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.

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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
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 apiicially 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
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 w1118 for our wild-type analyses. We used the following mutant stocks: N51, N55ec1 FRT101, FRT82B Delta^FvF10 (Heitzer and Simpson, 1991), FRT 82B Serrate^RX106 (Thomas et al., 1991), FRT 82B Delta^FvF70 Serrate^RX202 (double-mutant recombinant, a gift from Dr. S. Younger), Su(H)^3F FRT40A (strong loss-of-function allele, a gift from S. Blair), Su(H)^67F FRT40A (Morel and Schweisguth, 2000), and broad^w^4 FRT194 (Kiss et al., 1976). For our overexpression studies we used the following stocks: P[hs-MACRE/8-1 B2a2 (UAS-Notch^3-4)] (Doherty et al., 1996; Larkin et al., 1996); P[hs-MACRE/8-1 N[scer-UAS-So(1)] (gift from P. Simpson, Doherty et al., 1996).

For our analysis of rhomboid-lacZ expression in w1118, N55ec1 Delta^FvF10 Serrate^RX106, broad^w^4, UAS-DN-Delta, UAS-Notch full-length and UAS-DN-pangolin genetic backgrounds, we used the rho-lacZ2,2 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 stained 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, 1:200), and mouse monoclonal anti-Broad, anti-Notch intracellular domain and anti FAS3 (1:100, 1:20 and 1:35, respectively, Developmental Studies HybriDNA 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 N55ec1 FRT101/FM6B to ubiquitin-GFP FRT101; hsFLP/Cyo. To create clones of genes on 2R, we crossed Su(H)^3F FRT40A, and Su(H)^67F FRT40A to hs-FLP; ubiquitin-GFP FRT40. To create clones of genes on 3R, we crossed FRT82B DlRevF10 FRT82B Sc^RX106, and FRT82B DlRevF70 Ser^RX202 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^w^4 FRT194 to P[tubh-GAL80]L1 hs-FLP FRT194; UAS-mCD8-GFP tubGAL4/MKR5; flies.

To induce mitotic clones, we heat shocked F1 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 F1 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 N51 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\textsuperscript{−} 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\textsuperscript{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\textsuperscript{−} 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\textsuperscript{−} 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\textsuperscript{−} 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\textsuperscript{−} 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\textsuperscript{−} 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).

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\textsuperscript{−} clones in the dorsal anterior follicle cells (Regions 1 and 2), suggesting
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-NotchCA) 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-NotchCA 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 broadnpr-3 clones generated using the MARCM technique (Lee and...
 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).

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) hypomorphic allele) and Su(H) 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-DiDN) 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).

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. 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 expression 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.](Image 83x171 to 253x407)
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 EGF 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 (Home-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 gerarium 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 noteworthy. 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 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.
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.

References


