

Notch-mediated repression of bantam miRNA contributes to boundary formation in the *Drosophila* wing

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SUMMARY

Subdivision of proliferating tissues into adjacent compartments that do not mix plays a key role in animal development. The Actin cytoskeleton has recently been shown to mediate cell sorting at compartment boundaries, and reduced cell proliferation in boundary cells has been proposed as a way of stabilizing compartment boundaries. Cell interactions mediated by the receptor Notch have been implicated in the specification of compartment boundaries in vertebrates and in *Drosophila*, but the molecular effectors remain largely unidentified. Here, we present evidence that Notch mediates boundary formation in the *Drosophila* wing in part through repression of bantam miRNA. bantam induces cell proliferation and we have identified the Actin regulator Enabled as a new target of bantam. Increased levels of Enabled and reduced proliferation rates contribute to the maintenance of the dorsal-ventral affinity boundary. The activity of Notch also defines, through the homeobox-containing gene *cut*, a distinct population of boundary cells at the dorsal-ventral (DV) interface that helps to segregate boundary from non-boundary cells and contributes to the maintenance of the DV affinity boundary.

KEY WORDS: *bantam*, Enabled, Notch, Compartment, *Drosophila*

INTRODUCTION

The imaginal discs of *Drosophila* and the vertebrate central nervous system provide well-characterized experimental systems in which subdivision of the tissue into adjacent compartments depends on mechanisms that limit cell mixing in order to produce stable boundaries (Garcia-Bellido et al., 1973; Xu et al., 1999; Zeltser et al., 2001). Activation of the Notch signaling pathway plays a major role in both developmental contexts (Cheng et al., 2004; Micchelli and Blair, 1999; Rauskolb et al., 1999), but the molecular effectors downstream of Notch and contributing to maintaining these affinity barriers remain so far largely unknown.

The *Drosophila* wing imaginal disc provides a suitable model system to identify these effectors. The wing primordium is subdivided into dorsal (D) and ventral (V) compartments by the restricted expression of the LIM-homeodomain protein Apterous in D cells (Blair et al., 1994; Diaz-Benjumea and Cohen, 1993). Cell interactions between D and V cells result in the activation of Notch along the dorsal-ventral (DV) boundary (Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996; Doherty et al., 1996). Apterous induces expression of the Notch ligand Serrate in D cells and restricts expression of Delta, another Notch ligand, to V cells (Diaz-Benjumea and Cohen, 1995; Milán and Cohen, 2000). Dorsally expressed Serrate and ventrally expressed Delta activate Notch symmetrically in cells on both sides of the DV compartment boundary. Expression of the glycosyltransferase Fringe makes D cells more sensitive to Delta and less sensitive to Serrate (Brückner

et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000), thus contributing to polarization of Notch activation towards the DV boundary. Notch activation induces expression of the secreted protein Wingless and the homeodomain transcription factor Cut in cells along the DV boundary (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Micchelli et al., 1997).

The establishment and maintenance of the DV affinity boundary evolves from early to late stages of wing development and relies on distinct molecular actors. Whereas restricted expression of the transmembrane proteins Capricious and Tartan in D cells contributes to the establishment of the DV affinity boundary (Milan et al., 2001a), Notch signaling plays a major role in its maintenance (Micchelli and Blair, 1999; Rauskolb et al., 1999). During late stages of wing development, Notch activity defines the so-called zone of non-proliferating cells (ZNC) (Herranz et al., 2008; O'Brochta and Bryant, 1985), which was proposed to decrease cell mixing at the DV boundary and to contribute to its maintenance. More recently, an actomyosin barrier has been shown to inhibit cell mixing at the DV boundary (Major and Irvine, 2005; Major and Irvine, 2006). Similar barriers have been reported in the embryonic ectoderm and at the interface between anterior and posterior compartments in the *Drosophila* wing, where they increase mechanical tension (Landsberg et al., 2010) and inhibit cell mixing (Monier et al., 2010). So far, the link between these actomyosin-based barriers and Notch signaling remains elusive and the contribution of reduced cell proliferation to the maintenance of the DV affinity barrier remains controversial (Blair, 1993).

Here, we identify two molecular effectors that mediate the role of Notch in maintaining the DV affinity boundary. We show that Notch exerts this activity in part through repression of bantam micro-RNA (miRNA) at the DV interface. bantam miRNA regulates G1-S transition (Brennecke et al., 2003; Herranz et al., 2008) and we have identified the Actin regulator Enabled (Ena) as a new target of bantam. We present evidence that increased levels of Ena and reduced proliferation rates contribute to the maintenance of the DV affinity boundary. Finally, we demonstrate

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that the activity of Notch also defines, through Cut, a distinct population of boundary cells at the DV interface and helps to segregate boundary from non-boundary cells. Our data indicate that this later subdivision contributes to the maintenance of a stable DV affinity barrier.

MATERIALS AND METHODS

Drosophila strains

trn-lacZ (Milan et al., 2001a); *ap^{Gal4}* (Milan and Cohen, 1999); *msh^{lacZ} Δ69* (referred to as *msh-lacZ*) (Isshiki et al., 1997); *UAS-N^{dsRNA}* (Presente et al., 2004), *UAS-dMyc* (Johnston et al., 1999); *bantam^{Δ1}*, *bantam-sensor-GFP* and *UAS-bantam-GFP* (Brennecke et al., 2003); *sqh-GFP* (Royou et al., 2002); *UAS-FP4-mito* (Gates et al., 2007); *UAS-ena-RNAi* (ID number: 43056 and 106484), *UAS-cut-RNAi* (ID number: 4138) and *UAS-dicer-RNAi* (ID number: 11429) are from Vienna *Drosophila* RNAi Center; other stocks are described in FlyBase.

Genetic mosaics and temperature shifts

The following *Drosophila* genotypes were used to generate loss-of-function clones by the FLP/FRT system (Xu and Rubin, 1993):

hs-FLP; M(3L) ubi-GFP FRT80B/ban^{Δ1} FRT80B

FRT19/FRT19 GFP; hs-Flp

cut^{l45} FRT18 / FRT18 arm-lacZ; hs-Flp.

Heat-shock was induced 3, 4 or 5 days after egg laying and late third instar wing discs were dissected. *Notch^{ts}* larvae were raised at 29°C for 48 hours and wing discs were subsequently dissected and labeled.

Immunohistochemistry

Antibodies used were: mouse anti-Cut (2B10), mouse anti-Wg (4D4), rat anti-BrdU (G3G4) and mouse anti-Ena (5G2) (all from Developmental Studies Hybridoma Bank); rabbit anti-GFP (Upstate); rabbit anti-β-Gal (Cappel); guinea-pig anti-Sens (Nolo et al., 2000); and rat anti-Ci (Motzny and Holmgren, 1995). F-Actin was labeled with Alexa Fluor 488- or Alexa Fluor 546-conjugated phalloidin (Molecular Probes) as described by Major and Irvine (Major and Irvine, 2005). BrdU staining and in situ hybridization were performed as described previously (Milan et al., 1996). A Digoxigenin-RNA Labeling Kit (Roche) was used to synthesize a probe of *enabled*.

3'UTR constructs

The *ena*-3'UTR was amplified by PCR from the SD08336 (Berkeley *Drosophila* Genome Project) plasmid with the following primers: Ena Sensor-Fwd: 5'-ATCGGCCGATCAAAATGCTGTCACG-3'; Ena Sensor-Rev: 5'-CCGCTCGAGTCTTAAATTGAGTCGTATGAGATCGTGTGCTTAGG-3'; digested with *XhoI*/*EagI* and cloned into the control sensor plasmid (P-Casper-tub-GFP) via the *XhoI*/*NotI* site. In order to prepare the *ena*-3'UTR with the mutated or deleted *bantam* site, the following reverse primers were used, respectively: Ena Sensor-Rev Mut: 5'-CCGCTCGAGTCTTAAATTGAGTCGTATGCTTCTCGTGTGCTTAGG-3'; Ena Sensor-Rev Del: 5'-CCGCTCGAGGTGCTTAGGAAATGTTGTGG-3'.

Generation of a *bantam* sponge

The *bantam* sponge sequence [GCGGCCGCA(ATCAGCTTT-CTTTTGATCTCAGCGCA)₂ATCAGCTTTCTTTTGATCTCATCTAGA] contains ten binding sites that are complementary to the mature *bantam* sequence and has a central bulge to prevent direct mRNA cleavage. The DNA sequence was synthesized by Mr Gene (www.mrgene.com), cloned (*NotI*/*XbaI*) downstream of a *dsRed* coding sequence into a pUAST vector. Transgenic flies were generated using P element-based random integration. The pairing between *bantam* and sponge sequences is shown in Fig. 2F.

Quantification of the smoothness of the DV affinity boundary

We quantified the degree of smoothness of a given DV boundary by measuring an index which was calculated as the ratio between the smoothest length of a given DV boundary and the real measurement. An index value close to one meant that the DV boundary was smooth. Both lengths were measured using ImageJ software and along the entire membrane interface of DV boundary cells located in the wing pouch. In

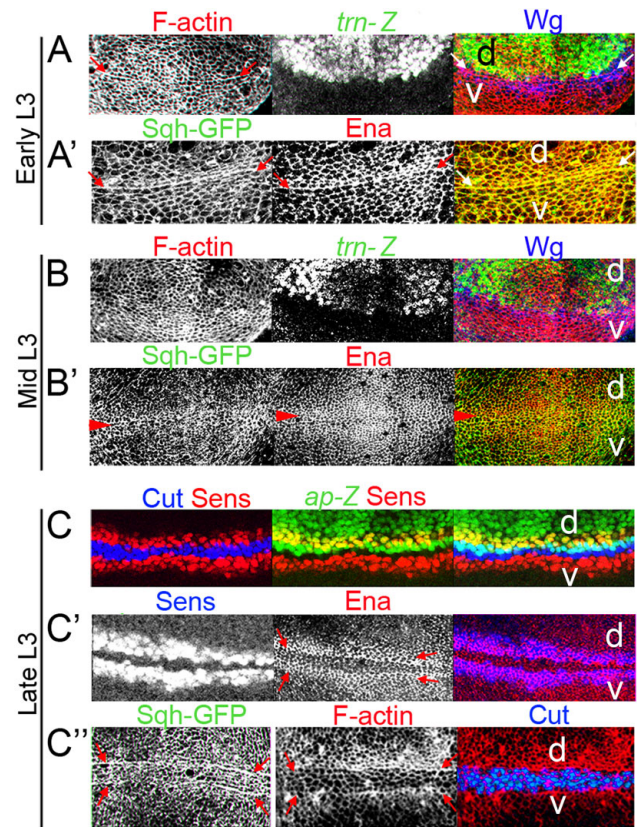


Fig. 1. Establishment and maintenance of the actomyosin barriers at the dorsal-ventral (DV) interface. Actin organization and signaling at the DV compartment boundary of early (A), mid- (B) and late (C) third instar (L3) *Drosophila* wing discs. (**A,A'**) In A, early L3 wing discs labeled to visualize *tartan-lacZ* (*trn-Z*, antibody to β-Gal) expression (green or white), F-Actin (phalloidin staining, red or white) and Wingless (Wg) protein (blue); in A', expression of Sgh-GFP (green or white) and Enabled (Ena) protein (red or white). Arrows point to the expression of Wg (A, right panel) or to the cable enriched in F-Actin, Sgh and Ena at the boundary between dorsal (d) and ventral (v) cells. (**B,B'**) In B, mid-L3 wing discs labeled to visualize *trn-Z* expression (green or white), F-Actin (phalloidin staining, red or white) and Wg protein (blue); in B', expression of Sgh-GFP (green or white) and Ena protein (red or white). Red arrowheads point to the apical accumulation of Ena and Sgh observed at the DV boundary. (**C-C''**) In C, late L3 wing discs labeled to visualize Cut protein (blue), Senseless (Sens) protein (red) and expression of *apterous-lacZ* (*ap-Z*, antibody to β-Gal, green); in C', expression of Ena protein (red or white) and Sens (blue or white); in C'', expression of Sgh-GFP (white), F-Actin (red or white) and Cut protein (blue). Ena protein is accumulated apically in the Sens-expressing cells (red arrows in C') and elevated levels of apical Sgh and F-Actin can be observed forming two actomyosin cables at the interface of boundary (Cut-expressing) and non-boundary (Sens-expressing) cells (red arrows in C'').

fixed wing discs, membrane interfaces corresponding to DV boundary cells were identified by the expression of *ap-lacZ* or *msh-lacZ* in D cells. Measurements were performed on one confocal section corresponding to a basolateral plane of the wing pouch. Images were captured using a 40× objective on a Leica SP5 confocal microscope.

RESULTS

In *Drosophila* tissues, local regulation of actomyosin contractibility induces cell bond tension at compartment boundaries (Landsberg et al., 2010) and plays a role in cell

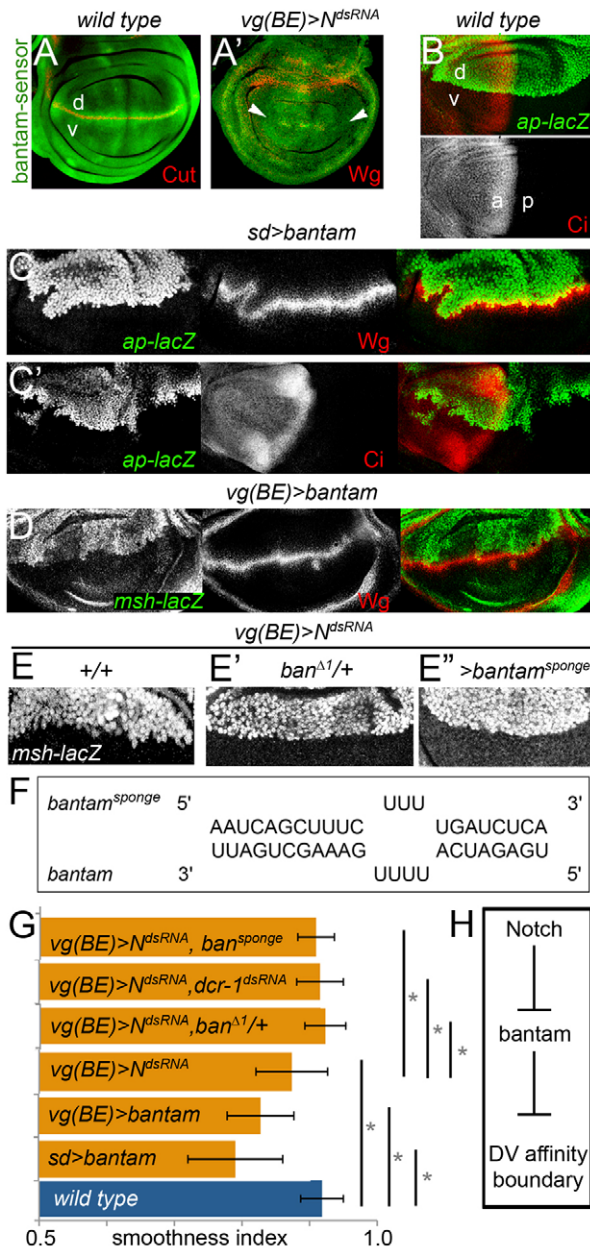


Fig. 2. Notch-mediated downregulation of bantam activity contributes to dorsal-ventral (DV) boundary formation.

(A,A') Wild-type (A) and *vg(BE)-Gal4, UAS-N^{dsRNA}* (A') late third instar *Drosophila* wing discs labeled to visualize expression of a *bantam*-GFP-sensor (green), and Cut (A) or Wg (A') protein (red). White arrowheads in A' point to the dorsal-ventral compartment boundary. (B-D) Wild-type (B), *sd^{Gal4}, UAS-bantam* (C,C') and *vg(BE)-Gal4, UAS-bantam* (D) wing discs labeled to visualize the DV boundary by expression of *apterous-lacZ* (*ap-lacZ*, antibody to β-Gal; B-C') or *msh-lacZ* (antibody to β-Gal; D) in dorsal cells (green or white), the anterior-posterior boundary by Ci protein expression in anterior cells (B,C'; red or white) and activity of Notch by Wg protein expression (C,D; red or white). (E-E') *vg(BE)-Gal4, UAS-N^{dsRNA}* (E), *vg(BE)-Gal4, UAS-N^{dsRNA}, bantam^{Δ1/+}* (E') and *vg(BE)-Gal4, UAS-N^{dsRNA}, UAS-bantam^{sponge}* (E') late third instar wing discs labeled to visualize expression of *msh-lacZ* (antibody to β-Gal, white). (F) Cartoon depicting the pairing between the sequences of bantam and bantam^{sponge}. (G) Histogram plotting the smoothness index (see Materials and methods for details) of the DV compartment boundary in late third instar wing discs of different genotypes. Error bars indicate s.d. This index was significantly reduced in *bantam-(sd>bantam: P<10⁻⁸ and vg(BE)>bantam: P<10⁻⁶)* or *N^{dsRNA}- (P<10⁻⁵)* expressing wing discs when compared with wild-type controls. Reducing the levels of bantam activity partially rescued the reduction in the smoothness index caused by *N^{dsRNA}* expression (in all cases, *P<10⁻³*). Average smoothness index values: wild type=0.91±0.03 (*n*=15); *sd>bantam*=0.78±0.069 (*n*=38); *vg(BE)>bantam*=0.82±0.048 (*n*=21); *vg>N^{dsRNA}*=0.86±0.037 (*n*=26); *vg>N^{dsRNA}, ban^{Δ1/+}*=0.92±0.03 (*n*=16); *vg>N^{dsRNA}>dcr-1^{dsRNA}*=0.91±0.034 (*n*=15); *vg>N^{dsRNA}>bantam^{sponge}*=0.91±0.027 (*n*=16). (H) Scheme depicting the role of Notch-mediated downregulation of bantam activity in the maintenance of the DV affinity boundary. a, anterior; d, dorsal; p, posterior; v, ventral.

sorting (Major and Irvine, 2005; Major and Irvine, 2006; Monier et al., 2010). During the establishment of the DV affinity boundary, filamentous Actin (F-Actin) accumulates at the adherens junction of boundary cells to form a long Actin cable (Major and Irvine, 2005; Major and Irvine, 2006) (Fig. 1A) and this accumulation correlates with increased junctional levels of Enabled (Ena), a cytoskeletal regulator that facilitates continued Actin polymerization at the barbed ends of Actin filaments, and Spaghetti Squash (Sqh), the regulatory light chain of Myosin II, activity of which is required to maintain cell sorting (Major and Irvine, 2005; Major and Irvine, 2006) (Fig. 1A'). The enrichment in F-Actin, Ena and Sqh observed at the interface between D and V cells coincides in time with high expression of the transmembrane proteins Caps and Tartan in the D compartment (Fig. 1A; data not shown). Later in development, the expression of Caps and Tartan starts to drop in the D compartment (Milan et al., 2001a) (Fig. 1B) and no major enrichment of F-Actin, Ena

or Sqh is found at the DV interface (Major and Irvine, 2005) (Fig. 1B,B'). However, Ena protein levels were generally increased in those cells close to the DV boundary (Fig. 1B' and see below). Maintenance of the DV affinity boundary during the late stages of development relies on Notch activity (Micchelli and Blair, 1999; Rauskolb et al., 1999). At these stages of development, two distinct cell populations are distinguished at the DV boundary: boundary cells expressing the transcription factor Cut and adjacent non-boundary cells expressing the transcription factor Senseless (Fig. 1C). F-Actin and Sqh are enriched at the interface between boundary and non boundary cells in two parallel stripes, whereas Ena is enriched in cells expressing Senseless (Major and Irvine, 2005; Major and Irvine, 2006) (Fig. 1C',C''). In this study, we aim to characterize those molecular effectors downstream of Notch that contribute to the generation of these actomyosin cables and to the maintenance of the DV affinity barrier.

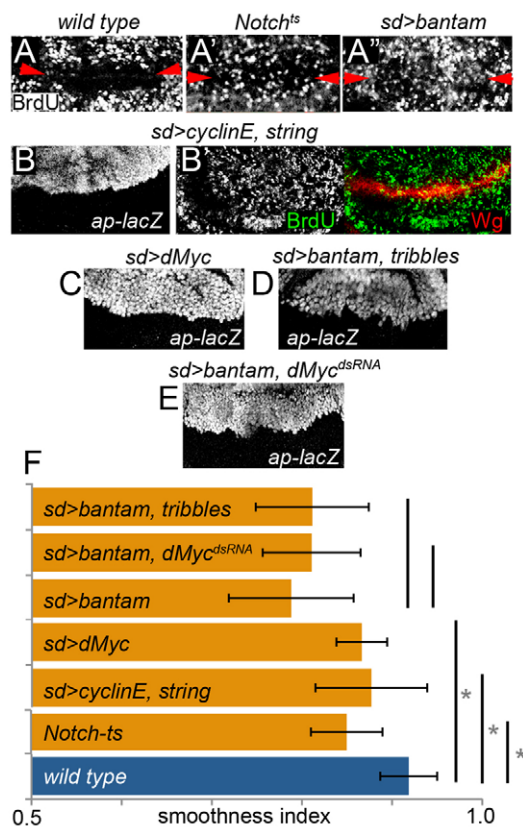


Fig. 3. A role of the zone of non-proliferating cells (ZNC) in the maintenance of the dorsal-ventral (DV) affinity boundary.

(A-A'') Wild-type (A), *Notch^{ts}* (A') and *sd>bantam* (A'') late third instar *Drosophila* wing discs labeled to visualize cells in S-phase by BrdU (white) incorporation. Red arrowheads point to the DV boundary. (B,B') Late third instar *sd^{Gal4}>UAS-CycE, UAS-string* wing discs labeled to visualize expression of *ap-lacZ* (antibody to β -Gal, white; B), or BrdU incorporation (white and green; B') and Wg protein expression (red; B'). (C-E) *sd^{Gal4}>UAS-dMyc* (C), *sd^{Gal4}>UAS-bantam, UAS-tribbles* (D), *sd^{Gal4}>UAS-bantam, UAS-dMyc^{dsRNA}* (E) late third instar wing discs labeled to visualize expression of *ap-lacZ* (antibody to β -Gal, white). (F) Histogram plotting the smoothness index (see Materials and methods for details) of the DV compartment boundary in late third instar wing discs of different genotypes. Error bars indicate s.d. This index was significantly reduced in *Notch^{ts}* mutant wing discs ($P<10^{-7}$) and *string-* and *cyclin-E-* ($P=0.02$) and *dMyc-* ($P<10^{-5}$) overexpressing wing discs. Co-expression of either *tribbles* or *dMyc^{dsRNA}* did not rescue the reduction in the smoothness index caused by *bantam* overexpression (*sd>bantam, tribbles*: $P=0.34$ and *sd>bantam, dMyc^{dsRNA}*: $P=0.28$). Average smoothness index values: wild type = 0.91 ± 0.03 ($n=15$); *Notch^{ts}* = 0.85 ± 0.04 ($n=29$); *sd>cyclin-E, string* = 0.87 ± 0.06 ($n=25$); *sd>dMyc* = 0.86 ± 0.03 ($n=21$); *sd>bantam* = 0.78 ± 0.069 ($n=37$); *sd>bantam, tribbles* = 0.81 ± 0.06 ($n=10$); and *sd>bantam, dMyc^{dsRNA}* = 0.81 ± 0.05 ($n=14$).

Notch-mediated repression of bantam contributes to DV boundary formation

Notch activity at the DV boundary induces a reduction in the activity levels of bantam miRNA (Herranz et al., 2008). Thus, a bantam-sensor that expresses GFP under the control of a ubiquitously active tubulin promoter and having two perfect bantam target sites in its 3'UTR shows increased levels of GFP at the DV boundary of wild-type wing discs (Fig. 2A) (Brennecke et al., 2003). This increase in

GFP was barely detectable when a dsRNA form of Notch (*N^{dsRNA}*) was expressed in boundary cells with the *vg(BE)-Gal4* driver (Fig. 2A'), indicating that Notch represses bantam activity in these cells (see also, Herranz et al., 2008). Because increased levels of GFP were already observed at the DV boundary of mid-third instar wing discs (see below), we questioned whether reduced bantam activity participates, downstream of Notch, in the maintenance of the DV affinity boundary during the mid- and late stages of wing development. To address this issue, we expressed *bantam* in the whole wing pouch, including DV boundary cells, using the *scalloped^{Gal4}* (*sd^{Gal4}*) driver. We then analyzed the impact on the DV affinity boundary, visualized with the *apterous-lacZ* or *msh-lacZ* [*msh* (*Dr* – FlyBase) is a target gene of Ap] (Milan et al., 2001b) reporters to label D cells. We focused our attention on late third instar wing discs and quantified the degree of smoothness of the resulting DV interfaces by calculating the ratio of the putative smoothest length of a given DV boundary and the true measurement (see Materials and methods). As shown in Fig. 2C,C' and quantified in 2G, the DV affinity boundary was greatly disturbed and, interestingly, the anterior-posterior (AP) compartment boundary, visualized with antibodies against Cubitus Interruptus (Ci) to label the A compartment, was straight and smooth (Fig. 2C', compare with 2B). These observations indicate that the phenotype observed is specific to the DV boundary and not due to a general disorganization of the wing disc. Ectopic expression of *bantam* at the DV boundary, using the *vg(BE)-Gal4* driver, also disrupted the DV affinity boundary (Fig. 2D,G). Similar defects were observed in mid-third instar wing discs (data not shown). Note that in all cases Notch signaling, monitored by the expression of the Notch-regulated gene *wingless* (*wg*), was not compromised (Fig. 2C,D). These results indicate that ectopic expression of *bantam* in boundary cells compromises the maintenance of the affinity boundary without altering the Notch activity levels. As bantam activity is downregulated at the DV boundary by Notch signaling, we then hypothesized that Notch maintains the DV affinity boundary in part by downregulating bantam activity. Wing discs expressing *N^{dsRNA}* with the *vg(BE)-Gal4* driver showed defects in the DV affinity boundary (Fig. 2E, see quantification in 2G). These defects were largely rescued by halving the doses of *bantam* (Fig. 2E',G), by expressing a dsRNA form of *Dicer-1* (*Dcr-1^{dsRNA}*) (data not shown; Fig. 2G), which is involved in the biogenesis of most miRNAs (Lee et al., 2003), or by expressing a *bantam* sponge (Fig. 2E',G), which contains ten binding sites that are complementary to the mature *bantam* sequence (Fig. 2F) and reduces bantam activity by sequestration (data not shown) (Loya et al., 2009). These results indicate that Notch controls the maintenance of the DV affinity boundary in part by repressing bantam activity (Fig. 2H).

A role of the zone of non-proliferating cells in DV boundary formation

bantam regulates G1-S transition in wing disc cells and reduced bantam activity at the DV boundary mediates the formation of the zone of non-proliferating cells (ZNC) (Brennecke et al., 2003; Herranz et al., 2008), which is characterized by its failure to incorporate BrdU (Fig. 3A). The ZNC can be observed in mid- and late third instar wing primordia (Fig. 3A; data not shown). Interestingly, reduced cell proliferation has been proposed to decrease cell mixing at the DV boundary and to contribute to its maintenance (O'Brochta and Bryant, 1985). Consistent with this proposal, reduced Notch activity or ectopic expression of *bantam* induced boundary cell proliferation (Fig. 3A',A'') and the DV affinity boundary was compromised (Fig. 3F). We then tested

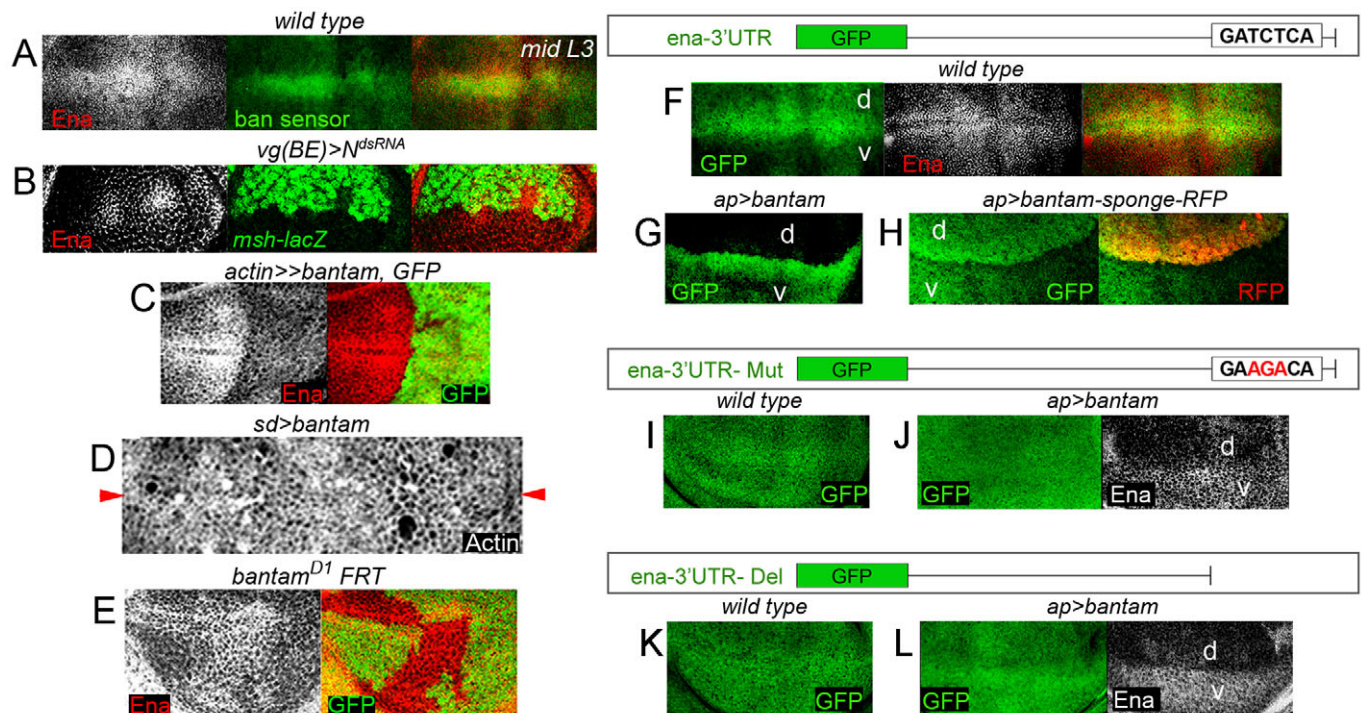


Fig. 4. *enabled* is a direct target of bantam. (A,B) Wild-type (A) and *vg(BE)>N^{dsRNA}* (B) *Drosophila* wing discs labeled to visualize expression of a *bantam*-GFP-sensor (green; A), Ena protein (red or white; A,B) and *msh-lacZ* (antibody to β -Gal, green; B). (C,D) Wing discs over-expressing *bantam* and GFP in clones of cells (C) or in the whole wing pouch (in *sd>bantam* larvae, D) and labeled to visualize Ena protein (red or white), GFP (green) and F-actin (white). Red arrowheads in D point to the dorsal-ventral compartment boundary. (E) Wing disc with clones of cells lacking bantam activity (*bantam^{Δ1}*), marked by the absence of GFP (green), and labeled to visualize Ena expression (red or white). (F-H) Wild-type (F), *ap^{Gal4}; UAS-bantam* (G) and *ap^{Gal4}; UAS-bantam-sponge-RFP* (H) wing discs showing expression of a tubulin-EGFP transgene carrying the *ena* 3'UTR (*ena* 3'UTR sensor, green) and labeled to visualize Ena protein (red or white in F) or RFP protein (red in H) expression. (I-L) Wild-type (I,K) and *ap^{Gal4}; UAS-bantam* (J,L) wing discs showing expression of a tubulin-EGFP transgene carrying the *ena* 3'UTR (green) with the *bantam* site mutated (I,J) or deleted (K,L) and labeled to visualize Ena protein (white in J,L). d, dorsal; v, ventral.

whether induction of cell proliferation by other means was also able to cause defects in the DV affinity boundary. For this purpose, we expressed either the G1-S and G2-M rate-limiting factors Cyclin E and String (Cdc25) in boundary cells (Edgar and O'Farrell, 1989; Knoblich et al., 1994) or the proto-oncogene *dMyc* (*dm* – FlyBase), also known to induce G1-S transition in wing cells (Herranz et al., 2008; Johnston et al., 1999). In both situations, the DV affinity boundary was compromised (Fig. 3B-C, see quantification in 3F). These results indicate that the ZNC contributes to the maintenance of this boundary. However, the strength of the phenotype caused by these cell cycle regulators was much lower than the one observed upon *bantam* overexpression (Fig. 3F). Moreover, expression of a dsRNA form of *dMyc* (*dMyc^{dsRNA}*) or Tribbles, a post-transcriptional repressor of String (Mata et al., 2000), did not rescue the defects in boundary formation caused by ectopic expression of *bantam* (Fig. 3D-F). These observations suggest that bantam targets other molecular effectors involved in the maintenance of the DV affinity boundary from mid- to late third instar stages.

***enabled* is a target of bantam that is required to maintain the DV affinity boundary**

In mid-third instar stages, the protein levels of Ena were increased at the DV boundary and this increase correlated with low bantam activity, visualized with the bantam sensor (Fig. 4A). Interestingly, the 3'UTR of *ena* contains a binding site for bantam (Stark et al.,

2003), suggesting that bantam is responsible for keeping Ena protein levels low in wing blade cells. Consistent with this proposal, over-expression of *bantam* led to a reduction in Ena levels (Fig. 4C) and clones of cells mutant for *bantam* showed increased levels of Ena (Fig. 4E). An *ena* 3'UTR sensor transgene consisting of the *ena* 3'UTR cloned into the tubulin-promoter-EGFP reporter plasmid recapitulated the expression pattern of Ena protein (Fig. 4F). This sensor was subject to regulation by bantam as over-expression of *bantam* in the D compartment reduced expression of the *ena* 3'UTR sensor transgene (Fig. 4G) and expression of a *bantam-sponge* induced an increase in GFP levels (Fig. 4H). By contrast, two transgenes carrying the *ena* 3'UTR lacking the *bantam* site or bearing a mutated *bantam* site (see Materials and methods for details) showed uniform expression of GFP (Fig. 4I,K) and expression of GFP was largely unaffected upon over-expression of *bantam* (Fig. 4J,L). These results indicate that *ena* is a direct target of bantam in wing disc cells.

In mid-third instar wing discs, no distinct accumulation of Actin or Myosin II was observed at the DV interface (Fig. 1B,B'), suggesting that increased levels of Ena protein might be required only in subsequent stages. Consistent with this proposal, we noted that the distinct accumulation of F-Actin observed at the DV boundary of late third instar wing discs (Fig. 1C'') was not observed upon overexpression of *bantam* (Fig. 4D) or upon expression of a dsRNA form of *ena* (*ena^{dsRNA}*) (Fig. 5E, compare with Fig. 1C''). In the latter case, the resulting boundary showed

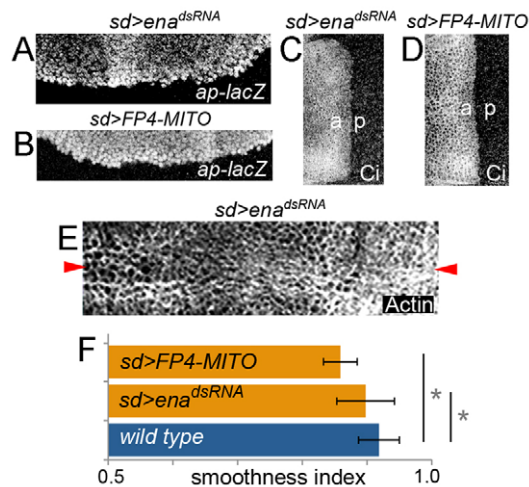


Fig. 5. Enabled contributes to the maintenance of the dorsal-ventral (DV) affinity boundary. (A-E) *sd>ena^{dsRNA}* (A,C,E) and *sd>FP4-mito* (B,D) late third instar *Drosophila* wing discs labeled to visualize expression of *ap-lacZ* (antibody to β -Gal; A,B), *Ci* (C,D) and F-Actin (E). Red arrowheads in E point to the DV compartment boundary. Anterior (a) and posterior (p) compartments are marked in C and D. (F) Histogram plotting the smoothness index (see Materials and methods for details) of the DV compartment boundary in late third instar wing discs of different genotypes. Error bars indicate s.d. This index was significantly reduced upon expression of an *ena^{dsRNA}* transgene or the fusion protein FP4-MITO when compared with wild-type control wing discs (*sd>ena^{dsRNA}*: $P=0.1$ and *sd>FP4-MITO*: $P<10^{-5}$). Average smoothness index values: wild type = 0.91 ± 0.03 ($n=15$); *sd>ena^{dsRNA}* = 0.89 ± 0.04 ($n=19$); *sd>FP4-MITO* = 0.86 ± 0.02 ($n=18$).

an irregular shape (Fig. 5A,F). Similar defects were observed upon expression of FP4-MITO (Fig. 5B,F), a fusion protein known to deplete Ena from its normal subcellular location, by sequestering it on mitochondria and neutralizing its function (Goh et al., 2002). The anterior-posterior compartment boundary was not affected upon expression of *ena^{dsRNA}* or FP4-MITO (Fig. 5C,D), consistent with the previous observation that overexpression of *bantam* did not affect this boundary (Fig. 2C'). Together, these results indicate that downregulation of bantam activity by Notch is required for the establishment of the ZNC and for the accumulation of Ena protein at the DV boundary, two features required for the maintenance of a stable DV affinity boundary. Indeed, in the absence of Notch signaling, cells at the DV boundary showed increased bantam activity (Fig. 2A'), reduced Ena protein (Fig. 4B) and increased BrdU incorporation (Fig. 3A').

A boundary cell population specified by the activity of Cut

In late third instar stages, the distinct accumulation of Ena at the DV boundary dropped in Cut-expressing cells (Fig. 6A, see also Fig. 1C'). This drop corresponded in time with the establishment of the Cut expression domain and the actomyosin cables observed at the interface between Cut-expressing and non-expressing cells (Fig. 1C'). We thus analyzed the contribution of Cut to reducing Ena levels in boundary cells and the role of Cut in the establishment of the two actomyosin cables. In wild type, Cut was expressed and Ena protein levels were reduced in dorsal and ventral boundary cells (Fig. 6C,D). In *cut* mutant wing discs, Ena

protein levels were no longer decreased in boundary cells (Fig. 6B), and when a dsRNA form of *cut* (*cut^{dsRNA}*) was expressed in dorsal cells (Fig. 6E,F), the reduction in Ena protein levels took place only in ventral cells expressing Cut (Fig. 6F, compare with 6D). These results indicate that Cut is required to reduce Ena levels at the DV boundary. This regulation appears to be transcriptional as *ena* mRNA levels were reduced in Cut-expressing cells (Fig. 6A') and this reduction was not observed in *cut* mutant wing discs (Fig. 6B'). Interestingly, the distinct localization of F-Actin and Sqh at the interface of boundary and non-boundary cells was disturbed in mature *cut* mutant or *cut*-depleted wing discs (compare Fig. 6H-J with Fig. 1C'). Moreover, ectopic expression of Cut was able to induce an ectopic actin cable at the interface of Cut-expressing and non-expressing cells (Fig. 6K). The early actin cable observed in early wing discs was not affected in a mutant condition for *cut* (Fig. 6G), consistent with the fact that *cut* is expressed only in boundary cells of mature wing discs (Buceta et al., 2007; Micchelli et al., 1997). These results indicate that Cut reduces the levels of Ena in boundary cells and suggest that this reduction contributes to the establishment of the two actomyosin cables observed at the interphase of boundary and non-boundary cells in late wing discs.

We next addressed whether the enrichment in F-actin and Sqh observed in late third instar wing discs at the interface of boundary and non-boundary cells reflects a process of cell sorting between these two types of cells. We noted that this interface was rather smooth (Fig. 6A), and thus analyzed the topological location of clones of wild-type cells with respect to the interface between boundary and non-boundary cells (Fig. 7C). Clones were induced at different stages of larval development and visualized in late third instar discs, and boundary cells were marked by the expression of Cut or Wingless. Most of the clones located close to the boundary and induced in second instar stages violated the interface between boundary and non-boundary cells and respected the dorsal-ventral lineage restriction boundary. A larger percentage of clones induced at later stages of development respected the interface between boundary and non-boundary cells (Fig. 7A, see quantification in 7C). The role of Cut in inducing this cell sorting was then tested. When clones were mutant for *cut*, the frequency of clones induced in second instar or at later stages and respecting the interface between boundary and non-boundary cells was largely increased (Fig. 7B, see quantification in 7C). Thus, cells mutant for *cut* tended to be excluded from the Cut-expressing domain. These results indicate that Cut activity contributes to sorting boundary from adjacent non-boundary cells and that this differential cell sorting is probably reflected by the presence of the two actomyosin cables observed at late stages of wing development (Fig. 1C'). It is worth noting that this is not a strict lineage restriction boundary as Cut expression requires the continuous activity of Notch to be induced (Micchelli et al., 1997).

Finally, we analyzed the role of Cut and this later subdivision to the stability of the DV compartment boundary. Depletion of Cut induced an irregular DV affinity boundary at late stages of wing development (Fig. 7D,E, see quantification in 7F). Even though Cut is required for the maintenance of the Notch signaling center (Buceta et al., 2007; Micchelli et al., 1997), the defects at the DV affinity boundary caused by *cut* depletion were observed before Notch signaling was compromised (Fig. 7D). These results indicate that Cut contributes to the role of Notch in maintaining the DV affinity probably by defining the later subdivision of boundary and non-boundary cells.

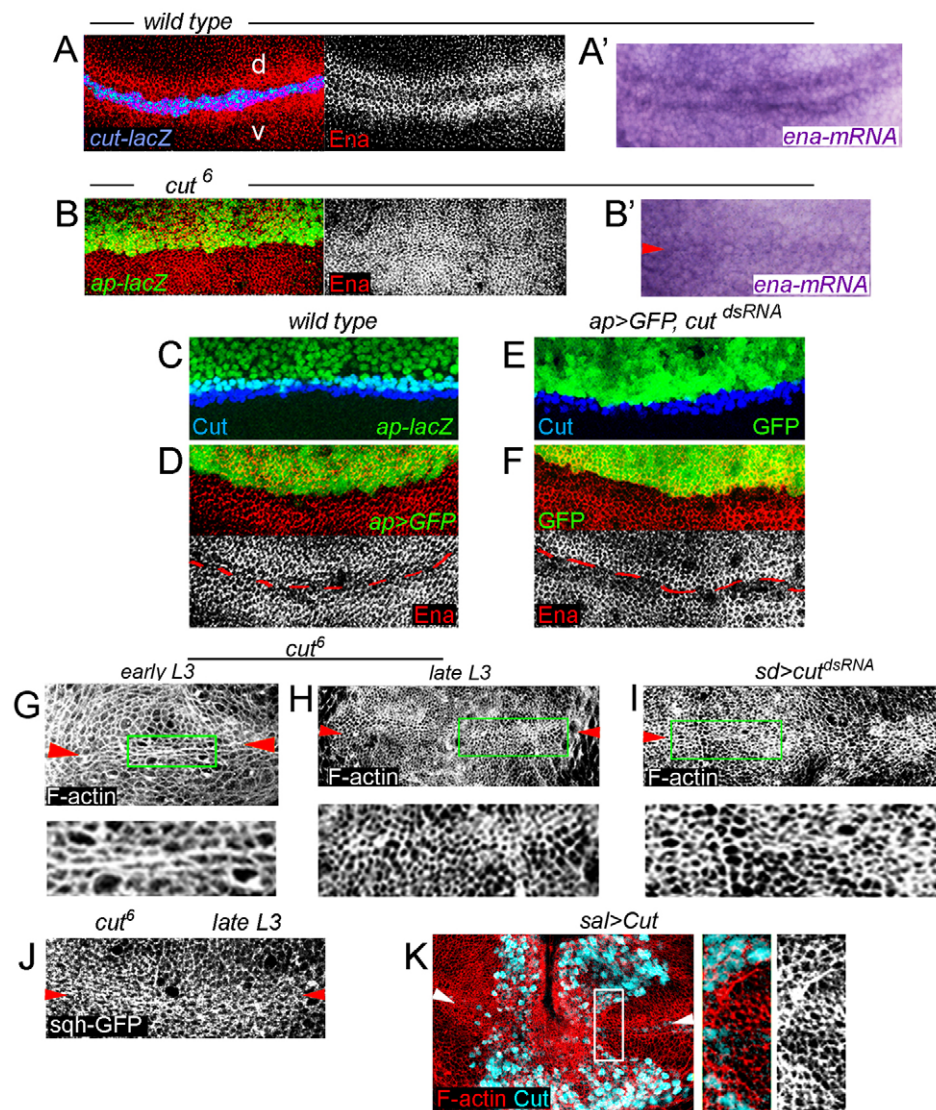


Fig. 6. A late role of Cut in regulating Enabled protein levels. (A-F) Wild-type (A,A',C), *cut⁶* (B,B'), *ap^{Gal4}; UAS-GFP* (D) and *ap^{Gal4}; UAS-GFP; UAS-cut^{dsRNA}* (E,F) late third instar *Drosophila* wing discs labeled to visualize expression of *cut-lacZ* (antibody to β -Gal, blue in A), Ena protein (red or white in A,B,D,F), *ena* mRNA (purple in A',B'), Cut protein (blue in C,E), GFP protein (green in D-F) and *ap-lacZ* (antibody to β -Gal, green in B,C). Dorsal (d) and ventral (v) compartments are shown in A and the dorsal-ventral compartment boundary is marked by a dashed red line (in D,F) or red arrowhead (in B'). (A-B') Although Ena protein and mRNA levels were reduced in Cut-expressing cells (A,A'), this reduction was not observed upon depletion of Cut in the whole wing disc (B,B'). Upon expression of a dsRNA form of Cut in the dorsal compartment (E,F), Cut protein levels were strongly reduced in those dorsal cells abutting the boundary (E) and downregulation of Ena protein was not observed in these cells (F, compare with D). (G-I) Early (G) or late (H,I) third instar *cut⁶* mutant (G,H) or *sd^{Gal4}; UAS-cut^{dsRNA}* (I) wing discs labeled to visualize F-Actin. Higher magnifications of the green boxes are shown below. (J,K) Late third instar *cut⁶* mutant (J) or *sal^{Gal4}; UAS-cut* (K) wing discs labeled to visualize Sqh-GFP (white in J), F-Actin (red or white in K) and Cut (blue in K) protein expression. Higher magnification of the white box in K is shown. Arrowheads in G-K point to the dorsal-ventral compartment boundary.

DISCUSSION

Here, we have identified two downstream effectors of Notch involved in the maintenance of the DV compartment boundary: *bantam* miRNA and the homeodomain-containing transcription factor Cut. The identification of these molecular actors provides new insights, at the molecular level, of boundary formation in animal development.

Cell divisions lead to cell rearrangements that may challenge straight and sharp compartment boundaries (Monier et al., 2010). The DV boundary of mid- and late third instar wing primordia is characterized by a reduced rate of cell proliferation which defines the zone of non-proliferating cells (ZNC). The contribution of the ZNC to the maintenance of the DV affinity boundary was proposed many years ago (O'Brochta and Bryant, 1985) but this notion was subsequently questioned (Blair, 1993). Here, we provide evidence that the ZNC does indeed play a role in boundary formation. *bantam* miRNA positively modulates the activity of the E2F transcription factor and drives G1-S transition in *Drosophila* tissues (Herranz et al., 2008). Notch-mediated downregulation of *bantam* miRNA defines the ZNC (Brennecke et al., 2003; Herranz et al., 2008) and contributes to maintain a stable DV affinity boundary (Fig. 8A). Induction of proliferation in boundary cells by the ectopic expression

of *bantam*, the cell cycle regulators Cyclin E and String, or the proto-oncogene *dMyc*, which is known to drive G1-S transition (Johnston et al., 1999), compromises the formation of a smooth DV affinity boundary. A similar reduction in proliferation rates is observed at the rhombomere boundaries in the developing hindbrain (Guthrie et al., 1991), suggesting that reduced rate of cell proliferation might often be used in compartment boundary formation.

Notch-mediated downregulation of *bantam* activity is not only required to define the ZNC but also to establish the actomyosin cables observed at the interface between boundary and non-boundary cells. We have identified Ena, a regulator of Actin elongation, as a direct target of *bantam* that is involved in DV boundary formation. The multiple roles of *bantam* in promoting G1-S transition and tissue growth, blocking apoptosis (Brennecke et al., 2003; Herranz et al., 2008) and regulating Actin dynamics unveil a new molecular connection between these three processes that might have relevance in growth control and tumorigenesis.

Intriguingly, *bantam* miRNA has no major role in the maintenance of the anterior-posterior compartment boundary of the developing wing and this boundary is not affected upon depletion of Ena protein levels. Thus, different regulators of actin elongation might be at work to regulate the actomyosin cytoskeleton and direct cell sorting in

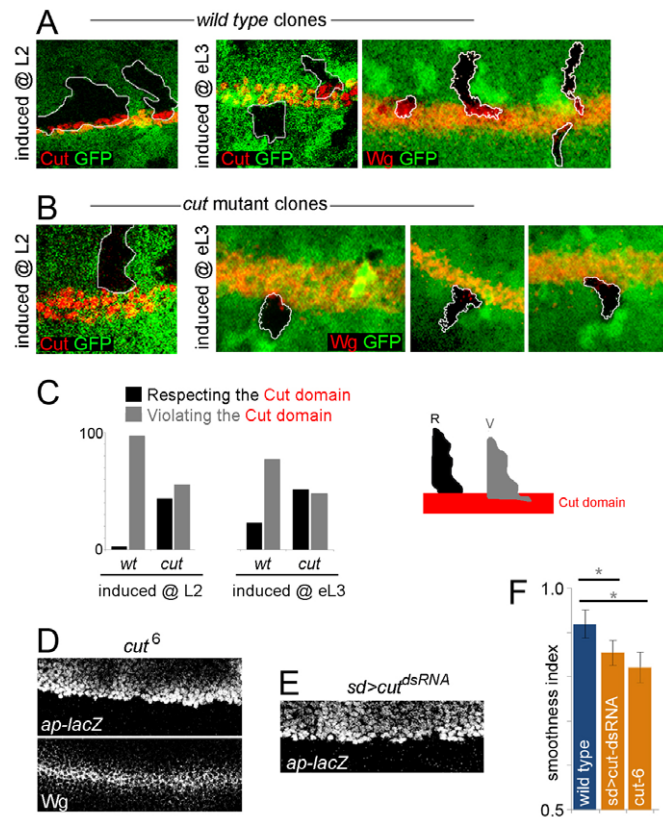


Fig. 7. A late role of Cut in sorting boundary from non-boundary cells. (A,B) Clones of wild-type (A) or *cut*¹⁴⁵ mutant (B) cells induced in early second (L2) or early third (eL3) larval stages, dissected in late third instar, marked by the absence of GFP (green) and labeled to visualize expression of Cut or Wingless protein (red). (C) Histogram plotting the percentage of wild-type and *cut*¹⁴⁵ mutant clones induced at different stages and respecting (black bars) or violating (gray bars) the Cut-expressing domain, and diagram depicting the behavior of clones respecting (R) or violating (V) the Cut domain (red). Only those clones at the dorsal-ventral compartment boundary were taken into consideration. Percentage of clones respecting/violating the Cut-expressing domain: wild type (L2)=2.7/97.3 (*n*=37); wild type (eL3)=22.95/77.05 (*n*=61); *cut*¹⁴⁵ (L2)=43.61/56.4 (*n*=39); *cut*¹⁴⁵ (mL3)=51.1/48.9 (*n*=45). (D,E) Late third instar *cut*⁶ mutant (D) and *sd*^{Gal4}; *UAS-cut*^{dsRNA} (E) *Drosophila* wing discs labeled to visualize expression of *ap-lacZ* (D,E; antibody to β -Gal, white) and Wingless protein (D; white). (F) Histogram plotting the smoothness index (see Materials and methods for details) of the dorsal-ventral compartment boundary in late third instar wing discs of different genotypes. Error bars indicate s.d. This index was significantly reduced upon expression of a *cut*^{dsRNA} transgene or in *cut*⁶ mutant wing discs when compared with wild-type control wing discs [*sd*>*cut*^{dsRNA}: *P*<10⁻⁴ and *cut*⁶: *P*<10⁻⁸]. Average smoothness index values: wild type=0.91±0.03 (*n*=15); *sd*>*cut*^{dsRNA}=0.85±0.02 (*n*=8); *cut*⁶=0.82±0.02 (*n*=13).

diverse developmental contexts. Whether reduced levels of bantam miRNA and increased levels of Ena protein are required to maintain differential cell sorting in the embryonic ectoderm or other imaginal tissues remains to be elucidated.

Cut is a late target of Notch that is expressed in boundary cells and is required to induce a stable Notch signaling center (Buceta et al., 2007; Micchelli et al., 1997). Here, we demonstrate that Cut activity has also a specific function in reducing Ena mRNA

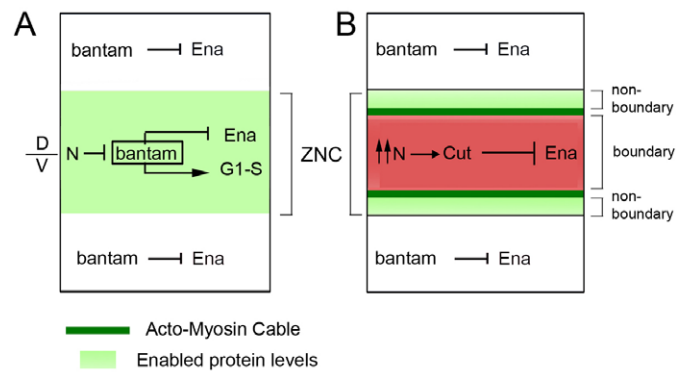


Fig. 8 The roles of bantam miRNA and Cut in maintaining the dorsal-ventral (DV) affinity boundary. (A) In mid-third instar (mid-L3) *Drosophila* wing discs, low levels of Notch (N) activity (green) reduces the activity of bantam, thus alleviating bantam-mediated targeting of the *ena* 3'UTR and reducing cell proliferation rates at the DV boundary. Note that no distinct accumulation of Actin or Myosin II is observed at the DV interface of mid-third instar wing discs and that increased levels of Ena protein might be required only in subsequent stages (see panel B). It is likely that reduced cell proliferation in boundary cells plays a major role in the maintenance of the DV affinity boundary during these stages. (B) In late third instar (late L3) wing discs, two distinct cell populations, boundary cells (red) and non-boundary cells (light green) can be observed. High levels of Notch activity induce the expression of Cut in boundary cells. Repression of Ena protein level by the activity of Cut is required to establish the actomyosin cables at the interface between boundary and non-boundary cells (dark green). Differential cell sorting can also be observed at this interface and is due to the activity of Cut. Repression of bantam activity is still required during these stages to establish the zone of non-proliferating cells (ZNC) and alleviate Ena repression, two features that contribute to the maintenance of the DV affinity boundary.

and protein levels in boundary cells (Fig. 8B). Although depletion of Cut compromises the formation of the actomyosin cables at the interface of boundary and non-boundary cells and the maintenance of a stable DV affinity boundary, cell lineage and clonal analysis of wild-type and *cut* mutant cells reveal that Cut plays a major role in sorting boundary from non-boundary cells. Our finding that the Notch signaling pathway defines, through Cut, a distinct population of boundary cells at the DV interface reinforces the mechanistic similarities in the maintenance of compartment boundaries within the vertebrate hindbrain and the *Drosophila* wing (Cheng et al., 2004). In both developmental contexts, Notch defines a distinct population of boundary cells and contributes to segregating boundary from non-boundary cells. Although Cut mediates the role of Notch in the *Drosophila* wing, the molecular effectors mediating the role of vertebrate Notch in boundary formation remain uncharacterized. Our data indicate that the later subdivision into boundary and non-boundary cells contributes to the maintenance of a stable DV affinity barrier in the mature wing primordium.

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Competing interests statement

The authors declare no competing financial interests.

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