The ubiquitin ligase *Drosophila* Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta

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Summary

The receptor Notch and its ligands of the Delta/Serrate/LAG2 (DSL) family are the central components in the Notch pathway, a fundamental cell signaling system that regulates pattern formation during animal development. Delta is directly ubiquitinated by *Drosophila* and *Xenopus* Neuralized, and by zebrafish Mind bomb, two unrelated RING-type E3 ubiquitin ligases with common abilities to promote Delta endocytosis and signaling activity. Although orthologs of both Neuralized and Mind bomb are found in most metazoan organisms, their relative contributions to Notch signaling in any single organism have not yet been assessed. We show here that a *Drosophila* ortholog of Mind bomb (D-mib) is a positive component of Notch signaling that is required for multiple Neuralized-independent, Notch-dependent developmental processes. Furthermore, we show that D-mib associates physically and functionally with both Serrate and Delta. We find that D-mib uses its ubiquitin ligase activity to promote DSL ligand activity, an activity that is correlated with its ability to induce the endocytosis and degradation of both Delta and Serrate (see also Le Borgne et al., 2005). We further demonstrate that D-mib can functionally replace Neuralized in multiple cell fate decisions that absolutely require endogenous Neuralized, a testament to the highly similar activities of these two unrelated ubiquitin ligases in regulating Notch signaling. We conclude that ubiquitination of Delta and Serrate by Neuralized and D-mib is an obligate feature of DSL ligand activation throughout *Drosophila* development.

Key words: Mind bomb, Neuralized, Serrate, Delta, Notch signaling, Endocytosis

Introduction

The Notch pathway is a signal transduction cascade that mediates cell-cell communication and is widely used to determine cell fate and cell behavior throughout the Metazoa (Lai, 2004). Indeed, there is scarcely a developmental process that does not involve Notch signaling in some reasonably direct fashion. Its central cell surface components are a Delta/Serrate/LAG-2 (DSL)-type ligand and the receptor Notch, both of which are type I transmembrane proteins that interact via their extracellular domains. Ligand-induced activation of Notch triggers the cleavage of the intracellular domain of Notch, which subsequently translocates to the nucleus and functions as a transcriptional co-activator for a CSL-type DNA-binding protein (Lai, 2004).

There are two DSL ligands in *Drosophila*, Delta and Serrate, which have both overlapping and distinct functions during development. For example, lateral inhibition of neural precursors is mediated largely by Delta (Heitzler and Simpson, 1991), whereas embryonic segmental patterning is mediated by Serrate (Wiellette and McGinnis, 1999). However, asymmetric cell divisions during peripheral sense organ development (Zeng et al., 1998) and leg joint specification (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999) require both ligands. Distinct patterns of DSL ligand expression help to explain why some settings require one or the other ligand. In fact, rescue experiments involving ectopically expressed ligands demonstrate a certain degree of functional interchangeability between Delta and Serrate (Gu et al., 1995; Klein and Arias, 1998). In certain settings, however, Delta and Serrate are co-expressed but have non-overlapping function. For example, lateral inhibition amongst proneural clusters of the adult peripheral nervous system requires only Delta, even though proneural clusters express both ligands.

The degree to which components of Notch signaling are regulated at the post-translational level has only become fully apparent in the last few years. Various components are subject to proteolysis, glycosylation, ubiquitination and phosphorylation, which collectively have a tremendous range of consequences on the efficacy of Notch signaling (Schweisguth, 2004). This variety is well illustrated by ubiquitination, a functionally versatile protein modification that can promote protein degradation, influence protein

localization, or modulate protein activity (Hershko and Ciechanover, 1998; Hicke and Dunn, 2003; Zhang, 2003).

Ubiquitination is mediated by the stepwise activity of several enzymes (Hershko and Ciechanover, 1998). First, an ubiquitin-activating enzyme (E1) activates the 76-amino-acid ubiquitin molecule by an ATP-dependent mechanism and transfers it to an ubiquitin-conjugating enzyme (E2). Then, an ubiquitin ligase (E3) facilitates transfer of ubiquitin from E2 to the appropriate substrate. Three protein motifs display intrinsic, biochemically demonstrable, ubiquitin ligase activity: the HECT domain, the RING finger and a structural relative of the RING finger termed the U box (Hatakeyama and Nakayama, 2003; Jackson et al., 2000; Joazeiro et al., 1999; Scheffner et al., 1993). As the E3 is responsible for target specificity, the number of E3 enzymes is far greater than the number of either E1 or E2 enzymes. A typical eukaryotic genome encodes a single E1 and perhaps ~20 E2s, but >100 E3s. In some cases, including in the prototypical SCF complex, ubiquitin ligase and substrate recognition domains reside in different proteins that associate as a multisubunit E3. In most cases, though, the E3 is a single protein that binds the substrate and catalyzes its ubiquitination.

At least five different components of the Notch pathway are regulated by ubiquitination [including the ligand Delta, the epsin Liquid facets (Lqf), the receptor Notch, the Notch regulator Numb, and some bHLH repressor-encoding products of the HES genes], and ubiquitination can either negatively- or positively-regulate Notch signaling depending on the particular substrate and situation (Chen et al., 2002; Hirata et al., 2002; Itoh et al., 2003; Lai, 2002). In some cases, the same component is directly targeted by multiple E3 ubiquitin ligases. For example, membrane-localized Notch is regulated by Su(dx)/Itch (Cornell et al., 1999; Qiu et al., 2000) and possibly by Sel-1 (Grant and Greenwald, 1997), whereas nuclear Notch^{intra} is regulated by Sel-10/Ago (Gupta-Rossi et al., 2001; Hubbard et al., 1997; Oberg et al., 2001; Wu et al., 2001).

In recent years, two types of E3 ubiquitin ligase were shown to target Delta and regulate its localization and signaling activity (Le Borgne and Schweisguth, 2003a). Both E3s were identified through the study of neurogenic mutants, which display excess neural differentiation. This phenotypic class includes mutations in all central components of Notch signaling. *neuralized* (*neur*) is a fly neurogenic that was discovered over twenty years ago (Lehmann et al., 1983; Wieschaus et al., 1984). Neur is absolutely required in some settings of Notch signaling in flies, but is dispensable in others. For example, Neur restricts neural precursors, R8 photoreceptors and muscle precursors, and controls asymmetric cell divisions within neural lineages, but is dispensable for wing margin specification, eye growth and restriction of wing vein thickness (Corbin et al., 1991; Lai and Rubin, 2001a; Lai and Rubin, 2001b; Lehmann et al., 1983; Yeh et al., 2000). *Mind bomb* (*mib*) is a fish neurogenic that also displays defective Notch signaling in certain other developmental settings, including somite formation and vascular development (Jiang et al., 1996; Lawson et al., 2001; Schier et al., 1996; van Eeden et al., 1996). Neur and Mib each contain a RING finger at their respective C termini, and both have been biochemically demonstrated to directly ubiquitinate Delta (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Price et al., 1993). Aside from this motif, however, these proteins are completely unrelated.

As is the case for a number of other transmembrane proteins, monoubiquitination of Delta induces its endocytosis and subsequent degradation (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Pavlopoulos et al., 2001). Curiously then, Neur and Mib non-autonomously stimulate Notch activation by Delta-expressing, signal-sending cells. The evidence for this is that *mib*-mutant fish cells are deficient in their ability to send a lateral inhibitory signal in neural tube cell transplantation experiments (Itoh et al., 2003), that *neur* mutant fly cells are preferentially inhibited from adopting the neural fate at mosaic clone borders (Pavlopoulos et al., 2001) and display non-autonomous defects during asymmetric neural lineage divisions (Le Borgne and Schweisguth, 2003b), and that co-expression of Neur with Delta potentiates the ability of a cell to activate Notch signaling in neighboring cells (Pavlopoulos et al., 2001).

Although both Neur and Mib have been evolutionarily conserved in diverse metazoans, a genetic requirement for both ubiquitin ligases in Notch signaling in any single organism has not yet been demonstrated. In addition, it has not yet been established whether signaling by the *Drosophila* DSL ligand Serrate is regulated by endocytosis. In this study, we characterize the *Drosophila* ortholog of *Mind bomb* (*D-mib*). We find that *D-mib* is essential for a large number of *neur*-independent, Notch pathway-mediated developmental processes, allowing us to classify it as a vital component of *Drosophila* Notch signaling. We find that D-mib directly associates with and targets both Serrate and Delta for endocytosis and degradation, and is able to generally influence Notch signaling through its ability to regulate DSL ligand activity (see also Le Borgne et al., 2005). Finally, we show that ectopic D-mib is able to rescue multiple aspects of the *neur* mutant phenotype, demonstrating that Neur and D-mib have highly overlapping functions in vivo.

Materials and methods

Drosophila genetics

Gal4-UAS binary expression system

The following Gal4 and UAS strains have been previously described: *sca-Gal4*, *ey-Gal4*, *GMR-Gal4*, *bx-Gal4*, *dpp-Gal4* (FlyBase, 2003); *UAS-neur*, *UAS-neurΔRF* (Lai and Rubin, 2001a).

D-mib structure-function analysis

We amplified the desired portions of *D-mib*, using the cDNA SD05267 as template (gift of the Berkeley Drosophila Genome Project), and cloned them into TOPO-D-ENTR (Invitrogen). Primer sequences are available upon request. Following sequence verification, these DNAs were cloned into UAS-HM-Gate, a Gateway-compatible vector that creates N-terminal in-frame fusions to 6×His and 3×Myc tags (gift of Cynthia Hsu and Brian McCabe). These were injected into *Drosophila* embryos using standard protocols, and multiple independent insertions were established and analyzed for each construct.

Exogenous Delta assay

These experiments used the *hs-Delta* construct of Struhl (Struhl and Adachi, 1998). We selected *Tb*$^+$ larvae of the following crosses: *hs-Delta*×*dpp-Gal4, UAS-D-mib*/*SM-TM6B, Cy, Tb* and *hs-Delta*×*UAS-D-mibΔRF*; *dpp-Gal4*/*SM-TM6B, Cy, Tb*. Larvae were heat-shocked at 38°C in a circulating water bath and then allowed to recover at 25°C for the desired length of time.

Development

Development

Neur rescue experiments

We made *neur* mutant clones using the *FRT82B*, *neur*^{A101} and *FRT82B*, *neur*^{IF65} chromosomes (Lai and Rubin, 2001a), using *ubx-FLP* and the MARCM system (Lee and Luo, 2001), and drove expression of transgenes within mutant clones using *sca-Gal4*. *ubx-FLP* reliably creates mutant notum clones in FRT-homozygous flies, so that 50% of the appropriate progeny will carry mutant clones; the *neur* mutant phenotypes are themselves completely penetrant. We inferred rescue of *neur*^{A101} by *UAS-neur* from the observation that 100% of the appropriate progeny were wild type. A similar observation applies to the rescue of *neur*^{IF65} by *UAS-D-mib*, although in this case, the presence of rescued, mildly tufted bristles is also a positive assessment of amelioration of the *neur*^{IF65} balding phenotype. Crosses were as follows.

neur rescue of *neur*^{A101}: *yw, ubx-FLP/+; sca-Gal4, UAS-pon-GFP, UAS-tau-GFP/CyO; FRT82B, tub-Gal80/TM6B, P(y*$^{+}$*)* × *w/Y; UAS-neur/+; FRT82B, neur*^{A101}.

D-mib rescue of *neur*^{IF65}: *yw, ubx-FLP/+; sca-Gal4, UAS-pon-GFP, UAS-tau-GFP/CyO; FRT82B, tub-Gal80/TM6B, P(y*$^{+}$*)* × *w, UAS-D-mib/Y; +/+; FRT82B, neur*^{IF65}*/+*. Female *Cy*$^{+}$, *Tb*$^{+}$ adults were selected.

Note that in second cross, only female progeny will show rescue, as an X-linked *UAS-D-mib* insertion was used. We exploited this in the analysis of *D-mib* rescue of *neur* mutant pupal clones, by separating male from female larvae in this cross, and selecting 36 hours after puparium formation (APF) pupae for patches of GFP expression on the notum, which indicated the presence of *neur*^{IF65} mutant MARCM clones. These were then fixed and stained.

D-mib:Delta and D-mib:Serrate co-immunoprecipitation

The construct for expressing Delta with two C-terminal polyoma tags was described previously (Lai et al., 2001). A similarly tagged Serrate expression construct was generated by PCR using LP24305 (Berkeley Drosophila Genome Project) and cloned into pcDNA3.1/TOPO (Invitrogen). Myc-tagged D-mib expression vectors contained the coding regions of UAS-HM-D-mib constructs cloned into pcDNA3.1/TOPO using PCR (Invitrogen). Plasmids were transiently transfected into 293T cells using Fugene 6 (Roche). After incubation for 48 hours, cells were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 0.5% Triton X-100 and EDTA-free Complete Protease Inhibitors (Roche). 200 μg or 250 μg protein lysate was used in Serrate and Delta co-immunoprecipitations, respectively; lysates were incubated with mouse anti-Myc, mouse anti-Delta C594 (Developmental Studies Hybridoma Bank, DSHB) or agarose-conjugated goat anti-Myc (NOVUS, San Diego) in 1 ml lysis buffer. The captured proteins were then precipitated with protein A/G-plus agarose (Santa Cruz biotechnology) and recovered by boiling in Laemmli sample buffer. The immunoprecipitated proteins or 5 μg total protein lysate were separated on SDS-PAGE gels (BioRad) and transferred to Hybond-C extra membrane (Amersham Pharmacia Biotech). The membranes were probed with anti-Myc, anti-Delta or anti-polyoma, and detected with ECL-plus reagents (Amersham Pharmacia Biotech).

Immunofluorescence

We used the following primary antisera, all of which were previously described: guinea pig anti-Senseless (1:5000, gift of Hugo Bellen), rat anti-Su(H) (1:1500, gift of Francois Schweisguth), mouse anti-Cut (1:100, DSHB), mouse anti-Delta (1:100, DSHB), guinea pig anti-Delta (1:2500, gift of Marc Muskavitch), rat anti-Serrate (1:2000, gift of Ken Irvine), mouse anti-Myc (1:5000, ascites), rat anti-ELAV (1:100). We detected proteins as described previously (Lai and Rubin, 2001a).

Results

D-mib is a ubiquitin ligase that regulates *Drosophila* Notch signaling

Itoh and colleagues noted the existence of homologs of zebrafish Mind bomb (Mib) in various metazoan species (Itoh et al., 2003). All Mib proteins share a common domain structure that we divide into three regions (Fig. 1). The N-terminal region consists of a zz zinc finger that is flanked by a pair of domains shared between Mib and HERC2 (Mib/HERC2 domains), followed by a repeated sequence specific to Mib (the Mib domain). The middle region is characterized by eight ankyrin repeats. Finally, the C-terminal region contains three RING finger domains, of which only the final RING finger fully resembles the canonical sequence known to mediate ubiquitin ligation. There are two predicted Mib homologs in *Drosophila*. Of these, CG5841 is more closely related to Mind bomb at the amino acid level and is thus the true *Drosophila* ortholog of Mind bomb (D-mib). We

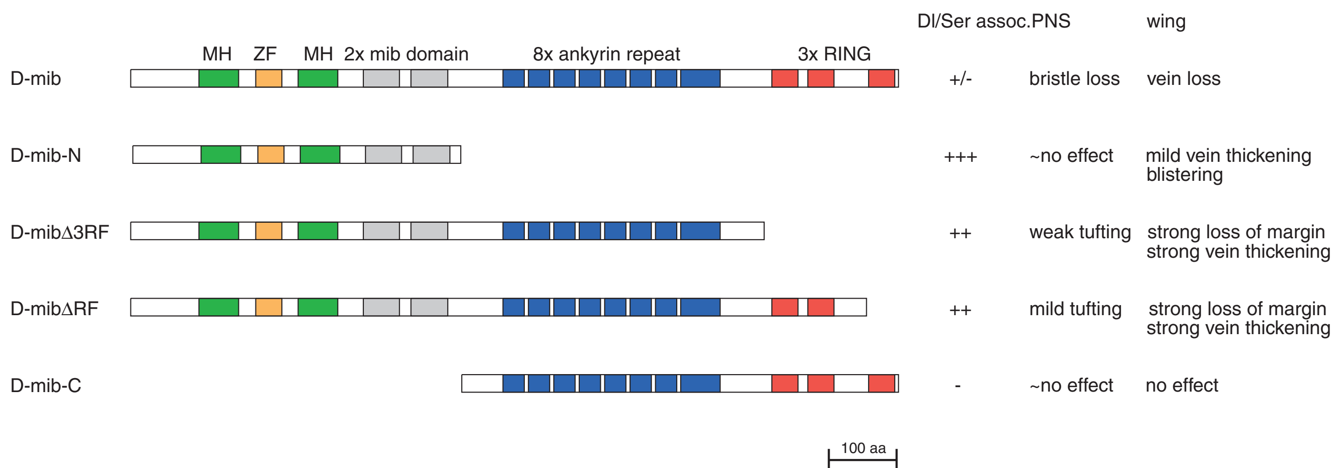

Fig. 1. Summary of D-mib structure-function studies. All Mib proteins display the same domain structure seen in D-mib. The different domains are color-coded as follows: Mib/Herc2 domains (MH), green; zz zinc finger (ZF), orange; Mib domain, light grey; ankyrin repeats, blue; RING fingers, red. We assayed the activities of the depicted portions of D-mib in vivo and in vitro, and the results are summarized on the right; selected data are shown in Figs 2-4. Dl, Delta; Ser, Serrate.

refer to CG17492 as *Drosophila* Mind bomb-like (D-mibl), but will not consider it further in the present study.

A pupal-lethal P element insertion in the 5′ UTR of *D-mib* (EY09760) was recently isolated by the *Drosophila* Gene Disruption Project (Bellen et al., 2004). We could revert its lethality by precise excision of the transposon, indicating that this insertion affects *D-mib* function. Accordingly, we refer to it as *D-mib*^{1}. Concurrent studies by the Schweisguth group further indicate that *D-mib*^{1} behaves as a genetic and protein null allele, and can be rescued by transgenic expression of D-mib (Le Borgne et al., 2005). We examined homozygous pharate *D-mib*^{1} flies and found that they were eyeless, had vestigial wings, and displayed squat legs lacking joints (Fig. 2A-F). We examined the basis of the wingless phenotype by staining for markers of wing development in the third instar imaginal disc. *D-mib*^{1} mutant discs displayed a severely reduced wing pouch, as marked by Nubbin (Fig. 2G,H). This is due to a loss of Cut expression in the wing pouch, indicating a failure to specify the wing margin (Fig. 2I,J). These phenotypes closely resemble those caused by Notch pathway loss of function, thus strongly implicating D-mib as a component of *Drosophila* Notch signaling. However, lateral inhibition was largely unaffected in *D-mib*^{1} discs, as most sensory organ precursors (as marked by Senseless) were singularized normally (Fig. 2K,L). Therefore, D-mib is required for only a subset of Notch-dependent processes. Interestingly, the ubiquitin ligase Neuralized (Neur) is essential for lateral inhibition of sensory precursors, but is not required for wing margin, leg joint or eye specification (Lai and Rubin, 2001a; Lai and Rubin, 2001b; Yeh et al., 2000). Therefore, Neur and D-mib appear to have complementary functions in regulating *Drosophila* Notch signaling.

In situ hybridization showed *D-mib* to be ubiquitously expressed in the wing imaginal disc (data not shown); D-mib protein expression is similarly ubiquitous (Le Borgne et al., 2005). This contrasts with the highly restricted expression of *neur* in sensory organ precursors of the wing imaginal disc (Boulianne et al., 1991). To gain further insight into the activity of D-mib, we ectopically expressed full-length and truncated D-mib proteins in transgenic *Drosophila* (Figs 1, 3). The activities of full-length D-mib and D-mibΔRF (lacking only its most C-terminal RING finger) exactly parallel those of corresponding Neur proteins (Lai and Rubin, 2001a; Lai and Rubin, 2001b) in that the full-length proteins hyperactivate Notch signaling, while RING-finger deleted versions inhibit Notch signaling. Thus, misexpression of D-mib induces mild loss of macrochaete bristles and campaniform sensilla (Fig. 3B,H), loss of sensory organ precursors (as marked by Sens expression, Fig. 3K), as well as loss of wing veins (Fig. 3E). By contrast, D-mibΔRF induces mild bristle tufting and multiplication of sensory precursors (Fig. 3C,I,L), potently inhibits wing margin development (Fig. 3F), eliminates expression of wing margin markers (including Cut, Fig. 3O), inhibits restriction of wing vein thickness (Fig. 3F,I), and inhibits eye disc growth (data not shown). Some effects of D-mibΔRF phenocopy *D-mib* loss of function (such as loss of wing margin and absent retinal development); however, D-mib and D-mibΔRF affect other settings of Notch signaling that only weakly require, or are independent of, *D-mib*. Therefore, as is the case for Neur (Lai and Rubin, 2001a; Lai and Rubin, 2001b), D-mib can generally affect Notch signaling when expressed ectopically. This is particularly so for the RING-finger deleted, dominant-negative isoforms, NeurΔRF and D-mibΔRF.

The extreme dominant-negative activity of D-mibΔRF is consistent with the observation that the only two missense alleles of zebrafish Mib alter amino acids in the most C-terminal RING finger of Mib (Itoh et al., 2003), which we may

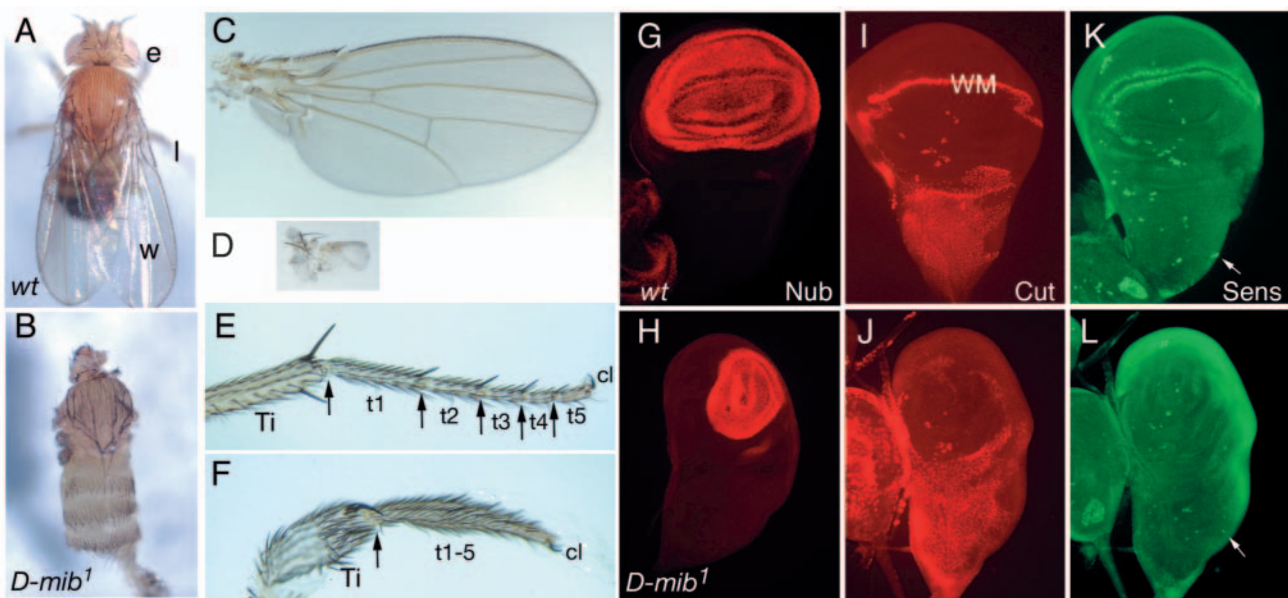

Fig. 2. *D-mib* is required for multiple Neur-independent, Notch-dependent developmental processes. (A,C,E,G,I,K) Wild-type; (B,D,F,H,J,L) *D-mib*^{1} homozygotes. *D-mib*^{1} pharate adults are largely eyeless (B) and wingless (D). *D-mib* mutants (F) also display defective leg development and lack joints (arrows compare with E). Leg structures are abbreviated as follows: Ti, tibia; t1-t5, the five tarsal segments; cl, claw. (G-L) Wing imaginal discs stained for Nubbin (G,H), Cut (I,J) and Senseless (K,L). Note that wing margin (WM) expression of Cut and Senseless is absent in *D-mib*^{1} discs, but that sensory organ precursors (arrows) are singularized normally in this mutant.

Development

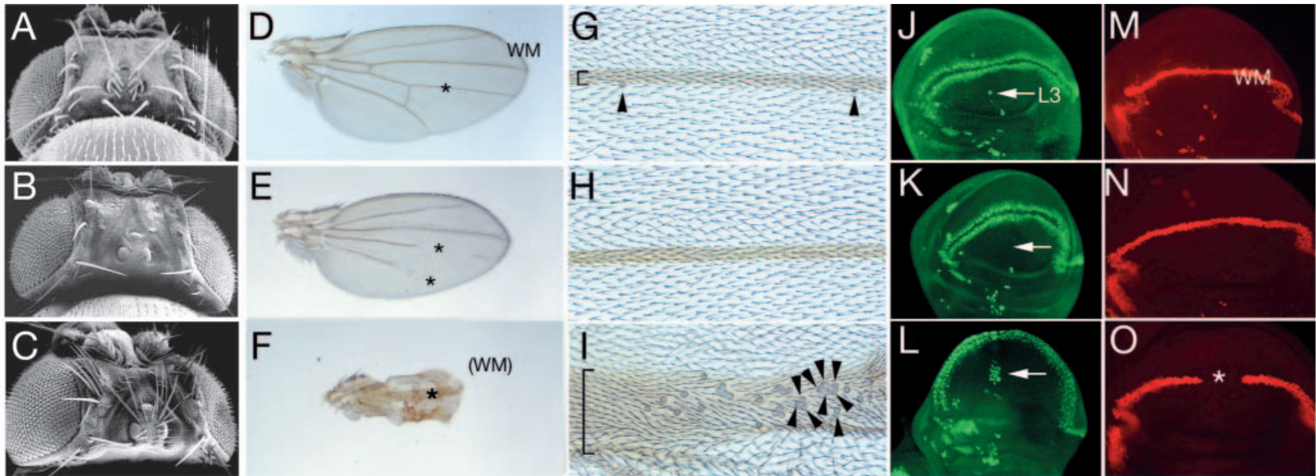

Fig. 3. Effects of ectopic D-mib and D-mibΔRF on Notch-regulated developmental patterning. (A) Wild-type adult head. (B) *sca-Gal4>UAS-D-mib* head is missing several macrochaetae. (C) *sca-Gal4>UAS-D-mibΔRF* exhibits macrochaetae tufting. (D) Wild-type wing. WM, wing margin; asterisk marks a wing vein. (E) *bx-Gal4/Y; UAS-D-mib* wing displays longitudinal vein breaks (asterisk) and lacks crossveins. (F) *bx-Gal4/Y; UAS-D-mibΔRF* is vestigial and completely lacks a wing margin; the remaining wing tissue present is composed mostly of severely thickened wing veins (asterisk). (G) Close-up of the L3 vein in a *dpp-Gal4/+* wing; arrowheads point to two campaniform sensilla. The normal thickness of vein is denoted with a bracket. (H) *dpp-Gal4, UAS-D-mib* wing lacks campaniform sensilla. (I) *dpp-Gal4, UAS-D-mibΔRF* wing displays an extremely thickened L3 vein and a vast surplus of campaniform sensilla; both features are indicative of failed Notch signaling. (J-L) Third instar wing imaginal discs stained for Sens; only the wing pouch region is shown. (J) In wild type, sensory organ precursors for L3 sensilla are indicated (arrow). (K) *sca-Gal4>UAS-D-mib* lacks some L3 sensory precursors. (L) *sca-Gal4>UAS-D-mibΔRF* shows ectopic L3 sensory precursors. As the sensory multiplication defect is more prominent at later times, the disc in panel L is slightly older than those of panels J and K. (M) Cut expression at the prospective wing margin (WM) in wild type. (N) *dpp-Gal4, UAS-D-mib* shows normal wing margin development. (O) *dpp-Gal4, UAS-D-mibΔRF* disc shows a gap in the wing margin in D-mibΔRF-expressing cells (asterisk).

infer to be the RING finger most critical for Mib function. A construct that lacks all three C-terminal RING fingers (D-mibΔ3RF) also strongly antagonizes Notch signaling, but its activity is less potent relative to D-mibΔRF in all settings examined (summarized in Fig. 1, and data not shown). The developmental effects of ectopic D-mib, D-mibΔRF and D-mib3ΔRF are confined entirely to Notch-regulated patterning events. However, further truncation of the ankyrin repeats resulted in an isoform (D-mib-N) with weakened ability to induce vein thickening, and also potentially nonspecific activity, manifested by wing blistering (Fig. 1 and data not shown). Finally, misexpression of the ankyrin repeats and RING finger domains had no detectable effects on development (Fig. 1 and data not shown).

D-mib physically associates with both Delta and Serrate

Our loss- and gain-of-function analyses indicate that the major function of D-mib is to regulate Notch signal transduction. As Delta is a bona fide substrate of zebrafish Mib (Chen and Corliss, 2004; Itoh et al., 2003), we tested for a physical association of D-mib and Delta by co-immunoprecipitation. We transfected 293T cells with Delta and various D-mib expression vectors, and performed co-immunoprecipitation in both directions. Although Delta did not successfully co-immunoprecipitate full-length D-mib, it did associate with all isoforms that contain the D-mib N terminus and lack the C-terminal RING finger (namely D-mib-N, D-mibΔ3RF and D-mibΔRF, Fig. 4A, lanes 2-4). Conversely, these same D-mib isoforms efficiently co-immunoprecipitated Delta (Fig. 4B, lanes 12-14); full-length D-mib also showed modest association with Delta in this direction (Fig. 4B, lane 11). We consistently observed that the presence of full-length D-mib reduced Delta levels (Fig. 4B, lane 16), which might account for why this interaction is poorly detected. Notably, D-mib-N showed the strongest interaction with Delta. In fact, immunoprecipitated D-mib-N brought down both full-length Delta and cleaved Delta^{IC} (Fig. 4B, lane 12), consistent with a direct interaction between the N terminus of D-mib and the intracellular domain of Delta. A truncated D-mib protein lacking the N-terminal domain (D-mib-C) showed no binding to Delta (Fig. 4A, lane 5, and Fig. 4B, lane 15), demonstrating that this region is crucial for association with Delta.

We also tested for physical association between D-mib proteins and Serrate. D-mib:Serrate interactions appeared to be somewhat weaker than D-mib:Delta interactions; however, the overall profile of the different D-mib truncations in association with Serrate and Delta was identical (Fig. 4C, lanes 21-25). These findings allow us to conclude that the N terminus of D-mib mediates physical association with both *Drosophila* DSL ligands. In addition, full-length D-mib similarly reduced the accumulation of Serrate (Fig. 4C, lane 26), indicating that D-mib downregulates both DSL ligands.

Our in vitro data correlate well with our in vivo studies, in that all RING-finger-deleted D-mib isoforms that retain the ability to associate with DSL ligands (D-mib-N, D-mibΔRF and D-mib3ΔRF) have at least some ability to inhibit Notch signaling. However, full specificity and activity of D-mib requires inclusion of the ankyrin repeats and the two non-canonical RING fingers. Curiously, there is no significant similarity at the primary amino acid level between the

intracellular domains of Delta and Serrate. In this regard, it is relevant to note that Xenopus Neur (X-Neur) robustly regulates *Drosophila* Delta in vivo (Deblandre et al., 2001), even though there is no significant similarity between the intracellular domains of Delta and X-Delta. D-mib and Neur may therefore recognize a more hidden, possibly structural, feature that is shared by DSL ligands.

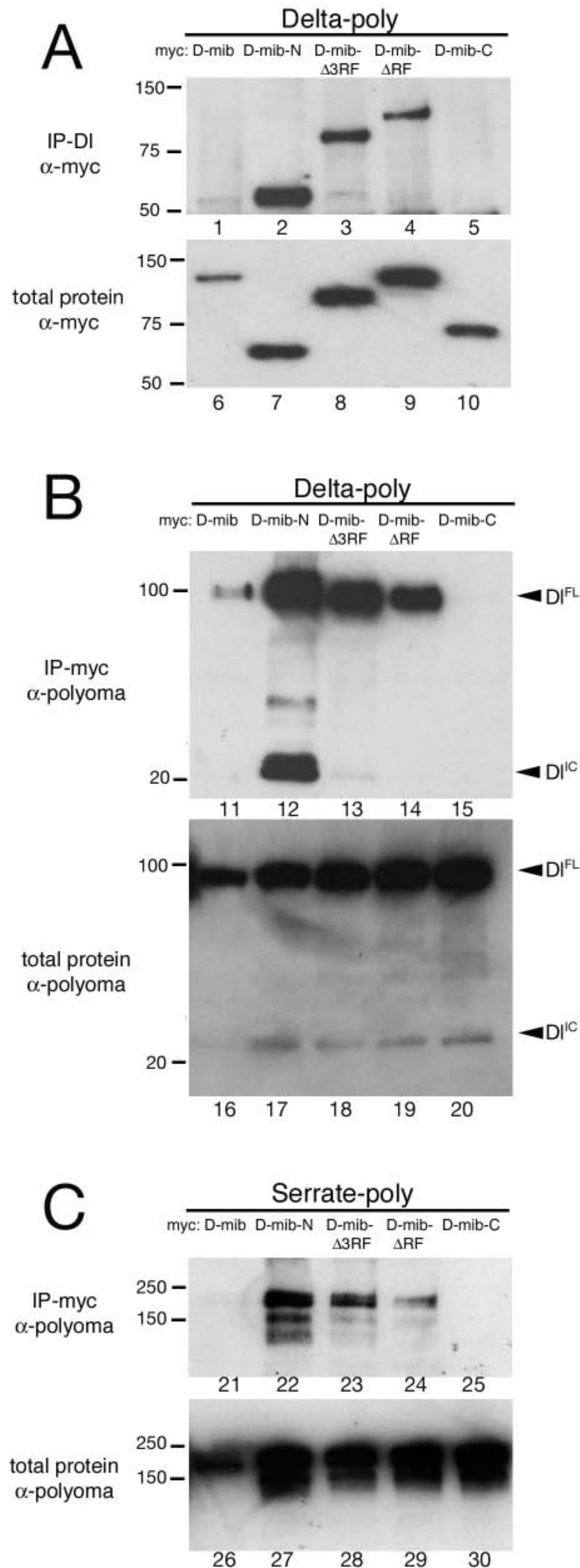

Fig. 4. The N terminus of D-mib mediates physical association with both Delta and Serrate. Co-immunoprecipitation was performed on lysates from 293T cells transfected with polyoma-tagged DSL ligands and Myc-tagged D-mib proteins. Structures of D-mib variants are depicted in Fig. 1. In transfected cells, D-mib proteins appear as single bands (A, lanes 6-10), Delta is present in full-length form and as a cleavage product corresponding to its intracellular domain (B, lanes 16-20), and Serrate appears as a series of relatively closely migrating bands (C, lanes 26-30). (A) Delta efficiently co-immunoprecipitates D-mib-N, D-mibΔ3RF and D-mibΔRF (lanes 2-4). (B) Delta is efficiently co-immunoprecipitated by D-mib-N, D-mibΔ3RF and D-mibΔRF (lanes 12-14); D-mib-N also associates with the cleaved intracellular domain of Delta (lane 12). Full-length D-mib interacts weakly with Delta (lane 11), but levels of Delta are also decreased in the presence of D-mib (lane 16). (C) Serrate is co-immunoprecipitated by D-mib-N, D-mibΔ3RF and D-mibΔRF (lanes 22-24), and D-mib reduces overall levels of Serrate (lane 26). In all cases, the interaction between DSL ligands and D-mib-N is strongest.

D-mib promotes the signaling activity of DSL ligands

We have shown that D-mib is positive component of *Drosophila* Notch signaling that physically interacts with both DSL ligands. To further explore its function in the Notch pathway, we assayed the ability of D-mib to influence DSL ligand activity in vivo. Misexpression of Delta along the anteroposterior compartment boundary of the wing disc using *dpp-Gal4* strongly induces disc overgrowth and ectopic wing margin in the dorsal wing pouch (Doherty et al., 1996; Panin et al., 1997) (Fig. 5A,B,G,H). By contrast, Delta does not induce ectopic margin ventrally (Fig. 5B). Co-misexpression of D-mib with Delta strongly potentiated Delta signaling, resulting in increased ventral disc overgrowth and ectopic wing margins that span the ventral compartment, both anterior and posterior to the *dpp-Gal4* domain (Fig. 5C,I). Conversely, co-misexpression of D-mibΔRF with Delta completely suppressed the activity of exogenous Delta, so that no ectopic margin or disc overgrowth was seen (Fig. 5D,J) In fact, misexpression of Delta was unable to rescue the loss of endogenous wing margin induced by D-mibΔRF (compare Fig. 3O with Fig. 5D). We conclude that D-mibΔRF simultaneously inhibits endogenous and exogenous Delta activity.

We also examined the ability of D-mib to influence Serrate signaling. As observed previously, ectopic Serrate efficiently promotes ectopic wing margin and disc overgrowth in the ventral compartment (Fig. 5E,K). We did not observe any consistent alteration in this phenotype when D-mib was co-misexpressed with Serrate; strong ectopic margins were present ventrally but none were ever found dorsally (data not shown). However, as seen with Delta, ectopic Serrate signaling was completely blocked by co-expression of D-mibΔRF (Fig. 5F,L). This is consistent with the observation that ectopic Serrate fails to rescue wing margin in *D-mib* discs (Le Borgne et al., 2005). Thus, both DSL ligands appear to be nonfunctional in the presence of D-mibΔRF, a finding that reinforces the essential nature of ubiquitination to DSL ligand activity in *Drosophila*.

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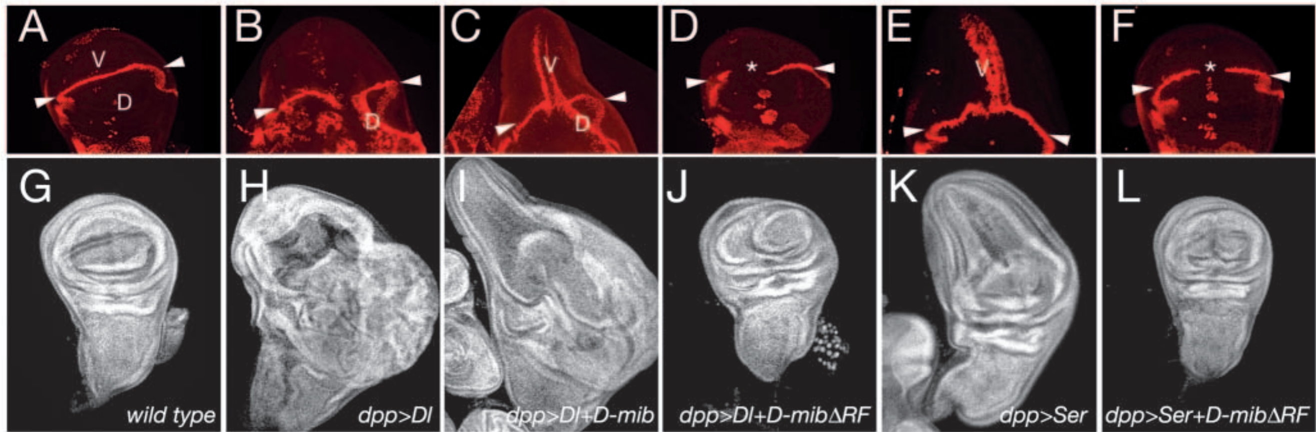

Fig. 5. D-mib promotes the signaling activity of DSL ligands. Shown are wing imaginal discs doubly stained for Cut (A-F) and DNA (G-L); only the wing pouch region is shown in (A-F). (A,G) Wild type. Expression of Cut at the wing margin is denoted with two arrowheads, and the ventral (V) and dorsal (D) regions of the wing pouch are marked. (B,H) *dpp-Gal4>UAS-Delta* disc is strongly overgrown (compare disc sizes of G and H). A strong ectopic margin is seen in the dorsal wing pouch (D) posterior to the *dpp-Gal4* domain. (C,I) *dpp-Gal4>UAS-Delta, UAS-D-mib* disc is similarly overgrown; however, strong ectopic margins are now induced in the ventral compartment (V) both anterior and posterior to the *dpp-Gal4* domain. (D,J) *dpp-Gal4>UAS-Delta, UAS-D-mibΔRF* shows complete suppression of Delta-induced disc overgrowth (compare J with H), and complete inhibition of Delta-induced margin development. In addition, there is a large gap in the endogenous wing margin in the *dpp-Gal4* domain (D, asterisk), as seen with *UAS-dpp-Gal4>UAS-D-mibΔRF* discs (see Fig. 6O). (E,K) *dpp-Gal4>UAS-Serrate* disc is strongly overgrown and shows ectopic ventral margins (V). (F,L) *dpp-Gal4>UAS-Serrate, UAS-D-mibΔRF* shows complete suppression of Serrate-induced disc overgrowth and margin induction, and a gap in the endogenous wing margin can be seen (F, asterisk).

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D-mib induces DSL ligand internalization and degradation

Le Borgne and colleagues have recently shown that *D-mib* mutant cells display a selective defect in DSL ligand internalization. Specifically, their antibody uptake assays demonstrated that *D-mib* is required for Serrate, but not Delta, endocytosis in living epithelial cells of wing imaginal discs (Le Borgne et al., 2005). In addition, they observed that *D-mib* cells show elevated accumulation of Serrate at the apical plasma membrane and a decrease in Serrate^{+} vesicles, whereas no corresponding alteration in Delta accumulation or localization was seen (Le Borgne et al., 2005). We verified that *D-mib*^{1} imaginal discs show a primary defect in Serrate, but not Delta accumulation (Fig. 6A-D).

Nevertheless, the fact that D-mib physically associates with and modulates the signaling activity of both Serrate and Delta suggests that it regulates the internalization of both DSL ligands. This led us to examine the response of DSL ligands to ectopic D-mib. Here, we focused on the wing pouch region, where both ligands have characteristic expression in developing wing veins. The presumptive L3 wing vein expression of both Serrate and Delta is contained within the domain of *dpp-Gal4* activity (Fig. 6E-H, asterisk), and misexpression of Neur using this driver strongly reduces the overall steady state level of Delta (Lai et al., 2001). We find that D-mib similarly decreases the overall level of endogenous Delta, particularly at the apical plasma membrane (Fig. 6J). D-mib decreases the level of Serrate to a lesser extent; nevertheless, it was clear that the level of Serrate at the apical plasma membrane is reduced in the presence of ectopic D-mib (Fig. 6I). Conversely, D-mibΔRF strongly increased the steady state levels of both Delta and Serrate (Fig. 6K,L), demonstrating that D-mib requires ubiquitin ligase function to induce the internalization of DSL ligands.

Although an abnormally large amount of Serrate and Delta accumulates at the apical plasma membrane in the presence of D-mibΔRF, their internalization was not completely blocked. Consistent with this, it was recently shown that vesicular Serrate can still be detected in *D-mib* mutant cells (Le Borgne et al., 2005). We also note that DSL ligands showed distinct behavior in the presence of D-mibΔRF, as Serrate accumulates in extremely large apical intracellular aggregates that co-localize only partially with Delta (Fig. 6K,L, insets). As Serrate and Delta are nonfunctional in the presence of D-mibΔRF (Fig. 5), the internalization of DSL ligands does not strictly correlate with their activation.

The observation of changes in the steady-state levels and localization of DSL ligands does not by itself distinguish between transcriptional, post-transcriptional and post-translational mechanisms. For example, the increase in Delta induced by D-mibΔRF is likely to be partly a consequence of the associated neurogenic defect (Fig. 3), which should be associated with increased transcription of *Delta* (Schweisguth and Posakony, 1994). Even if the observed effects are the result of post-translational modification of DSL ligands by D-mib, one cannot confidently distinguish between mechanisms whereby internalization of membrane-localized Delta is specifically affected, as opposed to aberrant trafficking of Delta from the endoplasmic reticulum directly to endosomes. Previous studies of zebrafish Mib employed static assays in transfected tissue culture cells (Chen and Corliss, 2004; Itoh et al., 2003), and also do not distinguish these possibilities.

We therefore employed a dynamic assay using exogenously expressed Delta under the control of a heat-shock promoter. When induced with a 40-minute heat shock, high levels of Delta accumulate at the plasma membrane of all cells (Fig. 6M), and remain detectable there for many hours. In the presence of Neur, exogenously expressed Delta is correctly

trafficked to the plasma membrane, but is rapidly internalized into vesicles and is subsequently degraded (Lai et al., 2001). We find that D-mib has identical activity to Neur in this assay. Thirty-five minutes into the heat-shock regime, ectopic Delta could be detected at apical cell membranes in the presence of exogenous D-mib (see Fig. S1 in the supplementary material), indicating that trafficking of Delta is normal in the presence of elevated levels of D-mib. Strikingly, Delta is quickly internalized in the D-mib-expressing domain, so that all of the Delta protein is vesicular by 40 minutes after heat shock-mediated induction of Delta expression (Fig. 6N). By 90 minutes post-induction, almost all of the ectopic Delta has been degraded (Fig. 6O). The effect of D-mib on DSL ligands is relatively specific, as double staining experiments showed bulk localization of the Notch receptor to be largely unaffected at time points when large amounts of Delta were actively being internalized and degraded (see Fig. S2). As with the endogenous Delta assay, D-mibΔRF is unable to mediate Delta endocytosis in this assay, and high levels of Delta persist even at 120 minutes post-induction (Fig. 6P). Therefore, D-mib and Neur have identical abilities to induce the internalization and degradation of Delta in a RING finger-dependent fashion.

Neur and D-mib are functionally interchangeable

Our studies, together with those of others, collectively demonstrate that Neur and Mib possess very similar activities with respect to the regulation of DSL ligands and to Notch signaling. However, it is a fact that the two have no sequence or domain similarity apart from their C-terminal RING fingers. Moreover, their in vivo requirements for DSL ligand internalization appear to be distinct, with Neur being more important for Delta endocytosis and D-mib being more

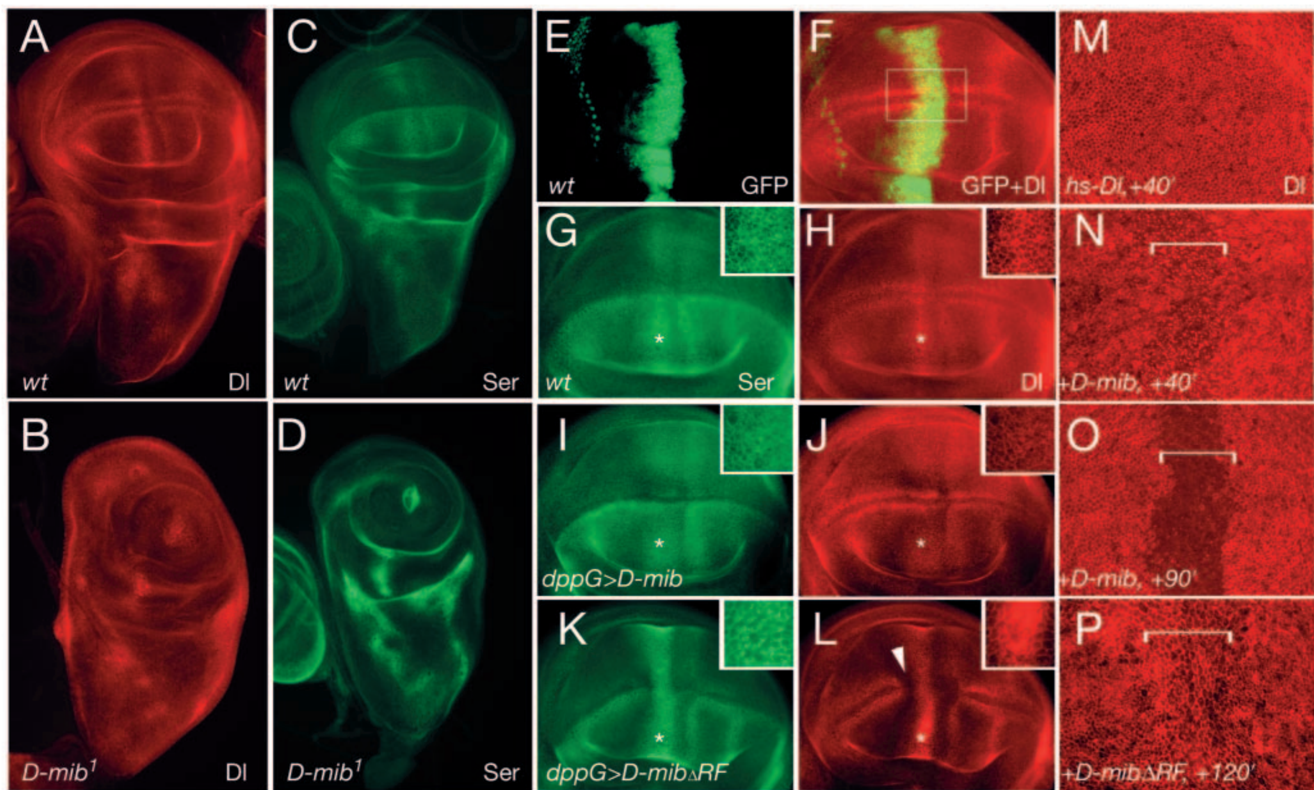

Fig. 6. In vivo regulation of Serrate and Delta localization and stability by D-mib. In all discs excepting E and F, Delta is in red and Serrate in green. (A-D) Tests of endogenous D-mib function. Wing imaginal discs from wild-type (A,C) and *D-mib¹* (B,D). Apart from the defective wing pouch development, Dl expression is fairly normal in *D-mib* discs (B), but elevated levels of Serrate are present and localized primarily to the plasma membrane (D). Imaging of Delta and Serrate in A-D was performed identically, so that relative protein levels between discs is comparable. (E-P) Tests of ectopic D-mib function. In these experiments, D-mib isoforms are expressed in a stripe at the anteroposterior compartment boundary using *dpp-Gal4*. (E,F) *dpp-Gal4, UAS-nGFP* double stained for GFP (E) and GFP + Delta (F). The L3 wing vein expression of Delta is contained within the domain of *dpp-Gal4* activity. (G-L) Wing discs double stained for Serrate and Delta. In all cases, the L3 wing vein domain is marked with an asterisk, and insets depict magnified apical views from this region. (G,H) Wild type. (I,J) *dpp-Gal4, UAS-D-mib* discs show a reduction of Serrate from the apical membrane and a strong decrease in both the apical and total level of Delta. (K,L) *dpp-Gal4, UAS-D-mibΔRF* disc displays strongly increased levels of both Serrate and Delta. Endogenous margin-specific expression of Serrate and Delta is also interrupted (arrowhead). Note the large apical intracellular aggregates of Serrate (K, inset). (M-P) Effect of D-mib proteins on exogenous Delta. In these panels, one copy of *hs-Delta* is present, and animals were heat-shocked at 38°C for 40 minutes, then allowed to rest for the indicated period of time prior to dissection and fixation. The regions shown in (N-P) correspond to the boxed region in F. (M) *hs-Delta/+*, 40 minute rest. Delta is present at the plasma membrane of all cells. (N) *hs-Delta, dpp-Gal4, UAS-D-mib*, 40 minute rest. All Delta within the D-mib-expressing domain is vesicular. (O) *hs-Delta, dpp-Gal4, UAS-D-mib*, 90 minute rest. All Delta within the D-mib-expressing domain has been degraded. (P) *hs-Delta, dpp-Gal4, UAS-D-mibΔRF*, 120 minute rest. D-mibΔRF fails to efficiently induce either the internalization or degradation of Delta.

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important for Serrate endocytosis. Therefore, we were interested to directly test the functional interchangeability of these two proteins and we approached this in two ways.

The first assay tested the ability of ectopic Neur and D-mib to genetically suppress the effects of their respective RING finger-deleted, dominant-negative counterparts. When activated using *dpp-Gal4*, the full-length proteins cause mild loss of veins and campaniform sensilla (Fig. 7A-C), whereas the dominant-negative proteins cause vein thickening, ectopic campaniform sensilla, and loss of wing margin (Fig. 7D,G); the effects of D-mib and D-mibΔRF are stronger than those of Neur and NeurΔRF, respectively. Ectopic Neur rescues the phenotype of *dpp-Gal4>NeurΔRF* flies, as does ectopic D-mib (Fig. 7E,F). Conversely, both ectopic D-mib and Neur can suppress the phenotype of *dpp-Gal4>D-mibΔRF* flies, although higher doses of Neur are required for rescue of *dpp-Gal4>D-mibΔRF* back to wild type (Fig. 7G-I and data not shown). The latter result is especially notable because, as shown in Fig. 5, neither ectopic Delta nor Serrate are able to rescue the wing margin defect induced by D-mibΔRF. In summary, both of these ubiquitin ligases can rescue the mutant phenotypes induced by their respective dominant-negative derivatives.

The second assay is more stringent, and tests the ability of ectopic Neur and D-mib to functionally rescue cell specification defects of *neur* mutant cells. *neur* rescue has proven challenging, possibly due to specific requirements in how *neur* is deployed spatially. For example, we failed to observe rescue of the neurogenic phenotype of *neur* mutant embryos by expressing Neur in stripes using a *ptc-Gal4* driver (data not shown). Here, we tested the ability of transgenes to rescue adult *neur* clonal phenotypes when activated with *sca-Gal4*. Neur is expressed in adult sensory organ precursors and in their lineages (Yeh et al., 2000), We reasoned that *sca-Gal4* might direct spatially relevant expression of transgenes, as its activity is elevated or exclusive to microchaete sensory precursor cells and persists in the sensory lineage (Abdelilah-Seyfried et al., 2000).

Neur is required at multiple steps during the development of peripheral sensilla. It is first required to restrict the sensory precursor fate amongst proneural cluster cells, and is subsequently required to direct multiple asymmetric cell fates in the sensory lineage. In the adult notum, clones of the hypomorphic allele *neur*^{A101} display tufted bristles (Fig. 8A) as a result of a mild defect in sensory precursor restriction, and subsequent development of supernumerary sensilla with normal cell complements. By contrast, similar clones of the null allele *neur*^{IF65} are bald (Fig. 8C) because of the combined effects of strongly defective lateral inhibition, followed by mis-specification of all sensory lineage cell fates as neurons (Lai and Rubin, 2001a; Yeh et al., 2000). We made notum clones of both alleles using the MARCM system and *ubx-FLP*, and tested the ability of full-length *UAS-neur* and *UAS-D-mib* to rescue *neur* mutant clones that have activated *sca-Gal4*. In Fig. 8A,B, we show that Neur completely rescues the bristle tufting phenotype of *neur*^{A101} clones, demonstrating the efficacy of this rescue strategy.

Using these scheme, we observed that D-mib rescues bald clones of the null allele *neur*^{IF65} back to a mild bristle tufting phenotype similar to *neur* hypomorphic clones, with 1-3 bristle organs at each position (Fig. 8C,D). We may infer rescue of multiple functions of *neur* by this phenotype. First, the presence of outer support cells (including socket and shafts) indicates that D-mib can rescue the pIIA-pIIb and the socket-

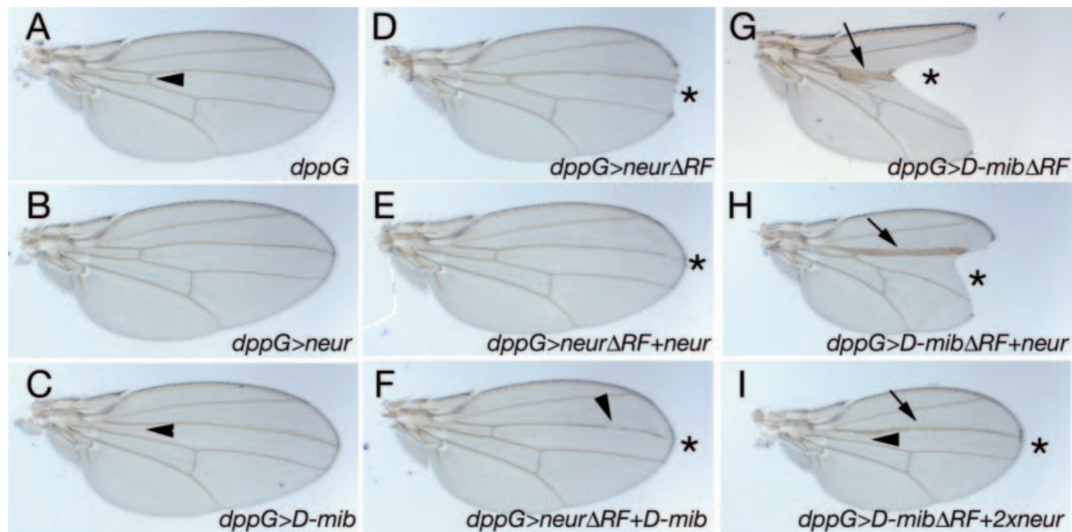

Fig. 7. Mutual phenotypic suppression of wild-type Neur and D-mib proteins with their RING-deleted counterparts. Shown are adult wings of the depicted genotypes, with a focus on wing vein determination and wing margin development. (A) *dpp-Gal4/+* wing. Arrowhead denotes the anterior crossvein and asterisk marks the distal wing margin. (B) *dpp-Gal4, UAS-neur* wing is mostly wild type. (C) *dpp-Gal4, UAS-D-mib* wing shows mild wing vein loss (arrowhead). (D) *dpp-Gal4, UAS-neurΔRF* wing exhibits a distal wing notch (asterisk) and a very mildly thickened L3 vein. Wing notching induced by neurΔRF is completely suppressed by the co-expression of *UAS-neur* (E, asterisk), as well as by *UAS-D-mib* (F, asterisk); wing vein loss is often observed in the latter (F, arrowhead). (G) *dpp-Gal4, UAS-D-mibΔRF* wing displays an enormous wing notch (asterisk) and a severely thickened L3 wing vein remnant (arrow). Both phenotypes are partially suppressed by the co-expression of one copy of *UAS-neur* (H, arrow and asterisk) and are almost completely suppressed by the co-expression of two copies of *UAS-neur* (I, arrow and asterisk). Note that some wing vein loss is even evident in the latter genotype (I, arrowhead).

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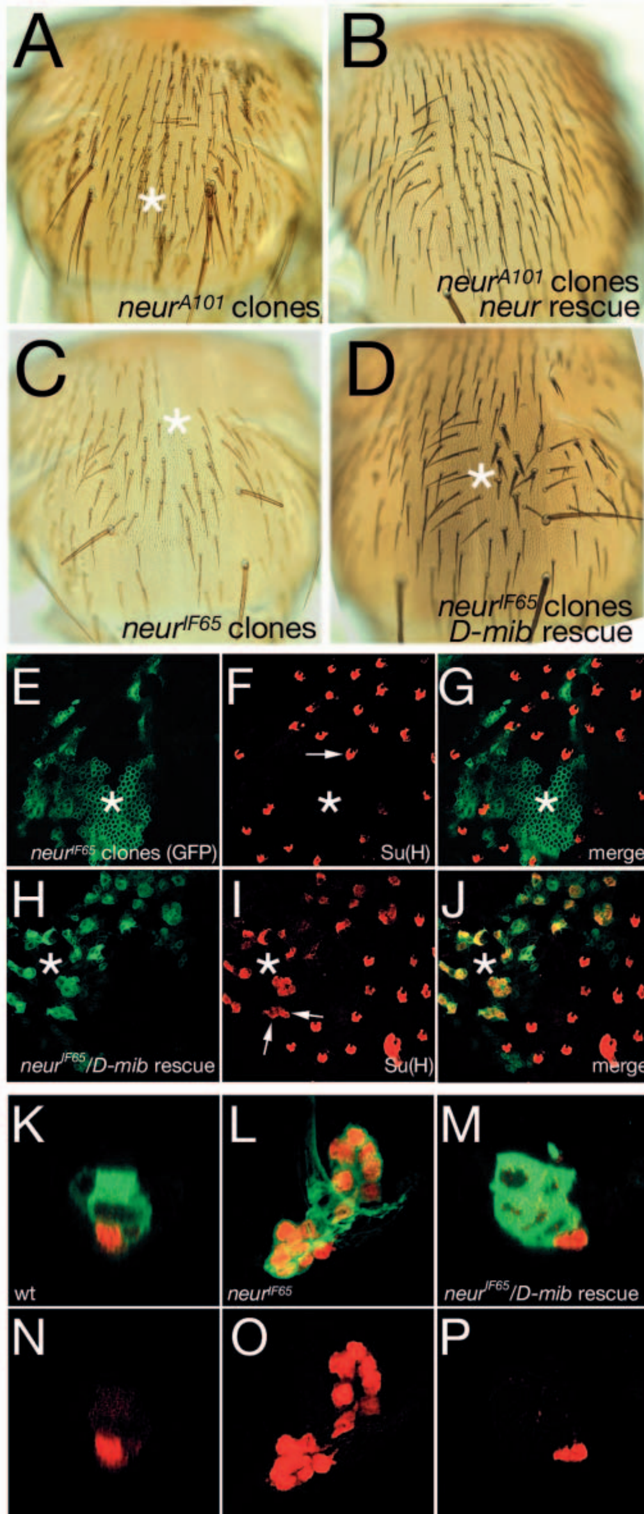
shaft cell fate decisions that are defective in the absence of *neur*. Second, the strong lateral inhibition defect of *neur*^{IF65} is largely corrected. We may be confident that rescue was obtained in these experiments given the appearance of a distinct, phenotypic class when the appropriate E3 transgene was present in the genetic background, and the fact that the balding phenotype of *neur*^{IF65} clones is 100% penetrant.



However, as the clone borders were not visualized in these experiments, we sought direct confirmation of phenotypic rescue of marked mutant cells.

We stained mutant sensory clusters that were positively marked by MARCM expression of *sca-Gal4>UAS-pon-GFP*. We first stained 36 hours after puparium formation (APF) pupae for Su(H), a marker of external socket sockets. *neur*^{IF65} mutant cells never express Su(H), whereas in the presence of ectopic D-mib, mutant sensory clusters displayed small groups of Su(H)$^+$ cells (Fig. 8E-J). We next stained for the neuronal marker Elav. Individual rescued sensory organs show a strongly reduced neurogenic phenotype in which two to three Elav$^+$ cells are present, instead of the large clusters present in *neur* mutant clusters (Fig. 8K-P, red). Finally, there is a strong reduction in the overall number of cells in each cluster (Fig. 8K-M, green). Together, these data directly demonstrate the rescue of pIIa specification (which gives rise to the outer sensory cells) and substantial rescue of the *neur* lateral inhibition defect (because clusters usually contained two to three cells of each lineage fate). We take the ability of D-mib to replace *neur* during multiple cell fate decisions in vivo as strong evidence for their functional similarity.

Discussion

D-mib is a ubiquitin ligase that regulates both *Drosophila* DSL ligands and promotes Notch signaling

Mind bomb was originally characterized in zebrafish through forward genetic studies of a novel locus that was absolutely required for Notch-mediated lateral inhibition of neural precursors (Itoh et al., 2003). The presence of a clear *Drosophila* ortholog of Mind bomb was somewhat of a surprise then, given that: (1) almost without exception, different species display similar functional requirements for evolutionarily

Fig. 8. Functional replacement of Neur by D-mib. Shown are dorsal thoraces from adult flies (A-D) or 36-hour APF pupae (E-P) containing *ubx-FLP*-generated MARCM clones of cells that are simultaneously mutant for *neur* and express *sca-Gal4* and relevant UAS-transgenes (please refer to the Materials and methods for details of the genetics). (A) Clones of the weak allele *neur*^{A101} exhibit a bristle tufting phenotype (asterisk), wherein multiple sensory organs are present at individual positions. (B) The tufting phenotype of *neur*^{A101} clones is completely rescued by expression of *UAS-neur*. (C) Clones of the stronger allele *neur*^{IF65} are bald (asterisk), due to conversion of outer sensory cell fates into neurons. (D) The balding phenotype of *neur*^{IF65} clones is rescued back to a mild-tufting phenotype (asterisk) by expression of *UAS-D-mib*. (E-G) *neur*^{IF65} sensory clusters marked by GFP expression (due to MARCM activation of *sca-Gal4>UAS-pon-GFP*: E, asterisk) fail to express Su(H), a marker of socket cell fate (red, F,G); arrow in F indicates a Su(H)$^+$ nucleus. (H-J) *neur*^{IF65} sensory clusters with sensory specific expression of D-mib show rescue of Su(H) expression. Note that small clusters of Su(H)$^+$ cells are usually seen, indicating a partial rescue of the strong *neur*^{IF65} lateral inhibition defect. (K-P) X-Z confocal sections through individual *neur* mutant sensory clusters expressing GFP and stained for ELAV, a neuronal marker. (K,N) A single neuron and the two large cell bodies of the socket and shaft cells are present in wild type. (L,O) A large mass of neurons is found in a *neur*^{IF65} sensory cluster, and large cell bodies indicative of outer cell fates are absent. (M,P) Rescue of the *neur*^{IF65} neurogenic defect and socket/shaft differentiation by ectopic D-mib.

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conserved components of the Notch pathway (Lai, 2004); (2) this locus never emerged from any of the extensive *Drosophila* genetic screens for neurogenic genes and components of the Notch pathway (Abdelilah-Seyfried et al., 2000; Fortini and Artavanis-Tsakonas, 1994; Go and Artavanis-Tsakonas, 1998; Verheyen et al., 1996; Xu and Artavanis-Tsakonas, 1990); and (3) it is a large locus that might be expected to have been relatively easily hit, as has proven to be the case in zebrafish (Golling et al., 2002; Jiang et al., 1996). It was therefore an open question as to whether D-mib was actually a component of Notch signaling in *Drosophila*, a question that our present studies allow us to answer in the affirmative.

Although *D-mib* mutants have in fact been previously isolated, they are only very weakly neurogenic (Le Borgne et al., 2005; Melendez et al., 1995). This might partially explain how it was missed in earlier screens for components of the Notch pathway. By contrast, *D-mib* is absolutely required for the execution of several other Notch-regulated development events, including wing margin specification, eye growth and leg joint specification. Misexpression of full-length and dominant-negative truncations of D-mib affects Notch-mediated pattern formation even more broadly, including many settings that do not normally require *D-mib*. Biochemical and genetic experiments demonstrate that D-mib associates with both *Drosophila* DSL ligands, and promotes their internalization and signaling activity. However, dominant-negative D-mibΔRF binds Delta and Serrate but interferes with their normal trafficking and inhibits their signaling capacity. We infer that D-mibΔRF binding to both Delta and Serrate occludes endogenous Neur and D-mib from ubiquitinating and activating DSL ligands, which likely underlies the broad capacity of D-mibΔRF to inhibit Notch activation in virtually all settings of Notch signaling.

Curiously, NeurΔRF potentiates Delta signaling during wing margin induction just as full-length Neur does (Pavlopoulos et al., 2001), even though ectopic NeurΔRF otherwise strongly inhibits Notch signaling (Lai and Rubin, 2001a; Lai and Rubin, 2001b). We lack an explanation for this difference between NeurΔRF and D-mibΔRF, but it might hint at a functional difference between these DSL-regulating ubiquitin ligases. In almost every other regard, however, the activities and functions of D-mib/D-mibΔRF are highly reminiscent of Neur/NeurΔRF. In fact, we showed that D-mib can functionally replace Neur in a series of developmental decisions in vivo. Conversely, contemporaneous studies show that Neur can functionally replace D-mib during wing margin specification (Le Borgne et al., 2005). Nevertheless, the essential endogenous requirements for *neur* and *D-mib* are quite distinct, in that they are genetically required for different developmental processes and the respective mutants have differential effects on DSL ligands. Despite potent effects of ectopic D-mib on Delta localization and activity, Delta is mislocalized primarily only in *neur* mutant tissue (Lai et al., 2001; Pavlopoulos et al., 2001), whereas Serrate is mislocalized primarily only in *D-mib* mutant tissue (Le Borgne et al., 2005).

This apparent specificity is unexpected, as *D-mib* is expressed ubiquitously, and is therefore present in all Delta-expressing cells. Does endogenous D-mib normally regulate Delta as implied by its ability to associate with Delta, induce Delta endocytosis, and potentiate Delta signaling activity? A close examination of *D-mib* mutants reveals certain phenotypes that are either stronger than those of *Serrate* mutants (i.e. leg truncation) or are more suggestive of *Delta* loss of function (i.e. wing vein deltas and a mildly neurogenic phenotype in the adult thorax) (Le Borgne et al., 2005). These observations collectively imply that another ubiquitin ligase may co-regulate Delta and thereby partially compensate for loss of *D-mib*. Neur is a possible, but relatively poor, candidate to supply this function. Although it has a demonstrated role in regulating Delta, *neur* expression in imaginal tissue is restricted mostly to neural precursors and photoreceptors (Boulianne et al., 1991). A more tantalizing candidate is D-mibl (CG17492), which we suspect may also prove to regulate DSL ligands. In support of this, systematic yeast two-hybrid screening has identified a specific interaction between D-mibl and Delta (http://pim.hybrigenics.com/pimriderext/droso/prflybase.html). Therefore, the in vivo function of D-mibl with regard to the regulation of DSL ligands deserves future investigation.

We have shown that both *Drosophila* DSL ligands are regulated by ubiquitin ligases that promote ligand endocytosis. Still, the mechanism by which endocytosis promotes DSL ligand activity is still unclear. An earlier proposal was that Delta endocytosis might facilitate Notch proteolytic processing by helping to unmask the S2 Notch cleavage site (Parks et al., 2000). Other models suggested that ligand endocytosis might promote ligand clustering or clearance of extracellular N^{ECD} (Le Borgne and Schweisguth, 2003a). Most recently, genetic studies of the epsin Liquid Facets (Lqf), an apparently DSL ligand-specific endocytic component (Overstreet et al., 2004; Wang and Struhl, 2004), have led to further insight into this mechanism. In particular, a provocative model was put forth suggesting that Lqf directs Delta into an endocytic recycling compartment, and that Delta recycling back to the plasma membrane is a prerequisite for ligand activation (Wang and Struhl, 2004). The finding that Serrate is similarly regulated by endocytosis via D-mib (this study) (Le Borgne et al., 2005) suggests further avenues for testing this model. For example, it will be informative to ask whether *lqf* shows defects in Serrate trafficking, or if the requirement of Serrate for *D-mib* can be bypassed by shunting it through an endocytic recycling pathway.

Even though Neur and D-mib promote DSL ligand activity by stimulating ligand endocytosis, they also efficiently induce ligand degradation. This might conceptually be at odds with the proposition that ligand recycling back to the plasma membrane underlies DSL ligand activation (Wang and Struhl, 2004). These activities might be reconciled if ubiquitination permits a portion of the DSL ligand pool to enter the select Lqf-mediated recycling pathway, but directs the bulk of DSL ligands for degradation. Consistent with this, Lqf is strictly required for DSL ligand activation, but is not required for bulk endocytosis of DSL ligands (Wang and Struhl, 2004). If ubiquitination is prerequisite for DSL ligand activation but also makes DSL ligands prone to degradation, this would prevent endless recycling of activated ligands and thereby limit the temporal extent of Notch pathway activation. The strategy of coupling activation with downregulation is seen with Notch itself. Ligand-induced Notch cleavage liberates activated $Notch^{intra}$, which is a potent regulator of gene expression as a nuclear co-activator for Su(H). However, nuclear $Notch^{intra}$ also becomes a substrate for ubiquitination by the ubiquitin ligase Sel-10, and is rapidly degraded (Gupta-Rossi et al., 2001;

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Hubbard et al., 1997; Oberg et al., 2001; Wu et al., 2001). Coupled activation and downregulation allows for precise temporal control of signaling by limiting the lifetime of activated signaling components (Schweisguth, 2004).

Evolutionary flux in regulation of DSL ligands by ubiquitin ligases

The presence of Neur and Mib homologs in both fly and vertebrate genomes suggests that both proteins were present and regulated DSL ligands in the ancient common ancestor of these species. We have demonstrated surprising functional overlap between these structurally unrelated ubiquitin ligases in regulating DSL ligand activity. What, then, was the rationale of evolving such different proteins to perform the same function?

As discussed earlier, the genetic implication that Neur and D-mib preferentially regulate Delta and Serrate, respectively, belies the ability of these enzymes to interact with and regulate the localization and signaling activity both DSL ligands. While it remains to be seen whether Neur regulates Serrate in addition to its documented substrate Delta, we showed that D-mib directly and efficiently regulates both Delta and Serrate. Therefore, these ubiquitin ligases did not obviously co-evolve with different classes of DSL ligands.

Another possible explanation lies in the curious observation that Neur is genetically required mostly in settings that involve 'lateral inhibitory' Notch signaling, wherein Notch restricts a cell fate amongst equipotent cells. By contrast, D-mib is required largely in settings that involve 'inductive' Notch signaling, which occurs between non-equivalent cell populations. This apparent division of labor raises the possibility that different ubiquitin ligases could help to specify the appropriate response to Notch activation in each developmental setting.

This hypothesis, however, is not particularly supported by the observations that D-mib and Neur can functionally replace each other in a variety of processes. Neither does this correlation hold up in other species, because Neur mediates lateral inhibition of neural precursors in flies, whereas Mib mediates lateral inhibition of neural precursors in fish. This latter finding highlights the plasticity in how these ubiquitin ligases have been deployed during evolution, and is consistent with a model in which fish Mib has subsumed the function of fly Neur during neurogenesis (or vice versa). This may have occurred by appropriate changes in the transcriptional regulation of these genes. Given this likely scenario, one wonders whether it might not have been more evolutionarily expedient to have diversified the function of duplicated, paralogous genes. There are indeed multiple *neur* and *mib* genes in vertebrates, and two *mib* genes in flies. Of course, it might be argued that a similar conundrum concerns the co-existence of the HECT domain and the RING finger/U box as unrelated protein domains that both catalyze E3 ubiquitin ligation.

How general is the requirement for DSL ligand endocytosis across evolution? The neurogenic mutant phenotypes of *Drosophila neur* and zebrafish *mib*, along with the involvement of *Xenopus neur* in lateral inhibition, show that DSL ligand ubiquitination is required in both invertebrates and vertebrates. However, thorough loss-of-function genetic studies are incomplete in any organism and are complicated by the duplication of *neur* and/or *mib* genes. For example, knockout of murine *Neur1* did not affect Notch signaling (Ruan et al., 2001; Vollrath et al., 2001), possibly due to functional overlap with *Neur2*.

Our present work clearly demonstrates that the vast majority of Notch-regulated settings during *Drosophila* development are strictly dependent on either Neur or D-mib. Thus, DSL ligand ubiquitination and endocytosis appears to be obligate in *Drosophila*. In light of this, the situation in nematodes provides an interesting possible counter-example. *C. elegans* lacks a recognizable *Mind bomb* ortholog, but does possess a single *Neur* gene. However, in contrast to what has been found in *Drosophila*, where intracellular deletions of the DSL ligands have a dominant-negative activity (Sun and Artavanis-Tsakonas, 1996; Sun and Artavanis-Tsakonas, 1997), the extracellular domains of the DSL ligands LAG-2 and APX-2 can fully rescue the *lag-2* mutant and can activate Notch signaling ectopically (Fitzgerald and Greenwald, 1995). A more recent analysis actually revealed a large family of putative secreted DSL ligands in the worm, at least one of which (DSL-1) is a bona fide DSL ligand (Chen and Greenwald, 2004). This suggests that nematodes may have dispensed with ubiquitination and endocytosis of DSL ligands in at least some settings of Notch signaling. Nevertheless, a nematode ortholog of epsin/Lqf (*Ce-epn-1*) participates in Notch signaling during germline development (Tian et al., 2004). The functional relationships amongst *Ce-epn-1*, nematode DSL ligands and any potential DSL-regulating E3s in nematodes remain to be determined.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/10/2319/DC1

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