Role of conserved intracellular motifs in Serrate signalling, cis-inhibition and endocytosis

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Notch is the receptor in a signalling pathway that operates in a diverse spectrum of developmental processes. Its ligands (e.g. Serrate) are transmembrane proteins whose signalling competence is regulated by the endocytosis-promoting E3 ubiquitin ligases, Mindbomb1 and Neuralized. The ligands also inhibit Notch present in the same cell (cis-inhibition). Here, we identify two conserved motifs in the intracellular domain of Serrate that are required for efficient endocytosis. The first, a dileucine motif, is dispensable for trans-activation and cis-inhibition despite the endocytic defect, demonstrating that signalling can be separated from bulk endocytosis. The second, a novel motif, is necessary for interactions with Mindbomb1/Neuralized and is strictly required for Serrate to trans-activate and internalise efficiently but not for it to inhibit Notch signalling. Cis-inhibition is compromised when an ER retention signal is added to Serrate, or when the levels of Neuralized are increased, and together these data indicate that cis-inhibitory interactions occur at the cell surface. The balance of ubiquitinated/unubiquitinated ligand will thus affect the signalling capacity of the cell at several levels.

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Introduction

Notch is the receptor in a highly conserved cell:cell signalling pathway that is essential in a multitude of developmental processes (Artavanis-Tsakonas *et al*, 1999; Schweisguth, 2004). Notch activity is also important in maintenance of many tissues, through its activity in stem cells and/or in regulating the production and fates of differentiated cell types. In the majority of processes, Notch receptors are

activated by a class of ligands referred to as DSL ligands, which are themselves type I integral membrane proteins (Fleming, 1998). Intriguingly, many of these ligands have the capacity to act as both agonists and antagonists of the receptor (e.g. de Celis and Bray, 1997; Klein *et al*, 1997; Micchelli *et al*, 1997; Sakamoto *et al*, 2002; Li and Baker, 2004).

The complex behaviours of Notch ligands have perhaps been best characterised in the *Drosophila* wing. *Drosophila* Notch has two ligands, Delta and Serrate. Like all members of this family, their extracellular domain consists largely of EGF repeats and an N-terminal region containing the conserved DSL motif. This motif is necessary for ligands to bind Notch and to confer full function *in vivo* (Muskavitch, 1994; Henderson *et al*, 1997; Fleming, 1998; Shimizu *et al*, 1999), although in one case a ligand lacking the DSL was reported to activate Notch (Kiyota and Kinoshita, 2002). In the developing wing the two ligands preferentially activate Notch in different compartments: Serrate activates Notch in ventral cells and Delta preferentially activates Notch in dorsal cells (Irvine, 1999). This difference in activating capacity occurs because of differential glycosylation of the Notch receptor by Fringe, which is present in dorsal cells and modifies sites in the receptor that alter its affinities for the two ligands (Irvine, 1999; Bruckner *et al*, 2000; Moloney *et al*, 2000; Okajima *et al*, 2003). At late stages in wing development, the domain of Notch activation is also limited by the expression of the ligands themselves, since the pathway becomes ectopically activated in clones of mutant cells that lack both ligands (Micchelli *et al*, 1997). This demonstrates that Notch can be inhibited by the presence of excess ligand in the same cell (cis-inhibition; de Celis and Bray, 1997; Klein *et al*, 1997; Micchelli *et al*, 1997; Sakamoto *et al*, 2002; Li and Baker, 2004).

Ligand signalling activity is regulated post-translationally through the actions of two E3 ubiquitin ligases, Mindbomb1 (Mib1) and Neuralized (Neur) (Lai *et al*, 2001, 2005; Pavlopoulos *et al*, 2001; Itoh *et al*, 2003; Koo *et al*, 2005; Le Borgne *et al*, 2005b). In *Drosophila* the two ligases can functionally substitute for one another in at least some processes, indicating that they perform similar functions even though they share no obvious sequence similarity apart from a canonical RING domain (Lai *et al*, 2005; Le Borgne *et al*, 2005b; Pitsouli and Delidakis, 2005). Mib1 and Neur also regulate trafficking of the Notch ligands, but how ubiquitination and/or trafficking renders the ligands active for signalling remains unclear (Overstreet *et al*, 2004; Lai *et al*, 2005; Le Borgne *et al*, 2005b; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). However, the importance of trafficking is underscored by the ability of an endocytic motif from an unrelated trans-membrane protein to substitute for the Delta intracellular domain (Wang and Struhl, 2004, 2005).

It is evident therefore that Notch ligands undergo complex regulation. Here, we have investigated what parts of Serrate are required for regulating its transport and signalling/

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inhibitory functions. To do this we have examined the effects of expressing altered Serrate ligands in the developing wing. Our results identify a conserved intracellular motif that is essential for Notch activation and for an interaction with Mib1 and Neur. We also show that Serrate mediated cis-inhibition requires the N-terminal/DSL domain, and occurs through Notch–Serrate interactions at the cell surface.

Results

Effects of ectopic Serrate on Notch activity

In the developing wing imaginal disc *Serrate* is expressed primarily in the dorsal compartment, from where it activates Notch at the d/v boundary to induce expression of genes such as *E(spl)mβ*, *cut* and *wingless* (Figure 1A; e.g. de Celis *et al*, 1996; Neumann and Cohen, 1996). To investigate which parts of Serrate are required for its functions, we expressed wild-type (*Ser*^{WT}) and mutant forms of the ligand in a stripe orthogonal to the d/v boundary (using *ptc::Gal4*; Figure 1B) and examined their effect on Notch activity. Two major effects were seen with *Ser*^{WT} as described previously (e.g. Speicher *et al*, 1994; Klein *et al*, 1997). Firstly, the ligand induced two stripes of ectopic Notch activation in the ventral compartment, as manifest by expression of *Cut* adjacent to cells with highest *Ser*^{WT} (trans-activation; Figures 1C and 2A). Ectopic Notch signalling is restricted to ventral cells

by the activity of Fringe in dorsal cells, which modifies the receptor making it refractory to activation by Serrate (Irvine, 1999). Secondly, in those cells where *Ser*^{WT} was present at higher levels it inhibited endogenous Notch activation at the d/v boundary (Figures 1C and 2A). This cell autonomous inhibitory effect is referred to as cis-inhibition.

To determine whether activation and cis-inhibition both require the conserved DSL domain, we generated a mutant ligand in which five residues were altered to alanines (*Ser*^{*DSL}; Figure 1D). *Ser*^{*DSL} had a similar cellular distribution to wild-type ligand, which is present in many intracellular vesicles as well as localizing to the subapical domain of the cell membrane (Figure 2E and F). However, *Ser*^{*DSL} failed to elicit *Cut* expression in adjacent cells (Figure 2B), demonstrating that it could no longer activate the receptor as predicted from the importance of the DSL domain for Notch binding (Muskavitch, 1994; Fleming, 1998; Shimizu *et al*, 1999). In addition, *Ser*^{*DSL} had lost the ability to significantly cis-inhibit as *Cut* was present throughout the ectopic domain of ligand expression (Figure 2B; the levels within the domain are slightly reduced but not dissimilar to variability seen in wild-type discs, see Figure 1A and B) and adult flies were phenotypically wild type. We also tested the effects of a range of other mutations within the DSL and adjacent conserved NT domain of Serrate (data not shown); all had similar consequences of eliminating both aspects of ligand function.

Figure 1 Wing disc assay for Serrate functions and details of mutant Serrate proteins tested. *Cut* (A–C, green) expression in wild type (A, B) and in discs ectopically expressing *Ser*^{WT} (C); ventral (v) and dorsal (d) are indicated. (A) Endogenous Serrate (red); (B) Src-GFP (red) expressed in the *ptc::Gal4* domain (*ptc::GAL4/UAS::Src-GFP*); (C, C') Ectopic *Cut* expression (trans-activation, arrows) is induced in cells flanking the domain with highest levels of *Ser*^{WT} (*ptc::GAL4/UAS::Ser*^{WT}, red), most evident at the posterior side (right) where there is a sharp boundary between *Ser*^{WT} expressing/non-expressing cells. Endogenous Notch signalling at the d/v boundary is inhibited due to cis-inhibition (arrowhead). *Note*: At the levels of detection used, endogenous Serrate expression is not visible in (B, C), or in most subsequent images. (D) Diagram depicting domains of Serrate as labelled: N-terminal (NT), green; DSL, yellow; EGF-repeats, grey; cysteine-rich region (Cys) black oval; transmembrane (TM), red. Sites of mutations are indicated. *DSL, RPRDD (residues 251–55) substituted with AAAAA; ΔC20, terminal 20 residues deleted; *LL, LL (1352–3) substituted with AA; Δint, residues 1269–1285 deleted; ΔT, truncation after residue 1290 fused to Serrate C-terminal tripeptide VMV; *KK, both K(1269) and K(1287) substituted with A; KKYL, addition of KKYL tetrapeptide to C-terminus. Table summarises results from expression in wing discs: + to +++ indicates increasing strength of effects; no, indicates no effect; * indicates there may also be inhibition of Notch on adjacent cells; ^ indicates possible weak inhibition of *Cut* at larval stages, this does not affect the adult wing. (E) ClustalW alignment of juxtamembrane region from the intracellular domains of the Serrate/Jagged proteins indicated: black shading, identities; grey shading, similarities; yellow shading indicates region with conservation between invertebrates and vertebrates; blue box, 17 amino-acids deleted in Δint; thicker blue lines highlight the NNL (aa1271–3) and NPL (aa1284–6) triplets; red boxes outline lysine residues mutated (K1269 and K1287); green triangle indicates ΔT truncation (T1290).

Figure 2 Differential effects of Serrate mutants on Notch activation. (A–D) Effects on Cut (green) when the indicated ligands are expressed using *ptc::Gal4*: arrows, ventral stripes of ectopic Cut (trans-activation); arrowheads, differing levels of inhibition. (E–H) Distribution of Serrate proteins (*x/z* sections), yellow line indicates *ptc::GAL4* domain: Ser^{WT} (E) and Ser^{*DSL} (F) are detected in intracellular puncta (arrowheads) and apically at the membrane (arrows); Ser^{*LL} (G) and Ser^{Δint} (H) are restricted apically at the membrane (arrows). (I–K) Similar activation characteristics (α -Cut, green) are observed when wild-type (I) and mutant (J, K) Serrate proteins are expressed in clones (red). In (K), Ser^{Δint} clones that touch the d/v boundary eliminate activity in both compartments, as occurs with *Notch* mutant clones (de Celis *et al*, 1996).

A Serrate intracellular motif is essential for activation

To investigate key functions within the Serrate intracellular domain, we looked for motifs that might confer important properties. The C-terminal residue of Serrate is valine (VMV). Since C-terminal valine is required for efficient maturation of proTGF α and many V-terminal motifs interact with PDZ domains (e.g. Briley *et al*, 1997; Sheng and Sala, 2001; Six *et al*, 2004; Wright *et al*, 2004), we tested the effects of removing 4 or 20 amino acids from the C-terminus of Serrate (Figure 1D). The resulting proteins (Ser^{ΔC4} and Ser^{ΔC20}) retained activating and cis-inhibitory functions, and showed normal subcellular distribution (data not shown). The C-terminal motif is thus not essential for either activation or cis-inhibition, at least when high levels of Serrate are expressed as in our assay.

A second sequence detected in the Serrate intracellular domain (RSSQILL) resembled a di-leucine motif ([D/E/R]_{x[3–5]} L[L/I]), which is involved in intracellular sorting and/or internalisation of proteins (Sandoval *et al*, 2000; Bonifacio and Traub, 2003). This motif is present in *Anopheles gambiae* Serrate (RNSQILL) and in *Danio rerio* Jagged 2 (RTKNGLI) and a related motif was found to regulate endocytosis of Lin12/Notch in *Caenorhabditis elegans* (SSQHSLL). Changing the two leucines to alanines (Ser^{*LL}; Figure 1D) altered ligand subcellular distribution in a manner that was consistent with the dileucine motif playing a role in endocytosis; Ser^{*LL} was predominantly localised at the cell surface and very little was detected as puncta inside the cell (Figure 2C and G). Surprisingly, this mutation had little effect on ligand function. Ser^{*LL} retained the capability to trans-activate Notch both when expressed using *ptc::Gal4* and when expressed randomly throughout the disc in *actin::Gal4* clones (Figure 2C and J). At all locations, robust cis-inhibition was detected. However, Ser^{*LL} differed from

Ser^{WT} in that it activated only on the posterior side of the *ptc::Gal4* expression domain. One possible explanation is Ser^{*LL} is less efficient at activating relative to cis-inhibition. The anterior stripe of Cut is induced within the region where *ptc::Gal4* expression gradually tails off towards the anterior. It occurs therefore at the transition between cells with high Ser^{WT} and those with lower levels that must be below the threshold for cis-inhibition so that the residual Ser^{WT} can transactivate. Therefore, if the balance between cis-inhibition and activation is altered in Ser^{*LL} as suggested above, this threshold for cis-inhibition may not be overcome. Nevertheless, Ser^{*LL} retains widespread ability to activate Notch despite the fact that the *LL mutation severely reduces its endocytosis.

Our analysis of the Serrate intracellular domain also identified a short motif conserved between invertebrate and vertebrate Serrate/Jagged proteins ([E/D][E/D]_{x[2–3]} NNx₅ Nx_[3–5] NP[L/I]; Figure 1E). A related motif [E/D]_{x[2–4]} NN[L/I] is present in the intracellular domain of Delta ligands, at a similar position relative to the transmembrane domain. We therefore generated a mutant form of Serrate that deleted this motif (Ser^{Δint}; Figure 1D and E). In this case, the mutant ligand completely lost the ability to trans-activate, since no ectopic Cut was detected when Ser^{Δint} was expressed in the wing disc (Figure 2D). In contrast to Ser^{*DSL}, however, Ser^{Δint} had enhanced inhibitory effects that most likely reflect a combination of trans and cis-inhibitory interactions (Figure 2D and K). Analysis of Ser^{Δint} protein distribution revealed that it was highly concentrated at the membrane but was not present in intracellular puncta (Figure 2H). Thus, this deletion had compromised both trans-activation and endocytosis of Serrate.

To further localise residues essential for Serrate function, a series of substitutions were made within the region

Figure 3 Role of the Serrate intracellular motif in Notch activation. (A, B, D, E) Cut expression (green) in wing discs expressing mutant Serrate proteins (red) as indicated. Ser^{NNL} (A) and Ser^{ΔT} (D) activate to varying extents (arrows) and retain cis-inhibition (arrowheads); Ser^{NNL/NPL} (B) and Ser^{KK} (E) no longer trans-activate but still inhibit (arrowhead). (C, F) x/z section showing that Ser^{NNL/NPL} (C) and Ser^{KK} (F) are predominantly localised apically at the cell membrane, no prominent intracellular staining is detected (although some Ser^{NNL/NPL} is visible basally). In (F), Src-GFP (green) expressed in the *ptc::Gal4* domain (*ptc::Gal4/UAS::Src-GFP*) labels cell membranes. Yellow line indicates *ptc::Gal4* domain in all panels.

delineated by Δ int (Figure 1E). Since many endocytic motifs require tyrosine residues, we first replaced the single tyrosine residue but found no effect on Serrate function (data not shown). Subsequently, the conserved NNL or NPL triplets were substituted with AAA (Figure 1E). Neither mutation alone eliminated function, although they both resulted in ligands with partially compromised signalling activity (Figure 3A and data not shown). In addition, Ser^{NNL} accumulated apically and retained robust cis-inhibition (Figure 3A). Combining the two mutations produced a ligand that mimicked the effects of Ser^{Δint}. Thus, Ser^{NNL/NPL} did not trans-activate signalling but it retained inhibition and strongly accumulated apically at the membrane of expressing cells (Figure 3B and C).

Previous experiments have shown that deletion of the intracellular domain compromises trans-activation by DSL ligands, although they retain inhibitory functions (e.g. Sun and Artavanis-Tsakonas, 1996; Shimizu *et al*, 2002; Itoh *et al*, 2003; Wang and Struhl, 2004). We therefore tested whether the ability to trans-activate was retained when Serrate was truncated just C-terminal to the Δ int motif (Ser^{ΔT}; Figure 1D and E) as predicted if this is the essential motif within the intracellular domain. In agreement, Ser^{ΔT} retained the ability to trans-activate (albeit weakly; Figure 3D) and to cis-inhibit (Figure 3D). These data therefore confirm the importance of the conserved motif for Serrate signalling activity and the dispensability of more C-terminal domains, including the di-leucine motif.

Ser^{Δint} defines a region required for an interaction with ubiquitin ligases Mib1 and Neur

DSL ligands in both vertebrates and invertebrates are regulated by the ubiquitin ligases Mib1 and Neur (Lai *et al*, 2001, 2005; Pavlopoulos *et al*, 2001; Itoh *et al*, 2003; Koo *et al*, 2005; Le Borgne *et al*, 2005b; Pitsouli and Delidakis, 2005). To investigate whether the inactivity of Ser^{Δint} could be explained by an impaired ability to interact with Mib1 or Neur, we employed an S2-cell co-expression assay (Figure 4). Ser^{WT} was highly enriched at the plasma membrane when expressed in S2 cells (Figure 4A). Co-expression with *Drosophila* Mib1 or Neur led to a dramatic relocalisation, such that little Ser^{WT} was detected on the cell surface (Figure 4D, G, J and K). Both Ser^{LL} and Ser^{Δint} were also enriched at the plasma membrane when expressed alone (Figure 4B and C), so we next compared effects of Mib1 and Neur on their cellular distribution. Like Ser^{WT}, Ser^{LL} was redistributed from the membrane in the presence of Mib1 or Neur (Figure 4E, H, J and K). In contrast, Ser^{Δint} remained at the cell surface in the presence of Mib1 or Neur (Figure 4F, I, J and K), indicating that it was no longer recognised by either ligase.

To confirm that Ser^{Δint} had lost sequences required for interaction with the E3 ligases, we tested whether Ser^{Δint} co-immunoprecipitated with Neur from *Drosophila* larval extracts. Ser^{WT}, Ser^{LL} or Ser^{Δint} were each expressed together with either Neur or Neur Δ RING (which retains ligand binding but is expected to lack ubiquitinating activity and is more stable than Neur; Pitsouli and Delidakis, 2005). We observed that total levels of Ser^{WT} and Ser^{LL} were lowered in the presence of Neur, whereas Ser^{Δint} was unaffected (Figure 4L, asterisks; the truncated Ser^{WT} fragment appears more susceptible to degradation), indicating that the latter is refractory to the activity of Neur. Furthermore, both Ser^{WT} and Ser^{LL} were co-immunoprecipitated with Neur Δ RING and weakly with Neur, but Ser^{Δint} failed to co-immunoprecipitate with either (Figure 4L). Thus, together these data confirm the importance of the conserved motif in an interaction with both Neur and Mib.

Finally, since Mib1 and Neur have E3 ubiquitin ligase activity which modifies lysine residues, we tested the consequences of mutating the two lysine residues most closely associated with the Δ int motif (Ser^{KK}, EEKSN...NPLK; Figure 1E). Ser^{KK} was incapable of trans-activation (Figure 3E) indicating that one or both of these lysine residues are likely targets for modification by Mib1/Neur (mutation of the second lysine alone, NPLK, had no effect on Serrate function; data not shown). As with the other mutations in the intracellular domain, Ser^{KK} accumulated at the membrane (Figure 3F) and strongly inhibited endogenous Notch signalling at the d/v boundary (Figure 3E).

Relationship between endocytosis and ligand functions

Most mutations that abolish Serrate's ability to signal (Ser^{Δint}; Ser^{NNL/NPL}; Ser^{KK}) correlate with a severe reduction in internalisation, as shown by the reduced number of intracellular puncta relative to surface ligand. However, in two cases (Ser^{LL}; Ser^{NNL}), trafficking is reduced without significantly compromising signalling, and in another (Ser^{DSL}) trafficking still occurs although signalling is abolished. We therefore investigated further the intracellular puncta and the effects of

Figure 4 The conserved intracellular motif confers an interaction with ubiquitin ligases. (A–K) S2 cells were transfected with the constructs indicated and the subcellular distribution of Serrate monitored. (A–C) All Serrate variants are predominantly localised at the cell surface when expressed alone. (D–I) Co-expression of Mib1 or Neur causes significant relocalisation of Ser^{WT} (D, G) and Ser^{*LL} (E, H), but not of Ser^{Δint} (F, I); cells were double labelled to confirm the presence of Mib1 or Neur (not shown). (J, K) Results from analysis of >50 cells for each combination, scored for high (black), low (green) or no (orange) detectable surface Serrate staining in the absence (–), dark shading) or presence (+, light shading) of Mib1 (J) or Neur (K). (L) Ser^{WT} and Ser^{*LL} but not Ser^{Δint} co-immunoprecipitate with NeurΔRING. Input: Serrate levels (detected as two bands) in extracts from larvae expressing Serrate and Neur constructs as indicated. Ser^{WT} and Ser^{*LL} are at reduced levels (asterisks) in the presence of full-length Neur, Ser^{Δint} is not affected. None show reduced levels in the presence of NeurΔRING. Extracts probed with antitubulin (α-Tub) as loading control. α-Neur IP: proteins complexed with Neur were immunoprecipitated from larval extracts expressing the constructs shown and probed with α-Neur (upper panel) or α-Ser (lower panel). Lower panel: levels of Ser^{WT}, Ser^{*LL} or Ser^{Δint} co-immunoprecipitated with Neur (arrowhead) or NeurΔRING (arrows). Ser^{WT} and Ser^{*LL} can be co-precipitated with NeurΔRING (black arrows) which does not promote ligand degradation, Ser^{Δint} does not co-precipitate even under these conditions (grey arrows).

altering endocytosis and/or intracellular transport on the ability of Serrate to signal.

First, we analysed the relationship between Serrate and Notch localisation within cells expressing Ser^{WT} (Figure 5A and B, and Supplementary Figure S1A). In wild-type cells, Notch was predominantly localised apically at the cell surface where it overlapped with E-Cadherin (Supplementary Figure S1D). However, little of the Notch remained at the cell membrane in cells producing Ser^{WT} (Figure 5A and B). Instead, Notch co-localised with Ser^{WT} in intracellular structures (Figure 5B and Supplementary Figure S1A) that were of endocytic origin, as they co-labelled with dextran in uptake assays (Supplementary Figure S1B) and were reduced in cells where endocytosis was compromised by a mutation in dynamin (*shits*; Supplementary Figure S1C). The amount of Notch in vesicles was increased 1.5-fold by the presence of Ser^{WT}. The distribution of Ser^{WT} and Notch was also altered by the expression of the small GTPases Rab5 and Rab7, which regulate endocytic and intracellular trafficking (Entchev *et al*, 2000; Supplementary Figure S1E–H). Co-expression of Rab5 with Ser^{WT} resulted in the accumulation of Notch and Ser^{WT} in large apical puncta (Supplementary Figure S1E and F), whereas co-expression with Rab7 led to their accumulation in Rab7 positive basal puncta (Supplementary Figure S1G and H). Together, these data indicate that a large proportion of Notch and Serrate travels through the Rab5/Rab7 endocytic pathway. Interestingly, however, neither increased Rab7 nor increased Rab5 had a significant effect on Ser^{WT} mediated signalling or cis-inhibition (data not shown). Likewise, the bulk of intracellular trafficking occurs independent of Notch signalling, since Ser^{*DSL} also colocalised with Notch in intracellular puncta even though it cannot make a productive interaction (Figure 5C and D). Thus, trafficking through these compartments does not appear to be limiting for Serrate mediated activation. However, in agreement with previous studies where endocytosis was linked to ligand activity (Parks *et al*, 2000; Wang and Struhl, 2004; Le Borgne *et al*, 2005a), co-expression of a dominant-negative Rab5 (Rab5SN) was able to severely reduce signalling by Ser^{WT} when larvae were maintained under conditions to maximise its dominant-negative effects (24 h at 29°C, data not shown).

Cis-inhibition by Serrate at the cell surface

When Ser^{WT} is expressed in the wing disc, it inhibits endogenous Notch signalling within the domain of highest ligand expression (Figures 1C and 2A). Ligand mediated cis-inhibition also plays a role in modulating Notch activation in wild-type wing discs, as illustrated by the ectopic Notch signalling detected within clones that are mutant for both ligands (Figure 6I; Micchelli *et al*, 1997). The change in Notch distribution elicited by Ser^{WT} (Figure 5A) initially suggested a model where cis-inhibitory interactions resulted in the down-regulation of Notch by promoting its endocytosis and degradation. However, several data suggest that depletion of Notch is not essential for cis-inhibition, and support an inhibitory interaction between Serrate and Notch taking place at the cell surface.

Firstly, we examined the distribution of Notch in the presence of Ser^{*LL} and Ser^{Δint}, which both accumulate at the cell surface and have inhibitory effects on signalling. In neither case was this inhibition accompanied by depletion of

Figure 5 Differential effects of Serrate mutants on Notch distribution. (A–K) Notch protein (A–H, anti-Notch^{ICD} white/green; I–K, anti-Notch^{ECD}) distribution in discs expressing Ser^{WT} (A, B, I), Ser^{DSL} (C, D), Ser^{LL} (E, F, J) and Ser^{Δint} (G, H, K). Yellow line marks *ptc::Gal4* domain, yellow arrows indicate d/v boundary. (A, C, E, G, I–K) Apical x/y sections: Notch is depleted from the membrane in the domain of ectopic Ser^{WT} but is enriched at the membrane in cells expressing Ser^{LL} or Ser^{Δint}. In (I–K), discs were not permeabilised so that only surface protein is detected. (B, D, F, H) x/z sections, arrowheads indicate Notch puncta which colocalise with Ser^{WT} (B, B') and Ser^{DSL} (D, D').

Figure 6 Cis-inhibition is compromised by an ER retention signal, enhanced by NeurΔRING and independent of Fringe. (A–C) Expression of Cut (green) in discs expressing Ser^{WT} (red, A) and Ser^{KKYL} (red, B, C). Addition of KKY motif results in reduced/no activation (arrows) and reduced cis-inhibition (arrowheads). (D, E, E') Ser^{KKYL} has different intracellular distribution from Ser^{WT}. In x/z sections, Ser^{WT} (D) is detected at the membrane (arrow) and in vesicles (arrowhead); Ser^{KKYL} (E, E') accumulates apically and basally within the cells (white lines). (F, G) Ser^{WT} (red, F) induces *E(spl)mβ::CD2* (green, F) in flanking ventral cells (arrows) and inhibits expression throughout the *ptc::GAL4/UAS::Ser* stripe (yellow line). Wild-type *E(spl)mβ::CD2* (G, white) is shown for comparison. To aid comparison, yellow asterisks indicate area of high expression around L3 sensilla, yellow line indicates position of *ptc::GAL4* domain and yellow arrows indicate d/v boundary expression. (H–K) Ectopic Notch activity as indicated by Cut expression (green) is detected in few *Df* (H) and in most *Df Ser* (I) mutant clones (red) in the wing disc (e.g. arrows). Expression of NeurΔRING (J, K) within the mutant cells prevents ectopic Cut expression in *Df* (J) but not in *Df Ser* (K) mutant clones (arrow). Data from multiple samples are presented in Table I.

Notch from the membrane (Figure 5E–H). Instead the receptor accumulated at the membrane where it co-localised with both Ser^{LL} and Ser^{Δint} (Figure 5E–H). In Ser^{LL} expressing cells the Notch was distributed in patchy domains, which

corresponded to sites of highest ligand accumulation (Figure 5E and F). We further confirmed that a significant proportion of Notch was on the cell surface in the presence of either mutant, by using an antibody to the Notch extracellular

domain (Notch^{ECD}) to stain non-permeabilised discs (Figure 5I–K). These observations suggest that Ser^{*LL} and Ser^{Δint} accumulate Notch via an interaction with the receptor at the cell surface and that cis-inhibition does not depend on Notch being depleted from the membrane.

Secondly, we tested whether Serrate could mediate cis-inhibition when it was prevented from reaching the cell surface via the addition of a strong C-terminal ER retention signal (KKYL; Zerangue *et al*, 2001; Figures 1D and 6A–E). Ser^{KKYL} differed in its distribution from Ser^{WT} (Figure 6D and E) and predominantly co-localised with a marker of the ER (data not shown), confirming that the bulk of the mutant ligand was retained within the secretory pathway. In many wing discs expressing Ser^{KKYL}, there was no ectopic Notch activation and cis-inhibition was severely compromised (i.e. there was little or no disruption to Cut expression at the endogenous d/v boundary; Figure 6C). Thus, in these discs Ser^{KKYL} appears to be fully retained within the cell and does not mediate cis-inhibition. In a fraction of wing discs analysed some Notch activation was detected (Figure 6B), thereby demonstrating that some Ser^{KKYL} had reached the cell surface and was functional. However, even under these conditions Ser^{KKYL} showed reduced levels of cis-inhibition (Figure 6B). The strength of cis-inhibition therefore correlated with the amount of residual trans-activation. This is consistent with both trans and cis interactions occurring at the cell surface and is the converse of what would be expected if Serrate inhibited Notch via an interaction within the ER/Golgi.

Thirdly, we investigated whether cis-inhibitory effects could be influenced by the availability of Neur/Mib1, which regulate endocytosis of ligand from the cell surface. If cis-inhibition is favoured by residence of Serrate on the cell surface, the levels of Neur/Mib1 might weaken this effect via their ability to promote ligand internalization. We took advantage of the observation that mutant cells lacking only Delta showed modest loss of cis-inhibition compared to mutant cells lacking both Delta and Serrate. This difference was manifest by the number of clones that exhibited ectopic Notch activity (28% of *Dl* mutant clones compared with 78%

of *Dl* Ser double mutant clones; Table I; Figure 6H and I) and can be explained by cis-inhibitory effects of Serrate in the *Dl* mutant clones. When ectopic Neur was expressed within *Dl* mutant clones, the frequency of clones displaying ectopic Cut expression rose from 28% to 50% (Table I). Conversely, expression of dominant negative Neur (Neur^{ΔRING}) eliminated ectopic Notch activity within *Dl* clones (0%; Table I; Figure 6J), suggesting that it had increased cis-inhibition by Serrate. Similar effects were seen with Ser clones where Delta was the residual ligand (Table I). In contrast, Neur ^{ΔRING} had no effect on the number of *Dl* Ser mutant clones displaying ectopic Notch activity (74%; Table I; Figure 6K), demonstrating that its effects were dependent on the presence of either ligand. In previous experiments, Neur ^{ΔRING} was shown to promote accumulation of ligands on the cell surface (Pavlopoulos *et al*, 2001). Thus, these data further support the model that cis-inhibitory interactions occur between ligand and Notch molecules at the surface of cells, and that affecting the rate of ligand clearance from the surface influences the extent of cis-inhibitory interactions.

Cis-interactions are not inhibited by Fringe

Our results suggest that cis-inhibition mediated by Serrate occurs after the ligand and receptor have reached the cell surface. However, this interaction is non-productive for signalling. One explanation could be that cis-inhibition involves different inter-molecular interactions between Serrate and Notch to those that mediate trans-activation. To investigate this, we asked whether the cis-inhibitory effects of Serrate, as monitored using the Notch reporter E(spl)mβ::CD2, are affected by the presence of Fringe in the dorsal part of the disc. E(spl)mβ::CD2 responds directly to Notch activation, with a different threshold/specificity than *cut*, and is expressed throughout the dorsal and ventral intervein regions as well as at the d/v boundary (Figure 6G; de Celis *et al*, 1998; Cooper *et al*, 2000). Expression of Ser^{WT} inhibits E(spl)mβ::CD2 expression strongly in dorsal and ventral cells despite the fact that ectopic Notch activation (measured as elevated E(spl)mβ::CD2 or ectopic Cut) is only detected in ventral cells (Figures 1C and 6F). Thus, the presence of Fringe

Table I Percentage of clones exhibiting ectopic Cut expression

Genotype	D%	Dn	<i>P</i>	V%	Vn	<i>P</i>	Σ%	Σn	<i>P</i>
<i>Dl</i> ^{-/-}	39	23		19	16		28	47	
<i>Dl</i> ^{-/-} ; UAS::neur	80	15	0.015	31	16	0.343	50	36	0.032
<i>Dl</i> ^{-/-} ; UAS::neur ^{ΔRING}	0	16	0.004	0	26	0.049	0	51	<0.001
Ser ^{-/-}	61	36		4	23		41	69	
Ser ^{-/-} ; UAS::neur	100	25	<0.001	80	20	<0.001	90	58	<0.001
Ser ^{-/-} ; UAS::neur ^{ΔRING}	19	27	0.001	8	10	0.521	12	50	<0.001
<i>Dl</i> ^{-/-} Ser ^{-/-}	96	22		65	23		78	55	
<i>Dl</i> ^{-/-} Ser ^{-/-} ; UAS::neur	96	22	0.756	70	23	0.500	80	65	0.682
<i>Dl</i> ^{-/-} Ser ^{-/-} ; UAS::neur ^{ΔRING}	100	12	0.647	50	14	0.896	74	34	0.777

D, dorsal; V, ventral; Σ, total.

% columns: percent of clones near d/v boundary that show ectopic Cut.

n columns: total number of clones near d/v boundary scored, difference between Dn + Vn and Σn is due to clones crossing boundary (included in the Σ scoring).

P: probability that this sample has the same frequency of ectopic Cut occurrence when compared with the equivalent sample without *neur* transgene expression (the shaded sample above it in each case). Using Fisher's exact test.

Note: *neur* expression suppresses, whereas *neur*^{ΔRING} expression enhances cis-inhibition in clones where a residual DSL ligand is present (Ser^{-/-} or *Dl*^{-/-}). The only exceptions are *Dl*^{-/-}; UAS::neur clones and Ser^{-/-}; UAS::neur^{ΔRING} clones in the ventral compartment, where endogenous levels of Ser are very low. In ventral *Dl*^{-/-} clones, the low levels of residual Ser may already be fully ubiquitinated so that UAS::neur does not cause significant affect; in the majority of Ser^{-/-} ventral clones the residual *Dl* is already inhibitory so that further enhancement by UAS::neur^{ΔRING} is not detectable.

is not able to deter the cis-inhibitory effects of Ser^{WT}, suggesting the interaction with Notch differs from that required for trans-activation, which is prevented by Fringe.

Discussion

Serrate is a type I transmembrane protein that has the capacity to activate Notch in adjacent cells and to inhibit Notch present in the same cell. Its ability to activate, like that of Delta, requires the E3 ubiquitin ligases Neur and Mib1 (Lai *et al*, 2001, 2005; Pavlopoulos *et al*, 2001; Itoh *et al*, 2003; Koo *et al*, 2005; Le Borgne *et al*, 2005b; Pitsouli and Delidakis, 2005). Here, we have identified a novel motif within the Serrate intracellular domain that is essential for its signalling activity and is necessary for the interaction with Neur and Mib1. Indeed, mutation of two lysine residues close to this region is sufficient to block signalling activity and internalisation of Serrate. Since ubiquitination typically occurs on lysine residues, this is consistent with an essential role for ubiquitination in conferring signalling activity on DSL ligands.

Motifs required for trans-activation

Localisation of both Mib1 and Neur interactions to the same region of Serrate is unexpected, because apart from a C-terminal RING domain, there is no obvious similarity between the two E3 ubiquitin ligases. However, the Neur/Mib1 interacting region encompasses a motif that is conserved between Serrate/Jagged ligands from different species, and also shows similarity with sequences in the intracellular domain of Delta. For Serrate/Jagged ligands, the most conserved features are NNL/V and NPL/I, and mutation of both triplets together mimicked the effects of deleting 17 amino acids spanning the conserved region. Mutation of either alone was not sufficient to eliminate signalling activity, although the Ser^{NNL} mutation did affect the subcellular distribution, causing the ligand to accumulate predominantly at the cell surface. Thus, the two triplets may combine to generate an interface for the stable binding of the different E3 ubiquitin ligases, or each may be a primary contact site for one.

Mutant forms of Serrate that no longer signal nor interact with Neur/Mib1 accumulate on the surface of expressing cells, and are not detected in intracellular vesicles (unlike the wild-type protein). This is similar to the behaviour of Serrate in mutant cells lacking Mib1 and/or Neur (Lai *et al*, 2005; Pitsouli and Delidakis, 2005; Le Borgne *et al*, 2005b) and implies a link between ligand endocytosis and Notch activation. Suggested mechanisms by which Mib1/Neur ubiquitination could provide this link are through promoting the redistribution and/or clustering of ligand into an endocytic microenvironment, or by stimulating its entry into a recycling pathway in which it is modified to enable signalling (Wang and Struhl, 2005; Le Borgne *et al*, 2005a). Our results do not distinguish between these models. However, they do demonstrate that a major component of endocytosis can be perturbed without significantly impairing signalling, since the latter is not eliminated by Ser^{LL}, a mutation that compromises bulk Serrate endocytosis but retains Neur/Mib1 interactions. Furthermore, most endocytosis is not a *consequence* of signalling, since the Ser^{DSL} mutant has lost both trans-activating and cis-inhibiting activities, but still trafficks normally.

Mutation of two lysines close to the conserved intracellular motif (Ser^{KK}) abolishes signalling activity and severely compromises ligand internalisation in a manner similar to the internal deletion (Ser^{Δint}). This is consistent with ubiquitination of Serrate *per se*—rather than an interaction with the ubiquitination machinery—being a prerequisite for both signalling and bulk endocytosis. In contrast, mutations in the ubiquitin-binding protein Epsin (*liquid facets*), prevent signalling but do not completely eliminate bulk endocytosis (Overstreet *et al*, 2004; Wang and Struhl, 2005). Hence, availability of Epsin (or an Epsin associated protein) may be a limiting factor downstream of Serrate ubiquitination, whereby the latter is routed away from the bulk endocytic pathway and acquires the capacity to signal.

Cis-inhibitory interactions

Our data argue that cis-inhibitory interactions take place between Notch and Serrate at the cell surface. Firstly, strong inhibition is retained by Serrate proteins such as Ser^{LL}, which are defective for endocytosis and accumulate Notch at the cell surface. Secondly, cis-inhibition is enhanced by NeurΔRING, which has lost the capacity to ubiquitinate and traps ligands at the cell surface (Pavlopoulos *et al*, 2001; Yeh *et al*, 2001). This also suggests that ubiquitination of Serrate is not required for the ligand to impart cis-inhibition. Thirdly, modifications that retain Serrate in the secretory pathway reduce its ability to cis-inhibit, arguing against an inhibitory interaction taking place before the two proteins reach the cell surface. This conclusion differs from one derived from a study using mammalian cell-culture, which proposed that cis-inhibition is mediated through interactions during transit in the secretory pathway that prevent functional receptor from reaching the cell surface (Sakamoto *et al*, 2002). It is possible that both types of interaction occur but in our *in vivo* assays the major cis-inhibitory interactions appear to take place at the cell surface.

The conclusion that cis-interactions occur at the cell surface raises the question why they do not promote Notch activation. A likely explanation is that cis-inhibition and trans-signalling involve different molecular interactions between Serrate and Notch. This could not be distinguished by mutations in the Serrate DSL domain, which abolished both cis-inhibition and trans-activation. However, the two functions could be distinguished by their sensitivity to Fringe; cis-inhibition was independent of Fringe activity whereas trans-activation was inhibited by it (Figure 6F; Irvine, 1999; Okajima *et al*, 2003). Thus, cis and trans interactions are not blocked by the same receptor modifications, implying that they involve different molecular contacts. Xu *et al* (2005) have proposed that ligand binding displaces intramolecular interactions between EGF-repeats within Notch to promote a change in conformation necessary for the activating cleavage. An attractive hypothesis therefore is that cis and trans interactions between Serrate and Notch differ in their phase or orientation of contacts and that the former are not sufficient to elicit an appropriate conformational change. This permits a model where competition exists between cis-inhibitory and trans-activating interactions. Under conditions in which Serrate is present at high levels inhibitory interactions are favoured, thereby reducing the amount of cell-surface receptor available for trans-activation.

A further consideration is whether the availability of Neur and Mib1 contributes to cis-inhibition. If Neur/Mib1 were limiting, some Serrate would remain un-ubiquitinated and as a consequence would reside for longer periods on the cell surface allowing more opportunity for cis-interactions. The observation that wild-type Neur suppresses cis-inhibitory effects while NeurΔRING enhances them supports this model (Table 1). The equilibrium between modified and unmodified ligands is likely therefore to contribute to the frequency of cis versus trans interactions and hence to the balance between the signal sending versus the signal receiving capacity of a cell.

Materials and methods

Drosophila strains and genetics

Drosophila strains are described in Flybase (<http://flybase.org>) unless otherwise indicated. Driver lines used for ectopic expression were *ptc::Gal4*, *hsp70::Gal4* and *hsFlp*; *act>stop>Gal4*. With the latter, larvae were incubated at 37°C for 10 min to induce clones. In addition to the Serrate constructs described below the following UAS lines were used: *UAS::Ser^{WT}* (Speicher *et al*, 1994); *UAS::Rab5*, *UAS::Rab7-GFP*, *UAS::Rab5^{SN}* (Entchev *et al*, 2000, but note new *UAS::Rab5^{SN}* transformant lines were made for this study); *UAS::Neur* and *UAS::NeurΔRING* (Pitsouli and Delidakis, 2005); *UAS::src-GFP* (gift from N Brown). *Gal4/UAS* fly crosses were routinely maintained at 25°C except *shibire^{ts1}* larvae were reared at 25°C and shifted to 34°C for 5 h immediately before dissection. The MARCM system (Lee and Luo, 2001) was used to generate positively marked loss of function clones expressing Neur or NeurΔRING (Figure 6). Crosses were between *y w hsFLP122 tub::Gal4 UAS::GFP-6xnl; FRT82B tub::Gal80/TM6B* and one of the following stocks:

(*DL-*) *w*; *UAS::neur* or *UAS::neurΔRING*; *FRT82B DL^{rev10}/T(2;3)SM5;TM6B*
(*Ser-*) *w*; *UAS::neur* or *UAS::neurΔRING*; *FRT82B e Ser^{RX106}/T(2;3)SM5;TM6B*
(*DL-, Ser-*) *w*; *UAS::neur* or *UAS::neurΔRING*; *FRT82B DL^{rev10}e Ser^{RX106}/T(2;3)SM5;TM6B*. Mosaics were induced during the first larval instar.

Generation of mutant Serrate transgenes

Starting plasmid contained Serrate cDNA in the *EcoRI* site of *pBSIIKS*.

Serrate intracellular domain mutants: *pBSIIKS-SerInt*, created by digesting *pBSIIKS-Serrate* with *SphI* and religating, was used as a template for Site-directed Mutagenesis (QuikChange[®] II Site-Directed Mutagenesis Kit, Stratagene) with primer pairs to introduce the desired mutation (details available on request). Mutated DNA was excised with *SphI-NheI* to release a 1180 bp fragment that was ligated to a 7350 bp *SphI* (partial digest)-*NheI* fragment excised from *pBSIIKS-Serrate*, thus replacing the corresponding wild-type sequence in full-length Serrate with the mutated version.

Ser^{*DSL}: two independent PCR reactions were performed on *pBSIIKS-Serrate* with primer pairs DSL1 (5'TTGCGGCCG²⁸TGATAGCCCCCTTTCTG⁴⁵3'; 5'TTGGATCC¹²²⁸GCAGGCGTAGTGA CCGAACTGAGCAGCAGCAGCAGCGCAGAAG¹¹⁸⁵3') or DSL2 (5'TTGGATCC¹²³⁵GAGGGTCAGAAGCTCTGCCTGAATGGC¹²⁶¹3'; 5'TTCTC GAG¹⁶⁷⁶ATGTGCGATACACTTGGGC¹⁶⁹⁴3'); superscript numbers refer to nucleotide position in Serrate cDNA M35759, the mutation is underlined. Products were ligated into pCR[®]II-TOPO[®] (Invitrogen)

and digested with *NotI-BamHI* and *BamHI-XhoI*, respectively. The fragments were ligated together into *NotI-XhoI* digested *pBSIIKS*. From this, an *NotI-NdeI* fragment was removed and used to replace the corresponding fragment in *pBSIIKS-Serrate*.

Full-length mutated Serrate cDNAs were subsequently ligated into *pUAST* for analysis in wing tissue, or into *pMTA-V5/His* (Invitrogen) for expression in S2-cells. All constructs were sequenced prior to use.

Transformant lines were obtained by injection into yellow white embryos following standard procedures. In all cases, multiple independent lines were examined. Results are shown for a representative line, since individual lines gave qualitatively similar effects, although the degree of activation/inhibition occasionally differed depending on levels of expression.

Immunofluorescent staining of wing imaginal discs

Antibody staining of imaginal discs was carried out as described (de Celis *et al*, 1996). In experiments analysing cell-surface Notch levels, Triton X-100 was omitted from all solutions. Dextran endocytosis assay was carried out under conditions described (Entchev *et al*, 2000; Pavlopoulos *et al*, 2001).

Primary antibodies were as follows: goat anti-Serrate 1/75 (Santa Cruz Biotechnology), rabbit anti-GFP 1/500 (Molecular Probes), mouse anti-CD2 1/50 (Serotec). Primary monoclonal antibodies were obtained from Developmental Studies Hybridoma Bank, University of Iowa and were as follows: mouse anti-Notch^{ICD} 1/20, mouse anti-Notch^{ECD12-120} 1/20, mouse anti-Cut 1/20, rat anti-DE-Cadherin 1/20. Secondary antibodies (Jackson Immunological) were used as follows: FITC conjugated anti-mouse/rabbit, 1/50; Cy3 conjugated anti-mouse/goat, 1/400.

S2 cell culture experiments

Transfections were carried out as described previously (Nagel *et al*, 2005) except that after transfection cells were removed from 12-well plates by gentle pipetting, lightly pelleted (4000 r.p.m., 30") and resuspended in prewarmed Schneider medium. Cells were then pipetted onto 13 mm diameter coverslips in a 24-well plate to achieve even distribution with minimal cell contact, and left to adhere for 3 h. Gene expression was induced by addition of CuSO₄ to 700 μM. Cells were incubated for a maximum of 24 h and subsequently fixed for immunofluorescence.

In addition to the Serrate constructs generated in this study, the following metallothienin (Mtn) inducible constructs were used: *Mtn::Gal4*, *pUAST::EGFP-Neur* (Pitsouli and Delidakis, 2005). For experiments with Mib1, an *Mtn::FLAG-Mib1* construct was generated by amplifying the Mib1 coding sequence from SD05287 (details available on request).

Immunoprecipitations

Immunoprecipitations were carried out as described by Pitsouli and Delidakis (2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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