Two Distinct E3 Ubiquitin Ligases Have Complementary Functions in the Regulation of Delta and Serrate Signaling in Drosophila

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Signaling by the Notch ligands Delta (Dl) and Serrate (Ser) regulates a wide variety of essential cell-fate decisions during animal development. Two distinct E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), have been shown to regulate Dl signaling in Drosophila melanogaster and Danio rerio, respectively. While the neur and mib genes are evolutionarily conserved, their respective roles in the context of a single organism have not yet been examined. We show here that the Drosophila mind bomb (D-mib) gene regulates a subset of Notch signaling events, including wing margin specification, leg segmentation, and vein determination, that are distinct from those events requiring neur activity. D-mib also modulates lateral inhibition, a neur- and Dl-dependent signaling event, suggesting that D-mib regulates Dl signaling. During wing development, expression of D-mib in dorsal cells appears to be necessary and sufficient for wing margin specification, indicating that D-mib also regulates Ser signaling. Moreover, the activity of the D-mib gene is required for the endocytosis of Ser in wing imaginal disc cells. Finally, ectopic expression of neur in D-mib mutant larvae rescues the wing D-mib phenotype, indicating that Neur can compensate for the lack of D-mib activity. We conclude that D-mib and Neur are two structurally distinct proteins that have similar molecular activities but distinct developmental functions in Drosophila.

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Introduction

Cell-to-cell signaling mediated by receptors of the Notch (N) family has been implicated in various developmental decisions in organisms ranging from nematodes to mammals [1]. N is well-known for its role in lateral inhibition, a key patterning process that organizes the regular spacing of distinct cell types within groups of equipotent cells. Additionally, N mediates inductive signaling between cells with distinct identities. In both signaling events, N signals via a conserved mechanism that involves the cleavage and release from the membrane of the N intracellular domain that acts as a transcriptional co-activator for DNA-binding proteins of the CBF1/Suppressor of Hairless/Lag-2 (CSL) family [2].

Two transmembrane ligands of N are known in Drosophila, Delta (Dl) and Serrate (Ser) [3]. Dl and Ser have distinct functions. For instance, Dl (but not Ser) is essential for lateral inhibition during early neurogenesis in the embryo [4]. Conversely, Ser (but not Dl) is specifically required for segmental patterning [5]. Some developmental decisions, however, require the activity of both genes: Dl and Ser are both required for the specification of wing margin cells during imaginal development [6,7,8,9,10]. These different requirements for Dl and Ser appear to primarily result from their non-overlapping expression patterns rather than from distinct signaling properties. Consistent with this interpretation, Dl and Ser have been proposed to act redundantly in the sensory bristle lineage where they are co-expressed ([11]; note, however, that results from another study have indicated a non-redundant function for Dl in the bristle lineage [12]). Furthermore, Dl and Ser appear to be partially interchangeable because the forced expression of Ser can partially rescue the Dl neurogenic phenotype [13]. Additionally, the ectopic expression of Dl can partially rescue the Ser wing phenotype [14]. The notion that Dl and Ser have similar signaling properties has, however, been recently challenged by the observation that human homologs of Dl and Ser have distinct instructive signaling activity [15].

Endocytosis has recently emerged as a key mechanism regulating the signaling activity of Dl. First, clonal analysis in Drosophila has suggested that dynamin-dependent endocytosis is required not only in signal-receiving cells but also in signal-sending cells to promote N activation [16]. Second, mutant Dl proteins that are endocytosis defective exhibit reduced signaling activity [17]. Third, two distinct E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), have recently been shown to regulate Dl endocytosis and N activation in Drosophila and Danio rerio, respectively [18,19,20,21,22,23,24,25]. Ubiquitin is a 76-amino-acid polypeptide that is covalently linked to substrates in a multi-step process that involves a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin–protein

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Abbreviations: Dl, Delta; D-mib, Drosophila mind bomb; DSHB, Developmental Studies Hybridoma Bank; D-V, dorsal-ventral; GFP, green fluorescent protein; Mib, Mind bomb; N, Notch; Neur, Neuralized; RING, really interesting new gene; Ser, Serrate; wg, wingless; YFP, yellow fluorescent protein

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lignase (E3). E3s recognize specific substrates and catalyze the transfer of ubiquitin to the protein substrate. Ubiquitin was first identified as a tag for proteins destined for degradation. More recently, ubiquitin has also been shown to serve as a signal for endocytosis [26,27]. Mib in D. rerio and Neur in Drosophila and Xenopus have been shown to associate with DI, regulate DI ubiquitination, and promote its endocytosis [18,19,20,22,25,28]. Moreover, genetic and transplantation studies have indicated that both Neur and Mib act in a non-autonomous manner [18,21,22,23,25,29], indicating that endocytosis of DI is associated with increased DI signaling activity. Finally, epsin, a regulator of endocytosis that contains a ubiquitin-interacting motif and that is known in Drosophila as Liquid facet, is essential for DI signaling [30,31]. In one study, Liquid facet was proposed to target DI to an endocytic recycling compartment, suggesting that recycling of DI may be required for signaling. Accordingly, signaling would not be linked directly to endocytosis, but endocytosis would be prerequisite for signaling [30]. How endocytosis of DI leads to the activation of N remains to be elucidated. Also, whether the signaling activity of Ser is similarly regulated by endocytosis is not known.

Neur and Mib proteins completely differ in primary structure. Drosophila Neur is a 754-amino-acid protein that contains two conserved Neur homology repeats of unknown function and one C-terminal catalyticreally interesting new gene (RING) domain. D. rerio Mib (also known as DIP-1 in the mouse [32]) is a 1,050-amino-acid protein with one ZZ zinc finger domain surrounded by two Mib/HERC2 domains, two Mib repeats, eight ankyrin repeats, two atypical RING domains, and one C-terminal catalytic RING domain. Both genes have been conserved from flies to mammals [18,19,33,34]. While genetic analysis has revealed that neur in Drosophila and mib in D. rerio are strictly required for N signaling, knockout studies of mouse NeuR1 has indicated that NEUR1 is not strictly required for N signaling [33,34]. One possible explanation is functional redundancy with the mouse NeuR2 gene. Conversely, the function of Drosophila mib (D-mib), the homolog of D. rerio mib gene, is not known.

To establish the respective roles of these two distinct E3 ligases in the context of a single model organism, we have studied the function of the Drosophila D-mib gene. We report here that D-mib, like D. rerio Mib, appears to regulate DI signaling during leg segmentation, wing vein formation, and lateral inhibition in the adult notum. We further show that D-mib is specifically required for Ser endocytosis and signaling during wing development, indicating for the first time, to our knowledge, that endocytosis regulates Ser signaling. Interestingly, the D-mib activity was found necessary for a subset of N signaling events that are distinct from those requiring the activity of the neur gene. Nevertheless, the ectopic expression of Neur compensates for the loss of D-mib activity in the wing, indicating that Neur and D-mib have overlapping functions. We conclude that D-mib and Neur are two structurally distinct proteins with similar molecular activities but distinct and complementary functions in Drosophila.

**Results**

**Isolation of D-mib Mutations**

The closest Drosophila homolog of the vertebrate mib gene is the predicted gene CG5841, D-mib [18]. The D-mib mutations identified are shown in Figure 1. A P-element inserted into the 5′ untranslated region of the D-mib gene was recently isolated (http://flypush.imgen bcm.tmc.edu/psearch/) (Figure 1A). Insertion of this P-element confers late pupal lethality. Lethality was reverted by precise excision of the P-element, suggesting that insertion of this P-element is a D-mib mutation, referred to as D-mib<sup>1</sup>. A 13.6-kb deletion that removes the entire D-mib coding region was selected by imprecise excision of this P-element. This deletion represents a null allele of D-mib and was named D-mib<sup>2</sup>. This deletion also deletes the 3′ flanking Rps531 gene (Figure 1A). The D-mib<sup>2</sup> and D-mib<sup>2</sup> mutant alleles did not complement the l(3)T2Cda<sup>12</sup> and l(3)T2Cda<sup>5</sup> lethal mutations that have been mapped to the same cytological interval as the D-mib gene [35]. This indicates that these two lethal mutations are D-mib mutant alleles, and they were therefore renamed D-mib<sup>1</sup> and D-mib<sup>2</sup>, respectively. The D-mib<sup>1</sup> and D-mib<sup>2</sup> mutations behave as generic null alleles (see Materials and Methods). In contrast, D-mib<sup>1</sup> is a partial loss-of-function allele because flies transheterozygous for D-mib<sup>1</sup> and any other D-mib null alleles are viable.

These four mutations identify the CG5841 gene as D-mib by the following evidence. First, lethality of homozygous D-mib<sup>1</sup> pupae is associated with the insertion of a P-element into the 5′ UTR of the D-mib gene. Second, genomic sequencing of the D-mib<sup>1</sup> allele revealed the presence of a stop codon at position 258 (Figure 1B). This allele is therefore predicted to produce a truncated protein devoid of the catalytic RING domain, consistent with D-mib<sup>1</sup> being a null allele. Genomic sequencing of the D-mib<sup>2</sup> allele showed that this mutation is associated with a valine-to-methionine substitution at a conserved position in the second Mib repeat (Figure 1B). Third, Western blot analysis showed that the D-mib protein was not detectable in imaginal disc and brain complex extracts prepared from homozygous D-mib<sup>1</sup> and D-mib<sup>1</sup>/D-mib<sup>2</sup> larvae (Figure 1C and C'). Fourth, the leaky, GAL4-independent expression of a UAS-D-mib transgene fully rescued the lethality of D-mib<sup>1</sup>/D-mib<sup>2</sup> flies (data not shown; see also Figure 1H). Thus, our analysis identified both complete and partial D-mib loss-of-function alleles.

**D-mib Regulates DI Signaling**

Complete loss of zygotic D-mib activity in homozygous D-mib<sup>1</sup> and D-mib<sup>2</sup> and trans-heterozygous D-mib<sup>1</sup>/D-mib<sup>2</sup>, D-mib<sup>1</sup>/D-mib<sup>2</sup> and D-mib<sup>2</sup>/D-mib<sup>2</sup> individuals led to late pupal lethality. Mutant pupae died as pharate adults showing ectopic macrochaetes, increased microchaete density on the dorsal thorax (Figure 1I and 1J), short legs lacking tarsal segmentation (Figure 1L and 1M), and nearly complete loss of eye and wing tissues (Figure 1D and 1E). Tissue losses were associated with a dramatic reduction in size of the eye field and of the wing pouch in mutant discs of third instar larvae (Figure 2A–2E). Hypomorphic D-mib<sup>2</sup>/D-mib<sup>2</sup> mutant flies only showed ectopic sensory organs, rough eyes, small wings, and thickened veins (Figure 1D, 1D′, 1G, and 1G′; data not shown).

All these phenotypes may result from reduced N signaling. More specifically, the bristle and leg phenotypes are likely to result from reduced signaling by DI (and not by Ser). Indeed, a reduction in DI-mediated lateral inhibition can result in ectopic sensory organs and increased bristle density on the body surface. In contrast, a complete loss of Ser signaling had no effect on bristle density (Figure 1K). Likewise, loss of DI
signaling has been shown to result in short unsegmented legs, similar to the ones seen in the absence of D-mib activity (Figure 1M), whereas a complete loss of Ser activity led to the formation of elongated unsegmented legs (Figure 1N) [36,37,38]. Finally, the vein phenotype seen in D-mib-hypomorphic flies is similar to the one seen in Dil mutant flies [39]. Together, these observations suggest that D-mib regulates Dil signaling in several developmental contexts. Consistent with this conclusion, we have shown that D-mib binds Dil and promotes Dil signaling and that overexpression of D-mib down-regulates the accumulation of Dil at the cell surface (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data).

**D-mib and neur Have Distinct Functions**

We then studied in more detail the function of D-mib during wing development. Growth of the wing pouch depends on the activity of an organizing center located at the dorsal-ventral (D-V) boundary [40,41]. This boundary is established in first instar larvae and is defined by the aterous expression boundary. Apterous activates the expression of the Ser and fringe genes in dorsal cells. High levels of Ser in dorsal cells activate N in trans in ventral cells and suppress N activation in cis in dorsal cells, whereas Fringe modifies N in dorsal cells such that dorsal cells located at the D-V boundary respond to Dil. Thus, composite signaling by Ser and Dil leads to symmetric N activation in margin cells located along the
The D-V boundary [8,9,42,43]. N then regulates the expression of the vestigial and wingless (wg) genes that cooperate to promote growth of the wing pouch. N also regulates expression of the cut gene in margin cells [44]. Thus, loss of N signaling results in a reduction in size of the wing pouch accompanied by the loss of cut and wg expression along the D-V boundary.

A complete loss of Cut and Wg accumulation and wg-lacZ expression was observed in the central region of third instar D-mib mutant wing discs (data not shown). Thus, the D-mib wing phenotype may result from defective N inductive signaling at the D-V boundary. We conclude that the activity of the D-mib gene is required for the specification of the wing margin and, hence, growth of the wing pouch. Interestingly, wing margin formation and expression of Cut are not affected by the complete loss of N activity (Figure 2F and 2F").

D-mib Co-Localizes with DI and Ser at the Apical Cortex

We next studied the subcellular localization of D-mib (Figure 3). Anti-D-mib antibodies were generated that specifically detected D-mib on Western blots (see Figure 1C) and on fixed tissues (Figure 3F–F'). Using these antibodies, we found that D-mib was detected in all imaginal disc cells (Figure 3A and 3B). We then examined D-mib subcellular distribution in epithelial cells located along the edge of the wing discs because cross-sectional imaging affords better resolution along the apical-basal axis. D-mib co-localized with Ser, DI, and N at the apical cortex (Figure 3B–3D''). DI and
Ser and D-mib were also detected in large intracellular vesicles that probably correspond to multivesicular bodies in that they also stained for hepatocyte growth factor-regulated tyrosine kinase substrate [46] (Figure 3B–3C"; data not shown). The intracellular dots seen with the anti-D-mib antibodies were distinct from the Dl- and Ser-positive dots and appeared to result from background staining (data not shown). The reduced cytoplasmic staining seen in D-mib mutant cells (Figure 3F–3F") suggests that D-mib is also present in the cytoplasm. A similar localization at the apical cortex and in the cytoplasm was seen for a functional yellow fluorescent protein (YFP):D-mib fusion protein (see Figure 6 below). These localization data suggest that D-mib may act at the apical cortex to regulate the activity of Dl and/or Ser.

**D-mib Regulates the Cell-Surface Level of Ser**

We next examined the potential role of D-mib in regulating Dl and Ser distribution in wing imaginal discs. We focused our analysis on the notum region since D-mib mutant discs have no wing pouch (Figure 4). Dl and Ser co-localized both at the apical cortex and in large intracellular vesicles in wild-type cells (Figure 4A–4C). The complete loss of D-mib activity in D-mib¹ mutant discs did not detectably change the subcellular localization of Dl (Figure 4C, 4C', 4F, and 4F'). In contrast, the accumulation of Ser at the apical cortex was strongly increased (Figure 4E) and Ser accumulation in Dl-positive vesicles was dramatically reduced (Figure 4E') in D-mib² mutant discs. Similar results were also obtained in D-mib² mutant clones, which showed strongly elevated levels of cortical Ser (Figure 4H) whereas the amount of Dl at the apical cortex was not detectably modified (see Figures 3F–3F", and 4J). Of note, loss of D-mib² activity in clones did not block the accumulation of Ser into intracellular dots (Figure 4H'). Thus, trafficking of Ser towards this intracellular compartment is, at least in part, D-mib-independent. We therefore conclude that the D-mib gene is required to regulate the level of Ser at the apical cortex of wing disc cells.

**D-mib Is Required for Ser Endocytosis**

To test whether this specific increase in the level of Ser at the apical cortex resulted from reduced Ser endocytosis in D-mib mutant cells, we followed the endocytosis of Ser in living imaginal discs using an antibody uptake assay. Briefly, dissected wing discs were cultured for 15 min in the presence of antibodies that recognize the extracellular part of Ser or Dl, then washed, cultured for another 45 min in medium without antibodies, and then fixed. The uptake of anti-Ser and anti-Dl antibodies was then assessed using secondary antibodies. The results are shown in Figure 5. Using this assay, we found that anti-Ser and anti-Dl antibodies were internalized in wild-type epithelial cells (Figure 5A–5C'}). The complete loss of D-mib activity in D-mib¹ wing discs did not significantly change the internalization of anti-Dl antibodies (Figure 5D", 5E", and 5F"), indicating that D-mib is not required for DI endocytosis in this tissue. However, the loss of D-mib activity strongly
inhibited the endocytosis of anti-Ser antibodies (Figure 5E'). Moreover, high levels of anti-Ser antibodies were seen at the apical surface (Figure 5D' and 5F'), confirming that D-mib mutant cells accumulate high levels of Ser at their surface. We therefore conclude that D-mib is specifically required for the endocytosis of Ser in wing discs.

Ubiquitin-mediated endocytosis is thought to depend on monoubiquitination. Thus, by analogy with the function of Mib in D. rerio [18,28], we suggest that D-mib may directly monoubiquitinate Ser. Consistent with this hypothesis, we show in a companion paper that D-mib binds Ser (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data). Moreover, a mutation in the C-terminal catalytic RING domain of D-mib abolished its ability to internalize Ser in transfected S2 cells (R. L. B. and F. S., unpublished data) implying that the E3 ubiquitin ligase activity of D-mib is required for Ser internalization. Biochemical analysis of the ubiquitination events regulated by D-mib will be needed to further define the mechanism by which D-mib regulates the endocytosis of Ser in vivo.

D-mib Regulates Ser Signaling

The regulation of Ser endocytosis by D-mib suggests that D-mib may regulate Ser signaling. Ser expression is restricted to dorsal cells in second instar wing imaginal discs [7,10,44,47,48]. Ser in dorsal cells signals across the D-V boundary to activate N in ventral cells [8,9]. If D-mib is required for Ser signaling during wing development, then loss of D-mib activity in dorsal cells should affect the specification of the wing margin in a non-autonomous manner. Loss of D-mib activity in large dorsal clones of D-mib mutant cells resulted in a loss of Cut expression at the D-V interface (Figure 6A and 6B). The lack of Cut expression in wild-type ventral cells abutting the D-V boundary indicates that D-mib is required for Ser signaling by dorsal cells and acts in a non-autonomous manner to activate N in ventral cells. Conversely, loss of D-mib activity in large ventral clones (Figure 6C and 6D) did not disrupt margin specification, indicating that D-mib is not strictly required for DI signaling by ventral cells. However, a narrowing of the Cut-positive margin was observed (Figure 6D), suggesting that D-mib contributes to regulating the level of DI signaling. Of note, ventral D-mib mutant cells expressed Cut, implying that D-mib is not required for N signal transduction.

We next tested whether expression of D-mib in dorsal cells is sufficient to rescue the D-mib wing phenotype. D-mib was expressed in dorsal cells of D-mib/D-mib mutant discs using Ser-GAL4. Similarly to the expression of the Ser gene, Ser-GAL4 expression is restricted to dorsal cells in secondarily third instar larvae and is weakly expressed in ventral cells in midlate third instar larvae, i.e., after margin cell specification [49,50]. Expression of D-mib in dorsal cells was sufficient to rescue growth of the wing pouch and of the expression of Cut in margin cells in D-mib mutant discs (Figure 7A). This result confirmed that D-mib regulates Ser signaling by dorsal cells.

A similar rescue was observed with a YFP::D-mib protein (Figure 7B–7B'), indicating that YFP::D-mib is functional. YFP::D-mib localized at the apical cortex and in the cytoplasm (Figure 7C–7D'), as seen for endogenous D-mib (see Figure 3). YFP::D-mib co-localized with DI and Ser at the apical cortex of cells expressing low levels of YFP::D-mib. However, cells expressing high levels of YFP::D-mib showed a strong reduction in the level of both DI and Ser at the cortex (Figure 7C–7C'), further indicating that D-mib down-regulates the levels of both Ser and DI at the apical cortex (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data).

D-mib Acts Downstream of Ser and Upstream of Activated N

The functional assay was then used to genetically position the requirement for the D-mib gene activity relative to Ser and N (Figure 8). Expression of an activated version of N, Ncdc10

![Figure 7](image-url) Expression of D-mib in Dorsal Cells Is Sufficient to Rescue the D-mib Mutant Phenotype

(A) Expression of D-mib (green) in dorsal cells, using Ser-GAL4, rescued the growth of the wing pouch and margin Cut (red) expression in D-mib/D-mib mutant discs.

(B–D) Ser-GAL4-driven expression of YFP::D-mib (green) rescued the D-mib/D-mib phenotype and strongly reduced the level of DI (blue in [B, B', C, C', D, and D']) and Ser (red in [B, B', C, C', D, and D']) in dorsal cells. (C–D') are high-magnification views (apical [C–C'] and basal [D–D']) of the disc shown in (B–B'). YFP::D-mib co-localized with DI and Ser at the apical cortex in cells expressing only low levels of YFP::D-mib.

Bar is 50 μm for (A–B') and 10 μm for (C–D').

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![Figure 8](image-url) Expression of Neur in Dorsal Cells Is Sufficient to Rescue the D-mib Mutant Phenotype

D-mib/D-mib mutant discs expressing GFP (A) (GFP staining not shown), Ser (B), Ncdc10 (C), or Neur (D) under the control of Ser-GAL4 were stained for Cut (red). Expression of Ser in dorsal cells did not rescue the D-mib/D-mib wing pouch mutant phenotype (compare B with A), consistent with D-mib being required for Ser signaling. By contrast, expression of Ncdc10, an activated version of N, led to the deregulated growth of the dorsal compartment and the expression of Cut in most dorsal cells (C), indicating that activated N acts downstream of D-mib. Expression of Neur in dorsal cells was sufficient to compensate for the loss of D-mib activity (D).

Bar is 40 μm for all panels.

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The forced ubiquitous expression of the contrast, D-mib appears to be uniformly expressed in margin cells, where it is not required, and appears to suppress the expression of Neur, using Neur-GAL4, restored growth of the wing pouch as well as the expression of Cut in margin cells in D-mib mutant discs (Figure 8D). We conclude that ectopic expression of Neur compensates for the loss of D-mib activity.

In a converse experiment, we found that the neur-driven expression of D-mib, using neurPGAL4, did not rescue the cuticular neurogenic phenotype of neurPGAL4/neur1F65 embryos. Three UAS-D-mib transgenic lines were tested, and none showed detectable rescue whereas the two UAS-neur lines used as positive controls either fully or partially rescued the cuticular neurogenic phenotype of neurPGAL4/neur1F65 embryos (data not shown). Moreover, expression of Neur in dorsal cells, using Ser-GAL4, was sufficient to rescue growth of the wing pouch as well as the expression of Cut in margin cells in D-mib mutant discs (Figure 8D). We conclude that ectopic expression of Neur compensates for the loss of D-mib activity.

Discussion

Many recent studies have revealed that endocytosis plays multiple roles in the regulation of N signaling (reviewed in [2]; see also [53,54]). Here, we show that the conserved E3 ubiquitin ligases Neur and D-mib have similar molecular activities in the regulation of DI and Ser endocytosis but distinct developmental functions in Drosophila.

Our analysis first establishes that D-mib regulates Ser signaling during wing development. First, clonal analysis revealed that the activity of the D-mib gene is specifically required in dorsal cells for the expression of Cut at the wing margin. Second, expression of D-mib in the dorsal Ser-signaling cells was sufficient to rescue the D-mib mutant wing phenotype. Third, results from an in vivo antibody uptake assay indicated that the endocytosis of Ser (but not of DI) was strongly inhibited in D-mib mutant cells. This inhibition correlated with the strong accumulation of Ser (but not DI) at the apical cortex of D-mib mutant cells. Thus, an essential function of D-mib in the wing is to regulate the endocytosis of Ser in dorsal cells to non-autonomously promote the activation of N along the D-V boundary. By analogy, the defective growth of the eye tissue may similarly result from the lack of Ser signaling and of N activation along the D-V boundary [55]. Because D-mib co-localizes with Ser at the apical cortex of wing disc cells, acts in a RING-finger-dependent manner to regulate Ser endocytosis in S2 cells (R. L. B. and F. S., unpublished results), and physically associates with Ser in co-immunoprecipitation experiments (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data), D-mib may ubiquitinate Ser and directly regulate its endocytosis.

Our analysis further suggests that endocytosis of Ser is required for Ser signaling. This conclusion is consistent with observations made earlier showing that secreted versions of Ser cannot activate N but instead antagonize Ser signaling [56,57]. Thus, endocytosis of both N ligands appears to be strictly required for N activation in Drosophila. Different models have been proposed to explain how endocytosis of the ligand, which removes the ligand from the cell surface, results in N receptor activation (discussed in [17,20,21,30]). Interestingly, the strong requirement for DI and Ser endocytosis seen in Drosophila is not conserved in Caenorhabditis elegans, in which secreted ligands have been shown to be functional [58,59]. Noticeably, there is no C. elegans Mib homolog, and the function of C. elegans neur (F10D7.5) is not known. We speculate that endocytosis of the ligands may have evolved as a means to ensure tight spatial regulation of the activation of N.

Our analysis also establishes that the activity of the D-mib gene is required for a subset of N signaling events that are distinct from those that require the activity of the neur gene. We have shown that the D-mib gene regulates wing margin formation, leg segmentation, and vein formation, whereas none of these three processes depend on neur gene activity ([45,60]; this study). Conversely, the activity of the neur gene is essential for binary cell-fate decisions in the bristle lineage [22] that do not require the activity of the D-mib gene (no bristle defects were seen in D-mib mutant flies). The activity of the neur gene is also required for lateral inhibition during neurogenesis in embryos and pupae [4,45,61]. This process is largely independent of D-mib gene activity since the complete loss of D-mib function only resulted in a mild neurogenic phenotype in the notum. These data thus indicate that the neur and D-mib genes have largely distinct and complementary functions in Drosophila. Whether a similar functional relationship between Neur and D-mib exists in vertebrates awaits the study of the D. rerio neur genes and/or of the murine Mib and Neur genes.

The functional differences observed between D-mib and neur cannot be simply explained by obvious differences in molecular activity and/or substrate specificity. First, both Neur and D-mib physically interact with DI ([20]; E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data) and promote the down-regulation of DI from the apical membrane when overexpressed (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data). Furthermore, DI signaling appears to require the activity of either Neur or D-mib, depending on the developmental contexts. We have shown here that specific aspects of the D-mib phenotype in legs and in the notum cannot simply result from loss of Ser signaling and are consistent with reduced DI signaling, suggesting that D-mib regulates DI signaling. Consistent with this interpretation,
overexpression studies indicate that D-mib up-regulates the signaling activity of Dl, whereas a dominant-negative form of D-mib inhibits it (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data). We note, however, that no clear defects in DI subcellular localization and/or trafficking were observed in D-mib mutant cells. It is conceivable that the contribution of D-mib to the endocytosis of DI is masked by the activity of D-mib-independent processes that may, or may not, be linked to DI signaling. We have also shown that, reciprocally, Neur and D-mib may similarly regulate Ser. Neur and D-mib were shown to similarly promote down-regulation of Ser from the cell surface when overexpressed (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data). Moreover, D-mib binds Ser (E. C. Lai, F. Roegiers, X. Qin, R. Le Borgne, F. Schweisguth, et al., unpublished data) and regulates Ser signaling (this study).

Whether endogenous Neur binds and activates Ser remains to be tested. However, the ability of Neur to rescue the D-mib mutant wing phenotype when expressed in dorsal cells strongly indicates that Neur can promote Ser signaling.

Together, these data indicate that Neur and D-mib have similar molecular activities.

D-mib and Neur may have identical molecular activities but distinct expression patterns, hence distinct functions at the level of the organism. Consistent with this possibility, D-mib is uniformly distributed in imaginal discs, whereas Neur is specifically detected in sensory cells [52]. Importantly, the rescue of the D-mib mutant phenotype by ectopic expression of Neur strongly supports this interpretation. This result further suggests that Neur can regulate Ser signaling. Consistent with this idea, overexpression of Neur in imaginal discs resulted in a strong reduction of Ser accumulation at the apical cortex (data not shown). Thus, despite their obvious structural differences, Neur and D-mib appear to act similarly to promote the endocytosis of DI and Ser. Nevertheless, our observation that D-mib could not compensate for the loss of neurf activity in the embryo indicates that D-mib and Neur have overlapping rather than identical molecular activities.

In conclusion, Neur and D-mib appear to have similar molecular activities in the regulation of DI and Ser endocytosis but distinct developmental functions in Drosophila. The conservation from Drosophila to mammals of these two structurally distinct but functionally similar E3 ubiquitin ligases is likely to reflect a combination of evolutionary advantages associated with: (i) specialized expression pattern, as evidenced by the cell-specific expression of the neur gene in sensory organ precursor cells [52]; (ii) specialized function, as suggested by the role of murine MIB in TNFα signaling [52]; (iii) regulation of protein stability, localization, and/or activity. For instance, Neur, but not D-mib, localizes asymmetrically during asymmetric sensory organ precursor cell divisions [22].

Materials and Methods

Flies. The D-mib1 mutation corresponds to the EY97600 P-element insertion generated by the Gene Disruption Project (http://flypush igen.bcm.tmc.edu/psecreren). The D-mib2 allele was selected as w D-mib- mutant derivative by imprecise excision of the EY97600 P-element. The precise breakpoints of the D-mib2 deletion were determined by sequencing a PCR fragment amplified from genomic DNA prepared from D-mib- homozygous larvae. The i(3)L2cad122 and i(3)L2cad1 alleles originally isolated by [35] failed to complement the D-mib1 and D-mib2 mutations and were renamed D-mib1 and D-mib2. The D-mib1, D-mib2, and D-mib3 alleles appear to be genetically null alleles since the phenotypes of D-mib1/D-mib1 and D-mib1/D-mib2 mutant pupae are indistinguishable from the ones seen in D-mib1/D-mib2 and D-mib2/D-mib2 pupae. Sequence analysis of the D-mib1 and D-mib2 alleles was carried on PCR products prepared from genomic DNA prepared from D-mib1/D-mib1 and D-mib2/D-mib2 mutant pupae. Genomic DNA from i(3)L2cad122/D-mib2 mutant pupae was used as control for polymorphism.

D-mib2 mutant clones were generated in y w hs-flp; FRT2A D-mib52 FRT2A M(3)ubi-nlsGFP larvae. neur1-165 mutant clones were generated as previously described [22].

UAS-D-mib and UAS-YFP; D-mib lines were generated via standard P-element transformation. These constructs were derived from the SD05267 CDNA obtained from ResGen (Invitrogen, Carlsbad, California, United States). Cloning details for these constructs are available upon request. UAS-DI (gift from M. Muskavitch), UAS-Neur (gift from R. Fleming), UAS-Neur (gift from C. Delidakis), UAS-Ncdc10 (gift of T. Klein), Ser-GAL4 lines, and Ser mutant alleles are described in FlyBase (http://flybase.bio.indiana.edu).

Antibodies. Dissected imaginal discs were fixed in 4% paraformaldehyde (15 min) and incubated with antibodies at room temperature in PBS 1X with 0.1% TritonX-100. Rabbit polyclonal anti-D-mib antibodies were raised against the CYNERKTDDSFLPSGN peptide (CovAlAb, Lyon, France). Immunopurified anti-D-mib antibodies (rabbit 5411) were used (immunofluorescence, 1:100; Western blot, 1:1,000). Other primary antibodies were mouse anti-Cut (2B10; Developmental Studies Hybridoma Bank [DSHB, Iowa City, Iowa, United States]) 1:500; rat anti-DE-Cadherin (gift from T. Uemura); 1:50; guinea pig anti-Discs-large (gift from P. Bryant; 1:3,000); anti-β-galactosidase (Cappel [MP Biomedicals, Irvine, California, United States]; 1:1,000); mouse anti-DeltaECED (C594.9B; DSHB; 1:1,000); mouse anti-NotchECED (C348.2H; DSHB; 1:1,000); rat anti-Ser (gift from K. Irvine; 1:2,000); rat anti-Ser (gift from S. Cohen; 1:290); rabbit anti-Ser (gift from E. Knust; 1:10); and guinea pig anti-Senseless (gift from H. Bellen; 1:3,000). Cy3-, Cy5-, and Cy5-coupled secondary antibodies were from Jackson Laboratory (Bar Harbor, Maine, United States). Alexa488-coupled secondary antibodies and phalloidin were from Molecular Probes (Eugene, Oregon, United States). Images were acquired on a Leica (Wetzlar, Germany) SP2 microscope and assembled using Adobe Photoshop (Adobe Systems, San Jose, California, United States).

Endocytosis assay. Third instar larvae wing discs were dissected in Schneider’s Drosophila medium (Gibco BRL, San Diego, California, United States) containing 10% fetal calf serum (Gibco BRL). Wing discs were cut between the wing pouch and the thorax to facilitate antibody diffusion. Wing discs were cultured for 15 min with mouse anti-DI(C594–9B at 1:100) and rat anti-Ser antibody (1:500; from K. Irvine). Following three medium changes and a 45-min chase period, wing discs were fixed and incubated with secondary antibodies.

Supporting Information

Accession Numbers

The FlyBase accession numbers for the gene products discussed in this paper are: (FBgn0000463), N (FBgn0004147), Neur (FBgn002932), P-element inserted into the 5′ untranslated region of the D-mib gene (FBgn0035588), and Ser (FBgn0014197). The NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/entrez) accession number for D. rerio Mib is NP_779533.

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