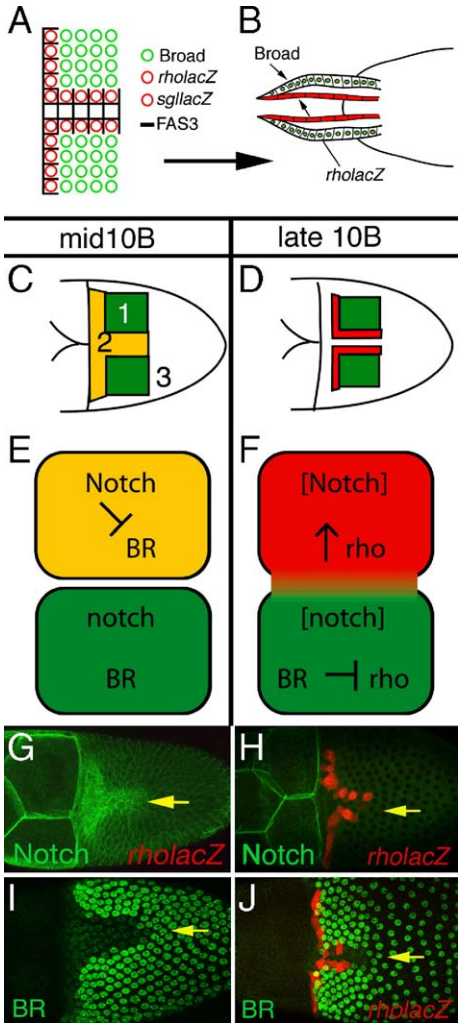



Fig. 1. High Notch levels accumulate prior to establishing dorsal appendage cell types. (A) Prior to tube formation, a single row of Rhomboid cells (*rho-lacZ*, red) flanks the anterior and dorsal margins of each cluster of Broad cells (green). The Rhomboid cells also express high levels of *sugarless-lacZ* (*sgl-lacZ*, red, Jordan et al., 2005). High levels of Fasciclin 3 (FAS3) accumulate in the Rhomboid cells as well as in the operculum cells (Ward and Berg, 2005). (B) After tube formation, the Rhomboid and Broad cells occupy stereotypic places within the tube (Dorman et al., 2004). (C, D) Summary of panels G through J: (C) At mid stage 10B, Notch (yellow) accumulates in a 'T' pattern in the dorsal anterior follicle cells. Broad expression (green) turns off in the 'T' region but is maintained in the roof-forming region of the dorsal appendage cells. Region 1 = Broad-expressing domain of the dorsal appendage cells, Region 2 = high-Notch cells, including the future Rhomboid cells, and Region 3 = all other columnar follicle cells. (D) At late 10B, a hinge-shaped row of Rhomboid cells (red) flanks each cluster of Broad cells (green). At this stage, little Notch protein is detectable in the dorsal anterior follicle cells. (E, F) Expanded view of two adjacent cells in Regions 2 and 1 to illustrate our hypothesis for boundary formation: (E) In Region 2 (yellow cell) at mid-10B, high levels of Notch are needed to repress Broad. In Region 1 (green cell), low levels of Notch allow high levels of Broad expression. (F) At late 10B, adjacent cells at the boundary have measured relative differences in Notch levels, indicated by a gradient of red to green between the two cells at the boundary. High [Notch] cells (red) respond by expressing *rhomboid*. Low [notch] cells (green) continue expressing high levels of Broad, which represses *rhomboid*. (G–J) Egg chambers stained with anti-β-galactosidase to reveal the *rhomboid* cells (red), (G, H) stained with anti-Notch (green) or (I, J) anti-Broad (green). Yellow arrow indicates dorsal midline. (G) At mid 10B, high levels of Notch accumulate in the dorsal anterior follicle cells in a 'T' pattern prior to *rhomboid* expression. The remaining follicle cells express low levels of Notch, except the stretch cells (squamous cells over the nurse cells), which express high levels of Notch. (H) By the time Rhomboid accumulates in the follicle cells at late 10B, Notch protein levels are dramatically reduced in the dorsal anterior follicle cells. (I) Initially, Broad is expressed in all the follicle cells over the oocyte (data not shown; Deng and Bownes, 1997). During stage 10B, Broad protein clears from a subset of dorsal anterior follicle cells. The cells that stop expressing Broad appear to correspond to the high-Notch cells (compare with G). (J) At late stage 10B, a hinge-shaped row of Rhomboid cells flanks the anterior/dorsal margins of each Broad cluster. Note: the dorsal appendage primordia appear closer together at late stage 10B due to cell shape changes.

and lateral dorsal cells, Broad levels increase and *rhomboid* levels decrease (Figs. 1C, E, I). Broad expression is needed to repress *rhomboid* in these cells (Fig. 1F). At the boundary between high and low Notch levels (indicated by a gradient of red to green in Fig. 1F), cells with high Notch induce a second round of *rhomboid* expression, forming a hinge-shaped row that flanks the high-Broad cells. Thus, we show that border formation between the Rhomboid and the Broad cells requires the Notch pathway. In particular, a difference in Notch levels in adjacent cells, rather than the absolute level of Notch, is critical for this process.

## Materials and methods

### *Fly strains*

We used $w^{1118}$ for our wild-type analyses. We used the following mutant stocks: $N^{ts1}$, $N^{55e11}$ *FRT101*, *FRT82B* $Delta^{RevF10}$ (Heitzler and Simpson, 1991), *FRT 82B* $Serrate^{RX106}$ (Thomas et al., 1991), *FRT82B* $Delta^{RevF10}$ $Serrate^{RX82}$ (double-mutant recombinant, a gift from Dr. S. Younger), $Su(H)^{8}$ *FRT40A* (strong loss-of-function allele, a gift from S. Blair), $Su(H)^{del47}$ *FRT40A* (Morel and Schweisguth, 2000), and $broad^{npr-3}$ *FRT19A* (Kiss et al., 1976). For our overexpression studies we used the following stocks: *P{w[+mC] = UAS-Dl: :N.ΔECN}B2a2 (UAS-*$Notch^{CA}$*)* (Doherty et al., 1996; Larkin et al., 1996); *P{w[+mC] = UAS-Dl.DN}TJ1 (UAS-Dl. DN)* (Huppert et al., 1997); *P{UAS-pan.dTCFΔN}5 (UAS-DN-pangolin)* (van de Wetering et al., 1997); and *UAS-Notch full-length (P{w[+mC]N[Scer\UAS.cSa]})* (gift from P. Simpson, Doherty et al., 1996).

For our analysis of *rhomboid-lacZ* expression in $w^{1118}$, $N^{55e11}$, $Delta^{RevF10}$, $Serrate^{RX106}$, $broad^{npr-3}$, *UAS-DN-Delta*, *UAS-Notch full-length* and *UAS-DN-pangolin* genetic backgrounds, we used the *rho-lacZ.2.2* strain carrying a promoter fusion construct encoding β-galactosidase under the control of a 2.2 kb HindIII—SspI fragment from the *rhomboid* regulatory region (Ip et al., 1992).

### *Immunofluorescence and antibodies*

Ovaries were fixed in PBSTwn (PBS, 0.1% Tween-20) plus 4% paraformaldehyde for 20 min and subsequently treated as described in Ward and Berg (2005). The primary antibodies were rabbit anti-β-galactosidase (Cappel, 1:6000), rabbit anti-GFP conjugated with AF488 (Molecular Probes, 1:2000), and mouse monoclonal anti-Broad, anti-Notch intracellular domain and anti FAS3 (1:100, 1:20 and 1:35, respectively, Developmental Studies Hybridoma Bank). The following secondary antibodies were used at the designated dilutions: Alexa 568 anti-rabbit and Alexa 488, 568 and 647 anti-mouse (1:200, Molecular Probes). Images were obtained using a BioRad Radiance 2000 MP confocal microscope and processed using Adobe Photoshop.

### *Loss of function clonal analysis*

We used both the standard FLP/FRT system and the MARCM technique to produce follicle cell clones (Xu and Rubin, 1993; Lee and Luo, 2001). To create *Notch* clones we crossed $N^{55e11}$ *FRT101/FM6B* to *ubiquitin-GFP FRT101*; *hsFLP/CyO*. To create clones of genes on 2R, we crossed $Su(H)^{8}$ *FRT40A*, and $Su(H)^{del47}$ *FRT40A* to *hs-FLP*; *ubiquitin-GFP FRT40*. To create clones of genes on 3R, we crossed *FRT82B* $Dl^{RevF10}$, *FRT82B* $Ser^{RX106}$, and *FRT82B* $Dl^{RevF10}$ $Ser^{RX82}$ to *hs-FLP; ; FRT82B ubiquitin-GFP/TM3*. We used the MARCM technique (Lee and Luo, 2001) to produce *broad* clones. For this technique, we crossed $broad^{npr-3}$ *FRT19A* to *P{tubP-GAL80}LL1 hsFLP FRT19A; ;UAS-mCD8GFP tubGAL4/MKRS*.flies.

To induce mitotic clones, we heat shocked $F_1$ adult females in empty glass vials for 60 min in a 37°C water bath. Typically, the flies were heat shocked once. Occasionally, however, to induce more frequent clones, the flies were heat shocked for 60 min 2 days in a row. After heat shock, the females (in the company of males) were reared on wet yeast at 25°C until dissection, 5 days after the first heat shock.

### *Ectopic expression studies*

To express Notch and Wingless pathway components ectopically in random clones, we crossed *y hsp70-FLP*; *Act > CD2 > GAL4*, *UAS-GFP* (Pignoni and Zipursky, 1997) to flies carrying the appropriate UAS construct. We heat shocked the $F_1$ progeny in empty glass vials at 37°C for 30 min, reared the flies at 25°C for 3 days, then dissected and analyzed ovaries.

## Results

### *Juxtaposition of cells with high Notch/low Notch corresponds to the Rhomboid/Broad boundary*

The dorsal appendage tubes are composed of two distinct cell types: the Rhomboid- and the Broad-positive cells (Dorman et al., 2004). In each dorsal appendage primordium, the Broad cells are flanked anteriorly and dorsally by a single row of cells expressing *rhomboid-lacZ* (*rho-lacZ*, hereafter referred to as 'Rhomboid' cells) (Fig. 1A). These two cell types are present in a flat epithelium prior to the morphogenetic movements that create the tube (Fig. 1A). The Rhomboid and Broad cells reorganize in highly stereotypic manners to produce a tube from an initially flat epithelium; the Rhomboid cells give rise to the ventral side of the tube while the Broad cells produce the dorsal and lateral sides of the tube (Fig. 1B). Despite these cellular rearrangements, the two cell types never intermingle, suggesting that a boundary separates the Rhomboid and the Broad cells (Ward and Berg, 2005). The molecular pathways necessary to produce this boundary are unknown. Since Notch is required for proper dorsal appendage formation (Xu et al., 1992; Jordan et al., 2000; Zhao et al., 2000), we investigated whether the Notch pathway is involved in forming the boundary between the distinct dorsal appendage cell types.

We employed a temperature-sensitive allele of *Notch* to ask whether Notch is required at this time in egg chamber maturation (Supplementary Fig. 1). The uniform loss of Notch produced by shifting $N^{ts}$ females to the restrictive temperature resulted in egg chambers that exhibited a variety of defects in Broad expression. These results prompted a detailed analysis of Notch expression relative to both of the dorsal appendage tube markers, Broad and *rhomboid*, and an in-depth study of these markers in *Notch* loss-of-function mutants.

During early stage 10B, Notch protein accumulates in all epithelial follicle cells. Interestingly, Notch protein accumulates in a much higher level in a restricted pattern in a subset of the dorsal anterior follicle cells (Fig. 1G). The high-Notch protein domain appears to be the reciprocal of the Broad expression pattern (Fig. 1I), suggesting that the Notch and Broad patterns are adjacent to one another (Fig. 1C). In addition, the high-Notch pattern precedes *rhomboid* expression (Figs. 1G, H). During stage 10B, Notch protein disappears from the epithelial follicle cells (Fig. 1H), presumably reflecting activation of the Notch signaling pathway in these cells. Consistent with this hypothesis, a reporter of Notch activity in the nucleus is expressed at this time in the dorsal anterior follicle cells (Jordan et al., 2000). Subsequent to Notch activation, *rhomboid* expression appears (Fig. 1H) in a single row of cells immediately adjacent to the Broad cells (Fig. 1J).

### *Notch*$^{-}$ clones induce an ectopic Rhomboid/Broad boundary

The juxtaposition of high/low Notch protein corresponds to the Rhomboid/Broad boundary, suggesting that differential levels of Notch activity define the boundary between the two cell types. To test this idea, we used the *Notch*$^{55e11}$ null allele to remove *Notch* from the follicle cells, thereby creating regions where cells with different Notch levels are adjacent to one another. We observed several changes in Broad and *rhomboid* expression. For clarity, we will discuss these two markers in separate sections.

Our clonal analysis reveals that Notch is required non-cell autonomously to prevent ectopic *rhomboid* expression in the dorsal anterior follicle cells. When Notch was removed from the dorsal anterior follicle cells (Region 1, Fig. 2), ectopic *rhomboid* expression was turned on non-cell autonomously in cells neighboring the clonal border, but not in those further away (Figs. 2A, 3B, Table 1, 68%, $n = 25$). Not all cells surrounding a *Notch*$^{-}$ clone expressed *rhomboid*. Generally, the ectopic *rhomboid* expression arose in cells situated on a side of the clone closest to the normal boundary. This bias might indicate that other signaling pathways influence ectopic *rhomboid* expression. Consistent with this hypothesis, we never observed ectopic Rhomboid cells associated with *Notch*$^{-}$ clones outside of the dorsal anterior follicle cells (Region 3, Fig. 2C, Table 1, 0%, $n = 13$). Thus, at the Notch border, defined as the location between two adjacent cells with different Notch levels, the cell with higher Notch will express *rhomboid*. *Notch*$^{-}$ clones introduce a new Notch border, which results in ectopic *rhomboid* expression in the cell with higher levels of Notch.

### *Notch is required both cell autonomously and non-cell autonomously for proper Broad expression*

The dorsal appendage cells express either *rhomboid* or Broad, but never both markers (Ward and Berg, 2005). To test whether the non-clonal cells that express ectopic *rhomboid* adjacent to Notch clones also express Broad, we triply labeled egg chambers for Broad, β-galactosidase (to mark the Rhomboid cells) and GFP. As expected, cells ectopically expressing *rhomboid* did not express Broad (Fig. 2A′, arrows, 100%, $n = 19$). Thus, when two cells with different levels of Notch are adjacent to one another, the cell with the higher Notch level simultaneously represses Broad and promotes *rhomboid* expression.

In addition, Notch is required cell autonomously for proper Broad expression in Regions 1 and 2. When Notch was removed from Region 1, Broad expression was lower than expression in the neighboring Broad cells in that region of the dorsal appendage primordium (Fig. 3A, Table 1, 100%, $n = 25$). Due to extra cell divisions, nuclei in *Notch*$^{-}$ clones were smaller than non-clonal nuclei, reflecting a failure to enter the endocycle at stage 6 (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Smaller nuclei in Region 1 do not contribute to the observed reduction in Broad expression, since Broad levels were not reduced in most clones outside of the dorsal anterior (Fig. 2C, Region 3). *Notch*$^{-}$ clones in Region 2, where the follicle cells normally express high levels of Notch, always expressed ectopic, low levels of Broad (Fig. 2B, Table 1, 100%, $n = 15$).

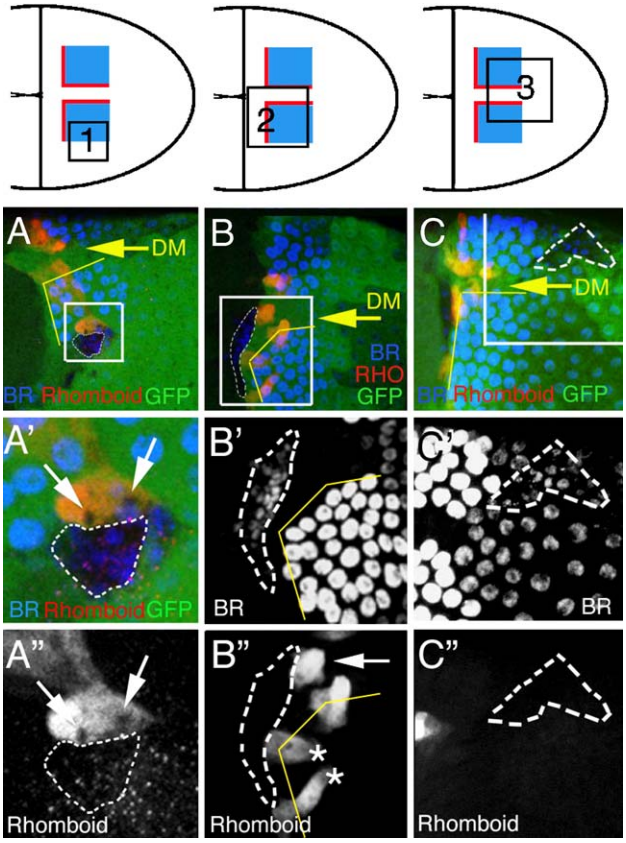


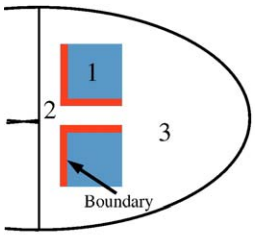

Fig. 2. Ectopic Rhomboid cells arise next to *Notch*$^{-}$ clones. (A–C) *Notch*$^{55e11}$ clones stained with anti-β-galactosidase (red) to reveal the Rhomboid cells, anti-Broad (blue) and anti-GFP (green). *Notch*$^{55e11}$ mutant cells appear black, since they do not express GFP. The location of each *Notch*$^{-}$ clone within the follicular epithelium is indicated in the sketch above each column, in which Rhomboid cells are red, Broad cells are blue, and numbers indicate regions within the follicular epithelium. Region 1 = Broad-expressing domain of the dorsal appendage cells, Region 2 = high Notch cells, including the Rhomboid cells, and Region 3 = all other columnar follicle cells. Dashed white lines outline each clone. Enlarged regions are indicated by white boxes. Anterior is to the left and the dorsal midline (DM) is indicated by a yellow arrow. (A) Two ectopic Rhomboid cells (white arrows) are associated with a *Notch*$^{-}$ clone in Region 1 of the left dorsal appendage primordium. These ectopic Rhomboid cells do not express Broad. (B) An ectopic Rhomboid cell (white arrow) is adjacent to a *Notch*$^{-}$ clone in Region 2. (The stretch follicle cells over the nurse cells are wild type; GFP staining in this region is weak.) The ectopic Rhomboid cell is 3–4 cells away from the Broad cells in the dorsal appendage primordium. Two other Rhomboid cells touch the clone; these cells, however, are part of the normal boundary, since each of these cells is adjacent to a Broad cell. Broad is ectopically expressed within the *Notch*$^{-}$ clone. Note: Due to continued cell divisions, the nuclei are smaller in *Notch*$^{-}$ clones. (C) Outside of the dorsal appendage primordia, no ectopic Rhomboid cells arise adjacent to *Notch*$^{-}$ clones. Broad expression is normal in Region 3, except for clones in the posterior of the egg chamber, where Broad expression is reduced (data not shown).

Thus, Notch is required to prevent Broad expression in this region after stage 10B. Finally, outside of the dorsal anterior in Region 3 (except in posterior follicle cells), Notch activity was not required for proper Broad expression (Fig. 2C, Table 1).

### *Notch is required for boundary formation*

Ectopic Rhomboid cells arose adjacent to *Notch*$^{-}$ clones in the dorsal anterior follicle cells (Regions 1 and 2), suggesting

Table 1
Clonal analysis of Notch function during late patterning of the follicular epithelium




| Region | Stage | *rhomboid* expression | Broad expression |
|---|---|---|---|
| Boundary | 10B | 100% absent ($n = 5$) | 100% ectopic ($n = 5$) |
| 1 | 10B-13 | 68% ectopic ($n = 25$) | 100% reduced ($n = 25$) |
| 2 | 10B-12 | 25%* ectopic ($n = 8$) | 100% elevated ($n = 15$) |
| 3 | 10B-12 | 0% ectopic ($n = 13$) | 100% normal** ($n = 12$) |

$n$ = Total number of clones counted.
Sketch shows the Broad (blue) and the Rhomboid (red) dorsal appendage cells; numbers indicate regions within the follicular epithelium. Region 1 = Broad domain of the dorsal-appendage-forming cells, Region 2 = high Notch cells, including the future Rhomboid cells, and Region 3 = all other columnar follicle cells. *Notch*$^{-}$ clones spanning the boundary do not express *rhomboid* and ectopically express Broad. In region 1, ectopic Rhomboid cells are associated with 17/25 *Notch*$^{-}$ clones. In region 2, an ectopic Rhomboid cell arises next to 2/8 *Notch*$^{-}$ clones. Importantly, these ectopic Rhomboid cells are in the twinspot ($N^{+}/N^{+}$). Finally, outside of the dorsal anterior follicle cells (region 3), no ectopic Rhomboid cells (0/13) were observed. In region 1, Broad expression is reduced in 25/25 clones. Conversely, in region 2, Broad expression is elevated in 15/15 clones. Outside of the dorsal anterior follicle cells in region 3, Broad expression is not changed in *Notch*$^{-}$ clones, except in the posterior of the egg chamber. Note: the numbers in the Broad expression column represent a combination of clones stained for both *rhomboid*/Broad and Broad alone. *Indicates that the ectopic Rhomboid cells in region 2 were in the twinspot. **Indicates that these region 3 clones do not include posterior follicle cells in which Broad is reduced.

that different levels of Notch activity can generate ectopic boundaries. When Notch clones span the normal boundary, the Rhomboid-positive cells were shifted to the edge of the *Notch*$^{-}$ mutant clone (Fig. 3B). Furthermore, Broad expression expanded across the normal boundary into the cells that would normally express *rhomboid* (Fig. 3A). Thus, Notch is required to establish the juxtaposition of Rhomboid and Broad at the boundary. That is, when the Notch activity border moves, the Broad/Rhomboid boundary also moves.

To investigate if Notch mis-expression is sufficient to disturb the boundary, we ectopically expressed two forms of Notch: the intracellular domain of Notch (*UAS-Notch*$^{CA}$) and full-length Notch (*UAS-Notch full-length*) (Larkin et al., 1996; Doherty et al., 1996). These two constructs differ from one another in the following ways: *UAS-Notch*$^{CA}$ activity is ligand independent and encodes the portion of Notch that is translocated to the nucleus. *UAS-Notch full-length* activity requires ligand binding and encodes the entire Notch protein. Overexpression of either construct in cells spanning the boundary resulted in mis-expression of Broad. Cells spanning the boundary ectopically expressed low levels of Broad and did not express *rhomboid*. Overexpression of *UAS-Notch full-length* in Region 1 lowered normal Broad expression (Fig. 3C) but never totally abolished Broad expression. Presumably, this low level of Broad protein prevents *rhomboid* expression (see below).

### *Broad represses rhomboid expression in the dorsal anterior follicle cells*

Both Rhomboid and Broad were mis-regulated in egg chambers with Notch clones in the dorsal anterior follicle cells. Within *Notch*$^{-}$ clones spanning the boundary, Broad was up-regulated and *rhomboid* was down-regulated (Fig. 3A). In cells adjacent to *Notch*$^{-}$ clones, Broad was totally repressed and *rhomboid* was up-regulated (Figs. 2A, B). These coordinate changes suggest a possible regulatory relationship between Broad and *rhomboid* expression. To test if these markers are independently or hierarchically regulated, we analyzed *rhomboid* expression in *broad*$^{npr-3}$ clones generated using the MARCM technique (Lee and

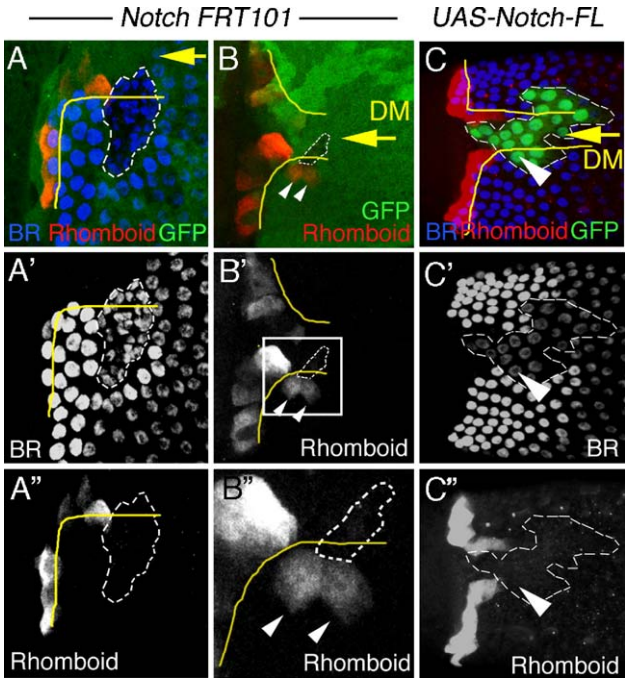



Fig. 3. Notch is required to establish the Rhomboid/Broad boundary. (A, B) *Notch*$^{-}$ clones in stage 10B egg chambers and (C) stage 11 egg chamber overexpressing Notch via *UAS-Notch-full-length*. (A–C) Egg chambers stained with anti-β-galactosidase (red) to reveal the Rhomboid cells, and with anti-GFP (green), and (A, C) anti-Broad (blue). Yellow arrows mark the dorsal midline (DM). Clones are outlined with dashed white lines. (A, B) *Notch*$^{55e11}$ mutant cells appear black, since they do not express GFP. Endogenous boundaries are marked by yellow lines. Enlarged region in panel B″ is indicated by a white box in B, (A) Notch activity is required to promote *rhomboid* and repress Broad at the boundary. The *Notch*$^{-}$ clone spanning the boundary does not express *rhomboid* and ectopically expresses Broad (Table 1, 5/5 clones). (B) A *Notch*$^{-}$ clone in the left primordium shifts the position of the boundary. A *Notch*$^{-}$ clone at the boundary does not express *rhomboid*. Two ectopic Rhomboid cells arise adjacent to the clone, thereby shifting the boundary. (C) Overexpression of *UAS-Notch-full-length* (GFP-positive cells) disrupts boundary formation (white arrowhead). Cells spanning the boundary and expressing *UAS-Notch-full-length* ectopically express Broad and do not express *rhomboid*.

Luo, 2001). Indeed, removal of Broad activity resulted in ectopic *rhomboid* expression in a cell-autonomous manner (Fig. 4A; 100%, $n$ = 17) and disrupted dorsal appendage formation (Fig. 4B′; 100%, $n$ = 9). Thus, Broad function is required to prevent *rhomboid* expression in the dorsal anterior follicle cells. Importantly, like normal Rhomboid cells, the ectopic Rhomboid cells present in small *broad*$^{-}$ clones expressed high levels of Fasciclin 3 (FAS3, Fig. 4A; 100%, $n$ = 17), and reorganized appropriately to form the ventral floor of an ectopic tube (Fig. 4B′; 100%, $n$ = 9). *broad*$^{-}$ clones outside of the dorsal anterior did not express ectopic *rhomboid*, consistent with previous reports suggesting that *rhomboid* expression depends on a combination of signals, including DPP and EGFR (Queenan et al., 1997; Deng and Bownes, 1997; Peri and Roth, 2000; Dequier et al., 2001). These findings show that in the dorsal anterior region, Broad is required to repress *rhomboid* expression in a cell-autonomous manner. Interestingly, the aberrant dorsal appendages produced by loss of *broad* resemble those present in egg chambers expressing dominant-negative Pangolin (Jordan et al., 2005).

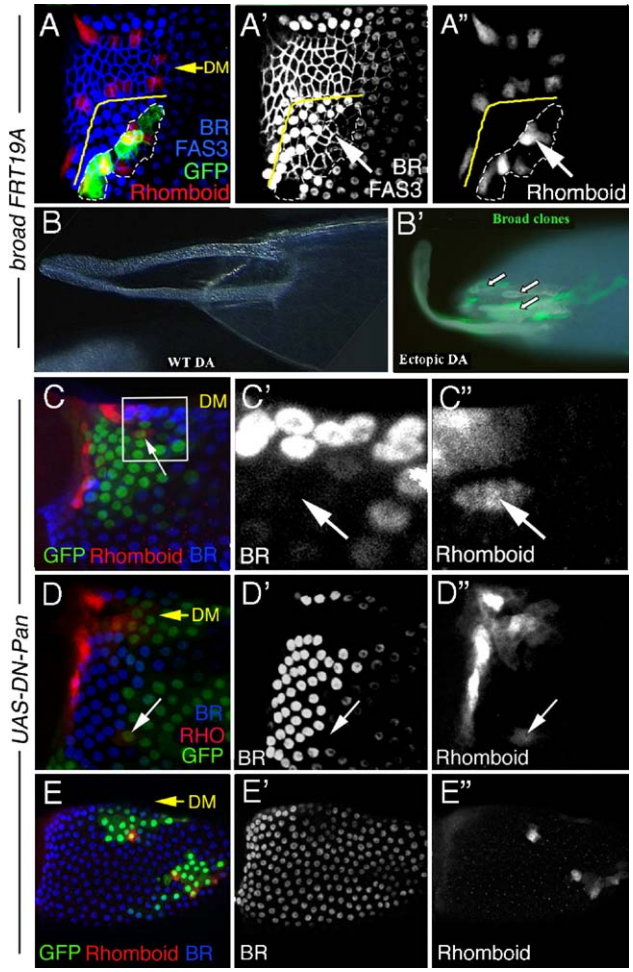



Fig. 4. Broad and Pangolin prevent Rhomboid expression. (A, C–E) Stage 10B egg chambers stained with antibodies to Broad (blue), GFP (green), anti-β-galactosidase to reveal the Rhomboid cells (red), and (A) anti-Fasciclin 3 (FAS3) (blue) to reveal apical membranes. Yellow arrow indicates the dorsal midline (DM). Clone in panel A is outlined with dashed white line; yellow lines show the endogenous Rhomboid/Broad boundary. White box in C shows enlarged area in C′, C″. (A, B) Analysis of *broad* function using positively marked *broad*$^{null}$ clones. (A) Broad prevents *rhomboid* expression. *broad*$^{npr\text{-}3}$ clones (identified by GFP-positive expression) do not express Broad but do express *rhomboid*. Furthermore, FAS3 expression reveals that these cells do not constrict apically. (B) *broad*$^{npr\text{-}3}$ *FRT19A/ + FRT19A* egg chambers produce wild-type dorsal appendages. (B′) Loss of *broad* in the dorsal appendage primordium disrupts dorsal appendage formation, yet *broad*$^{-}$ cells (green) can contribute to tube formation as floor cells (white arrows outlined in black). (C–E) *UAS-dominant-negative pangolin* expression (GFP-positive) reduces Broad expression in the follicle cells, occasionally allowing ectopic *rhomboid* expression (white arrows). (E) In Region 3, *UAS-dominant-negative pangolin* expression reduces Broad expression, thereby allowing *rhomboid* expression outside of the dorsal anterior follicle cells.

### *Pangolin, a component of the Wingless pathway, represses rhomboid expression*

Our data demonstrated that dorsal anterior follicle cells detect relative differences in Notch levels and respond by regulating Broad and *rhomboid* expression. As shown in Fig. 3A, Notch is required cell autonomously to repress Broad at the boundary, thereby allowing *rhomboid* expression at the boundary. The Notch pathway could directly repress Broad at the boundary or it could act by negatively regulating another pathway that is necessary to promote Broad expression in the follicle cells. Pangolin, a downstream transcription factor in the Wingless pathway, is required for Broad expression and for normal dorsal appendage formation (Jordan et al., 2005).

To test if Pangolin is required to prevent Rhomboid expression, we expressed *UAS-Dominant-Negative-Pangolin* (*UAS-DN-Pan*) in follicle cells. Figs. 4C and D show that Pangolin is required in a cell-autonomous manner to prevent *rhomboid* expression and ensure Broad expression in Region 1. Broad expression was down-regulated sufficiently to allow ectopic *rhomboid* in a subset of cells expressing *UAS-DN-Pan*; ectopic *rhomboid* usually appeared at the edges of the *DN-Pan* expression domains (Figs. 4C–E). Interestingly, *UAS-DN-Pan* reduced Broad expression and allowed ectopic *rhomboid* expression in Region 3 as well (Fig. 4E).

### *Notch acts via the canonical signaling pathway*

Our clonal analysis revealed that Notch is required for proper specification of the Rhomboid and Broad cells during dorsal appendage formation. To determine if Notch acts via the canonical signaling pathway during dorsal appendage formation, we analyzed *Suppressor of Hairless*, *Delta* and *Serrate* function at this time.

In nuclei of cells that have activated Notch signaling, the transcription factor Suppressor of Hairless (Su(H)) interacts with the *Notch*$^{-}$ intracellular domain, leading to expression of downstream target genes (Schweisguth, 2004). To determine if Notch regulates the Rhomboid/Broad cell types via this pathway, we analyzed Broad expression in both *Su(H)*$^{SF8}$ (hypomorphic allele) and *Su(H)*$^{D47}$ (null allele) clones. In Region 1 of the dorsal appendage primordium, *Su(H)* was required to ensure that Broad is expressed at a high level (Fig. 5A; 9/9 clones). Loss of *Su(H)* function had no effect on the

basal Broad expression in other follicle cells (Region 3, Fig. 5A). Since the *Su(H)* and *Notch*$^{-}$ mutant phenotypes are similar to one another, we conclude that Notch regulates dorsal appendage formation through the transcription factor Su(H).

In *Drosophila*, the two known Notch ligands are Delta and Serrate (Schweisguth, 2004). To determine if Notch signaling occurs via a canonical ligand-binding mechanism, we expressed *Dominant-negative Delta* (*UAS-Dl.DN*) in the follicle cells (Huppert et al., 1997). In Region 1 of the dorsal appendage primordium, Dominant-negative Delta expression produced ectopic Rhomboid cells (Fig. 5B, B′ arrow) in a non-cell autonomous manner, similar to that seen with *Notch*$^{-}$ clones. These ectopic Rhomboid cells did not express Broad. In addition, we occasionally observed reduced Broad expression in other cells expressing Dominant-negative Delta (data not shown). All these phenotypes resembled those observed with *Notch*$^{-}$ clones, suggesting that in this process, Notch is activated by a canonical ligand-binding mechanism. Surprisingly, however, *rhomboid* and Broad were expressed normally when Delta, Serrate, or both Delta and Serrate (null alleles) were removed from the follicle cells (Fig. 5C and data not shown). These results suggest that during boundary formation in the dorsal anterior follicle cells, Notch signaling might be regulated by a new, as yet uncharacterized Notch ligand, or, alternatively, that the germ line might provide a source of Delta (Deng et al., 2001; Lopez-Schier and St Johnston, 2001).

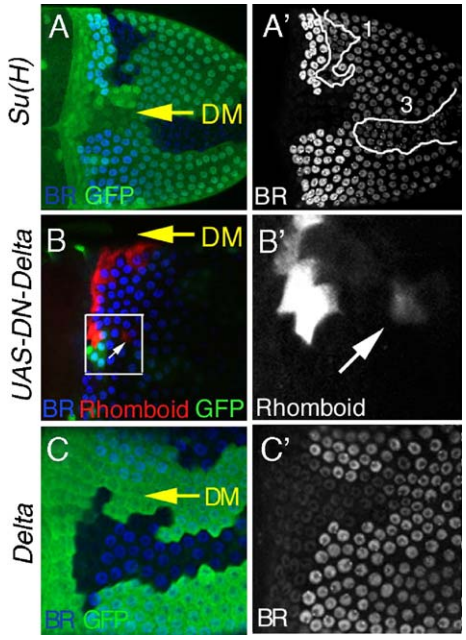



Fig. 5. Notch acts through the canonical signaling pathway. (A–C) Stage 10B egg chambers stained with anti-Broad (blue) anti-GFP (green) and (B) anti-β-galactosidase to reveal the Rhomboid cells (red). Yellow arrows indicate the dorsal midline (DM). Loss-of-function clones are outlined with a dashed white line. Enlarged area in panel B is indicated by a white box. (A) *Su(H)* clones reduce Broad expression in the dorsal appendage primordium (Region 1) but do not alter Broad levels in Region 3. (B) *UAS-Dominant-negative Delta* expression (marked by GFP staining) occasionally produces ectopic Rhomboid cells within Region 1 of the dorsal appendage primordium (white arrow). (C) Removal of *Delta* from the follicle cells (shown), or simultaneous loss of both *Delta* and *Serrate* (data not shown) does not alter Broad expression, indicating that neither of these ligands is required in the follicle cells for *Notch*-induced patterning.

## Discussion

Here, we show that the juxtaposition of cells with different Notch levels creates a boundary that promotes formation of the two dorsal appendage cell types (Fig. 6). At the boundary, cells with high Notch express *rhomboid*, whereas cells with lower Notch express Broad. A new boundary is established at *Notch*$^{-}$ mutant clone borders, where *Notch*$^{+}$ cells adjacent to *Notch*$^{-}$ cells ectopically express *rhomboid* and do not express Broad. Thus, in the dorsal anterior, when two cells with different Notch levels are adjacent to one another, the cell with higher Notch levels simultaneously represses Broad and promotes *rhomboid* expression. We found that *broad*$^{-}$ cells ectopically express *rhomboid*, indicating that Broad normally represses *rhomboid* expression. We infer that cells with higher Notch levels repress Broad, thereby allowing *rhomboid* expression. We now propose that when cells with different levels of Notch are located next to each other, the cells with high Notch repress Broad, allowing *rhomboid* expression. In contrast, cells with low Notch express Broad and therefore repress *rhomboid* expression.

### *Notch activity is required for correct dorsal appendage cell fate*

Notch, an important modulator of boundary function in other tissues, establishes the boundary that defines the Rhomboid and the Broad dorsal appendage cell types. When Notch is removed from cells that should span the boundary, *rhomboid* is not expressed, and Broad is ectopically expressed. Thus, at the boundary, Notch regulates the patterning of both Rhomboid and Broad cell types. When Notch activity is removed from Region 1, ectopic Rhomboid cells (*Notch*$^{+}$) arise adjacent to *Notch*$^{-}$ (Broad) cells, thus resembling the normal Notch border. We propose that these *Notch*$^{-}$ mutant clones produce ectopic borders of differential Notch activity, which in turn generate ectopic boundaries between Rhomboid and Broad domains.

Normally, Rhomboid cells arise all along the high–low Notch boundary in each dorsal appendage primordium. Based upon this observation, one might expect that Rhomboid cells would surround the *Notch*$^{-}$ clones. In our studies, however, we found that only those cells close to the normal boundary turned on ectopic *rhomboid*. Two factors probably contribute to this result. First, other signaling pathways, most notably EGFR and DPP, are involved in specifying and positioning the Rhomboid and Broad cell populations within the follicular epithelium (reviewed by Horne-Badovinac and Bilder, 2005; Berg, 2005). Presumably, these other signaling pathways influence Broad/*rhomboid* expression in cells adjacent to *Notch*$^{-}$ clones. Second, the ectopic Notch borders generated by *Notch*$^{-}$ clones arise within the Broad domain, which normally has low levels of Notch. Therefore, many cells at the ectopic border may not have sufficient Notch activity to repress Broad and activate *rhomboid*.

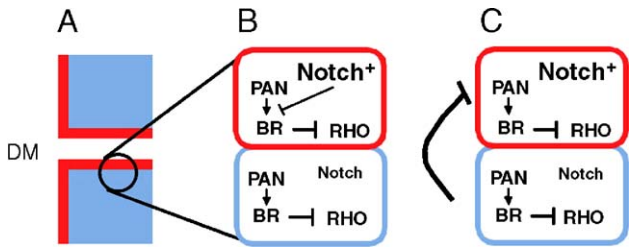



Fig. 6. Notch signaling establishes a boundary in the dorsal anterior follicle cells. Diagram showing the Rhomboid (red) and Broad (blue) dorsal appendage cell types. The juxtaposition of cells with different levels of Notch creates a boundary that facilitates patterning of the Rhomboid and Broad cells. At the boundary (A, expanded in B, C), cells with high Notch express *rhomboid* and do not express Broad. Conversely, cells with low Notch express Broad and do not express *rhomboid*. (B) Pangolin is necessary for Broad expression, and therefore repression of *rhomboid*. Since Broad is repressed in cells with High Notch, Notch may directly block Broad, or it may repress Broad by inhibiting Pangolin (or some other Wingless-pathway component). Repression of Broad allows activation of *rhomboid* at the boundary. (C) Alternatively, cells with lower Notch levels may secrete a Wingless pathway or Broad protein inhibitor that functions only in cells with high Notch.

### *Broad represses rhomboid expression*

Within the domain that would normally express Broad, loss of Notch causes the loss of Broad non-cell autonomously in adjacent cells and the appearance of ectopic *rhomboid* in these same cells. Furthermore, *Notch*$^{-}$ clones spanning the boundary ectopically express Broad and do not express *rhomboid*. These findings are consistent with previous results demonstrating that dorsal appendage cells express either *rhomboid* or Broad, but never both markers (Ward and Berg, 2005). In this work, we now show that *broad*$^{-}$ cells ectopically express *rhomboid*, suggesting that one function of Broad in the follicular epithelium is to directly or indirectly repress *rhomboid* expression. Such regulation must occur (at least in part) in the 2.2-kb fragment that drives *lacZ* expression in our reporter construct. CONSITE software detects twenty Broad binding sites clustered together in this region; all four zinc-finger isoforms have the potential to bind. Thus, high levels of Broad could directly regulate *rhomboid* in Region 1. Additional work is needed to test this hypothesis.

Other factors must also regulate *rhomboid* expression in Region 2. Within clones spanning the boundary, ectopic expression of Broad prevents *rhomboid* expression. In cells adjacent to *Notch* clones, loss of Broad expression allows ectopic *rhomboid* expression. Nevertheless, the simple absence of Broad is insufficient to induce *rhomboid* expression, since the majority of cells in Region 2 lack Broad expression and do not express *rhomboid*. Presumably, high levels of EGFR and DPP signaling prevent *rhomboid* expression in these cells (Ward and Berg, 2005).

### *High Notch*

The *Notch* loss- and gain-of-function data, as well as the Notch expression pattern, all suggest that juxtaposition of two cells with different Notch levels is critical for establishing the boundary between Rhomboid and Broad cell types. How, then, is Notch protein level regulated? The restricted pattern of Notch in the dorsal anterior follicle cells suggests that Notch expression is determined by a combination of patterning instructions from DPP along the anterior/posterior axis and EGFR signaling along the dorsal/ventral axis (Horne-Badovinac and Bilder, 2005; Berg, 2005).

The importance of regulating Notch protein levels is underscored by our data showing that overexpression of full-length Notch represses Broad expression throughout the follicular epithelium. Since the full-length Notch receptor must be bound by ligand to initiate Notch signaling, a Notch ligand is either present throughout the follicular epithelium or is presented to the follicle cells by the underlying germ line. The *Drosophila* genome encodes two known Notch ligands, Delta and Serrate, and several potential ligands, such as CG9138 (Grumbling and Strelets, 2006). The absence of both Delta and Serrate in the follicular layer did not affect Broad or *rhomboid* expression. The function of other potential ligands in follicle cells is not currently known. It is also possible that the ligand for this process is present in the germ line. Delta is expressed in the germ line at the appropriate time and functions in the germ line to regulate follicle cell processes, such as the pinching-off of egg chambers in the germarium and the mitotic-to-endocycle transition at stage 7 (Lopez-Schier and St Johnston, 2001; Deng et al., 2001; Schaeffer et al., 2004; Shcherbata et al., 2004; Jordan et al., 2006). Additionally, previous work demonstrates that *egghead* and *brainiac*, which encode modulators of Notch function, act in the germ line to pattern the dorsal anterior follicle cells (Goode et al., 1992, 1996). Regardless of the tissue distribution of the ligand, however, the ability to uniformly activate the Notch pathway throughout the follicle cell layer is note-worthy. This observation suggests that Notch *levels*, rather than *spatial* location of a ligand (or ligand modulator), determines where or how Notch signals in follicle cells of late stage egg chambers.

### *Non-autonomous Notch activity*

One of the most surprising aspects of the work presented here is that *Notch* clones act in a non-cell-autonomous manner to regulate Broad and *rhomboid* expression in adjacent cells. While surprising, non-cell-autonomous Notch activity occurs in the embryo (Baker and Schubiger, 1996), and most notably, at the D/V boundary in the wing disc (Rulifson and Blair, 1995; Micchelli et al., 1997). In the third-instar wing disc, Wingless is expressed in a 3- to 6-cell wide stripe spanning the D/V boundary, which separates the dorsal and ventral portions of the future wing blade. In this system, *wingless-lacZ* is repressed both within and adjacent to *Notch*$^{-}$ clones (Rulifson and Blair, 1995). Thus, *Notch*$^{-}$ clones act non-cell autonomously in two different tissues where boundaries act to distinguish different cell types.

What is the nature of the non-autonomous signal from the *Notch*$^{-}$ clones? We propose two potential mechanisms to explain this process (Fig. 6). First, Notch itself measures Notch levels in adjacent cells, either directly through homophilic adhesion or indirectly through interaction with *Notch*-binding proteins. When a *Notch*$^{-}$ clone occurs in the dorsal anterior,

adjacent cells sense the absence of Notch and respond as wild-type cells do when high-Notch cells neighbor low-Notch cells (Figs. 1E–H); they either repress Broad directly, or they repress Broad indirectly by affecting Pangolin (or some other component of the Wingless signaling pathway). Pangolin is needed to express Broad and therefore down-regulate *rhomboid* throughout the follicle cell layer (Figs. 4C–E, 6B). A second possibility is that when cells have little or no Notch activity, they might secrete an inhibitor of the Pangolin pathway that only affects cells with high Notch (Fig. 6C). We favor the first mechanism for its simplicity in accounting for *rhomboid* expression only at the border between high- and low-Notch-expressing cells.

### *Function of the boundary*

The establishment of a border between Rhomboid and Broad cells is important for preventing intermingling of these cell types during tube formation (Ward and Berg, 2005). It is not clear, however, what mechanism separates the Broad and Rhomboid cells from each other at the border. In some situations, the non-transcriptional branch of the Notch pathway regulates F-actin (Major and Irvine, 2005), which creates a "fence" that could help separate the two cell types from each other in the border. In dorsal anterior follicle cells, however, the canonical Notch pathway acts through the transcription factor Su(H). It is possible that in this cell type, the Notch pathway transcriptionally regulates a cell adhesion molecule or other component of an actin-binding protein complex, which in turn coordinates the cytoskeleton, thereby maintaining a separation between the Rhomboid cells and the Broad cells. Unlike cells at other boundaries in which an actin fence is evident, the Rhomboid and Broad cells undergo dramatic morphological changes and reorganize their actin networks to produce these effects. A fence that could maintain the separation of these cells during apical constriction, directed elongation, and convergent extension would be critical during these processes. One such *Notch*-interacting candidate gene that links to actin filaments is Echinoid (Rawlins et al., 2003; Wei et al., 2005; Lecuit, 2005). Future experiments will define whether Echinoid plays a role during border formation between Rhomboid and Broad cells.

Animals have a wide variety of organs containing different cell types arranged in a stereotypical manner. While the general morphogenesis of most organs has been described, little is known about the molecular mechanisms required to specify boundaries between diverse cell types and direct their subsequent reorganization to produce a functional structure. We have shown here that canonical Notch signaling is necessary to establish a boundary between the Broad and Rhomboid cells, which will form the dorsal and ventral portions of the dorsal appendage tube. Notch is also required in the vertebrate hindbrain for rhombomere boundary formation (Cheng et al., 2004; Amoyel et al., 2005). Thus, in simple and more complex organs, Notch specifies boundaries between distinct cell populations needed for organ formation. Generating a boundary through Notch signaling could be an evolutionarily conserved first step during organ formation in many tissues. The next challenge is to define the molecular nature of the physical power that keeps the two different cell types separated from each other in the border.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.05.021.

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