The interplay between DSL proteins and ubiquitin ligases in Notch signaling

Chrysoula Pitsouli[1,2] and Christos Delidakis[1,2,*]

[1]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, Heraklion, Greece
[2]Department of Biology, University of Crete, Heraklion, Greece
*Author for correspondence (e-mail: delidaki@imbb.forth.gr)

Accepted 12 July 2005

Development 132, 4041-4050
Published by The Company of Biologists 2005
doi:10.1242/dev.01979

Summary

Lateral inhibition is a pattern refining process that generates single neural precursors from a field of equipotent cells and is mediated via Notch signaling. Of the two Notch ligands Delta and Serrate, only the former was thought to participate in this process. We now show that macrochaete lateral inhibition involves both Delta and Serrate. In this context, Serrate interacts with Neuralized, a ubiquitin ligase that was heretofore thought to act only on Delta. Neuralized physically associates with Serrate and stimulates its endocytosis and signaling activity. We also characterize a mutation in *mib1*, a *Drosophila* homolog of *mind bomb*, another Delta-targeting ubiquitin ligase from zebrafish. Mib1 affects the signaling activity of Delta and Serrate in both lateral inhibition and wing dorsoventral boundary formation. Simultaneous absence of *neuralized* and *mib1* completely abolishes Notch signaling in both aforementioned contexts, making it likely that ubiquitination is a prerequisite for Delta/Serrate signaling.

Key words: *Drosophila*, DSL, Notch, *mind bomb*, *neuralized*, Lateral inhibition

Introduction

Notch signaling is a widely used cell-cell signaling pathway that modulates cell fate and function in a great number of processes at all developmental stages of metazoans (Lai, 2004; Schweisguth, 2004; Yoon and Gaiano, 2005). It revolves around the transmembrane receptor Notch and its transmembrane ligands, known as DSL proteins, from their characteristic extracellular Notch-binding domain – the Delta/Serrate/Lag-2 (DSL) domain. Ligand-receptor binding precipitates a series of proteolytic events culminating in the release of the intracellular domain of Notch from the plasma membrane and its subsequent import into the nucleus, where it acts as a transcriptional co-activator. A major question is how DSL protein-Notch interactions bring about activation of the receptor. A curious association between Notch activation and endocytosis has been noticed for some time (Seugnet et al., 1997a); recent work has started unraveling this connection and suggests that it is partly due to the stimulation of signaling by endocytosis of DSL proteins. A discovery that helped formulate this hypothesis was the existence of E3 ubiquitin ligases that ubiquitinate Delta (Dl) proteins and simultaneously promote Delta endocytosis and signaling (Chen and Corliss, 2004; Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Le Borgne and Schweisguth, 2003; Pavlopoulos et al., 2001; Yeh et al., 2001). Membrane protein ubiquitination can promote their endocytosis by association with endocytic adaptor proteins that recognize the ubiquitin moiety (Haglund et al., 2003). One such adaptor protein is epsin. The recent discovery that Liquid facets (Lqf), the *Drosophila* epsin, is needed for DSL protein function supports the hypothesis that DSL protein ubiquitination and endocytosis are crucial events in signal emission (Overstreet et al., 2003; Overstreet et al., 2004; Wang and Struhl, 2004).

DSL proteins from insects and vertebrates can be classified into two categories, Delta (Dl) and Serrate/Jagged (Ser/Jag), based on conserved structural features of their extracellular domains (Fleming, 1998). These two families have different expression patterns and consequently function in distinct Notch-dependent processes. Expression pattern differences, however, are not the sole distinguishing feature of DSL proteins; the two families appear to show strong preference for binding to differentially glycosylated forms of Notch receptors (Haines and Irvine, 2003; Okajima et al., 2003) – glycosylation of Notch by Fringe stimulates Dl signaling, whereas it inhibits Ser signaling. In terms of intracellular regulation, ubiquitin ligases had been described only for Dl proteins, until very recently (see below); yet, the need for Epsin in order for both Dl and Ser to emit their signal (Wang and Struhl, 2004) implicates ubiquitination also in Ser function. Two different E3 Ub ligases seem to affect Dl function: Neuralized (Neur) has been characterized in *Drosophila* (Lai et al., 2001; Pavlopoulos et al., 2001) and *Xenopus* (Deblandre et al., 2001); and Mind bomb (Mib) in zebrafish (Itoh et al., 2003). Both associate with Dl triggering its endocytosis. Apart from a catalytic RING domain at their C termini; Neur and Mib display no further similarity. During the course of 2005 (while this paper was under review), one *Drosophila* homolog of Mib, which we call Mib1, was initially characterized by two groups, who showed that it interacts with both Dl and Ser and variably affects their activity and endocytosis (Lai et al., 2005; Le Borgne et al., 2005). Vertebrate Mib homologs were also shown to associate with both Dl and Jag family members (Koo et al., 2005a;

Takeuchi et al., 2005). However, these papers did not make it clear whether different Ub ligases show preference for association with different DSL proteins, nor whether DSL proteins absolutely require Ub ligases in order to signal.

The present work and recent work done independently by Wang and Struhl (Wang and Struhl, 2005) have addressed both of these issues. Wang and Struhl (Wang and Struhl, 2005) conclusively showed that Mib1 is necessary for signal sending by both Dl and Ser in wing dorsoventral boundary establishment, a well-characterized instance of Notch signaling. In that context, absence of Mib1 can be rescued by ectopic provision of Neur. We have corroborated their findings and have further tested the role of Dl, Ser, Neur and Mib1 in a different instance of Notch signaling, lateral inhibition of neural precursors (Bray, 1998; Skeath and Thor, 2003). Lateral inhibition was heretofore thought to depend solely on Dl and Neur (Lai and Rubin, 2001; Lehman et al., 1983), with no input from Ser or Mib1 (Lai et al., 2005; Zeng et al., 1998). By contrast, wing DV boundary establishment requires both Dl and Ser (Irvine and Vogt, 1997); it also requires Mib1 but not Neur (Lai et al., 2005; Lai and Rubin, 2001; Le Borgne et al., 2005; Wang and Struhl, 2005). Lack of requirement of a factor in any given process may well be a result of its expression pattern; this seems to be the case for *neur*, which is not broadly expressed in wing cells during DV boundary establishment. Similarly, during embryonic neuroblast lateral inhibition, *Ser* is not expressed, making the process solely *Dl* dependent (Gu et al., 1995). We have focused on adult macrochaete SOP lateral inhibition, which takes place in the wing disk at the third larval instar, where all *Dl*, *Ser*, *neur* and *mib1* are expressed. Contrary to expectations, we show that both Dl and Ser participate in this process in a partially redundant fashion, and the same holds true for Neur and Mib1. More importantly, we show that simultaneous removal of *neur* and *mib1* results in a complete block of lateral inhibition. Our results lead us to conclude that (1) Ub ligases are absolutely required for DSL protein function (at least in the present contexts) and (2) either Ub ligase can activate either DSL protein. Our work, taken together with other recent papers (Wang and Struhl, 2004; Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005), is strongly in favor of a ubiquitin/epsin-mediated endocytosis pathway playing an indispensable role in the emission of DSL-Notch signals.

Materials and methods

Plasmids and transgenics

pUAST-EGFP-neur is a fusion of EGFP at the N terminus of Neur, which was generated by fusing a PCR product (primers available upon request) of *neur* in frame with *EGFP* from pEGFP-C1 (Clontech). The fusion site is DELYK-*SGLRSR*-GLSDIPANY (EGFP-*polylinker*-Neur).

pUAST-DlV5His was generated by subcloning an *Eco*RI/*Dra*I fragment containing the V5-tagged *Dl*-coding sequence from pIZ-DlV5His (Bland et al., 2003) into pUAST cut with *Eco*RI-*Xho*I (filled-in).

Antibodies and immunohistochemistry

Anti-Neur polyclonal antisera

pRSET-neur1050 was generated by cloning a PCR fragment encoding amino acids 11-360 of Neur in frame with the 6×His tag of the pRSET-C vector (Invitrogen). The fusion protein was expressed in *E. coli* and purified with Ni^{2+}-affinity chromatography under denaturing conditions (Qiagen). Rabbit antiserum production and affinity purification was carried out by Davids Biotechnologie.

Other antibodies

Mouse anti-Delta mAb9B (Qi et al., 1999)
Rabbit anti-Serrate (Klueg and Muskavitch, 1999)
Guinea pig anti-Senseless (Nolo et al., 2000)
Mouse anti-Cut (Blochlinger et al., 1990)
Mouse anti-Wg [developed by S. M. Cohen; obtained from DSHB (The Developmental Studies Hybridoma Bank was developed under the auspices of the NICHD and is maintained by The University of Iowa, Department of Biological Sciences)]
Mouse anti-V5 (Invitrogen)
Mouse anti-Myc mAb9E10 (developed by J. M. Bishop; obtained from DSHB)

Fluorescent and HRP-labeled secondary antibodies were from Molecular Probes and Jackson Immunoresearch, respectively. Immunohistochemistry was performed as described by Pavlopoulos et al. (Pavlopoulos et al., 2001).

Transient transfections and immunoprecipitation

Transient transfections of S2 cells were carried out with the calcium phosphate precipitation method. pIZ-DlV5His (Bland et al., 2003) and pRMHa3-Sermyc (gift of R. Fleming) were used to express Delta and Serrate, respectively. pUAST-EGFP-neur and pUAST-neurΔR-GFP were used to express Neur or NeurΔR in conjunction with mt-Gal4 (inducible by Cu^{2+}). Transfected cell lysate was used for immunoprecipitation with rabbit anti-Neur antiserum and protein A sepharose. One percent of the total extract was used as control (input). For larval immunoprecipitations, the lysate was prepared from 30 third-instar disk-CNS complexes.

Drosophila stocks

Gal4 lines

C253-Gal4 (FlyBase: P{GawB}C253)
hs-sev-Gal4 (FlyBase: P{GAL4-Hsp70.sev}2)
dpp-$Gal4^{40C6}$ (FlyBase: P{GAL4-dpp.blk1}40C.6)
act>CD2stop>Gal4 (FlyBase: P[GAL4-Act5C(FRT.CD2).P]S)

UAS lines

UAS-$srcGFP^{10}$ (FlyBase: P{UAS-src-GFP(S65T/I167T)})
UAS-fng^{22c} (FlyBase: P{UAS-fng.K})
UAS-Dl^{B41} (FlyBase: P{UAS-Dl.L})
UAS-$Sermyc^{IC}$ (FlyBase: P{UAS-Ser.G})
UAS-neur (FlyBase: P{UAS-neur.P})
UAS-neurΔRING-GFP (FlyBase: P{UAS-neur.DeltaRING::EGFP})
UAS-DlV5His (this work)
UAS-EGFP-neur (this work)

Fly stocks were either obtained via the Bloomington and Szeged Stock Centers or generously provided by colleagues.

Mosaic analysis

All alleles used are described in FlyBase. Mosaics were induced during the first larval instar using the conventional FLP/FRT technique (Xu and Rubin, 1993) or the MARCM system (Lee and Luo, 2001). For cross details see Table S1 in the supplementary material.

Results

Note on nomenclature

We use '*mind bomb1*' and '*mind bomb2*' for the *Drosophila* homologs of zebrafish *mind bomb*. We favor this over the recently proposed alternative nomenclature *D-mib* and *D-mibl*

(Lai et al., 2005; Le Borgne et al., 2005), as it has been agreed not to use the prefix D- for *Drosophila* genes. The symbol *mib* already exists in the fly gene collection for the gene *miniature bristles* (FlyBase FBgn0002744), whereas *mib1* and *mib2* are available.

Both Dl and Ser participate in lateral inhibition of macrochaete SOPs

To address the role of DSL proteins in lateral inhibition, we focused on the third instar notum, where nine SOPs arise in a well-defined pattern. We first confirmed that both Dl and Ser are expressed within the proneural clusters giving rise to these SOPs, although the Dl and Ser patterns are not entirely identical (see Fig. S1 in the supplementary material). To visualize SOPs we used the nuclear protein Sens (Nolo et al., 2000) as a marker. By counting the number of SOPs per position in different mutant mosaic clones, we could conclude about the extent of the lateral inhibition defect. Our first indication that Dl was not solely responsible for lateral inhibition in these regions was that *Dl* clones showed a much weaker defect than either *N* clones or doubly mutant *Dl Ser* clones (Fig. 1A-C; Table 1); the latter contained a lot more (typically more than 10) Sens-positive cells per SOP position, whereas *Dl* clones usually had two to four SOPs, and some were even wild-type in appearance (one SOP). Yet, *Ser* singly mutant clones did not affect SOP numbers (Fig. 1D). As the difference between our *Dl* and *Dl Ser* clones could conceivably be due to some background mutation(s) other than *Ser*, we sought an independent way to assay the role of Ser. One way to inactivate any Ser contribution in signaling is to overexpress *fringe*, as Fringe-modified Notch is refractory to Ser signaling. *Dl; UAS-fng* clones (using the very same *Dl* chromosome, which gave a mild phenotype) were generated using the MARCM system, which inactivates one gene while simultaneously overexpressing another within the same clone. These clones displayed a significantly higher number of SOPs per cluster than *Dl* alone (Fig. 1E, Table 1, $P<0.05$). The control experiment of overexpressing *UAS-fng* in a wild-type background produced no defect in SOP numbers. As two independent ways of blocking Ser activity enhanced the *Dl* mutant phenotype, we conclude that in normal tissue Ser contributes to lateral inhibitory Notch signaling.

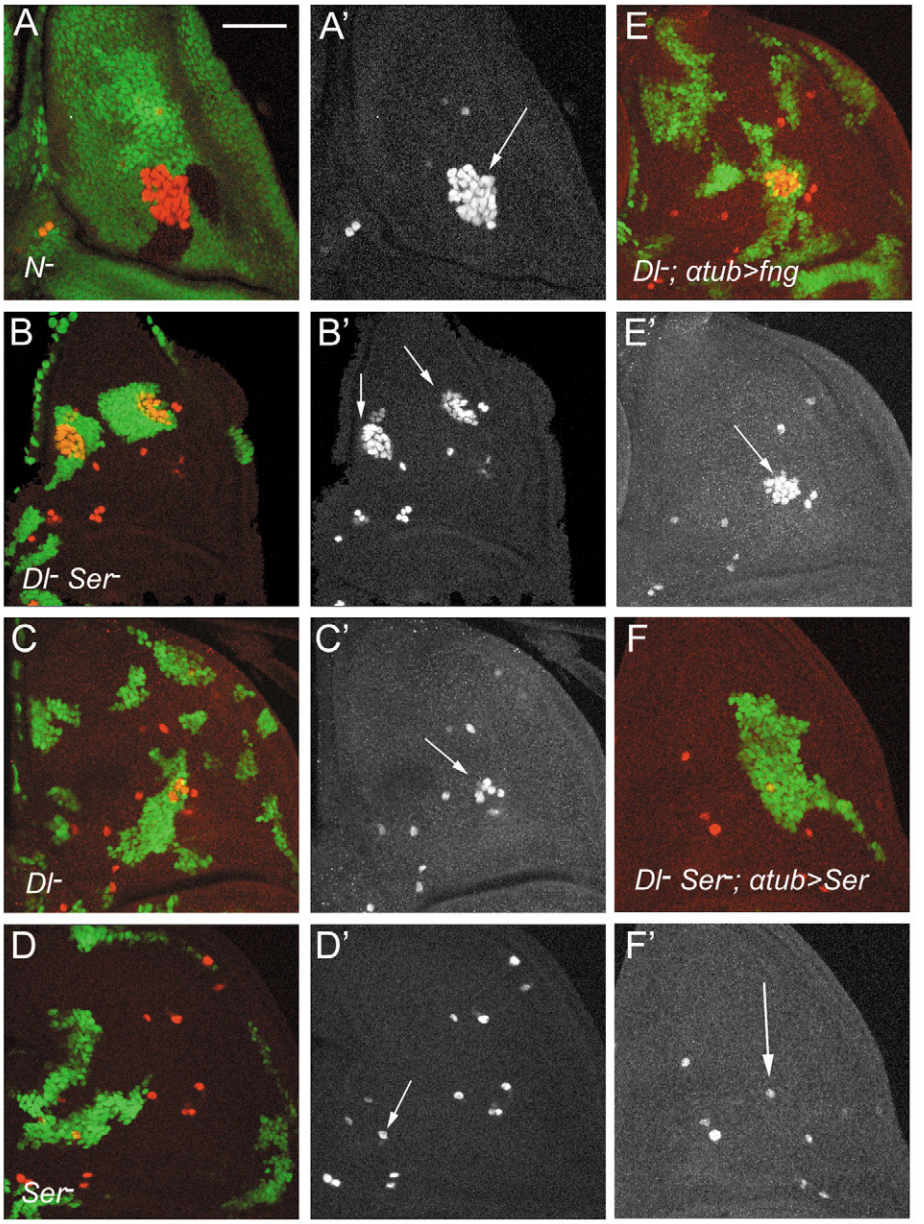

Fig. 1. Dl and Ser act redundantly during lateral inhibition. Third instar nota are stained for Senseless (red), to visualize SOPs. Proximal is upwards, anterior is towards the left. Scale bar: 40 μm. (A) *Notch* mutant cells marked by absence of GFP. (B-F) Mutant cells marked by presence of GFP. (A′-F′) Red/Sens channels of A-F, respectively; mutant areas are indicated by arrows. (A) *N* or (B) *Dl Ser* clones display a large number of clustered ectopic SOPs. This phenotype probably represents the complete loss of lateral inhibition. By contrast, (C) *Dl* mutant clones show only a few ectopic SOPs and (D) *Ser* mutant clones appear wild type. (E) *Dl* mutant clones simultaneously expressing *UAS-fng* display a more severe phenotype than *Dl* (C). (F) *Dl Ser* mutant clones expressing *UAS-Ser* appear wild type.

From the previous experiment it appears that whereas Dl is sufficient for lateral inhibition (Ser^{-}), Ser is not (Dl^{-}). This could be due to qualitative differences in the signal produced by each ligand; alternatively, the ligands could be interchangeable, but their expression levels might make one more essential. We therefore sought to increase the levels of either ligand in order to ask whether at sufficiently high levels either one would carry out lateral inhibition independently of

Table 1. Lateral inhibition defects in third instar nota

Genotype*	Strong†	Moderate†	Weak†	Wild type†	*n*
N^{5419}	44.5	26	26	3.5	27
Dl^{rev10} Ser^{RX106}	70.5	21.5	6	2	51
Dl^{rev10}	11	11	41	37	27
Ser^{RX106}	0	0	0	100	24
Dl^{rev10}+UAS-fng^{22c}	47	12.5	28	12.5	32
Dl^{rev10} Ser^{RX106}+UAS-Dl^{B41}	0	8.5	0	91.5	24
Dl^{rev10} Ser^{RX106}+UAS-Ser^{JC}	0	0	26	74	23
Dl^{rev10}+UAS-Ser^{JC}	0	0	0	100	14
UAS-fng^{22c}	0	0	0	100	11
UAS-Dl^{B41}	0	0	0	100	20
UAS-Ser^{JC}	0	0	0	100	42
$neur^{1}$	11	33.5	48	7.5	27
$neur^{1}$ Dl^{rev10}	50	35	15	0	20
$neur^{1}$ Ser^{RX106}	33.5	47	19.5	0	36
$mib1^{EY9780}$	0	0	0	100	18
$mib1^{EY9780}$ $neur^{1}$‡	82.5	17.5	0	0	17
$mib1^{EY9780}$ Dl^{rev10}‡	40	45	5	10	20
$mib1^{EY9780}$ Ser^{RX106}‡	0	0	0	100	24

Only clones in late larval nota were scored (SOP positions ASC, PSC, ADC, PDC, APA, tr1, PSA, ANP, PNP) (see Huang et al., 1991). *n*, number of SOP positions scored. No phenotypic preferences were seen depending on the specific SOP position. We therefore grouped all notum SOP positions for the statistical analysis. Some clones must have intersected proneural clusters (rather than wholly encompassing them) – these account for the rare occurrences of weak defects in genotypes known to completely abolish lateral inhibition (e.g. N^{5419} and Dl^{rev10} Ser^{RX106}). Additionally, N^{5419} clones had a partially penetrant growth defect; as a result some of the very small clones (two to seven cells) that were entirely composed of SOPs were placed in the weak/moderate categories – none of the other genotypes had any growth defects. *P*-values given in the text refer to pairwise comparisons using a χ-square test.

*Genotype refers to the homozygous genotype of mutant clones. *UAS* transgenes were expressed only within mutant clones using *αtub-Gal4*.

†Shown are percentages of SOP positions falling in different categories, which were defined as follows: wild type, one SOP; weak, between one and four SOPs; moderate, between four and eight SOPs; strong, at least eight SOPs.

‡Double combinations with $mib1^{EY9780}$ were generated as mosaic clones of the other allele ($neur^{1}$, Dl^{rev10} or Ser^{RX106}) in a uniform $mib1^{EY9780}$ genetic background.

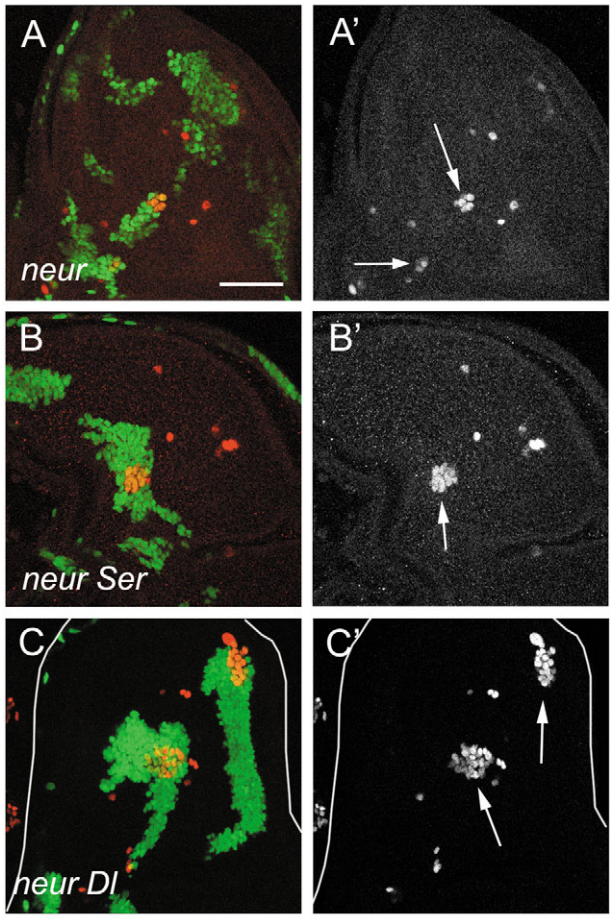

Fig. 2. Neur enhances *Dl* or *Ser* loss of function. (A-C) Nota stained for Senseless (red) to visualize SOPs; Sens channel shown separately in A′-C′. Mutant areas are indicated by arrows. Proximal is upwards, anterior is towards the left. Scale bar: 40 μm. Mutant cells express GFP. (A) *neur* mutant clones display weak/moderate SOP overcommitment. By contrast, (B) *neur Ser* clones and (C) *neur Dl* clones display a much more severe SOP overcommitment. In C the outline of the notum is drawn.

the other. This turned out true, as the excessive number of SOPs in *Dl Ser* clones could be rescued to the wild-type single SOP, when we provided uniform expression of either a *UAS-Dl* (Table 1) or a *UAS-Ser* transgene within the clone (Fig. 1F; Table 1). Furthermore, the milder excess-SOP phenotype of *Dl* clones could be rescued by a *UAS-Ser* transgene (Table 1), confirming that simply increasing the levels of Ser can compensate for the lack of Dl. As a control, clonal ubiquitous expression of *UAS-Dl* or *UAS-Ser* did not affect SOP number (Table 1), suggesting that the overexpression levels attained in these experiments were not so high as to result in cis-inactivation of Notch signaling (de Celis and Bray, 1997; Li and Baker, 2004; Micchelli et al., 1997). By image densitometry, we estimated the overexpression levels to be approximately two- to threefold (see Fig. S2A,B in the supplementary material) of the endogenous levels, using regions of strong endogenous expression as reference. In conclusion, it appears that Ser, as well as Dl, can sustain lateral inhibition alone, but endogenous levels of Ser are limiting, whereas Dl is in plentiful supply. An important corollary from this experiment is that transcriptional modulation of *Dl* (or *Ser*) is not a prerequisite for lateral inhibition, as we obtained a wild-type phenotype with either ligand expressed uniformly via *αtub-Gal4* (Fig. 1F, Table 1).

Neuralized modulates the activity of both Dl and Ser in lateral inhibition

We and others have previously shown that Dl activity is augmented by its association with Neuralized (Lai et al., 2001; Pavlopoulos et al., 2001). Any possible influence of Neur on Ser had so far remained an unanswered question. In fact, recent data has suggested that Neur may act primarily on Dl, whereas Mib1 may act primarily on Ser (Le Borgne et al., 2005). We generated doubly mutant clones between *neur* and *Dl* or *Ser* to assess the ability of *neur* to modify the DSL mutant phenotype. In both cases, *neur* enhanced the phenotype (Fig. 2B,C; Table 1), suggesting that either Dl alone (in *Ser* clones) or Ser alone (in *Dl* clones) is more active in the presence than in the absence of $neur^{+}$.

An interesting observation in this series of experiments was that in contrast to the severe phenotypes of *neur Dl* or *neur Ser* clones, *neur* clones displayed only a weak-moderate defect in

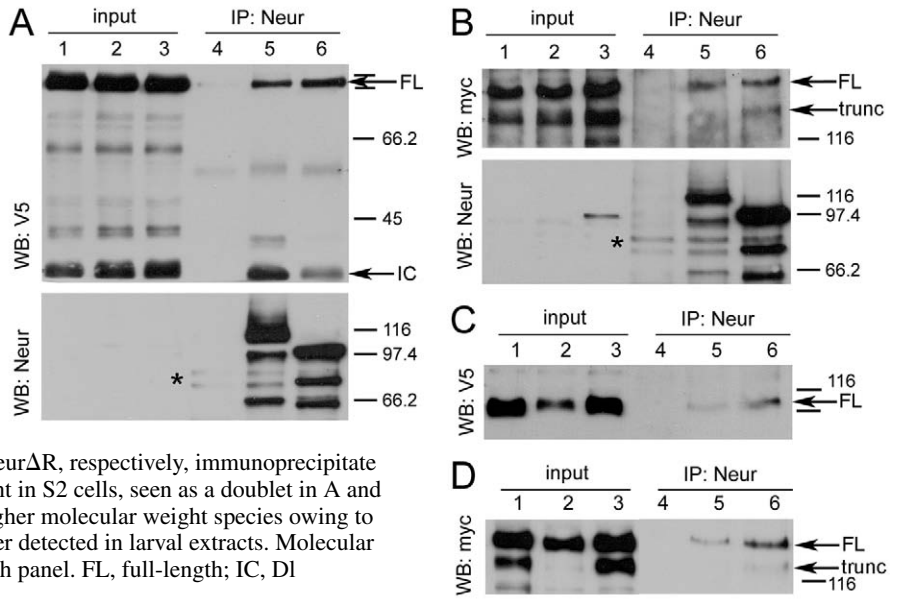

Fig. 3. Physical association between Neur and DSL proteins. (A,B) Immunoprecipitations from transfected S2 cells expressing V5-tagged Dl (A) or myc-tagged Ser (B), along with nothing (lanes 1,4), EGFPneur (lanes 2,5) or neurΔR-GFP (lanes 3,6). Lanes 1-3: cell extract (input). Lanes 4-6: anti-Neur immunoprecipitate.
(C,D) Immunoprecipitations from larval disk/CNS complexes. (C) *hs-Gal4; UAS-Dl-V5* along with another *UAS* transgene as follows: nothing (lanes 1,4), *UAS-EGFPneur* (lanes 2,5) or *UAS-neurΔR-GFP* (lanes 3,6). (D) *hs-Gal4; UAS-Ser-myc* plus another *UAS* transgene, as in C. In lanes 4 of all panels (no Neur expressed) no DSL protein is detected, showing the specificity of the immunoprecipitation. In lanes 5 and 6, Neur and NeurΔR, respectively, immunoprecipitate both Dl and Ser. Endogenous Neur protein is present in S2 cells, seen as a doublet in A and B (lane 4, asterisks). Transfected Neur produces higher molecular weight species owing to the GFP tags. Curiously the Dl_{IC} fragment was never detected in larval extracts. Molecular mass standards are shown in kDa to the right of each panel. FL, full-length; IC, Dl intracellular fragment; trunc, truncated Ser.

lateral inhibition (Fig. 2A), somewhat more severe than *Dl* clones. We concluded that each ligand has residual activity in the absence of Neur, which we subsequently showed to be dependent on Mib1 (see below).

Neur associates with Ser and affects its subcellular localization

The fact that Ser activity seems to be influenced by Neur (Fig. 2) prompted us to investigate whether Neur can associate with Ser and modify its subcellular localization in an analogous manner to its effects on Dl (Lai et al., 2001; Pavlopoulos et al., 2001). Association was assayed by co-immunoprecipitation. We used Schneider S2 cells or transgenic larval tissue, in both cases overexpressing epitope-tagged DSL protein and EGFP-tagged Neur. Immunoprecipitation of transfected S2 cell extracts using anti-Neur antiserum was able to specifically co-precipitate Dl protein, as well as its intracellular proteolytic product (Dl_{IC}), presumably cleaved extracellularly near the trans-membrane domain (Bland et al., 2003) (Fig. 3A,C). The Neur antiserum could similarly immunoprecipitate Ser (Fig. 3B,D). We were not able to detect a short Ser_{IC} fragment comparable with Dl_{IC}; instead two major high molecular weight bands were obtained, one consistent with the predicted size for the FL protein and the other apparently lacking part of the extracellular domain (truncated Ser). When a NeurΔR mutant was used, which lacks the RING domain, essentially the same results were obtained, suggesting that the RING domain is dispensable for association with DSL proteins. Negative controls were performed using *lacZ* or *groucho*: no β-galactosidase or Groucho was detected in the anti-Neur immunoprecipitates (data not shown). Even though association between either DSL protein and Neur was detected in both S2 cells and larval tissues, we were unable to observe any interactions between various fragments of Neur and the intracellular domains of Dl or Ser in a yeast two-hybrid approach (V. Baoussis and C.D., unpublished).

Like Dl, Ser is found both on the apical plasma membrane and in intracellular vesicles, both endogenously and when overexpressed in wing disk cells (Fig. 4A,C). This changed dramatically when a *UAS-neur* transgene was co-expressed; Ser was cleared from the apical surface (Fig. 4B,C). The subapical intracellular aggregates were not affected in the case of endogenous Ser, but were greatly increased in the case of overexpressed Ser. Using an *EGFP* tagged *neur* transgene (which behaves identically to our untagged *UAS-neur*; data not shown), we showed that most of the subapical Ser-overexpressing aggregates also accumulated Neur, which additionally remained ubiquitously cortical, mostly on the apical side (Fig. 4B,E). This cortical localization is what is normally observed for Neur in the absence of co-overexpressed DSL ligand (Fig. 4C,D), suggesting that the large number of Ser-positive/Neur-positive intracellular aggregates probably appear because of impaired trafficking caused by Ser overexpression. This response of endogenous and overexpressed Ser to Neur is identical to what has been previously described for Dl (Lai et al., 2001; Pavlopoulos et al., 2001). Using *Dl* or *Ser* mutant backgrounds, we showed that Neur elicits endocytosis of each DSL protein independently of the presence of the other (see Fig. S3 in the supplementary material); this refutes the possibility that the effects of Neur on Ser are due to Dl-Ser interactions.

Mind bomb1 acts redundantly with Neur in lateral inhibition

Despite its physical and functional association with both DSL proteins (Lai et al., 2001; Pavlopoulos et al., 2001) (this work), *neur* loss of function has only a mild lateral inhibition defect compared with *Dl Ser* loss of function (Table 1, Fig. 2). This led us to conclude that there is substantial Neur-independent DSL activity. The characterization of *mind bomb* as a Dl-targeting Ub ligase in zebrafish (Chen and Corliss, 2004; Itoh et al., 2003) made us wonder whether a possible *Drosophila* ortholog might be responsible for this activity. BLAST search identified two *Drosophila* genes with close similarity to zebrafish *mib*, *CG5841* and *CG17492*, which we henceforth call *mib1* and *mib2,* respectively. Of these, Mib1 has a better

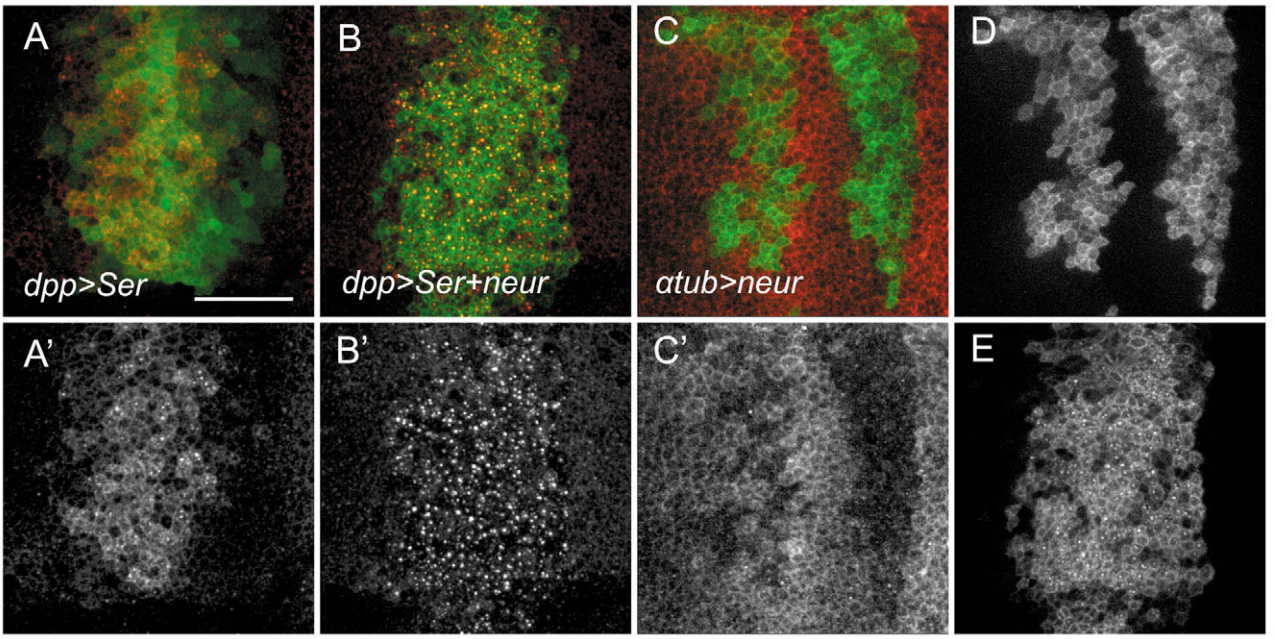

Fig. 4. Neur induces Ser endocytosis. Details of wing pouches are shown; anterior is towards the left, dorsal is upwards and the DV boundary is at the bottom of each panel. Scale bar: 20 μm. (A,B) *dpp-Gal4; UAS-Ser* with co-expression of *UAS-GFP* (A) or *UAS-EGFPneur* (B). GFP (green) marks the domain of overexpression and Ser (red) is shown separately in A′ and B′. Neur causes loss of pericellular staining and an increase in intracellular aggregates in B. (C) Effects of mosaic expression of *UAS-EGFPneur* on endogenous Ser (red, C′). In cells expressing EGFPneur (green), apical Ser staining is lost. The detection sensitivity for Ser is increased in C compared with A,B to image endogenous Ser levels. (D,E) Green (EGFPneur) channels of C,B, respectively. EGFPneur is cortical with little punctate accumulation when no DSL protein is co-overexpressed (D); when Ser is co-overexpressed there is additional accumulation into punctate structures, which also contain Ser (yellow dots in B). All images are projections of the apical-most 1.5-2 μm of the wing epithelium.

similarity to zebrafish Mib. In situ hybridization to embryos revealed a segmentally repeated stripe pattern of *mib1* mRNA at stage 9-10, which disappears later, whereas third instar wing disks showed low ubiquitous expression (data not shown) (see also Le Borgne et al., 2005). The *Drosophila* gene disruption project (Bellen et al., 2004) has generated a P-element insertion, *EY9780*, which disrupts the *mib1* gene in the 5′ UTR. *EY9780* homozygotes survive to pupal stage with a good percentage of pharate adult escapers. These have small, almost non-existent, eyes and wings, and short legs (data not shown) (Lai et al., 2005; Le Borgne et al., 2005). We could not detect any *mib1* mRNA in *EY9780* homozygotes by RT-PCR (data not shown). Based on this and on the fact that excision of the P-element reverted the lethality (data not shown), we concluded that this P-element represents a null allele of *mib1* and we designated it as $mib1^{EY9780}$ [see also complementary evidence elsewhere (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005)].

$mib1^{EY9780}$ pharate adults showed a mild increase in microchaete density and only occasional macrochaete duplications (data not shown); therefore, loss of *mib1* does not particularly affect lateral inhibition. When, however, we induced *neur* mutant clones in a homozygous *mib1* background, we observed a large number of ectopic SOPs (Fig. 5), a phenotype much more severe than that of *neur* clones and indistinguishable from that of *N* or *Dl Ser* clones (Table 1). It appears, therefore, that Neur and Mib1 have redundant roles in lateral inhibition. We took advantage of our finding that macrochaete lateral inhibition can be carried out by each individual Notch ligand, to a certain extent at least (Fig. 1), to ask whether Mib1 affects one or both DSL proteins. A *mib1* background

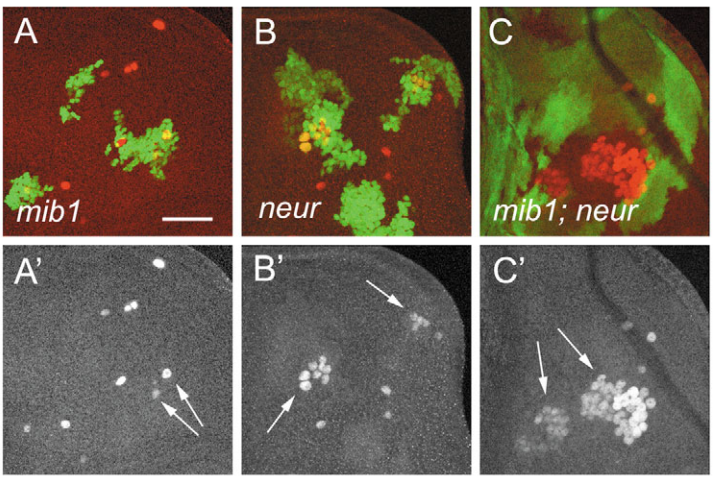

Fig. 5. *mib1* enhances the lateral inhibition phenotype of *neur*. (A-C) Nota stained for Sens (red, A′-C′) to reveal SOPs. (A,B) Mutant clones marked by GFP expression; (C) mutant clones marked by GFP absence. Arrows indicate mutant areas. (A) $mib1^{EY9780}$ clones do not produce supernumerary SOPs. (B) $neur^{1}$ mutant clones show mild defects. (C) $neur^{1}$ mutant clones in a $mib1^{EY9780}$ background show severe hyperplasia of SOPs. Scale bar: 40 μm.

enhanced the phenotype of *Dl* clones ($P<0.05$; Table 1), suggesting that Ser is less active when Mib1 is removed. However, *Ser* clones in a *mib1* background appeared wild type. Therefore, Dl retains full activity in the presence of only Neur, whereas Ser requires both Mib1 and Neur for full activity during lateral inhibition.

Mind bomb1 has similar activity to Neur

The enhancement of the *neur* phenotype by *mib1* may indicate that Neur and Mib1 act in parallel and have similar molecular functions, or they could have distinct functions in the same pathway, as partial block of signal flow in two steps along a pathway can result in an enhanced phenotype (e.g. *neur Dl* versus either *neur* or *Dl*). To address Neur-Mib1 interchangeability, we turned to wing DV boundary induction. When DSL proteins are expressed ectopically in the wing pouch, they induce ectopic Notch targets (e.g. *wg*) in a compartment-specific manner: Dl being active preferentially in the D compartment (Fig. 6A) and Ser being active exclusively in the V compartment (Fig. 6D). In *mib1* clones, overexpressed DSL proteins were unable to induce ectopic Wg (Fig. 6B,E) – even endogenous Wg was abolished. Therefore, Mib1 appears to be needed for signal emission by both DSL ligands in the wing pouch, where *neur* is not normally expressed. Reciprocally, Mib1 appears dispensable for signal reception: *mib1* cells were able to express Wg when they abutted a *mib1*$^{+}$ stripe of cells ectopically expressing *Dl* (Fig. 6I). When we co-expressed Neur with either DSL protein in *mib1* clones, the ligands regained their ability to induce Wg (Fig. 6C,F); in fact, Dl was hyperactivated, just as it is when co-expressed with Neur in a wild-type background (Pavlopoulos et al., 2001). Therefore, Neur can substitute for the lack of Mib1 activity during wing DV boundary specification in agreement with recent reports (Le Borgne et al., 2005; Wang and Struhl, 2005). These experiments clearly show that each Ub ligase can activate DSL signaling alone (in the absence of the other ligase). This is consistent with the two Ub ligases having similar molecular functions, as has also been suggested by their

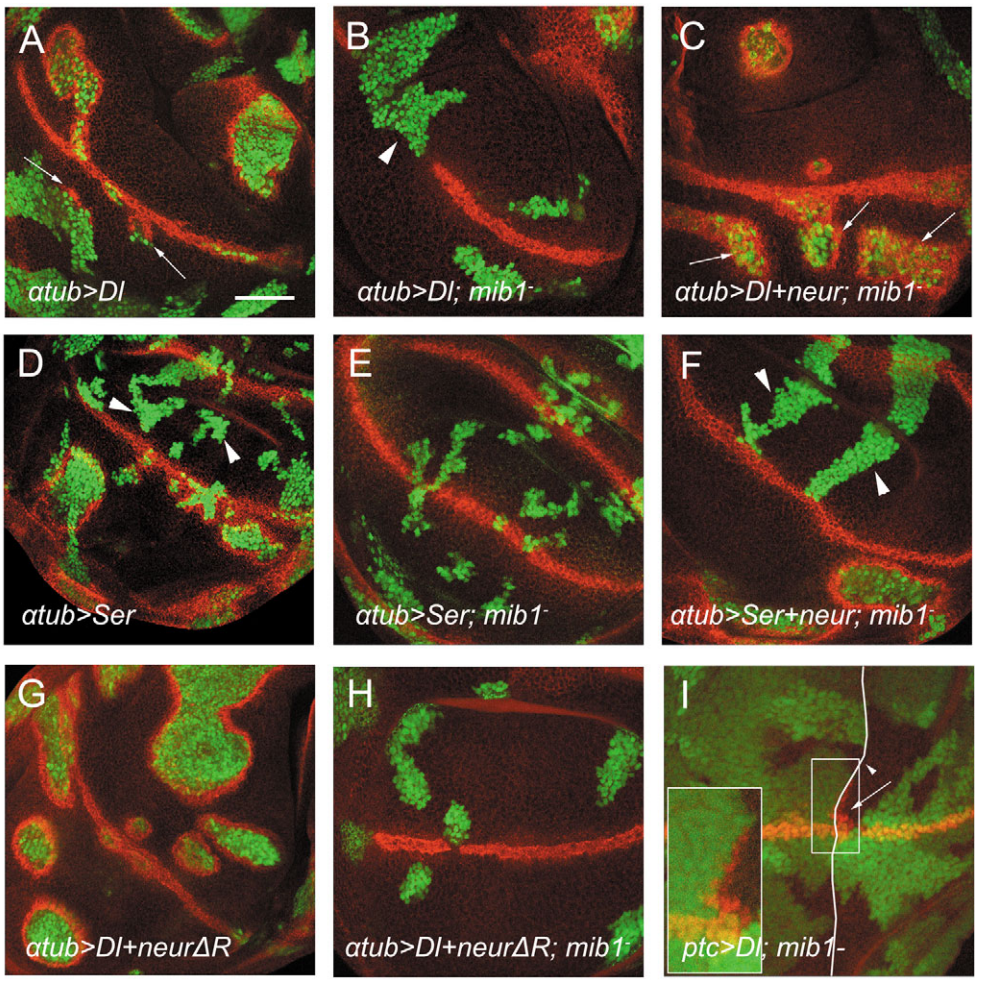

Fig. 6. Dl and Ser signaling in the wing pouch needs ubiquitin ligase activity. (A-H) Wg (red) in wing pouches carrying mutant clones marked by the expression of GFP (green). In all panels, ventral is downwards. Scale bar: 40 μm; 60 μm in D,G. (A,B) Clones overexpress *UAS-Dl* in a wild-type (A) or *mib1* (B) background. Whereas ectopic Wg is induced by dorsal and (less) by ventral (arrows) clones in A, no Wg induction is observed in B. The endogenous Wg stripe is abolished (arrowhead); this is also observed in *mib1* clones without *Dl* overexpression (data not shown). (C) *mib1* clones overexpressing *UAS-Dl* together with *UAS-neur* restore Wg induction; in fact Dl activity in the ventral compartment is enhanced (arrows, compare with A). (D,E) Clones overexpress *UAS-Ser* in a wild-type (D) or *mib1* (E) background. Ser induces Wg exclusively in the ventral compartment (D), but not when expressed in *mib1* cells (E). (F) Ser regains its activity to induce Wg in a *mib1* background, if it is co-expressed with *UAS-neur*. Ser cannot induce Wg in the dorsal compartment, irrespective of the presence of ubiquitin ligases (D,F; arrowheads), probably owing to high Fng levels. (G) Co-expression of *UAS-Dl* with *UAS-neurΔR* in a wild-type background efficiently induces ectopic Wg in both compartments, indicative of increased Dl activity (compare with A). (H) This is abolished by loss of *mib1*. (I) Cut is imaged (red), which is a nuclear marker for the DV boundary. *mib1* mutant clones are marked by the absence of GFP. *UAS-Dl* is overexpressed via *ptc-Gal4*, which drives expression just anterior of the anteroposterior boundary (white line). In the cells posteriorly adjacent to this expression domain (to the right of the white line), ectopic Cut is detected within *mib1* cells if they abut anterior wild-type cells (arrow), but not if they abut mutant anterior cells (arrowhead). Inset shows an enlargement of the boxed area; the Cut-positive nuclei, which do not contain GFP appear red. The presence of these Cut-positive *mib1* mutant cells suggests that *mib1*$^{+}$ is not needed for signal reception.

biochemical analysis (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Lai et al., 2005).

The mechanism via which Neur and Mib1 activate the DSL proteins has not been established; a likely hypothesis is that it involves one or more ubiquitination events, which target Dl and Ser for endocytosis. This is supported by the need for Epsin, a ubiquitin-binding endocytic adaptor protein, specifically for Notch signal emission but not signal reception (Overstreet et al., 2004; Wang and Struhl, 2004). We had previously challenged the view that Dl ubiquitination/endocytosis is necessary for its activity, as NeurΔR, which has lost the ubiquitin ligase catalytic domain, could hyperactivate Dl in a *wg* induction assay, behaving similarly to wild type Neur (Pavlopoulos et al., 2001). We assayed the ability of Dl and NeurΔR to induce *wg* when co-expressed in *mib1* clones (Fig. 6G,H). Unlike the high activity detected in wild-type background, this combination was completely inactive. The most likely explanation is that Dl cannot signal if ubiquitination is abolished. A further conclusion is that Neur(ΔR) has some additional Dl stimulatory activity (besides ubiquitination) that, however, can only be manifested when ubiquitination is feasible (in *mib1*$^{+}$ cells; compare Fig. 6G with 6A); it remains to be discovered what the molecular basis for this activity is.

Discussion

The roles of DSL proteins in lateral inhibition

Until now, it was thought that lateral inhibition in notum SOPs was solely mediated via Dl (Zeng et al., 1998) and that *Dl* transcriptional upregulation in the nascent neural precursor was crucial for a Dl-N negative feedback loop to establish the neural precursor fate within a group of equivalent cells (Heitzler and Simpson, 1991). Our data have refuted both of these models, as endogenous Ser has been shown to participate in lateral inhibition of macrochaete SOPs (Fig. 1) and either Dl or Ser uniformly expressed is able to produce a wild-type pattern of macrochaetes (Fig. 1, Table 1). Dl transcriptional upregulation in the absence of Notch signaling in proneural fields does occur (Koelzer and Klein, 2003; Schweisguth and Posakony, 1994), but this modulation does not appear to be a prerequisite for the specification of the wild-type neural precursor, at least in the case of macrochaetes (this work) and embryonic neuroblasts (Seugnet et al., 1997b). It is possible that the genetically detected N-Dl negative feedback loop may reflect Dl and N activity rather than transcription, although a transcriptional input has been documented (Heitzler et al., 1996). An exciting possibility, given the reliance of DSL activity on ubiquitin ligases, is that this feedback loop targets transcription of *neur*, rather than *Dl*. *mib1* is an unlikely target as it shows no transcriptional modulation within proneural regions.

Ubiquitin ligases and DSL protein function

Although Neur was known to affect Dl localization and function in some instances (Lai et al., 2001; Lai and Rubin, 2001; Le Borgne and Schweisguth, 2003; Pavlopoulos et al., 2001; Yeh et al., 2000), ubiquitin ligases were not considered as essential components of Notch signaling. The characterization of Mib1 described here and in recent papers (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005) points to a much more prominent role of these factors. *mib1* appears to be required in a large number of Notch-dependent processes where *neur* is not expressed, e.g. the wing DV boundary. The fact that *mib1 neur* double mutants appear to lose all ability to perform lateral inhibition (Fig. 5) strongly supports the hypothesis that Ub ligases may always be required for Dl/Ser signaling. A comprehensive survey of Notch-dependent events with respect to *neur* and *mib1* will test this hypothesis and may uncover additional E3 ligases with this activity; Mib2 represents a potential candidate.

The intimate relation between Neur/Mib1 and DSL proteins is generally assayed in three ways: (1) physical association, (2) effects on Dl/Ser endocytosis and (3) effects on Dl/Ser signaling. All of these had been well documented for the Neur-Dl combination (Lai et al., 2001; Pavlopoulos et al., 2001) and, more recently, for the Mib1-Dl and Mib1-Ser combinations (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005) (this work). In the present work we have added the final pair, Neur-Ser, using all of the above assays. The conclusion, stated simply, is that both Neur and Mib1 associate with and affect the endocytosis and function of both Dl and Ser.

Mechanism of Dl/Ser signaling

Ubiquitination of transmembrane proteins tags them for endocytosis, using a complex of adaptors, including epsin, which carry ubiquitin recognition domains (Haglund et al., 2003). The simplest scenario for the role of Neur/Mib1 in Dl/Ser signaling would be that they attach ubiquitin to Dl/Ser to trigger endocytosis. Signaling would ensue, either as a consequence of recruiting/clustering ubiquitinated DSL cargo to specialized plasma membrane domains conducive to signaling, or by more elaborate routes involving DSL protein recycling through the endocytic pathway as a prerequisite for their modification/activation (Wang and Struhl, 2004).

Alternatively, Neur/Mib1 need not ubiquitinate the DSL proteins directly. In the ubiquitin-dependent endocytosis pathway, many of the adaptor proteins are themselves ubiquitinated, possibly favoring the formation of interconnected cargo-adaptor complexes (Polo et al., 2002); Neur/Mib1 could have one or more of the adaptors, including themselves, as substrates. DSL protein chimaeras become Mib1 independent if their intracellular domains are substituted with ones bearing alternative internalization motifs (Wang and Struhl, 2005). Of two such artificial Mib1-independent versions of Dl, one is ubiquitination/epsin-independent (Dl-LDL-receptor fusion), whereas the other (Dl-random-peptide-R fusion) still curiously requires ubiquitination/epsin for activity (Wang and Struhl, 2004). Nothing is yet known about the native Dl/Ser intracellular domains, other than the puzzling fact that they are neither similar nor evolutionarily conserved, despite apparent conservation of recognition by Neur/Mib.

An even more puzzling observation in the light of our model is that some DSL proteins in *C. elegans* appear to be secreted (Chen and Greenwald, 2004). Secreted mutants of *Drosophila* Dl and Ser act as Notch antagonists (Mishra-Gorur et al., 2002; Sun and Artavanis-Tsakonas, 1997), consistent with a requirement for endocytosis in DSL signaling. Even *C. elegans* LAG-2 (a transmembrane DSL) needs EPN-1 (epsin ortholog), in order to signal to GLP-1 (Notch-like) during germline differentiation (Tian et al., 2004), which is hard to reconcile with secreted DSL proteins. Apparently, ubiquitination/

endocytosis can be bypassed in some contexts, allowing secreted DSL proteins to signal via a yet unknown process.

Whatever the molecular details and variations turn out to be, we are quickly coming to realize that ubiquination plays a prominent role in Notch signaling, in both sending and receiving cells. In the latter, Ub ligases downregulate Notch activity either at the membrane (Qiu et al., 2000; Sakata et al., 2004; Wilkin et al., 2004) or in the nucleus (Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). Besides downregulation, however, Notch ubiquitination is also needed for activation: ubiquitination apparently targets Notch to a compartment where it can be activated by γ-secretase cleavage (Gupta-Rossi et al., 2004). How two ubiquitination/trafficking events, activating DSL proteins in one cell and Notch in another, might be coordinated across the extracellular space is a mystery worth investigating in the future.

The authors thank Kiki Kanakousaki for help with staining and in situ, and Ioannis Livadaras and Giorgos Vrentzos for technical assistance. We also thank Gary Struhl, Sarah Bray, Matt Rand, Marc Muskavitch, Spyros Artavanis-Tsakonas, Hugo Bellen and Bob Fleming for generously providing, flies and reagents. This work was funded in part by the Herakleitos program of the Greek Ministry for Education and by IMBB intramural funds.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/18/4041/DC1

Note added in proof

Koo et al. (Koo et al., 2005b) have studied murine Mib1 and have come to a similar conclusion, namely that Mib1 associates with all Notch ligands (Dll1, Dll3, Dll4, Jag1 and Jag2) and is necessary for their activation.

References

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics* **167**, 761-781.

Bland, C. E., Kimberly, P. and Rand, M. D. (2003). Notch-induced proteolysis and nuclear localization of the Delta ligand. *J. Biol. Chem.* **278**, 13607-13610.

Blochlinger, K., Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). Patterns of expression of Cut, a protein required for external sensory organ development in wild-type and *cut* mutant *Drosophila* embryos. *Genes Dev.* **4**, 1322-1331.

Bray, S. (1998). Notch signalling in Drosophila: three ways to use a pathway. *Semin. Cell Dev. Biol.* **9**, 591-597.

Chen, N. and Greenwald, I. (2004). The lateral signal for LIN-12/Notch in C. elegans vulval development comprises redundant secreted and transmembrane DSL proteins. *Dev. Cell* **6**, 183-192.

Chen, W. and Corliss, D. C. (2004). Three modules of zebrafish Mind bomb work cooperatively to promote Delta ubiquitination and endocytosis. *Dev. Biol.* **267**, 361-373.

de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124**, 3241-3251.

Deblandre, G. A., Lai, E. C. and Kintner, C. (2001). Xenopus Neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. *Dev. Cell* **1**, 795-806.

Fleming, R. J. (1998). Structural conservation of Notch receptors and ligands. *Semin. Cell Dev. Biol.* **9**, 599-607.

Gu, Y., Hukriede, N. A. and Fleming, R. J. (1995). *Serrate* expression can functionally replace *Delta* activity during neuroblast segregation in the *Drosophila* embryo. *Development* **121**, 855-865.

Gupta-Rossi, N., Le Bail, O., Gonen, H., Brou, C., Logeat, F., Six, E., Ciechanover, A. and Israel, A. (2001). Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. *J. Biol. Chem.* **276**, 34371-34378.

Gupta-Rossi, N., Six, E., LeBail, O., Logeat, F., Chastagner, P., Olry, A., Israel, A. and Brou, C. (2004). Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. *J. Cell Biol.* **166**, 73-83.

Haglund, K., Di Fiore, P. P. and Dikic, I. (2003). Distinct monoubiquitin signals in receptor endocytosis. *Trends Biochem. Sci.* **28**, 598-603.

Haines, N. and Irvine, K. D. (2003). Glycosylation regulates Notch signalling. *Nat. Rev. Mol. Cell Biol.* **4**, 786-797.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**, 1083-1092.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the *Enhancer of split* and *achaete-scute* complexes are required for a regulatory loop between *Notch* and *Delta* during lateral signalling in *Drosophila*. *Development* **122**, 161-171.

Irvine, K. D. and Vogt, T. F. (1997). Dorsal-ventral signaling in limb development. *Curr. Opin. Cell Biol.* **9**, 867-876.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Klueg, K. M. and Muskavitch, M. A. (1999). Ligand-receptor interactions and trans-endocytosis of Delta, Serrate and Notch: members of the Notch signalling pathway in *Drosophila*. *J. Cell Sci.* **112**, 3289-3297.

Koelzer, S. and Klein, T. (2003). A Notch-independent function of Suppressor of Hairless during the development of the bristle sensory organ precursor cell of *Drosophila*. *Development* **130**, 1973-1988.

Koo, B. K., Yoon, K. J., Yoo, K. W., Lim, H. S., Song, R., So, J. H., Kim, C. H. and Kong, Y. Y. (2005a). Mind bomb-2 is an E3 ligase for Notch ligand. *J. Biol. Chem.* **280**, 22335-22342.

Koo, B.-K., Lim, H.-S., Song, R., Yoon, M.-J., Yoon, K.-J., Moon, J.-S., Kim, Y.-W., Kwon, M. c., Yoo, K. W., Kong, M.-P. et al. (2005b). Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development* **132**, 3459-3470.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-973.

Lai, E. C. and Rubin, G. M. (2001). *neuralized* functions cell-autonomously to regulate a subset of Notch-dependent processes during adult *Drosophila* development. *Dev. Biol.* **231**, 217-233.

Lai, E. C., Deblandre, G. A., Kintner, C. and Rubin, G. M. (2001). Drosophila Neuralized is a ubiquitin ligase that promotes the internalization and degradation of Delta. *Dev. Cell* **1**, 783-794.

Lai, E. C., Roegiers, F., Qin, X., Jan, Y. N. and Rubin, G. M. (2005). The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. *Development* **132**, 2319-2332.

Le Borgne, R. and Schweisguth, F. (2003). Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. *Dev. Cell* **5**, 139-148.

Le Borgne, R., Remaud, S., Hamel, S. and Schweisguth, F. (2005). Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. *PLoS Biol.* **3**, e96.

Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. *Trends Neurosci.* **24**, 251-254.

Lehman, R., Jimenez, F., Dietrich, U. and Campos-Ortega, J. A. (1983). On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*. *Roux Arch. Dev. Biol.* **192**, 62-74.

Li, Y. and Baker, N. E. (2004). The roles of cis-inactivation by Notch ligands and of *neuralized* during eye and bristle patterning in *Drosophila*. *BMC Dev. Biol.* **4**, 5.

Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of *cut* expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.

Mishra-Gorur, K., Rand, M. D., Perez-Villamil, B. and Artavanis-Tsakonas, S. (2002). Down-regulation of Delta by proteolytic processing. *J. Cell Biol.* **159**, 313-324.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* **102**, 349-362.

Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M. and Lendahl, U. (2001).

The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J. Biol. Chem.* **276**, 35847-35853.

Okajima, T., Xu, A. and Irvine, K. D. (2003). Modulation of Notch-ligand binding by protein O-fucosyltransferase 1 and Fringe. *J. Biol. Chem.* **278**, 42340-42345.

Overstreet, E., Chen, X., Wendland, B. and Fischer, J. A. (2003). Either part of a Drosophila epsin protein, divided after the ENTH domain, functions in endocytosis of Delta in the developing eye. *Curr. Biol.* **13**, 854-860.

Overstreet, E., Fitch, E. and Fischer, J. A. (2004). Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. *Development* **131**, 5355-5366.

Pavlopoulos, E., Pitsouli, C., Klueg, K. M., Muskavitch, M. A., Moschonas, N. K. and Delidakis, C. (2001). *neuralized* encodes a peripheral membrane protein involved in Delta signaling and endocytosis. *Dev. Cell* **1**, 807-816.

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P. and Di Fiore, P. P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416**, 451-455.

Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T. and Artavanis-Tsakonas, S. (1999). Processing of the Notch ligand Delta by the metalloprotease Kuzbanian. *Science* **283**, 91-94.

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T. and Liu, Y. C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J. Biol. Chem.* **275**, 35734-35737.

Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K. and Hayashi, S. (2004). Drosophila Nedd4 regulates endocytosis of notch and suppresses its ligand-independent activation. *Curr. Biol.* **14**, 2228-2236.

Schweisguth, F. (2004). Notch signaling activity. *Curr. Biol.* **14**, R129-R138.

Schweisguth, F. and Posakony, J. W. (1994). Antagonistic activities of *Suppressor* of *Hairless* and *Hairless* control alternative cell fates in the *Drosophila* adult epidermis. *Development* **120**, 1433-1441.

Seugnet, L., Simpson, P. and Haenlin, M. (1997a). Requirement for dynamin during Notch signaling in Drosophila neurogenesis. *Dev. Biol.* **192**, 585-598.

Seugnet, L., Simpson, P. and Haenlin, M. (1997b). Transcriptional regulation of *Notch* and *Delta*: requirement for neuroblast segregation in *Drosophila*. *Development* **124**, 2015-2025.

Skeath, J. B. and Thor, S. (2003). Genetic control of *Drosophila* nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.

Sun, X. and Artavanis-Tsakonas, S. (1997). Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in *Drosophila*. *Development* **124**, 3439-3448.

Takeuchi, T., Adachi, Y. and Ohtsuki, Y. (2005). Skeletrophin, a novel ubiquitin ligase to the intracellular region of Jagged-2, is aberrantly expressed in multiple myeloma. *Am. J. Pathol.* **166**, 1817-1826.

Tian, X., Hansen, D., Schedl, T. and Skeath, J. B. (2004). Epsin potentiates Notch pathway activity in *Drosophila* and *C. elegans*. *Development* **131**, 5807-5815.

Wang, W. and Struhl, G. (2004). Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. *Development* **131**, 5367-5380.

Wang, W. and Struhl, G. (2005). Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. *Development* **132**, 2883-2894.

Wilkin, M. B., Carbery, A. M., Fostier, M., Aslam, H., Mazaleyrat, S. L., Higgs, J., Myat, A., Evans, D. A., Cornell, M. and Baron, M. (2004). Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. *Curr. Biol.* **14**, 2237-2244.

Wu, G., Lyapina, S., Das, I., Li, J., Gurney, M., Pauley, A., Chui, I., Deshaies, R. J. and Kitajewski, J. (2001). SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol. Cell. Biol.* **21**, 7403-7415.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Yeh, E., Zhou, L., Rudzik, N. and Boulianne, G. L. (2000). *neuralized* functions cell autonomously to regulate *Drosophila* sense organ development. *EMBO J.* **19**, 4827-4837.

Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C. J. and Boulianne, G. L. (2001). Neuralized functions as an E3 ubiquitin ligase during Drosophila development. *Curr. Biol.* **11**, 1675-1679.

Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* **8**, 709-715.

Zeng, C., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1998). Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the *Drosophila* sensory organ lineage. *Genes Dev.* **12**, 1086-1091.