Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in *Drosophila*

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Summary

Ligands of the Delta/Serrate/Lag2 (DSL) family must normally be endocytosed in signal-sending cells to activate Notch in signal-receiving cells. DSL internalization and signaling are promoted in zebrafish and *Drosophila*, respectively, by the ubiquitin ligases Mind bomb (Mib) and Neuralized (Neur). DSL signaling activity also depends on Epsin, a conserved endocytic adaptor thought to target mono-ubiquitinated membrane proteins for internalization. Here, we present evidence that the *Drosophila* ortholog of Mib (Dmib) is required for ubiquitination and signaling activity of DSL ligands in cells that normally do not express Neur, and can be functionally replaced by ectopically expressed Neur. Furthermore, we show that both Dmib and Epsin are required in these cells for some of the endocytic events that internalize DSL ligands, and that the two *Drosophila* DSL ligands Delta and Serrate differ in their utilization of these Dmib- and Epsin-dependent pathways: most Serrate is endocytosed via the actions of Dmib and Epsin, whereas most Delta enters by other pathways. Nevertheless, only those Serrate and Delta proteins that are internalized via the action of Dmib and Epsin can signal. These results support and extend our previous proposal that mono-ubiquitination of DSL ligands allows them to gain access to a select, Epsin-dependent, endocytic pathway that they must normally enter to activate Notch.

Key words: *Drosophila*, DSL-Notch signaling, Delta, Serrate, Mindbomb, Epsin/Liquid facets, Neuralized, Endocytosis, Ubiquitination

Introduction

Delta/Serrate/Lag2 (DSL) ligands comprise a family of single pass transmembrane proteins that function as short-range signals to activate Notch family receptors on the surface of neighboring cells (reviewed by Greenwald, 1998; Artavanis-Tsakonas et al., 1999; Schweisguth, 2004). DSL ligands activate Notch by inducing two proteolytic cleavages in the receptor that release the cytosolic domain, a transcriptional activator, for entry into the nucleus (Mumm and Kopan, 2000; Struhl and Adachi, 2000). The first cleavage occurs between the ectodomain and the rest of the receptor. Shedding of the ectodomain then triggers the second cleavage, which occurs within the transmembrane domain, allowing the cytosolic domain to enter the nucleus.

Here, we focus on a critical but poorly understood aspect of the mechanism by which DSL ligands induce proteolytic processing of Notch, namely that DSL proteins must normally be endocytosed in signal-sending cells to activate Notch in signal-receiving cells (Parks et al., 2000; Struhl and Adachi, 2000; Le Borgne and Schweisguth, 2003a; Wang and Struhl, 2004). Two general classes of hypotheses have been put forward to explain why DSL ligands must be endocytosed to activate Notch (Le Borgne and Schweisguth, 2003a; Wang and Struhl, 2004). In the first class, entry of DSL ligands into coated pits or other specializations in signal-sending cells is proposed to create conditions essential for cleaving or shedding the ectodomain of Notch before internalization of the ligand. Such conditions could be mechanical stress of the intercellular bridge between the ligand and the receptor (Parks et al., 2000; Struhl and Adachi, 2000; Wang and Struhl, 2004), clustering of DSL ligands in coated pits (Le Borgne and Schweisguth, 2003a), or the recruitment of essential accessory factors to the same micro-environment (Wang and Struhl, 2004). In the second class, DSL signaling activity is thought to depend on events that take place after internalization of the ligand, such as enzymatic processing of the ligand from an inert to an active form (Wang and Struhl, 2004) or packaging into exosomes (Le Borgne and Schweisguth, 2003a): accordingly, signaling activity would require recycling of the converted or repackaged ligand to the cell surface.

The endocytic adaptor protein Epsin provides a potential key to understanding the role of endocytosis in DSL signaling. Epsins are conserved multidomain proteins that appear likely to interact with core components of the endocytic machinery (Clathrin, AP2, PIP2) as well as with mono-ubiquitin, and are required in some contexts for endocytosis of mono-ubiquitinated cargo proteins (Chen et al., 1998; Wendland, 2002). We have recently shown that *Drosophila* Epsin, encoded by the gene *liquid facets* (*lqf*) (Cadavid et al., 2000), has a remarkably specific role in normal cell signaling and physiology: it appears to be required solely for cells to send, but not to receive, DSL signals (Wang and Struhl, 2004) (see also Overstreet et al., 2004; Tian et al., 2004). Furthermore, we

obtained evidence that Epsin-dependent signaling depends on ubiquitination of DSL ligands (Wang and Struhl, 2004). These and related findings led us to propose: (1) that DSL ligands must normally be ubiquitinated to be targeted for Epsin-mediated endocytosis; and (2) that the ubiquitinated ligands must normally be internalized via the action of Epsin to activate Notch (Wang and Struhl, 2004).

Surprisingly, we were not able to detect any effect of the removal of Epsin on normal, bulk endocytosis of the DSL ligand Delta (Dl) (Wang and Struhl, 2004). Instead, our results suggested that Epsin mediates only a small subset of the endocytic events that internalize ubiquitinated forms of Dl. Thus, we further proposed that it is not endocytosis, per se, that is normally essential for DSL signaling, but rather entry of ubiquitinated DSL ligands into a select Epsin-dependent endocytic pathway (Wang and Struhl, 2004).

In experiments described in this paper, we present in vivo experiments that test two predictions of this hypothesis: (1) that DSL ligands must be ubiquitinated to signal; and (2) that both ubiquitination and Epsin are required for the normal internalization of DSL ligands.

To test the first prediction, we have sought to block ubiquitination of DSL ligands by removing the relevant ubiquitin ligase(s). Previous work had demonstrated that two RING-domain-containing E3 ubiquitin ligases, Neuralized (Neur) in *Drosophila* and Mind bomb (Mib) in zebrafish promote DSL endocytosis and signaling (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001; Itoh et al., 2003), implicating them in the normal pathway of Notch activation by DSL ligands and suggesting that they perform homologous functions in their respective organisms (Le Borgne and Schweisguth, 2003a). However, Neur is normally expressed and required in only a subset of DSL signaling cells in *Drosophila* (Boulianne et al., 1991; Yeh et al., 2000; Lai and Rubin, 2001), indicating either that ubiquitination of DSL ligands is not essential for activating Notch in all signaling contexts, or that other ubiquitin ligase(s) may be responsible for ubiquitinating DSL ligands in cells that do not express Neur.

The *Drosophila* genome contains an ortholog of the zebrafish *mib* gene, *dmib* (Itoh et al., 2003), raising the possibility that *dmib* encodes one such alternative ligase. Here, using newly obtained loss-of-function mutations of *dmib*, we demonstrate an absolute requirement for Dmib in sending, but not receiving, DSL signals in cells that normally do not require or express Neur. Furthermore, we find that we can bypass the requirement for Dmib either by ectopically expressing Neur in these cells, or by expressing a chimeric DSL ligand in which the cytosolic domain is replaced by a heterologous substrate for ubiquitination. Together, these findings support the proposal that DSL ligands must be ubiquitinated to signal.

To test the second prediction, that signaling activity of DSL ligands depends on their being endocytosed in a ubiquitin- and Epsin-dependent fashion, we asked whether removing either Dmib or Epsin impairs the normal bulk endocytosis of Dl and Ser in cells that depend on Dmib function. We show that bulk endocytosis of Ser is severely impaired in Dmib-deficient cells and partially impaired in Epsin-deficient cells, whereas that of Dl is not detectably affected in either case. We infer that DSL ligands are normally internalized via multiple, distinct endocytic pathways, only some of which depend on mono-ubiquitination and only a further subset of these which depend on Epsin. Most Ser appears to be internalized via Epsin-dependent pathways, whereas most Dl is not. Nevertheless, only those DSL ligands that are internalized via the action of Epsin activate Notch.

Materials and methods

dmib mutant alleles

$dmib^{L53}$ and $dmib^{L70}$ are EMS-induced stop codons at Q1163 and Q954, respectively. $dmib^{EY09780}$ is an EP transgene inserted 96 nucleotides upstream of the putative *dmib* translation start site; the promoter of the EP transgene points away from the *dmib* coding sequence and does not confer *dmib* gene function under the control of Gal4 drivers [Berkeley *Drosophila* Genome Project (BDGP), Flybase; Fig. 2]. All three alleles fail to complement each other as well as deficiencies that remove the *dmib* locus [e.g., *Df(3L)brm11*; Flybase]. Hemizygous larvae, as well as $dmib^{EY09780}$ homozygotes, have abnormally small wing and eye imaginal discs and die as pupae; the same is true for $dmib^{L53}/dmib^{L70}$ transheterozygotes. $dmib^{L53}$ and $dmib^{L70}$ homozygotes die as first instar larvae, which we attribute to second site mutations on the same chromosome.

Genetic materials

The following transgenes and mutations were used: *UAS-Dl, UAS-*Dl^{R+}, lqf^{1227}(Wang and Struhl, 2004); *Tubα1-Gal4, UAS-GFPnls* (Struhl and Greenwald, 1999; Struhl and Greenwald, 2001); *nub-Gal4*, *UAS-*y^+ (Calleja et al., 1996); *UAS-CD8-GFP*, *Tubα1-G80* (Lee and Luo, 1999); *UAS-Ser* (Panin et al., 1997); *UAS-neur* (Lai and Rubin, 2001); *ptcG4*, *UAS-lacZ*, *arm-lacZ* (Bloomington stock center).

dmib RNAi and overexpression constructs

To generate the *UAS-*$dmib^{RNAi}$ transgene, a *dmib* cDNA fragment corresponding to nucleotides 3626-4027 of the *dmib* cDNA (clone SD05267;BDGP) was generated by PCR amplification and inserted in opposite orientations into a pUAST-RNAi intron vector (Lee and Carthew, 2003). *UAS-*$dmib^{RNAi}$*/ptc-Gal4* and *UAS-*$dmib^{RNAi}$*/nub-Gal4* transheterozygous larvae were maintained at 30°C. The *UAS-dmib* transgene was generated by inserting the full-length *dmib* coding sequence from SD05267 into the pUAST vector (Brand and Perrimon, 1993).

Genotypes employed

Clones of *dmib*$^-$, *lqf*$^-$, or wild-type cells marked by GFP and/or *y*$^+$ and expressing *UAS-X* transgenes (MARCM technique)

y hsp70-flp Tubα1-Gal4 UAS-GFPnls/y hsp70-flp; UAS-y/+; [*dmib*$^-$, *lqf*$^-$ or wild type] *FRT2A/Tubα1-Gal80 FRT2A, UAS-X.* *dmib*$^-$=$dmib^{L53}$, $dmib^{L70}$ or $dmib^{EY09780}$; *UAS-X* was provided either on II in trans to *UAS-y*, or on III, in trans to *Tubα1-Gal80 FRT2A*.

Clones of *dmib*$^-$ or *lqf*$^-$ cells in discs expressing *UAS-Dl* or *UAS-Ser* in all prospective wing cells under *nub-G4* control

y hsp70-flp; *nub-Gal4/UAS-Dl* (or *UAS-Ser*); *dmib*$^-$ (or *lqf*$^-$) *FRT2A/arm-lacZ FRT2A*. Clones were generated by heat shocking first or second instar larvae at 37°C for 60 minutes.

Immunofluorescent staining

Imaginal discs were fixed and stained as described previously (Wang and Struhl, 2004), using mouse α-Dl (Developmental Studies Hybridoma Bank, DSHB), Ginuea pig α-Dl (Parks et al., 2000), Ginuea pig α-Hrs (Lloyd et al., 2002); mouse α-Wg (DSHB), mouse α-Cut (DSHB), rat α-Ser (Panin et al., 1997), and rabbit α-βGal (Cappel). To monitor cell surface accumulation of Ser, living discs were incubated for 20-30 minutes at room temperature with rat α-Ser antisera in *Drosophila* tissue culture media, and then rinsed and fixed

in the absence of detergent, prior to executing the standard staining protocol. We could not distinguish any difference in Ser staining associated with the apical cell surface in such living discs, compared with that in discs fixed in detergent prior to incubation with rat α-Ser, as in our standard protocol.

Results

Loss of function mutations of *Drosophila mib* (*dmib*)

The *Drosophila* gene *CG5841* encodes the *Drosophila* ortholog of the zebrafish *mind bomb* (*mib*) gene (Itoh et al., 2003; Chen and Casey Corliss, 2004); we tentatively call this gene *dmib* (*Drosophila mind bomb*) to avoid confusion with the pre-existing *Drosophila* gene *miniature bristles* (abbreviated *mib*; Flybase). We have identified three loss-of-function alleles of *dmib*. Two, *L53* and *L70*, were isolated in previous screens for mutations that cause wing pattern phenotypes in mutant clones (Jiang and Struhl, 1995); both are associated with stop codons in the *dmib* coding sequence (T. Correia and K. Irvine, personal communication; data not shown; Fig. 1A). The third is an EP transgene insertion EY09780 positioned just upstream of the presumed start site of *dmib* (BDGP, Materials and methods; Fig. 1A). All three mutations behave as amorphs (Materials and methods) and cause indistinguishable loss-of-function phenotypes in clones of homozygous mutant cells (Fig. 1B,D; Materials and methods); we refer to them, below, as *dmib*$^{-}$ mutations. Furthermore, these phenotypes are rescued by expression of a *UAS-dmib* transgene (data not shown) and can be phenocopied by expression of a *UAS-dmib*^{RNAi} transgene (Fig. 1E, Fig. 2H).

Requirement for *dmib* during DSL-Notch signaling

During development of the wing imaginal disc, DSL-Notch signaling is required for inductive interactions across the dorsoventral (D-V) compartment boundary to specify a thin stripe of prospective wing margin cells; these 'border' cells express the transcription factor Cut and the morphogen Wingless (Wg) and organize growth and patterning of the wing blade (reviewed by Blair, 2000). DSL-Notch signaling is also essential for prospective wing vein cells to inhibit surrounding cells from choosing the vein fate (reviewed by De Celis, 2003), and for specification of sensory organ precursor cells (SOPs) as well as their clonal descendents that form the mechanosensory bristles and chemosensory sensilla of the adult wing and mesonotum (reviewed by Simpson, 1997).

Clones of *dmib*$^{-}$ cells are associated with large wing notches and thickened veins (Fig. 1D), both hallmark phenotypes associated with loss of DSL-Notch signaling. However, the formation of bristles on the mesonotum is only weakly affected. Both macro and microchaetes develop normally,

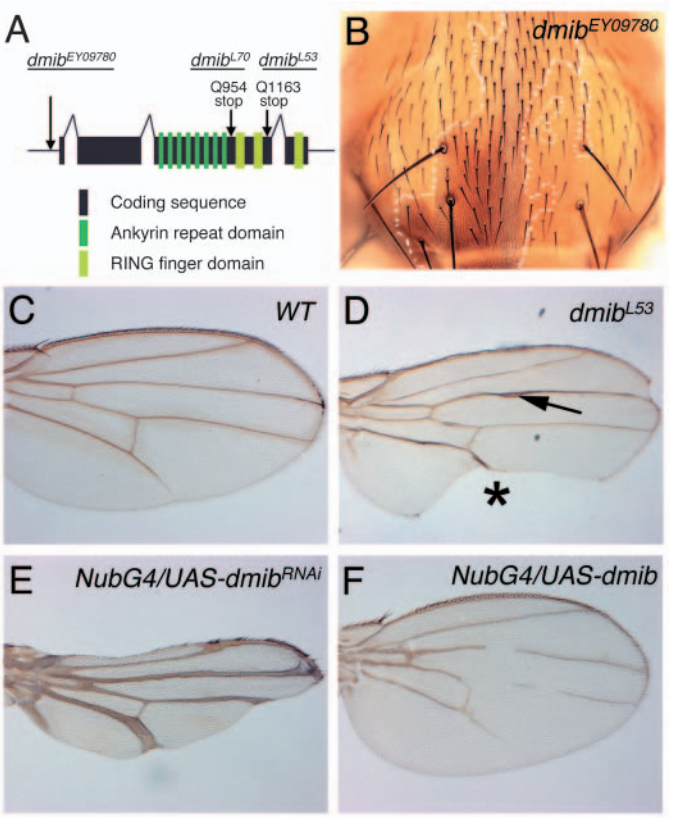

Fig. 1. *dmib* is required for normal DSL-Notch signaling during wing development. (A) Predicted transcript and protein product of the *Drosophila mind bomb* (*dmib*) locus. The *dmib* coding sequence (thick black line) is present on four exons interrupted by three introns (thin lines). The Dmib protein contains conserved Ankyrin repeats (dark green) and RING finger domains (light green). *dmib*^{EY09780} is an EP transgene that is inserted 96 bp upstream of the inferred translation start site; *dmib*^{L70} and *dmib*^{L53} are EMS-induced stop codons at Q954 and Q1163 deleting all three, and/or just the third, RING finger domain(s), respectively. All three *dmib* alleles behave as amorphs (Materials and methods), and all three gave indistiguishable results in each of the various experiments shown in this and subsequent Figures. (B) Adult mesonotum with several clones of *dmib*^{EY09780} cells. The mutant cells are marked by expression of a *UAS-y*$^{+}$ transgene, which darkens cuticle, especially bristles; the mutant territories are outlined in white. *dmib* mutant cells form normal patterns of macro- and micro-chaetes, except that the density of microchaetes is somewhat higher than normal and some macrochaetes (particularly scutellar bristles) are occasionally duplicated (not shown); however, each bristle organ itself appears morphologically normal. (C) Wild-type wing. (D) Wing with clones of *dmib*^{L53} cells (marked by *UAS-y*$^{+}$ expression) associated with wing notching (asterisk) and vein thickening (arrow), both phenotypes indicating a loss of DSL-Notch signaling activity. We note here that entirely mutant, *dmib*$^{-}$ animals can survive to the pupal stage (Materials and methods), and that some of these differentiate as pharate adults; the same is also true of *dmib*$^{-}$ animals obtained from *dmib*$^{-}$ female germ cells. As expected from the phenotype of mutant clones, these show severely truncated wings, consisting only of small stumps of wing hinge tissue, as well as an abnormally high density of microchaetes and occasional duplication of macrochaetes on the mesonotum. In addition, such mutant animals develop only rudimentary eyes containing around ~25-50 ommatidia and form legs with truncated and fused leg segments. All of these phenotypes indicate deficiencies in well-characterized DSL-Notch signaling events during wing, eye and leg development. (E) Wing derived from a *nub-Gal4/UAS-dmib*^{RNAi} wing imaginal disc, in which the *UAS-dmib*^{RNAi} transgene was expressed uniformly throughout the prospective wing, causing severe wing notching and vein thickening. (F) Wing derived from a *nub-Gal4/UAS-dmib* wing imaginal disc. Over-expression of *dmib* throughout the prospective wing partially suppresses vein formation, the reciprocal phenotype to that caused by expression of *dmib*^{RNAi}, indicative of an abnormal gain in DSL-Notch signaling.

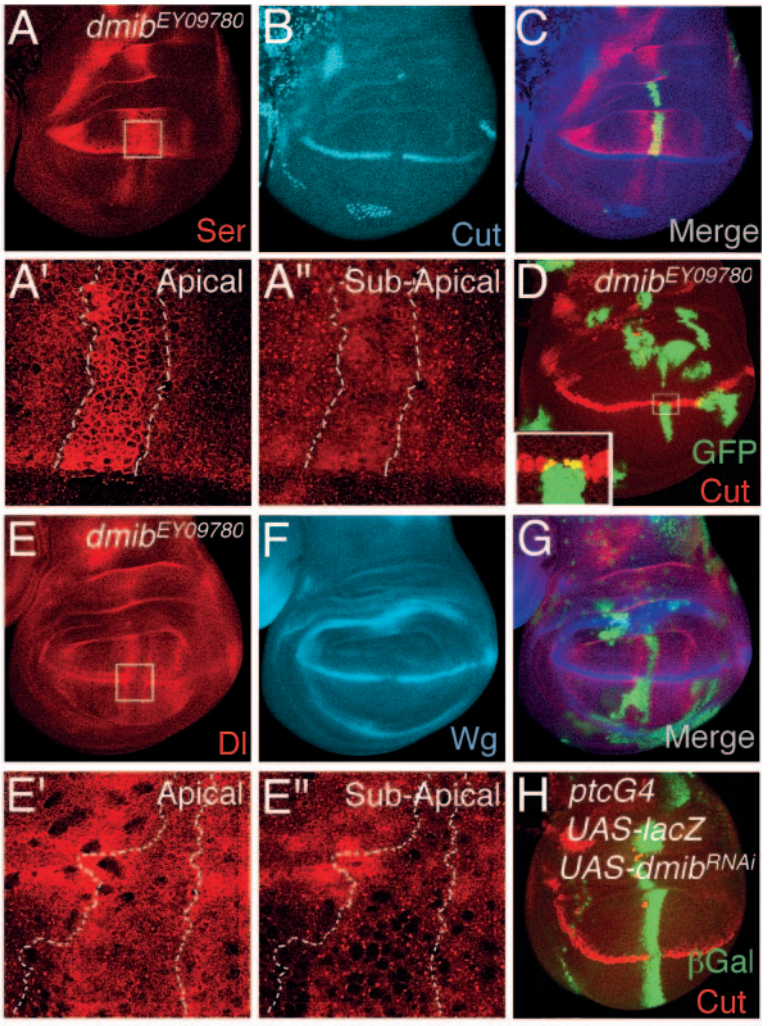

Fig. 2. Requirements for *dmib* activity in DSL endocytosis and signaling across the dorsoventral compartment boundary. (A-C) Wing disc containing a clone of *dmib*^{EY09780} cells [here, and in all subsequent figures, clones are marked by nuclear GFP expression (green) unless otherwise stated, the discs are oriented dorsal side up and anterior to the left]. (A,C) Ser (red) is normally expressed at moderate levels throughout the dorsal compartment (upper half) and is upregulated in cells neighboring the dorsoventral (D-V) compartment boundary (horizontal stripe) and in pro-vein cells (vertical stripes). (B,C) Cut (blue) is expressed in dorsal and ventral cells flanking the D-V compartment boundary in response to Ser signaling from dorsal cells and Dl signaling from ventral cells. DSL-Notch signaling across the D-V compartment boundary also upregulates Dl and Ser expression on each side, creating a positive feedback loop necessary for for peak activation of Notch and Cut expression on both sides. The *dmib*^{EY09780} clone abuts the D-V boundary and blocks Cut expression on both sides (B), indicating a failure in DSL-Notch signaling. (A′,A″) Apical and sub-apical planes of section of the region boxed in white in A are shown at higher magnification; Ser accumulates at abnormally high levels in association with the apical cell surface in *dmib*^{EY09780} cells and at the expense of Ser-positive cytosolic puncta within the cells (the clone border is outlined in white). (D) Wing disc containing multiple clones of *dmib*^{EY09780} cells (green). As in A-C, *dmib*^{EY09780} clones that abut the D-V boundary are associated with a loss in Cut expression (red); however, as shown at higher magnification in the boxed inset, *dmib*^{EY09780} cells next to wild-type cells along the clone border express Cut (appears as yellow) indicating that they received DSL signals sent from neighboring wild-type cells. (E-G) Wing disc containing clones of *dmib*^{EY09780} cells (green). (E,G) Dl (red) is normally expressed at moderate levels in all cells in both compartments, and is upregulated along the D-V boundary and in pro-veins. (F,G) Wingless (Wg) expression (blue), like Cut, is induced in response to DSL-Notch signaling across the D-V boundary; a *dmib*^{EY09780} clone that crosses the D-V boundary blocks Wg expression on both sides. (E′,E″) Apical and sub-apical planes of section of the region boxed in white in E are shown at higher magnification; Dl accumulation on the apical cell surface and in cytosolic puncta appears unchanged in *dmib*^{EY09780} cells relative to neighboring wild-type cells (the clone border is outlined in white, as in A′,A″). (H) Wing disc in which *UAS-dmib*^{RNAi} and *UAS-lacZ* (green) are co-expressed along the antero-posterior compartment border under the control of *ptcG4*; Cut expression (red) is abolished in *dmib*^{RNAi} *lacZ* expressing cells flanking the D-V boundary.

except that the density of microchaetes is somewhat higher than normal and some macrochaetes are occasionally duplicated (Fig. 1B; data not shown). Nevertheless, each individual bristle organ appears to be morphologically normal. Thus, although we can detect evidence that the process of SOP segregation is compromised, albeit modestly, the subsequent, Neur-dependent segregations of cell types comprising each bristle sense organ appear unaffected.

D-V border cells are normally induced to express Cut and Wg by either Delta (Dl) or Serrate (Ser), depending on their compartmental provenance: dorsal cells respond to Dl sent from ventral cells, whereas ventral cells respond to Ser sent by dorsal cells (Fleming et al., 1997; Panin et al., 1997). Activation of Notch in both dorsal and ventral cells upregulates signal production, creating a positive auto-feedback loop necessary to maintain peak levels of Dl and Ser signaling across the compartment boundary. *dmib*$^{-}$ clones that abut or cross the D-V boundary cause a loss in Cut and Wg expression on both sides, the expected result if mutant cells cannot send, or cannot receive, DSL signals (Fig. 2B,C,F,G). A similar phenotype results from expression of a *UAS-dmib*^{RNAi} transgene in a thin stripe of cells along the anterior-posterior (A-P) compartment boundary (Fig. 2H). However, *dmib*$^{-}$ cells resemble *Dl*$^{-}$ *Ser*$^{-}$ cells (which cannot send signals) but differ from *N*$^{-}$ clones (which cannot receive signals) in that they can be induced to express both Cut and Wg if they are positioned near or next to wild-type border cells (Fig. 2D).

Dmib is required in signal-sending cells to activate Notch in signal-receiving cells

To determine unequivocally whether Dmib is required in signal-sending cells to activate Notch in signal-receiving cells, we used the MARCM technique (Lee and Luo, 1999) to generate clones of *dmib*$^{-}$ cells that ectopically express high levels of either Dl or Ser under *Gal4* control. Normally, clones that ectopically express Dl but are otherwise wild type, induce Cut expression in adjacent, non-expressing cells only when

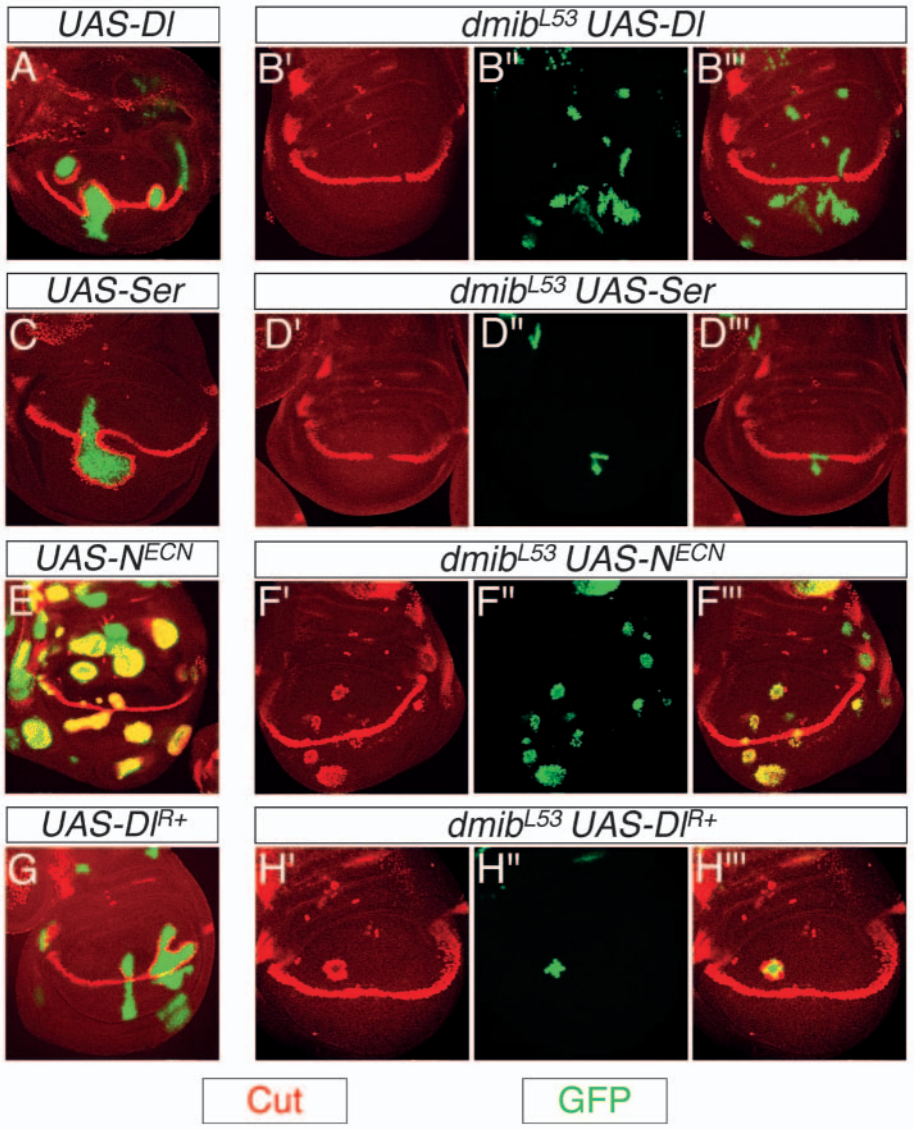

Fig. 3. Dmib is required for sending, but not receiving, DSL signals that induce Cut expression in the wing disc. (A) Clones of cells ectopically expressing Dl (green) induce Cut expression (red) in adjacent wild-type cells in the dorsal compartment (upper half). The abnormally high levels of Dl expression generated under these conditions autonomously inhibits Notch transduction and Cut expression by cells within the clones. (B′-B‴) Clones of *dmib*^{L53} cells that ectopically express Dl fail to induce ectopic Cut expression and interrupt normal Cut expression when they abut the D-V boundary. Ectopic Dl expressing *dmib*$^{-}$ cells, unlike simple *dmib*$^{-}$ cells, do not express Cut even when adjacent to wild-type, Cut-expressing border cells along the D-V boundary, presumably because Dl over-expression in these cells autonomously inhibits Notch transduction, as it does in otherwise wild-type cells. (C) Clones of cells ectopically expressing Ser induce Cut expression in wild-type cells in the ventral compartment (lower half); Cut expression within the clone is inhibited, as in A. (D′-D‴) Clones of *dmib*^{L53} cells that ectopically express Ser fail to induce ectopic Cut expression and interrupt normal Cut expression when they abut the D-V boundary. Cut expression is autonomously inhibited within the clones, as in B. (E) Clones of cells expressing N^{ECN} activate Cut expression autonomously (appears yellow). (F′-F‴) Clones of *dmib*^{L53} cells that over-express N^{ECN} activate Cut expression autonomously, as in E. (G) Clones of cells expressing Dl^{R+} activate ectopic Cut expression in adjacent wild-type cells in the dorsal compartment; Cut expression within such clones is inhibited, as in A,C. (H′-H‴) Clones of *dmib*^{L53} cells that express Dl^{R+} induce ectopic Cut expression ectopically in adjacent wild-type cells within the dorsal compartment, as in G, indicating that they bypass the requirement for Dmib.

located in the dorsal compartment, whereas clones of ectopic Ser-expressing cells induce Cut expression, but only when located in the ventral compartment (Fig. 3A,C) (Fleming et al., 1997; Panin et al., 1997). By contrast, clones of *dmib*$^{-}$ cells that ectopically express either Dl or Ser fail to induce Cut expression, no matter where they are located. Furthermore, Dl and Ser expressing *dmib*$^{-}$ clones that abut or cross the D-V boundary block normal Cut expression on both sides (Fig. 3B′-B‴, D′-D‴).

We also used the MARCM technique to assay further the capacity of *dmib*$^{-}$ cells to transduce DSL signals. In particular, we generated clones of *dmib*$^{-}$ cells that express Notch^{ECN}, a truncated, constitutively active form of Notch that mimicks the state of the receptor following ligand-induced extracellular cleavage and ectodomain shedding (Rebay et al., 1993; Struhl and Adachi, 1998; Struhl and Adachi, 2000). We find that clones of such Notch^{ECN} expressing *dmib*$^{-}$ cells constitutively express Cut (Fig. 3F′-F″), corroborating our observation that *dmib*$^{-}$ cells can transduce DSL signals if located next to wild-type cells (Fig. 2D). Thus, Dmib does not appear to be required downstream of the initial DSL-Notch signaling interactions that result in cleavage and shedding of the Notch ectodomain, notably those that execute transmembrane cleavage, nuclear entry, or transcriptional regulation.

We conclude that in the context of inductive Notch signaling across the D-V compartment boundary, Dmib is selectively required for sending, but not receiving, DSL signals.

A chimeric form of Delta with heterologous sites for ubiquitination bypasses the requirement for Dmib

The ability of cells to send DSL signals across the D-V compartment boundary also depends on Epsin [encoded by the *liquid facets* (*lqf*) gene (Cadavid et al., 2000)]: like clones of *dmib*$^{-}$ cells, clones of *lqf*$^{-}$ cells in the wing primordium can receive, but not send, DSL signals that specify the border cell fate (Wang and Struhl, 2004). Epsin is thought to target mono-ubiquitinated cargo proteins for endocytosis (Hofmann and Falquet, 2001; Wendland, 2002); hence, *dmib*$^{-}$ wing cells may not be able to send DSL signals because they normally depend

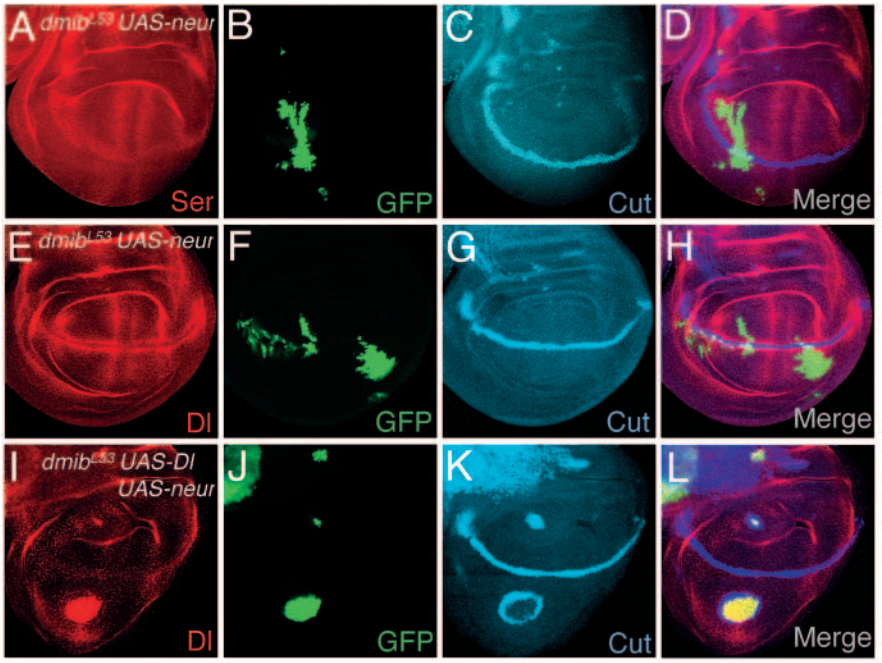

Fig. 4. Ectopic Neur can substitute for Dmib during wing disc development. (A-D) A clone of *dmib*^{L53} cells that ectopically expresses Neur (green) does not abolish Cut expression (blue) where it crosses the D-V compartment boundary. Ser expression (red) also appears normal in these cells. (E-H) Clones of *dmib*^{L53} cells that ectopically express Neur (green) do not block Cut expression (blue) when they abut or cross the D-V boundary. Normal Dl expression (red) is observed within the clones. (I-L) Clones of *dmib*^{L53} cells (green) that ectopically co-express Neur and Dl (red) induce ectopic Cut expression (blue) in adjacent wild-type cells in both dorsal and ventral compartments.

on Dmib to ubiquitinate Dl and Ser and thereby target them for Epsin-mediated endocytosis. To test this, we assayed whether Dmib is required for signaling by a chimeric form of Dl, Dl^{R+}, which appears to contain one or more heterologous sites for ubiquitination.

The Dl^{R+} protein contains a random 50 amino acid peptide 'R^{+}' in place of the normal cytosolic domain of Dl, but behaves indistinguishably from native Dl in its ability to activate Notch and its dependence on Epsin for signaling activity (Wang and Struhl, 2004). The R^{+} peptide includes two Lysine residues and replacement of both residues by Arginine blocks endocytosis and signaling. Similarly, replacement of the Dl cytosolic domain with Ubiquitin itself, but not with a mutant form of Ubiquitin that lacks the capacity to serve as an internalization signal, also yields a chimeric Dl ligand that can be internalized and signal (Itoh et al., 2003; Wang and Struhl, 2004). These data suggest that the R^{+} peptide mediates endocytosis and signaling by the Dl^{R+} protein by serving as a substrate for ubiquitination.

We generated clones of *dmib*$^{-}$ cells that express Dl^{R+}. Such clones induce Cut in surrounding cells (Fig. 3H′), just like clones of wild-type cells that express Dl^{R+} (Fig. 3G), but in contrast to clones of *lqf*$^{-}$ cells that express Dl^{R+} (Wang and Struhl, 2004). Hence, we infer that expression of the chimeric Dl^{R+} protein bypasses the requirement for Dmib because it is ubiquitinated by some other ubiquitin ligase, thereby providing the necessary signal for Epsin-mediated internalization and signaling.

Ectopic expression of Neur bypasses the requirement for Dmib

Although *dmib* and *lqf* are both essential for wing cells to send DSL signals, the requirement for Dmib is context dependent, whereas that for *lqf* appears general. Specifically, *dmib* has only a modest role during the establishment of bristle SOPs, and little or no role in the segregation of their descendents, both contexts in which DSL signaling depends on the action of Neuralized (Neur) (Lai and Rubin, 2001). Hence, Neur and Dmib might constitute functionally related ligases that normally ubiquitinate DSL proteins in different signaling contexts.

To test this, we first asked whether ectopic expression of Neur, which is normally not expressed during wing development except in SOPs and their lineal descendents, can rescue the ability of *dmib*$^{-}$ cells to send DSL signals that specify Cut-expressing border cells and wing veins. We find that ectopic Neur expression does indeed rescue the ability of clones of *dmib*$^{-}$ cells to signal in both contexts, as indicated by the rescue of Cut expression on both sides of the D-V boundary in the wing disc (Fig. 4A-H), as well as by the rescue of the wing notching and vein thickening phenotypes that would otherwise result from the loss of *dmib* activity (data not shown). Second, we asked whether co-expression of Neur can rescue the ability of clones of *dmib*$^{-}$ cells that ectopically express Dl to activate Cut. As shown in Fig. 4I-L, we find that this is also the case. In fact, clones of *dmib*$^{-}$ cells that ectopically co-express Neur and Dl exhibit enhanced signaling activity in that they activate Cut expression even in ventral compartment cells (Fig. 4K); clones of otherwise wild-type cells that ectopically co-express Neur and Dl similarly activate Cut in ventral, as well as dorsal, cells (Pavlopoulos et al., 2001). Thus, ectopic Neur activity can substitute functionally for the absence of Dmib activity, suggesting that both proteins can execute a common ubiquitin ligase activity necessary for DSL signaling.

Dmib is required for normal bulk endocytosis of Ser but not Dl

Previously, we found that Epsin is essential for cells to send DSL signals. However, we were not able to detect any effect of removing Epsin on the bulk endocytosis of Dl in otherwise wild-type cells (Wang and Struhl, 2004). Hence, we hypothesized that if Dl must enter a select endocytic pathway mediated by Epsin to acquire signaling activity, this pathway would constitute a relatively small subset of the available pathways by which it can be internalized. Similar results were also obtained with the chimeric Dl^{R+} ligand: like native Dl, Dl^{R+} is strictly dependent on Epsin for signaling activity; however we could not detect any difference in bulk endocytosis of Dl^{R+} protein in clones of *lqf*$^{-}$ cells. Hence, even if one considers only those pathways that target ubiquitinated Dl for endocytosis, only a small subset might be Epsin-dependent and responsible for conferring signaling activity.

To determine the extent to which Dl and Ser internalization might depend on Dmib activity, we examined the subcellular distribution of both ligands in clones of *dmib*⁻ cells. As we describe below, we observe a pronounced and abnormal cell surface accumulation of Ser in *dmib*⁻ cells, but can not detect a change in Dl.

In mature wing discs, Dl protein is expressed at low level throughout the wing primordium, but accumulates at higher level in two stripes flanking the D-V boundary and along the 'pro-veins' that will give rise to the longitudinal veins of the wing (Fig. 2E). Ser protein shows a similar expression pattern, except that its expression is much weaker in the ventral compartment (Fig. 2A). Within cells, Dl appears to accumulate primarily on the apical cell surface, but is also readily detected in intracellular puncta. By contrast, we find that the majority of Ser appears to be localized in intracellular puncta (Fig. 2A′), raising the possibility that it might normally be cleared from the apical cell surface more efficiently than Dl. For both Dl and Ser, we observe significant co-staining of labelled cytosolic puncta with an antisera directed against Hepatocyte growth factor-regulated tyronine kinase substrate (Hrs), an endosomal marker protein (Lloyd et al., 2002), indicating that at least some of these puncta are endosomal [data not shown; confirmed for Dl by independent experiments (Wang and Struhl, 2004)].

In general, the distribution of endogenous Dl appears unchanged in clones of *dmib*⁻ cells relative to neighboring wild-type cells (Fig. 2E′, E″). However, as described previously for clones of *lqf*⁻ cells (Wang and Struhl, 2004), *dmib*⁻ clones that abut or cross the D-V boundary show reduced expression of Dl and clones in pro-vein regions show increased expression; both these effects can be attributed to alterations in Dl transcription resulting from the loss of DSL-Notch signaling. In general, we could not detect a change in the subcellular distribution of Dl in *dmib*⁻ cells relative to neighboring wild-type cells, although a small enhancement of staining at the apical cell surface was occasionally apparent in pro-vein regions; this difference could be due to either enhanced transcription of *Dl* or an abnormal surface accumulation of Dl protein.

By contrast, endogenous Ser protein accumulates apically to abnormally high levels in clones of *dmib*⁻ cells (Fig. 2A′,A″). We obtained similar results whether or not the discs were fixed in the presence of detergent prior to incubation with αSer antisera (Materials and methods; data not shown); hence, it appears that Ser accumulates abnormally on the apical cell surface of *dmib*⁻ cells. In addition, the number of intracellular Ser-positive puncta sometimes appears to be significantly reduced in *dmib*⁻ cells relative to surrounding wild-type cells (Fig. 2A′,A″). Both effects are more obvious when the mutant clones are located in or near pro-vein regions, where *Ser* transcription is likely to be upregulated as a consequence of the loss of DSL-Notch signaling. However, we also detect abnormally high levels of cell surface accumulation of Ser in intervein regions where such transcriptional upregulation is not expected to occur. Moreover, the level of surface accumulation of Ser in *dmib*⁻ cells exceeds that of their wild-type neighbors even within provein regions. Hence, the abnormal accumulation of Ser in *dmib*⁻ cells appears to result primarily from altered Ser trafficking and/or stability rather than from an abnormal upregulation of Ser transcription.

To determine unequivocally whether the cell surface accumulation of Dl and Ser is altered in *dmib*⁻ cells, we examined *dmib*⁻ clones in wing discs in which the ligands were over-expressed at uniformly high levels under Gal4 control. Under these conditions, the great majority of protein derives from Gal4 driven expression of the *UAS-Dl* or *UAS-Ser* transgene, obviating a significant contribution from altered transcription of the endogenenous gene.

For over-expressed Dl, we were not able to detect any difference in subcellular distribution between *dmib*⁻ cells and their wild-type neighbors (Fig. 5A-C″). In both populations of cells, the over-expressed Dl protein is localized mostly at the

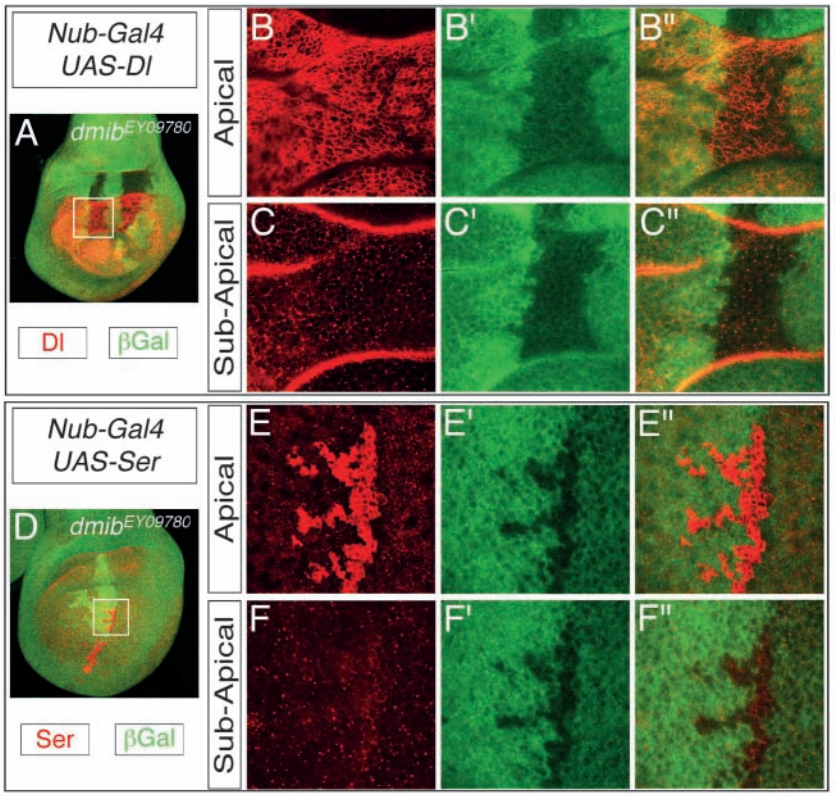

Fig. 5. Dmib is required for normal bulk endocytosis of Ser but not Dl. (A) Clones of *dmib*^{EY09780} cells (marked black by the absence of βGal, green) in a wing disc in which Dl (red) is over-expressed in the wing primordium under *nub-Gal4* control. (B,C) Higher magnification of the region boxed in white in A; apical and sub-apical planes of focus are shown in B and C, respectively. Accumulation of Dl on both the apical cell surface, as well as in cytosolic puncta, appears unaffected by the absence of Dmib. (D) Clones of *dmib*^{EY09780} cells (marked as in A) in a wing disc in which Ser (red) is over-expressed in the wing primordium under *nub-Gal4* control. Surface accumulation of Ser is dramatically enhanced by the absence of Dmib. (E-E″) Higher magnification of the region boxed in white in D, showing enhanced surface accumulation of Ser on the apical surface of *dmib*^{EY09780} cells. (F-F″) Little, or no, change is apparent in the accumulation of Ser in cytosolic puncta in *dmib*^{EY09780} cells.

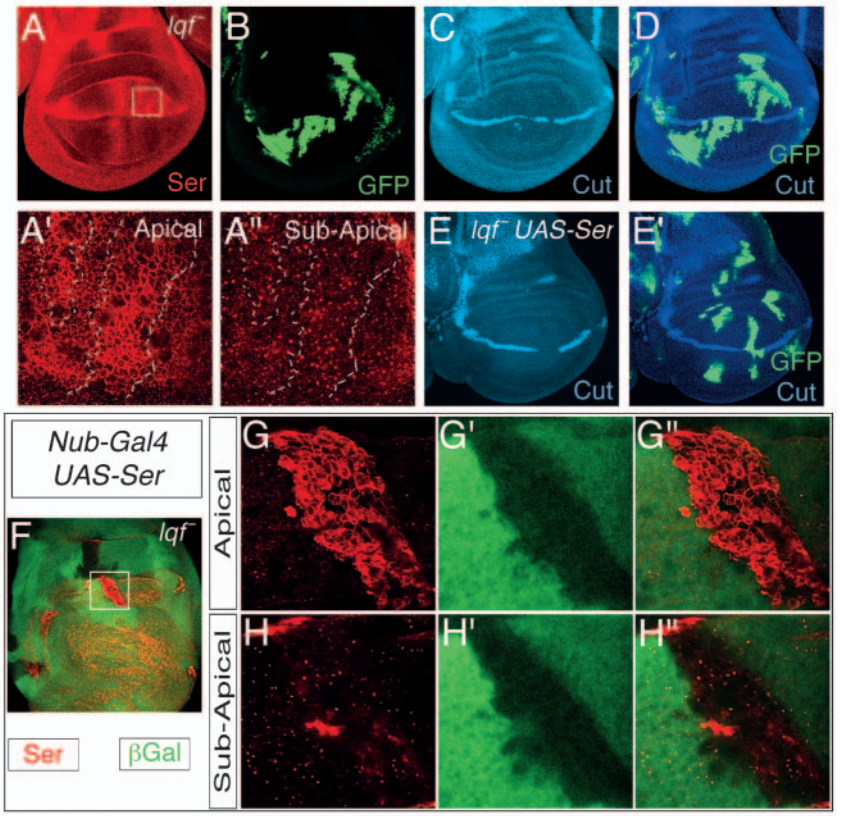

Fig. 6. Epsin is required for normal bulk endocytosis of Ser. (A-D) Wing disc stained for endogenous Ser expression (red) containing clones of *lqf*⁻ cells (green). *lqf*⁻ clones that abut or cross the D-V boundary impair Cut expression (blue). (A′,A″) Apical and sub-apical planes of section of the region boxed in white in A are shown at higher magnification, and the borders of two clones of *lqf* cells are outlined in white; Ser shows enhanced accumulation in *lqf*⁻ cells relative to neighboring wild-type cells at the apical cell surface. (E,E′) Clones of *lqf*⁻ cells that ectopically express Ser (green) fail to induce ectopic Cut expression (blue) and interrupt normal Cut expression when they abut the D-V boundary. (F) Clones of *lqf*⁻ cells (marked black, by the absence of βGal, green) in a wing disc in which Ser (red) is over-expressed in the wing primordium under *nub-Gal4* control. Surface accumulation of Ser is dramatically enhanced by the absence of Epsin. (G,H) Apical and sub-apical planes of section of the region boxed in white in F are shown at higher magnification in G and H, respectively. Ser accumulates dramatically at the apical cell surface of *lqf*⁻ cells, but no change is apparent in the number or intensity of staining of Ser-positive puncta within the cells (the small island of strong Ser staining in H is due to surface staining in a local fold in the disc).

cell surface (Fig. 5B″,C″). Hence, bulk Dl endocytosis does not appear to be affected in *dmib*⁻ cells, as we previously observed for *lqf*⁻ cells. By contrast, a different result was obtained for over-expressed Ser. Here, as observed for endogenous Ser in wild-type discs, we again detected a dramatic accumulation of Ser protein at the apical surface of *dmib*⁻ cells (Fig. 5D-F″), consistent with a block in the clearance of Ser from the cell surface.

Thus, it appears that bulk endocytosis of Ser is largely dependent on Dmib activity and hence that Dmib-dependent ubiquitination constitutes the main internalization signal responsible for targeting Ser for endocytosis. Conversely, Dl appears to be targeted for endocytosis primarily by other signals. Nevertheless, both ligands still depend critically on Dmib for signaling activity, as *dmib*⁻ cells that ectopically express high levels of either Dl or Ser fail to induce Cut expression in neighboring cells (Fig. 3B′,D′).

Epsin is required for normal bulk endocytosis of Ser

Our finding that normal bulk Ser endocytosis depends on Dmib activity raises the possibility that it may also depend on Epsin. Hence, we investigated whether Ser endocytosis is affected in *lqf*⁻ mutant cells. We first examined endogenous Ser expression in clones of *lqf*⁻ cells in otherwise wild-type wing discs. As in *dmib*⁻ cells, we observed an enhanced accumulation of Ser on the apical cell surface (Fig. 6A-A″). However, the degree of enhancement was less dramatic than that observed for *dmib*⁻ cells (Fig. 2A′,A″) making it less easy to attribute the abnormal accumulation to impaired endocytosis, as opposed to transcriptional upregulation. To resolve this uncertainty, we repeated the experiment in wing discs over-expressing uniformly high levels of exogenous Ser under Gal4 control. As in clones of *dmib*⁻ cells obtained under the same conditions, we find that Ser accumulated at dramatically higher levels along the apical surface of *lqf*⁻ cells (Fig. 6F-H″).

Thus, Ser differs from Dl in that it does not normally accumulate to high levels on the apical cell surface, but instead appears to be cleared from the cell surface by the actions of both Dmib and Epsin. Notably, the effects of abolishing Epsin activity on bulk endocytosis of Ser are less severe than those of abolishing Dmib, suggesting that Epsin mediates only a subset of the endocytic events that internalize ubiquitinated forms of Ser. However, as is the case for Dl, it is the subset of Epsin-mediated events that are essential for signaling activity (Fig. 6E) (Wang and Struhl, 2004).

Over-expression of Dmib enhances endocytosis and signaling activity of DSL ligands

Ectopic expression of Neur in presumptive wing cells has previously been shown to enhance both endocytosis and signaling activity of ectopically expressed Dl, suggesting that under these conditions, the rate of ubiquitination is limiting. Accordingly, over-expression of Dmib might be expected, similarly, to enhance DSL endocytosis and signaling, and we have found evidence that this so.

First, we find that ectopic expression of Dmib enhances endocytosis of ectopically expressed Dl and Ser. For example, when Dl is ectopically expressed along the A-P compartment boundary under the control of *ptc-Gal4*, it is localized predominantly at the apical cell surface, while Ser shows only a modest cell surface accumulation and is found, instead, mostly in intracellular vesicles (Fig. 7A-A‴,C-C‴). However, when coexpressed with exogenous Dmib, the subcellular distributions of both Dl and Ser shift towards localization in

intracellular puncta at the expense of accumulation at the cell surface (Fig. 7B-B‴,D-D‴, data not shown). Second, we observe that uniform over-expression of Dmib in the wing primordium under *nub-Gal4* control suppresses vein formation (Fig. 1F). This phenotype is reciprocal to the vein-thickening phenotype caused by loss of *dmib*, *lqf*, or *Dl* activity but similar to that caused by ectopic activation of Notch. Hence, the level of endogenous Dmib activity in wing cells appears to limit both the strength of DSL-Notch signaling, as well as the rate of internalization of DSL ligands.

Discussion

Previous studies have established that DSL ligands must be endocytosed in signal-sending cells to activate Notch in signal-receiving cells, and have suggested that to activate Notch, DSL proteins must normally be ubiquitinated, and thereby targeted to enter a select internalization pathway mediated by the endocytic protein Epsin (Parks et al., 2000; Wang and Struhl, 2004) (see also Le Borgne and Schweisguth, 2003b; Overstreet et al., 2004; Tian et al., 2004). Here, we present genetic evidence that ubiquitination and Epsin-dependent endocytosis are indeed required in vivo for DSL signaling activity. Our results also reveal unexpected differences in the roles of the ubiquitin ligases Dmib and Neur, as well as in the endocytic behavior of Ser and Dl.

Distinct roles for Epsin and the ubiquitin ligases Dmib and Neur in sending DSL signals

To date, two E3 ubiquitin ligases have been implicated in DSL signaling: zebrafish Mind bomb (Mib) and *Drosophila* Neuralized (Neur). Both proteins have been shown to promote DSL ubiquitination, endocytosis and signaling (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001; Itoh et al., 2003). Moreover, loss-of-function mutations in zebrafish *mib* and *Drosophila neur* cause essentially the same hallmark phenotype exemplifying a failure of DSL-signaling in their respective organisms; namely, a dramatic hyperplasia of the embryonic nervous system at the expense of the epidermis (Lehmann et al., 1983; Jiang et al., 1996; Schier et al., 1996). These observations have led to the suggestion that zebrafish Mib and *Drosophila* Neur are functional homologs (Le Borgne and Schweisguth, 2003a). Yet, the two proteins show only limited sequence homology; moreover they appear to be members of distinct Mib and Neur ubiquitin ligase families, each having true orthologs in both vertebrate and invertebrate genomes. As a consequence, the relative roles of Mib and Neur are not known in any animal system and this uncertainty complicates the use of mutations in these genes to assay the role of ubiquitination in DSL endocytosis and signaling.

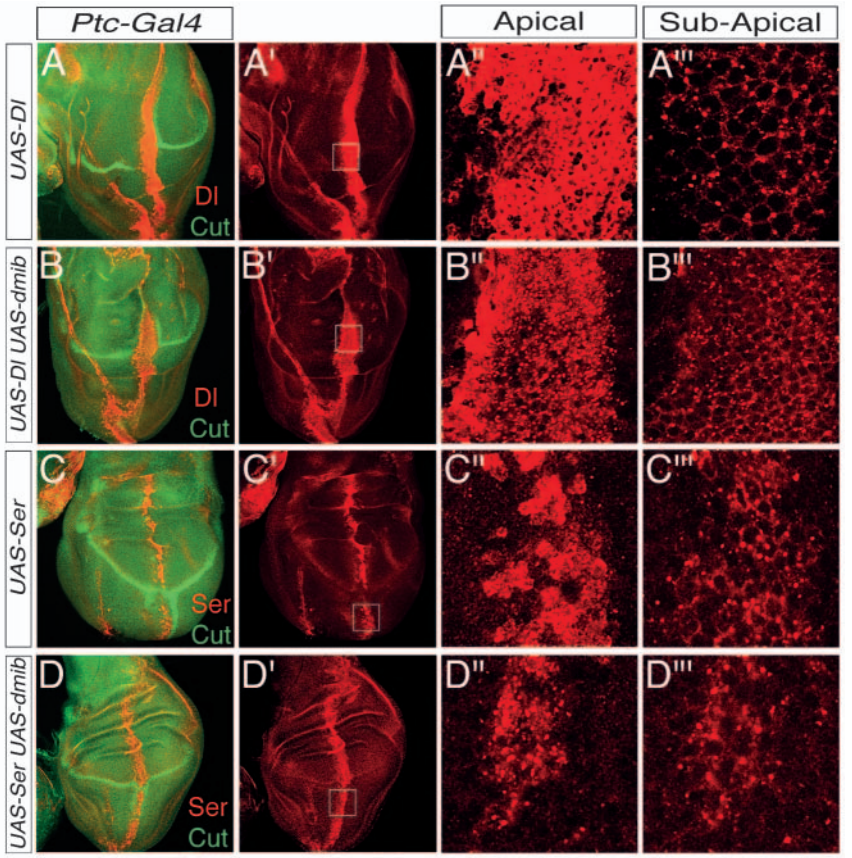

Fig. 7. Over-expression of Dmib enhances endocytosis of DSL ligands. (A-A‴) Wing disc in which Dl (red) is over-expressed in anterior cells along the A-P compartment boundary under the control of *ptc-Gal4*, inducing ectopic expression of Cut (green) in neighboring posterior cells in the dorsal compartment. A″ and A‴ show the region boxed in A′ at higher magnification at apical and sub-apical planes of focus: Dl accumulates predominantly at the cell surface. (B-B‴) As in A, except that Dmib is co-over-expressed with Dl; under these conditions, Dl accumulation at the cell surface is depleted (compare A″ and B″), allowing Dl-positive puncta to be readily detected just below the apical cell surface in B″. (C-C‴) As in A, except that Ser (red) rather than Dl is over-expressed under *ptc-Gal4* control, and ectopic Cut expression (green) is induced in the ventral compartment. Note the cell surface accumulation of Ser in C″. (D-D‴) As in C, except that Dmib is co-over-expressed with Ser; under these conditions, Ser accumulation at cell surface is depleted (compare C′ and D″), and cytosolic puncta visible just beneath the apical surface in C″.

Using newly isolated mutations in *dmib*, we have found evidence that Dmib and Neur constitute functionally related ubiquitin ligases that are normally required for DSL signaling in different developmental contexts. In the developing *Drosophila* wing disc, where we have focused our analysis, we find that Dmib is required for inductive signaling across the D-V compartment boundary, as well as for the refinement of wing vein primordia, both contexts in which Neur is normally not required or expressed. However, Dmib plays only a modest role in specifying sensory organ precursor (SOP) cells, and little or no role in the subsequent segregation of distinct cell types that form each sensory organ. Instead, Neur appears to provide the essential ubiquitin ligase activity required for DSL signaling in these latter two contexts. Similarly, in the embryo, where Neur is required for most DSL signaling events (Lehmann et al., 1983; Corbin et al., 1991; Hartenstein et al., 1992; Martin-

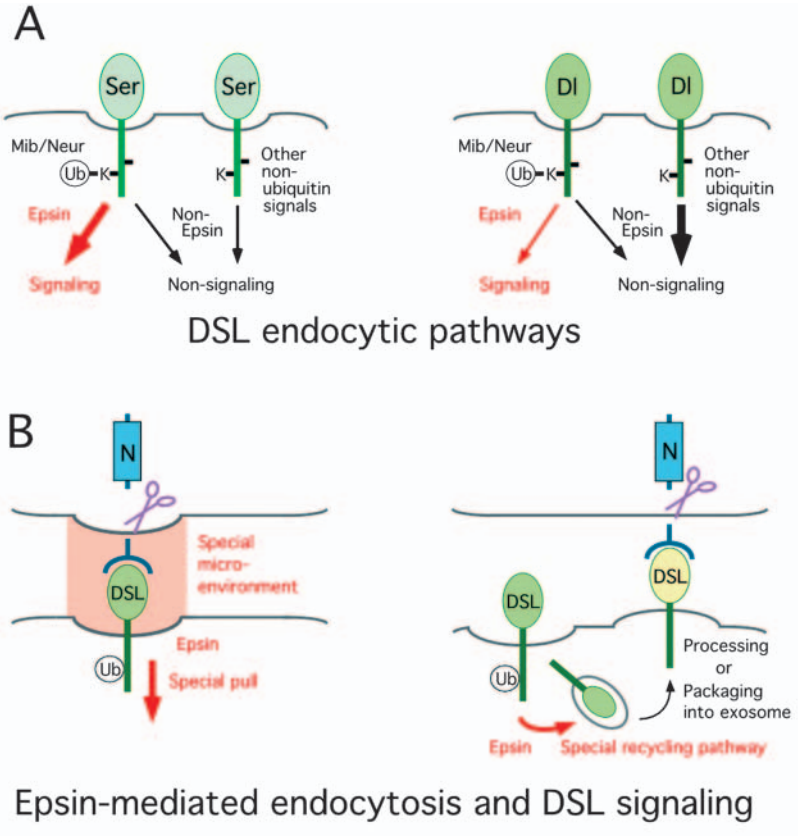

Fig. 8. Roles of Dmib, Neur, Epsin and endocytosis in DSL signalling. (A) Different utilization of distinct endocytic pathways by Ser and Dl. Both ligands can enter the cell by at least three internalization pathways (arrows). Dmib/Neur ubiquitinates Lysine residues (K) in the cytosolic domains of both ligands, targeting them for endocytosis via the action of Epsin (red arrows) or by other factors, defining two distinct routes. Both ligands also contain additional internalization signals that target them for endocytosis in a ubiquitin- and Epsin-independent manner, defining a third route. As indicated by the thickness of the arrows, Ser normally enters the cell predominately via the two ubiquitin-dependent pathways, and mostly via the Epsin-dependent pathway. By contrast, Dl does so predominately via the remaining, ubiquitin-independent pathway. Nevertheless, only those molecules of Ser and Dl that enter via the Epsin pathway can signal. (B) Models for Epsin-mediated activation of Notch by DSL ligands. Two general classes of models are shown, distinguished by whether the Epsin-mediated endocytic event required for signaling occurs before or after the ligands are internalized. In the first class (left), Epsin-mediated clustering of DSL ligands into coated pits or other specializations, or Epsin-mediated invagination of these structures into the cell, might provide a particular micro-environment (red shading) or mechanical stress (red arrow) that is essential for inducing cleavage or shedding of the ectodomain of Notch. In the second class (right), Epsin might direct, or accompany, DSL proteins into a particular recycling pathway (red arrow) that is essential to convert or repackage them into ligands that can activate Notch upon return to the cell surface.

Bermudo et al., 1995), we find that Dmib has little or no apparent role. Indeed, embryos devoid of Dmib activity hatch as viable first instar larvae; moreover they develop into pharate adults that show only a limited subset of Notch-related mutant phenotypes, each of which appears to reflect the failure of a particular DSL signaling event that does not, normally, depend on Neur (Fig. 1 legend). Thus, we infer that Dmib and Neur share a common ubiquitin ligase activity that is essential for DSL ligands to signal.

While this paper was under review, Le Borne et al. (Le Borgne et al., 2005) published similar findings indicating a role for Dmib in DSL signaling, and the capacity of ectopic Neur to substitute for Dmib during wing development. Our results differ from theirs, however, in that our analysis of clones of *dmib*$^{-}$ cells that express Ser, Dl, or the Dl^{R+} chimera appears to indicate an absolute requirement for Dmib in sending both *Drosophila* DSL signals, Dl and Ser (Fig. 3). By contrast, Le Borgne et al. interpret their data as evidence for a regulatory rather than an obligatory role of Dmib in sending DSL signals, as well as for a lesser role of Dmib in Dl signaling compared with Ser signaling. Differences in experimental design, particularly in the means used to define or infer the identity of DSL signaling and Dmib-deficient cells, could account for the different conclusions reached.

We note that if Mib and Neur ligases have overlapping molecular functions in all animal systems, as we find them to have in *Drosophila*, there is no compelling reason why they would need to be deployed in the same way in different animal species. Instead, any given DSL-signaling process might depend on Mib in one animal system but on Neur in another, as appears to be the case for neurogenesis in zebrafish and *Drosophila*.

In contrast to the selective requirement for Dmib and Neur in overlapping subsets of DSL signaling contexts, Epsin is required for most or all DSL signaling events (Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2004). This difference is expected if ubiquitination of DSL ligands by either Dmib or Neur is normally a prerequisite for Epsin-mediated endocytosis, and hence for signaling activity.

Do Dmib and Neur directly bind and ubiquitinate DSL ligands and thereby confer signaling activity by targeting them for Epsin-mediated endocytosis? Although ectopic Dmib and Neur activity are both associated with enhanced DSL ubiquitination, endocytosis and signaling, there is still no compelling evidence that either ligase directly binds and ubiquitinates DSL proteins, or that Dmib/Neur-dependent ubiquitination of DSL ligands confers signalng activity. However, we show here that the obligate requirement for Dmib for Dl-signaling by wing cells can be bypassed by replacing the cytosolic domain of Dl with a random peptide, R^{+}, that may serve as the substrate for ubiquitination by an unrelated ubiquitin ligase (Wang and Struhl, 2004). This result provides in vivo evidence that Dmib/Neur-dependent ubiquitination of DSL ligands is normally essential to confer signaling activity. Moreover, the failure of the chimeric Dl^{R+} ligand to bypass the requirment for Epsin (Wang and Struhl, 2004), supports the interpretation that ubiquitination of DSL ligands confers signaling activity because it targets them for Epsin-mediated endocytosis.

Bulk endoctyosis of Ser, but not Dl, depends on Dmib and Epsin

During wing development, Ser and Dl both serve as unidirectional signals that specify the 'border' cell fate in cells across the D-V compartment boundary, and we find that both are equally dependent on Dmib and Epsin function for signaling activity. However, we find the two ligands differ in the extent to which they accumulate on the cell surface, and to which they are cleared from the surface as a consequence of Dmib and Epsin activity. Specifically, we find that most Ser accumulates in cytosolic puncta rather than on the cell surface, whereas the reverse is the case for Dl. Furthermore, removing either Dmib or Epsin activity results in a dramatic and abnormal retention of Ser on the cell surface, whereas it has no detectable effect on the surface accumulation of Dl. Similar results for Dmib were also obtained by Le Borgne et al. (Le Borgne et al., 2005). Thus, it appears that most Ser is efficiently cleared from the cell surface by the actions of Dmib and Epsin, whereas most Dl remains on the cell surface, irrespective of Dmib and Epsin activity. This unexpected difference provides two insights.

First, in our previous analysis of the role of Epsin, we focused almost exclusively on Dl endocytosis and signaling (Wang and Struhl, 2004) and failed to obtain direct evidence that Epsin is required for normal DSL endocytosis, despite the obligate role for Epsin in sending both Dl and Ser signals. Instead, we could only detect such a requirement in experiments in which we abnormally enhanced surface clearance of over-expressed Dl by ectopically co-expressing Neur, or infer it from experiments in which we bypassed the requirement for Epsin by replacing the cytosolic domain of Dl with the well-characterized endocytic recycling signal from the mammalian low density lipoprotein (LDL) receptor. By contrast, the different endocytic behavior of Ser has now allowed us to obtain direct evidence that Dmib and Epsin are both required for normal DSL endocytosis.

Second, we find that even though bulk endocytosis of Ser depends on both Dmib and Epsin activity, neither requirement appears absolute. Instead, we can still detect the accumulation of Ser in cytosolic puncta in both Dmib- and Epsin-deficient cells. Moreover, we can detect a difference in the abnormal cell surface accumulation of Ser in Dmib-deficient versus Epsin-deficient cells; significantly more Ser appears to accumulate in the absence of Dmib than in the absence of Epsin. As diagrammed in Fig. 8A, we infer that both Dl and Ser are normally internalized by multiple endocytic pathways, only some of which depend on ubiquitination of the ligand, and only a subset of these that depends on Epsin. However, the two ligands normally utilize these pathways to different extents, most Ser being internalized by ubiquitin- and Epsin-dependent pathways, and most Dl being internalized by alternative pathways. We presume that this difference reflects the presence of different constellations of internalization signals in the two ligands, especially the presence of signals in Delta, but not Ser, that target the great majority of the protein for internalization pathways that do not depend on ubiquitination or Epsin. Nevertheless, only those molecules of Ser and Dl that are targeted by ubiquitination to enter the Epsin-dependent pathway have the capacity to activate Notch; all other routes of entry that are normally available appear to be non-productive in terms of signaling. These results reinforce our previous evidence (Wang and Struhl, 2004) that endocytosis of DSL ligands, per se, is not sufficient to confer signaling activity; instead, DSL ligands must normally be internalized via the action of Epsin to signal.

Epsin-dependent endocytosis and DSL signaling activity

Why must DSL ligands normally be internalized by an Epsin-dependent endocytic mechanism to activate Notch? We can distinguish two general classes of explanation (Fig. 8B) (see also Wang and Struhl, 2004). In the first, Epsin confers signaling activity by regulating an early event in DSL endocytosis that occurs before internalization. For example, Epsin might cluster DSL ligands in a particular way or recruit them to a select subset of coated pits or other endocytic specializations. Alternatively, Epsin-mediated invagination of these structures might control the physical tension across the ligand/receptor bridge linking the sending and receiving cell, creating a sufficiently strong or special mechanical stress necessary to induce Notch cleavage or ectodomain shedding. In the second class of models, Epsin acts by regulating a later event in DSL endocytosis that occurs after internalization. For example, Epsin might direct, or accompany, DSL proteins into a particular recycling pathway that is essential to convert or repackage them into ligands that can activate Notch upon return to the cell surface. In both cases, internalization of DSL ligands via the other endocytic routes normally available to them would not provide the necessary conditions, even in the extreme case of Dl, which appears to be internalized primarily by these other pathways.

Our present results do not distinguish between these models. However, recent studies of Epsin-dependent endocytosis in mammalian tissue culture cells suggest that Epsin may direct cargo proteins to different endocytic specializations or pathways, depending on their state of ubiquitination (Chen and De Camilli, 2005; Sigismund et al., 2005). They also suggest that interactions between Epsin and components of the core Clathrin endocytic machinery normally regulate where and how Epsin internalizes target proteins. Both properties might govern how DSL proteins are internalized, allowing the ligands to gain access to the select endocytic pathway they need to enter to activate Notch.

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Note added in proof

Lai et al. present complementary findings that similarly support a direct role for Dmib in ubiquitination, internalization and signaling by DSL ligands (Lai et al., 2005).

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