Down-regulation of Delta by proteolytic processing

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Notch signaling regulates cell fate decisions during development through local cell interactions. Signaling is triggered by the interaction of the Notch receptor with its transmembrane ligands expressed on adjacent cells. Recent studies suggest that Delta is cleaved to release an extracellular fragment, DlEC, by a mechanism that involves the activity of the metalloprotease Kuzbanian; however, the functional significance of that cleavage remains controversial. Using independent functional assays in vitro and in vivo, we examined the biological activity of purified soluble Delta forms and conclude that Delta cleavage is an important down-regulating event in Notch signaling. The data support a model whereby Delta inactivation is essential for providing the critical ligand/receptor expression differential between neighboring cells in order to distinguish the signaling versus the receiving partner.

Introduction

Notch signaling defines a conserved mechanism that controls the acquisition of cell fate in metazoans through local cell interactions (Kimble and Simpson, 1997; Egan et al., 1998; Greenwald, 1998; Weinmaster, 1998; Artavanis-Tsakonas et al., 1999). Signals transmitted into a cell through the Notch surface receptor are activated by ligands expressed in adjacent cells. Two ligands, Serrate and Delta, have been identified with certainty. Both are single-pass transmembrane proteins comprised of a single Delta, Serrate, Lag-2 (DSL)* domain followed by EGF-like repeats, a short juxtamembrane domain, a transmembrane segment, and a short cytoplasmic tail (Fleming et al., 1998; Kopczynski et al., 1990). Molecular genetic evidence suggests that the ligands interact via their DSL domain with a specific region in the extracellular domain of Notch to trigger a series of molecular events that eventually result in the transmission of a signal from the surface to the nucleus (Rebay et al., 1991, 1993a,b; Fitzgerald and Greenwald, 1995). Not much is understood about the molecular mechanisms that regulate Notch activation at the cell surface, but over the past few years, several studies have presented evidence suggesting that the proteolytic processing of both the receptor and the Delta ligand is important for signaling (Pan and Rubin, 1997; Wen et al., 1997; Jarriault et al., 1998; Klueg and Muskavitch, 1999; Qi et al., 1999; Ye and Fortini, 1999; Brou et al., 2000; Klein, 2002; Lieber et al., 2002; Mumm et al., 2000).

Genetic interaction studies led to the identification of numerous modifiers of Notch signaling activity, among them is the ADAM metalloprotease, Kuzbanian (Fambrough et al., 1996; Rooke et al., 1996; Pan and Rubin, 1997; Sotillos et al., 1997; Lieber et al., 2002). The full role of Kuzbanian in Notch signaling remains controversial, but in vitro and in vivo evidence clearly shows that Kuzbanian can mediate Delta processing (Klueg et al., 1998; Qi et al., 1999); it is not known if Kuzbanian acts on Delta by direct or indirect mechanisms. Fractions enriched in soluble forms of Delta obtained from conditioned medium of Delta expressing cells have been shown to be capable of activating Notch signaling (Qi et al., 1999). These observations raise the possibility that this cleavage is an activating event generating a potential soluble ligand. Such a conclusion is challenged by transgenic analyses involving the overexpression of similarly truncated Delta and Serrate molecules, which demonstrate that these molecules behave as antagonists of Notch signaling (Sun and Artavanis-Tsakonas, 1996; Hukriede et al., 1997). However, phenotypes elicited by a dominant-negative form of Kuzbanian can be suppressed by extra copies of WT Delta, consistent with the notion that the Kuzbanian-dependent Delta cleavage event is essential for signaling (Qi et al., 1999).

We sought to clarify the functional role of the proteolytic processing of Delta by characterizing the specific activity of the cleaved product in vitro and in vivo. Our findings indicate that the purified cleaved Delta fragment from cells, and the ectopically expressed soluble Delta in flies is biologically

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*Abbreviations used in this paper: DSL, Delta, Serrate, Lag-2; E(spl)-C, enhancer of split complex.

Key words: Delta; Kuzbanian; Notch; cleavage; down-regulation

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inactive, leading to the conclusion that cleavage is a downregulating event in ligand activity. We discuss the developmental significance of this cleavage and suggest a functional role for Kuzbanian activity in Notch signaling.

Results

Characterization of soluble Delta fragments

Western analysis of extracts from tissues and cultured *Drosophila* S2 cells show that the ligand Delta is cleaved at an extracellular site close to the transmembrane domain, shedding a fragment that encompasses most of the extracellular domain (DlEC; Qi et al., 1999). Conditioned medium from S2 cells stably expressing Delta (S2-Dl) was used to purify DlEC to homogeneity by affinity chromatography using the 9B monoclonal antibody (C594.9B; Qi et al., 1999; Fig. 1 A).

Resolution of the highly purified product by SDS-PAGE and silver staining demonstrates two species migrating as a doublet of 63 and 65 kD, with the 63 kD species being the predominant form. NH_2-terminal sequence analysis revealed a single sequence consistent with the putative NH_2 terminus resulting from the signal peptide cleavage (unpublished data; Qi et al., 1999). Direct chemical COOH-terminal sequence analysis determined that the COOH-terminal residue of both isoforms is alanine. These results were corroborated by tryptic digestion followed by mass spectrometry, which revealed the existence of two peptides ending in the sequence LTNA and . . . QYGA. We conclude that Delta is cleaved at two distinct sites: COOH-terminal to Ala581 and Ala593, respectively. Henceforth, we refer to these two isoforms as $DlEC^{581}$ and $DlEC^{593}$.

In our attempts to explore the functional significance of the two extracellular cleavages in Delta, we generated truncated soluble molecules mimicking the $DlEC^{581}$ and $DlEC^{593}$. In addition, we mutagenized the Ala581 and Ala593 amino acids to serine (henceforth Ala581Ser and Ala593Ser; Fig. 1 B). The constructs were transfected into *Drosophila* S2 cells, which endogenously express Kuzbanian (Pan and Rubin, 1997) but not Delta. Transfection of the $DlEC^{581}$ and $DlEC^{593}$ constructs effectively generated soluble secreted products, with $DlEC^{593}$ exhibiting a slightly different molecular mass, consistent with the 11-amino-acid difference in their COOH-termini (Fig. 2 A). When expressed in S2 cells, both the Ala581Ser and Ala593Ser mutants were cleaved to generate a product of essentially the same size as DlEC (Fig. 2 B). In addition, we generated an Ala581,593Ser double mutant which was also cleaved to generate a product similar to DlEC (unpublished data). We also assessed the cis or trans requirement of Kuz in Delta cleavage using the S2 cell-culture system. S2 cells stably expressing either WT Kuz or dominant-negative Kuz, when mixed with S2-Dl cells, do not affect Delta cleavage (unpublished data); Kuz effects are seen only when it is cotransfected with Delta into the same cell (unpublished data; Qi et al., 1999).

DlEC–Notch interaction

It has been established that cells expressing Notch aggregate with Delta-expressing cells (Fehon et al., 1990). Although a rigorous, in vivo demonstration that this interaction is direct is still lacking, we do know that specific regions in the extracellular domain of Notch and Delta are necessary and sufficient for aggregation (Rebay et al., 1991; Muskavitch, 1994; Shimizu et al., 1999, 2000). We sought to examine whether WT DlEC, $DlEC^{581}$, and $DlEC^{593}$ interact with Notch and thus inhibit the normal Notch–Delta-mediated cell aggregation.

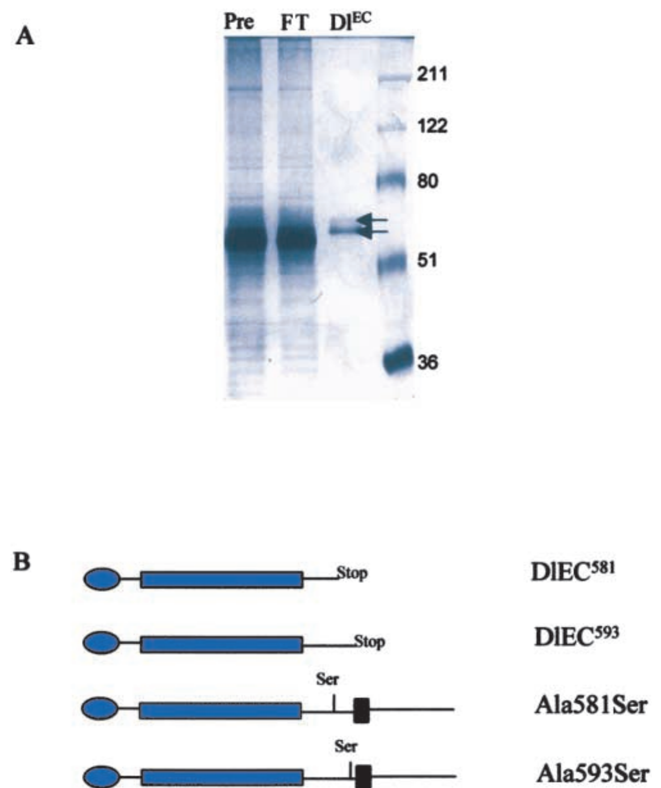


Figure 1. **Purification of DlEC and schematic of Delta constructs.** (A) DlEC was purified from conditioned medium from S2 cells stably expressing Delta using a Delta antibody (9B) affinity column. The purified form runs as a doublet as seen by SDS-PAGE analysis and silver staining. (B) Schematic representation of Delta constructs used to analyze the function of DlEC.

Preincubation of S2-N cells with concentrated conditioned medium from S2-Dl cells causes a >60% inhibition of aggregation rate (Fig. 3 A; Qi et al., 1999). In contrast, concentrated conditioned medium from S2 cells stably expressing each of the mutant forms of soluble Delta (Fig. 3 A) showed essentially no inhibitory effect in the aggregation assay.

The medium from S2-Dl–expressing cells was fractionated on an anti-Delta (9B) antibody affinity column, and selected fractions were tested for their inhibitory effect in the aggregation assay. We find all the inhibitory activity in the flowthrough of the affinity column. Further, the purified DlEC fractions showed essentially no inhibitory activity (Fig. 3 B). A mild inhibition in aggregation (<20%) was seen at concentrations of DlEC >0.5 μM (unpublished data), indicating that DlEC has a very weak affinity for Notch and is not an effective competitive inhibitor of Notch–Delta aggregation. Western blot analysis of the different fractions during purification of DlEC showed that the flow through contains full-length Delta, (unpublished data; Fig. 3) suggesting that the inhibitory effect could be either attributed to this Delta protein species or to an un-

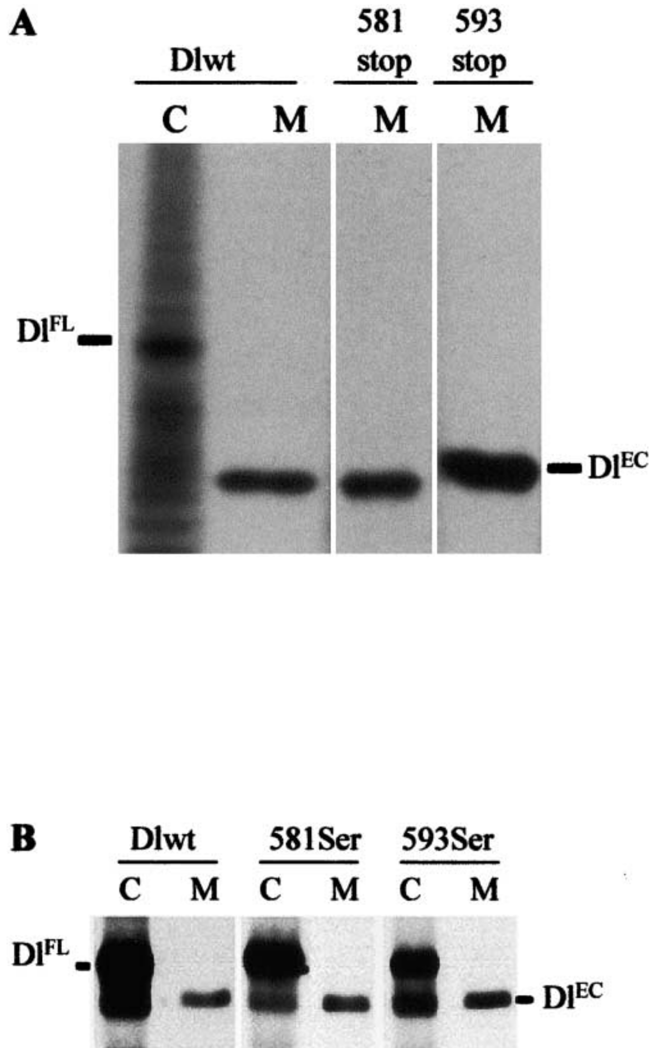


Figure 2. **Expression of Delta mutant constructs.** (A) Both the DlEC^{581} and DlEC^{593} truncated mutant constructs elicit a soluble protein into the medium. DlEC^{593} is slightly larger than DlEC^{581} consistent with the predicted 11 amino acid difference in their sequence. (B) The mutant constructs of Delta transfected into S2 cells and analyzed for Delta cleavage show that Serine mutants of Delta undergo cleavage like WT Delta. Full-length Delta (DlFL) was detected in the cell lysates (C), whereas DlEC is detected in the medium (M) by Western analysis with 9B antibody.

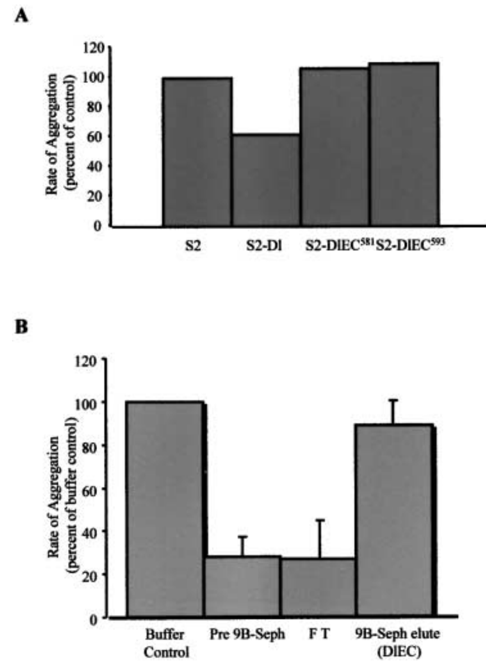


Figure 3. **DlEC does not interact with Notch in S2-N cells.** (A) Preincubation of S2-N cells with concentrated conditioned medium from S2 cells stably expressing DlEC^{581} or DlEC^{593} does not affect the rate of aggregation of S2-N and S2-Dl cells. Preincubation with concentrated conditioned medium from S2-Dl cells significantly affects the rate of aggregation of S2-N and S2-Dl cells. (B) The pre, flow-through (FT) and DlEC fractions (of 9B-affinity purification, Fig. 1 A) were analyzed in the N-Dl cell aggregation as described in the methods. Essentially all of the inhibitory activity of the precolumn sample is seen in the F.T. DlEC shows no significant inhibition of aggregation at the highest concentrations examined (up to 0.58 μM).

known activity which copurified with it. In any case, we note that these experiments reveal the existence of a Notch agonist activity, other than DlEC, in the supernatant of the S2-Dl cells.

We further examined the S2-Dl–derived inhibitory activity by size exclusion chromatography in neutral aqueous buffer to avoid the harsh elution conditions of the affinity column (i.e., pH 2.8). When S2-Dl conditioned medium was fractionated on a Sephadex-200 HR FPLC size exclusion column, all of the inhibitory activity was seen to elute in the void volume of the column (Mr $>$ 600 kD; Fig. 4, A and C). Western blot analysis also demonstrated that this fraction was devoid of DlEC, which eluted in subsequent fractions (Fig. 4 B). It is important to note that a band corresponding in size with full-length Delta is seen in the void-volume fractions. These data corroborate the notion that the DlEC fragment does not compete with the Notch–Delta interaction mediating the cell aggregation.

Assessment of DlEC activity by in vitro activity assays

We extended this analysis by examining the activity of the soluble Delta molecules using three independent in vitro assays of Notch activation.

Cortical neuron neurite retraction assay. It has previously been shown that Notch activation can directly influence neurite morphology by preventing neurite outgrowth from the outset as well as inducing neurite retraction (Qi et al., 1999; Sestan et al., 1999). The presentation of exogenous ligand to neurons, which express the Notch receptor, results in the activation of the receptor and the retraction of the neurites. When primary cortical neurons isolated from E14 mice and cultured in vitro were treated with conditioned medium from S2-Dl we observed extensive retraction, whereas neurons treated with purified (see above) DlEC, DlEC^{581}, or DlEC^{593} exhibited minimal or no retraction of neurites, suggesting that these soluble forms cannot activate the Notch signaling pathway (Fig. 5 A). Western analysis of the S2-Dl medium shows the presence of full-length Delta in addition to the DlEC (Fig. 5 B).

N2A neurite outgrowth assay. An independent neurite outgrowth assay using the Neuro2a mouse neuroblastoma cell line (N2a) confirmed these observations. Withdrawal of serum from the N2a cells triggers neurite formation in ~40% of the

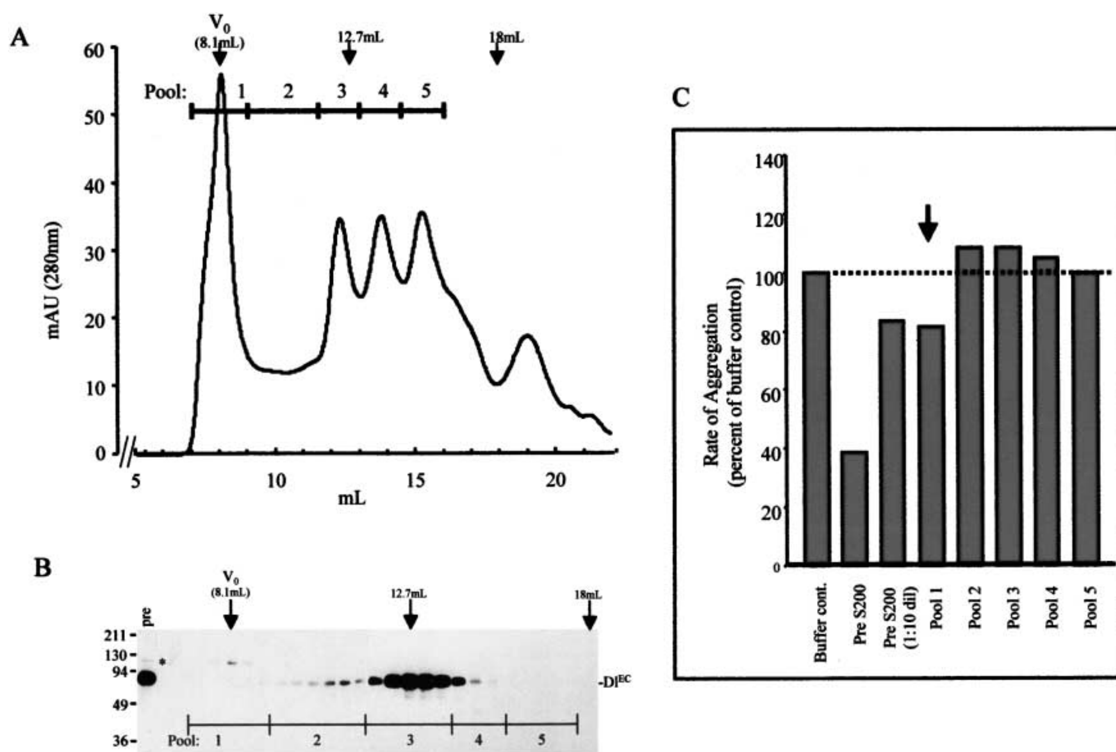


Figure 4. **Fractionation of N-Dl cell aggregation inhibitory activity.** (A) A sample of concentrated S2-Dl cell condition medium was fractionated on a Superdex 200HR size exclusion column. (B) Fractions analyzed by Western blotting with the 9B antibody reveal the DlEC protein in the precolumn sample and eluted fractions with a peak at ∼12.7 ml. A faint band consistent in size with full-length Dl is seen in the precolumn sample (*) and elutes in the void volume (V_0). The V_0 fraction contains proteins in the size range of 600 kD. (C) Pooled fractions were analyzed for inhibition of aggregation in the N-Dl cell aggregation assay. The precolumn sample shows significant inhibition of aggregation. Inhibition is still seen when the precolumn sample is diluted 1:10. Only pool 1, containing the V_0 peak and the full-length Dl, showed inhibition of aggregation. This inhibition is equivalent to the 1:10 diluted precolumn sample, which is consistent with the approximate 10-fold dilution that the sample experiences during the run of the column. Fractions containing DlEC elicit no activity in the aggregation assay.

cells in 16–24 h. The simultaneous activation of the Notch pathway in N2a has been shown to inhibit the outgrowth of neurites (Franklin et al., 1999). Addition of S2-Dl concentrated medium to the N2a cells at the point of serum withdrawal causes a significant inhibition in neurite outgrowth (Fig. 6, A and B). In contrast, concentrated conditioned medium from the $DlEC^{581}$ and $DlEC^{593}$ cells has no effect.

E(Spl)m3 expression assay. Finally, we monitored the effect that soluble Delta forms have on Notch-dependent transcriptional activity. For this we established that the HLH gene m3 of the enhancer of split complex (E[spl]-C) is transcriptionaly responsive to Notch activation in S2 cells (Fig. 7 A). In the same cells, the HLH mβ and mγ E(spl)-C transcripts are constitutively expressed or remain unresponsive, respectively, to Notch activation in S2 cells (Fig. 7 A). rp49, which encodes the ribosomal protein L32, was used as an internal control for the RT-PCR (Al-Atia et al., 1985). The effects of full-length and soluble Delta on Notch signaling were analyzed using the up-regulation of m3 expression as a reporter. To distinguish between the juxtacrine (full-length) and paracrine (soluble) effects of Delta, a transwell system was used as has previously been used for studies in EGF signaling (Nakagawa et al., 1996). Although aggregation of S2-N and S2-Dl cells leads to an up-regulation of m3, such an effect is not seen when S2-N cells are cocultured with S2-Dl cells in a transwell. We subjected S2-Dl cells to formalin fixation to stabilize the full-length form of Delta on the cell surface. Aggregation of S2-N with formalin-fixed S2-Dl cells resulted in an induction of m3 expression, which interestingly, does not decline over time as seen with live Delta cells (Fig 7 B). Treatment of S2-N cells with 5× concentrated conditioned medium from S2-Dl cells leads to the expression of m3. However, Western analysis of the conditioned medium shows the presence of full-length Delta in the medium (Fig 7 C).

In conclusion, all of the in vitro assays we employed consistently indicate that the soluble forms of Delta are not active, and support the notion that cleavage of Delta corresponds to an inactivation of the ligand. On the other hand, they also corroborate the existence of a soluble agonist activity in fractions containing small amounts of full length Delta.

Activity of soluble and mutant Delta constructs in vivo

The in vitro studies were extended by an assessment of the activity of the mutant constructs in transgenic flies. Flies carrying the various Delta mutants were generated and the effects of expression of the different DlEC isoforms and Delta mutants were analyzed in vivo. Expression was driven by the eye specific glass (pGMR) promoter, which is active in all cells posterior to the morphogenetic furrow. Flies expressing $DlEC^{581}$ and $DlEC^{593}$ exhibited mild eye phenotypes (Fig. 8, top) similar to those that we reported earlier (Sun and Arta-

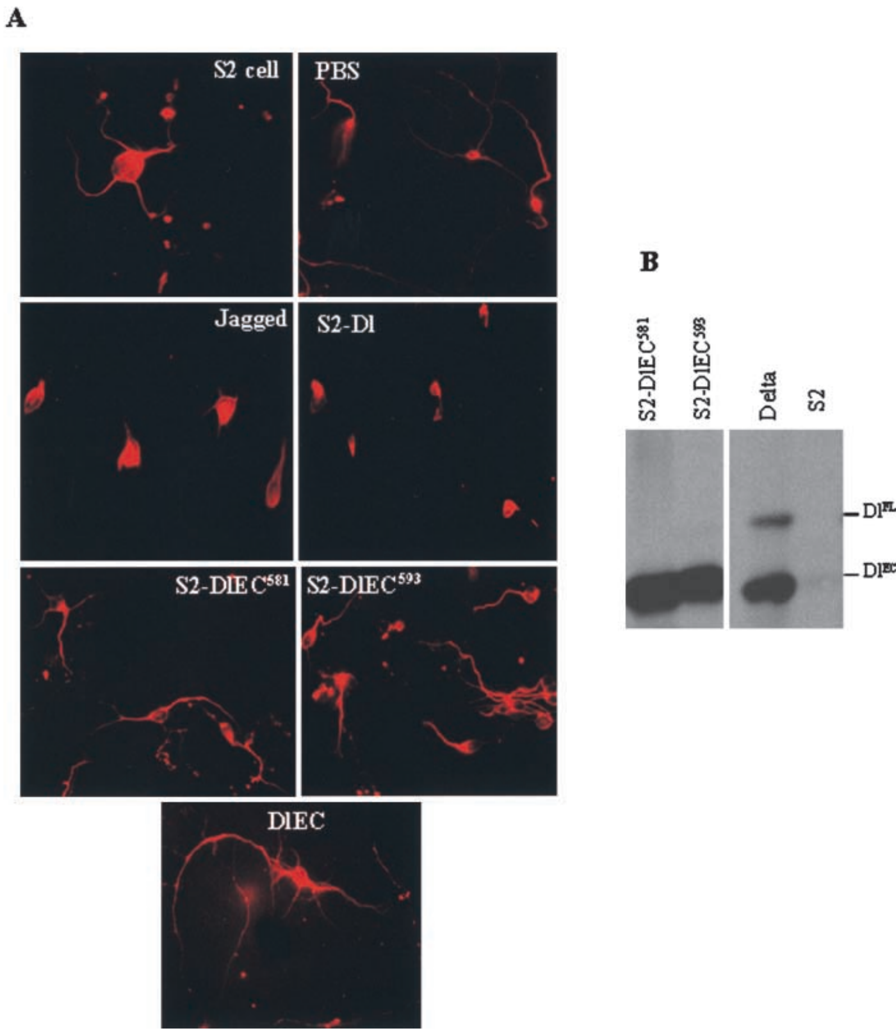


Figure 5. **DlEC does not induce neurite retraction in cultured cortical neurons.** (A) The effect of DlEC on primary cultured cortical neurons is shown in the representative images. There was no effect seen with 5× concentrated conditioned medium from S2 cells or PBS alone. Extensive neurite retraction associated with Notch activation is seen when the neurons are cocultured with cells expressing the Jagged ligand or treated with 5× concentrated conditioned medium from S2-Dl cells. No effect on neurite length is seen when neurons are treated with 5× concentrated conditioned medium from S2-DlEC^{581} or S2-DlEC^{593} or affinity purified DlEC. (B) Western analysis with anti-Delta 9B antibody on the conditioned medium from S2-Dl cells shows the presence of full length WT Delta in the medium.

vanis-Tsakonas, 1997). We do not understand the underlying mechanism of the weak effects associated with soluble ligand expression; however, the severity of phenotypes of the various transgenic lines varies from mild to no phenotype, suggesting a link with the level of over expression. This may correlate with the slight inhibition of aggregation we observe with micromolar amounts of purified DlEC in the in vitro aggregation assay (unpublished data).

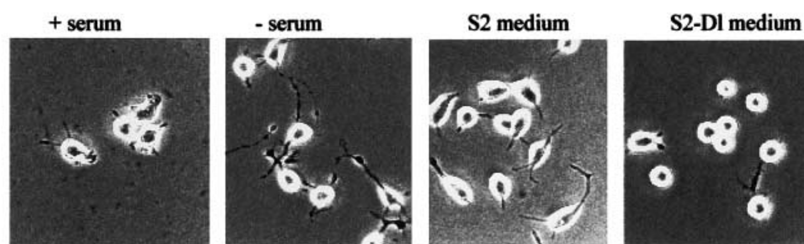
In contrast, a severe glassy eye phenotype was exhibited with the Ala581Ser and Ala593Ser mutants. Significantly, very similar results are seen with pGMR driven overexpression of WT Delta, consistent with the notion that the cleavage site mutations, in addition to being ineffective at preventing cleavage, do not significantly alter the biological activity of Delta.

Because the effects of Notch signals are highly dependent on the developmental context, we sought to corroborate the effect of the mutants in another tissue and assayed expression of the mutants in the developing wing using the UAS-Gal4 system. The two drivers used were Vg-Gal4, which drives expression along the D/V boundary of the developing wing disc and the A9-Gal4, which drives expression predominantly in the dorsal wing compartment. The severity of phenotypes associated with the different mutants is similar to those observed in the eye. Expression of DlEC^{581} and DlEC^{593} with the vestigial driver did not affect wing development, whereas the A9-gal4 driver resulted in the formation of mild wing deltas (Fig. 8, bottom). Transgenic flies expressing either Ala581Ser, Ala593Ser, or WT Delta with the vestigial or A9 driver could not eclose and pharate adults exhibited a severe wing phenotype. Raising the flies at 18°C helped in obtaining a few escapers all of which exhibited rudimentary wings.

In summary, the in vivo activity of the mutant Delta molecules corroborates the results of the cell-based analysis. It is important to note that unlike the strong phenotypes associated with the overexpression of the WT ligand, or the full-length mutant ligands that are normally cleaved in the aforementioned cell based assays, the soluble forms have mild effects. The expression of the soluble Delta isoforms in every context examined could at best elicit only mild phenotypes consistent with the notion that these molecules are inactive.

Discussion

The past few years have brought an increasing appreciation of the importance of proteolysis in the Notch signaling pathway, but the exact mechanistic role of these events is not well established and is often contradictory (Pan and Rubin, 1997; Sotillos et al., 1997; Jarriault et al., 1998; Klueg et al.,



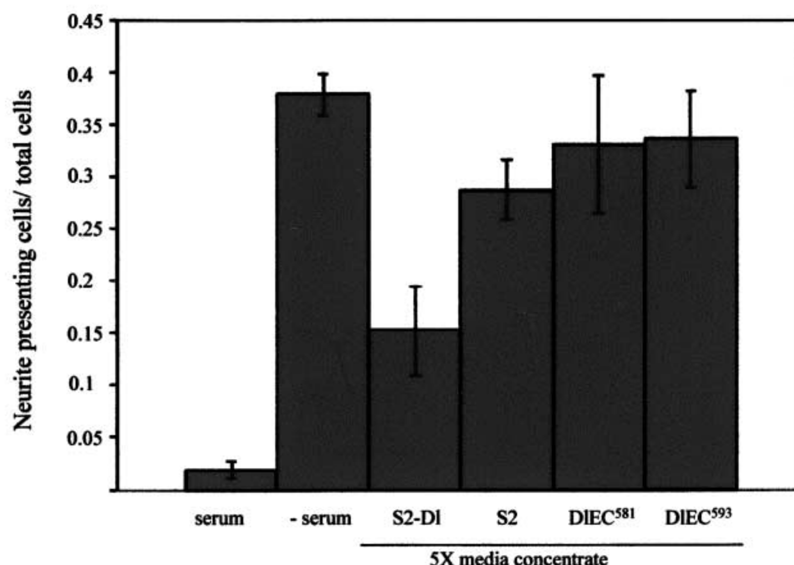


Figure 6. **DlEC does not inhibit neurite outgrowth in neuroblastoma N2A cells.** (A) Withdrawal of serum from actively growing N2A cells results in neurite outgrowth. This response is associated with Notch activation and shown in representative cultures in A. Addition of 5× concentrated conditioned medium from S2-Dl cells at the time of serum withdrawal inhibits neurite outgrowth. (B) The number of neurite presenting cells relative to the total number of cells in the plate is represented in the graph. Conditioned medium from S2, S2-DlEC^{581} and DlEC^{593} had a very mild effect on neurite outgrowth as compared with the effect of conditioned medium from S2-Dl cells.

1998; Qi et al., 1999; Kramer, 2001; Lai and Rubin, 2001a, 2001b; Lai et al., 2001; Klein, 2002; Lieber et al., 2002). The finding that the proteolytic processing of Delta releases soluble DlEC raised the obvious question of the functional significance of this cleavage. Even though several studies have addressed this question, either directly or indirectly, in flies, nematodes and vertebrates, it is unclear whether this is an antagonistic or agonistic event in Notch signaling (Fitzgerald and Greenwald, 1995; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997; Qi et al., 1999). Our initial characterization of soluble fractions of Delta suggested an agonistic function for the DlEC. More rigorous biochemical characterization presented here clearly shows Delta proteolysis yields more than one DlEC (DlEC581 and DlEC593), neither of which exhibit significant biological activity. Furthermore, the previously reported soluble activity is most likely attributed to trace levels of full-length Delta in the cell culture media. Our conclusion is that the proteolytic processing of Delta is a step that renders this Notch ligand inactive.

Our previous studies demonstrated a central role for Kuzbanian in Delta processing both in cell-based assays and in vivo by mutant analysis (Qi et al., 1999). The existence of two DlEC products (DlEC581/593) indicates more than one cleavage event occurs in the extracellular domain of Delta. It is important to note that the DlEC581 product is far more abundant as compared to DlEC593 and experiments using KuzDN result mainly in the reduction of the 581 form (unpublished data). Whether or not Kuzbanian alone or additional enzyme activity is responsible for these cleavages requires further investigation. Regardless of the mechanism of cleavage, both of the Delta products have proven to be biologically inactive. Therefore, it is reasonable to conclude that processing in general results in ligand inactivation.

Based on the results we report here, we suggest that the agonistic activity, previously reported by us to be associated with the medium from Delta expressing cells, was not due to the activity of DlEC. However, we note that the present study detected the presence of a “soluble” activity in the medium, raising the possibility that such an activity may after all exist in vivo. Formally at least, this activity can be attributed to the detectable quantities of full length Delta in the medium or to another yet-to-be-determined molecule. It is not inconceivable that soluble, full-length, membrane-associated Delta may in fact be secreted into the medium even if only to act on a neighbor rather than over long distances.

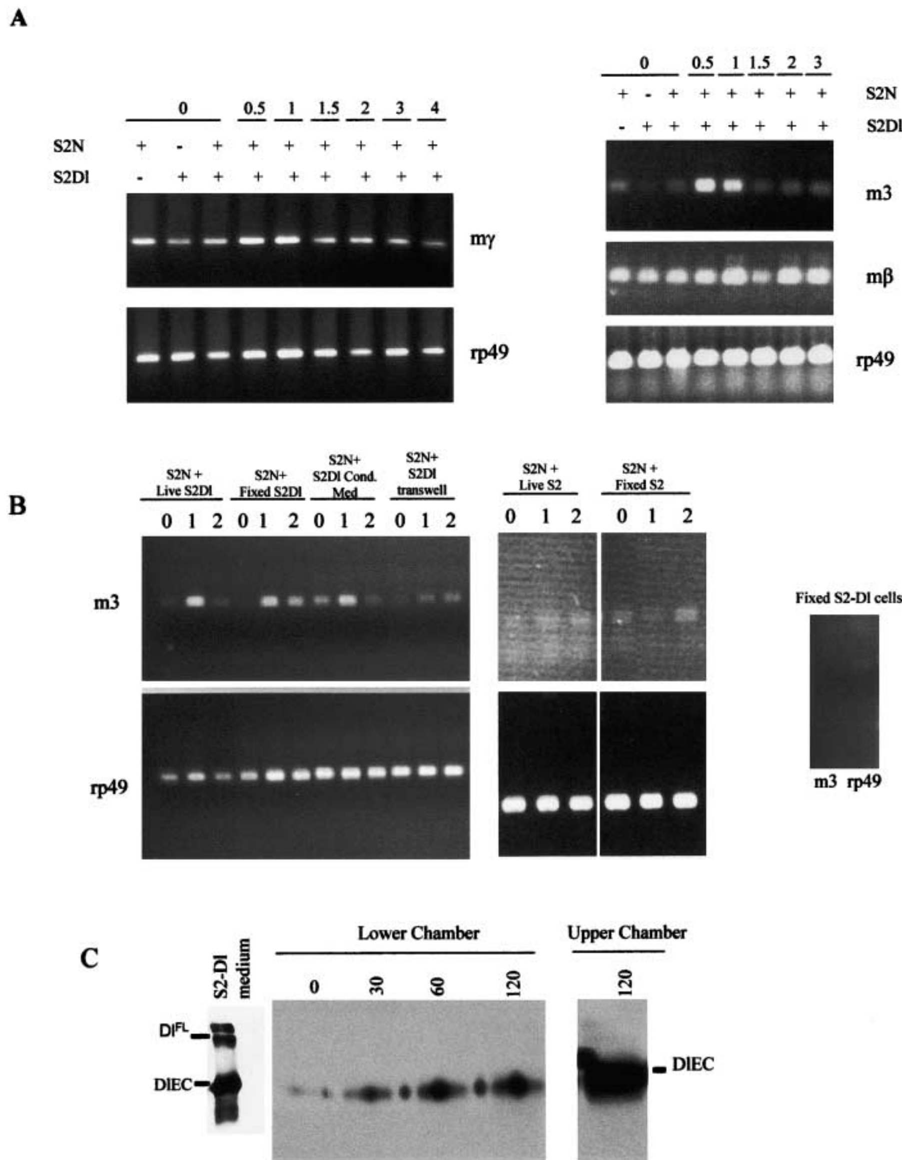
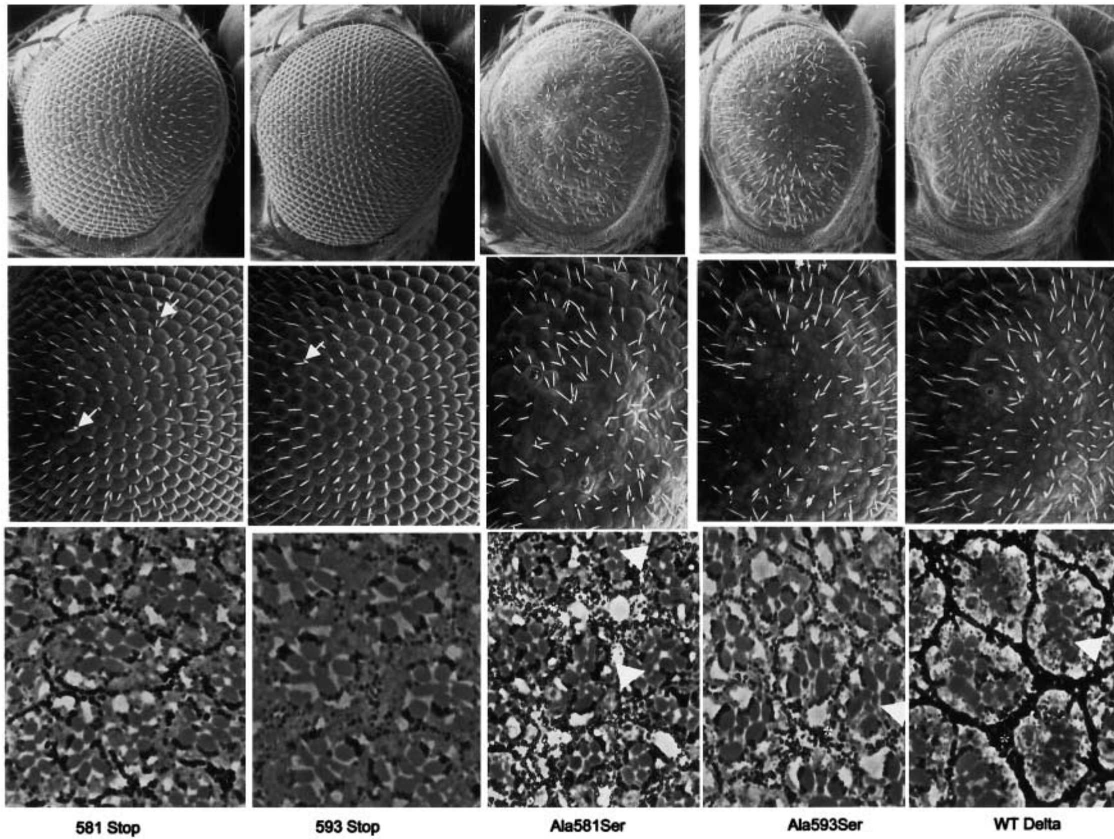


Figure 7. **Full-length Delta, but not DlEC, induces *E(Spl)m3* expression in S2-N cells.** (A) S2-N cells were aggregated with live S2-Dl cells for various time periods as described in Materials and methods. The cells were harvested and RNA was used for RT-PCR for either *mβ*, *mγ*, or *m3* expression. The *rp49* gene was used as an internal control. Unlike *mβ* and *mγ* only *m3* transcription was seen to be specifically up-regulated in S2-N cells after 30 min of exposure to Delta expressing cells. (B) S2-N cells were aggregated with live S2-Dl cells or formalin fixed S2-Dl cells. The S2-N cells were also treated with 5× concentrated conditioned medium from S2-Dl cells or cocultured with S2-Dl cells in a transwell system. *m3* expression was induced only when S2-N cells were aggregated with either live or fixed S2-Dl cells. There was no induction of *m3* in the transwell system. The *m3* induction with S2-Dl conditioned medium is correlated to the presence of full-length Dl in the medium (C). No *m3* induction is seen when S2-N cells are aggregated with either live or fixed S2 cells. (C) Western analysis with anti-Delta 9B antibody of the S2-Dl conditioned medium and the medium in the lower and upper chambers of the transwell system. DlEC is found in the lower chamber with the S2-N cells within 30 min of culturing.

For instance, in the case of wingless, the existence of membrane exovesicles as a vehicle for wingless delivery has been documented (Greco and Eaton, 2001). Whether a soluble, biologically significant Delta activity can be generated by exocytic events remains to be tested, but we suggest that it is worth considering.

Despite the uncertainty of the role of ligand processing, several studies have attempted to use soluble forms of the ligand as an agonist of the receptor with variable success. However, a common element in these studies is that the soluble forms display activity only if they are forced into an oligomeric state either via Fc fusions (Wang et al., 1998; Shimizu et al., 1999) or by immobilization on a matrix (Varnum-Finney et al., 2000). We find that the biologically inactive DlEC fragment secreted in the medium does not have a natural tendency to oligimerize because it exists in a monomeric state (as judged by gel filtration and centrifugal/sedimentation studies [unpublished data]). Furthermore, the inactivity of DlEC expressed in vivo indicates that a biologically relevant mechanism for immobilization of the DlEC so as to make it active is nonexistent. Therefore, it is of utmost importance to consider the physiological relevance of continued attempts to employ soluble ligands as Notch agonists.

Irrespective of the potential requirement for oligomerization or immobilization as an essential activation step for the ligand, Muskavitch (1994) has proposed that endocytosis of the dissociated Notch extracellular domain bound to Delta into the Delta-expressing cells (transendocytosis) is a critical part of the Notch signaling mechanism (Parks et al., 2000). If such a mechanism is essential for Notch activation, then the blocking of an endocytic event may result in inhibition of signaling. This notion is also compatible with the in vivo analysis which demonstrates that membrane-tethered forms of either Delta or Serrate lacking the intracellular domain cannot undergo effective endocytosis, and hence behave as antagonists of Notch signaling (Sun and Artavanis-Tsakonas, 1996; Klueg et al., 1998). On the other hand, the present analysis shows that Delta molecules fixed on the cells, similar to a molecule immobilized on a matrix, is still capable of activating the Notch receptor. This observation



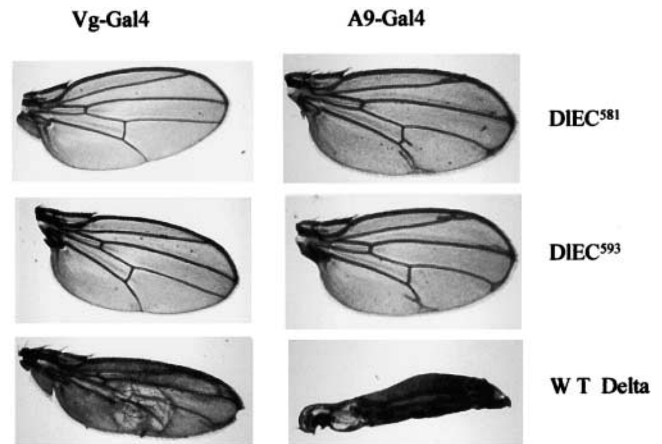


Figure 8. **Mild eye and wing phenotypes associated with DlEC in transgenic flies.** (top) Scanning electron micrograph (SEM) of adult eyes. Expression of DlEC^{581} and DlEC^{593} driven by the GMR promoter yields a phenotype similar to a WT eye with occasional miss positioning or duplication of bristles (arrows). Expression of Ala581Ser, Ala593Ser, and WT Delta with the same driver results in duplication of bristles and melting and smoothing of lens material. Tangential sections show extra photoreceptors (arrowheads) as well as extra interommatidial pigment cells (*) in the Ala581Ser, Ala593Ser, and WT Delta eyes. (bottom) Adult wing phenotypes resulting from expression of Delta mutants: DlEC^{581} and DlEC^{593} exhibited WT wings with the Vg-Gal4 driver, whereas mild wing deltas and occasional extra vein material was seen with the A9-Gal4 driver. Expression of WT Delta under either the Vg- or A9-driver resulted in rudimentary wings. The Ala581Ser or Ala593Ser mutants resulted in phenotypes identical to those seen with WT Delta (not depicted).

would then favor the hypothesis that endocytosis of Delta may be a facilitating but not necessarily an essential part of Notch signaling.

In assessing the developmental significance of Delta cleavage, the activity of Kuzbanian needs to be examined more closely. Although the initial link between Notch signaling and Kuzbanian was reported to involve Notch processing, genetic data show that multiple copies of Delta can suppress the phenotypes associated with dominant-negative Kuzbanian (KuzDN) expression. This observation is compatible with the notion that Delta cleavage produces an active soluble ligand (Qi et al., 1999). However the mechanism of action of KuzDN is not known and it may be equally plausible to consider that KuzDN acts by sequestering Delta, such that the addition of more WT Delta molecules suppress the KuzDN phenotype. It is also worth emphasizing that whereas the dominant-negative forms of Kuzbanian inhibit Delta cleavage, and that Delta cleavage products are not detected in loss of function *kuz* embryos (Qi et al., 1999), it is quite possible that the Kuzbanian–Delta interaction is indirect. The original proposal that Kuzbanian is involved in the proteolytic processing of Notch has been challenged by subsequent experimentation (Qi et al., 1999; Brou et al., 2000; Mumm et al., 2000). Indeed, recent reports documenting Kuzbanian cleavage of Notch rely on deletion mutants of the receptor that are susceptible to cleavage, bringing further uncertainty to the physiological relevance of Kuzbanian acting on Notch directly (Lieber et al., 2002).

A model to explain the role of Delta down-regulation by proteolysis must consider the mechanism of action of the

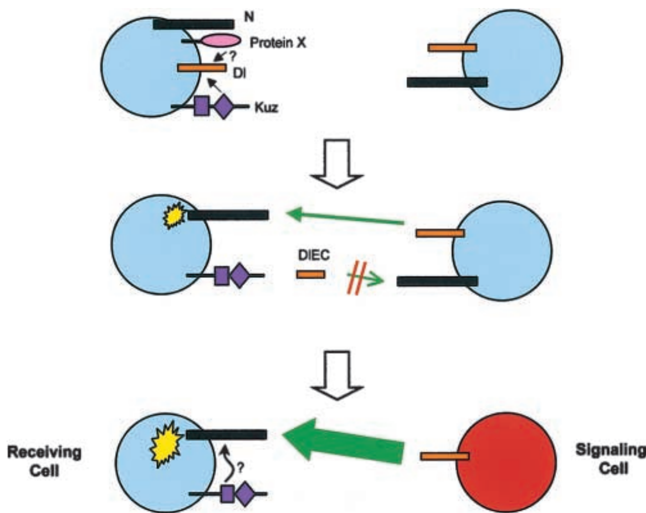


Figure 9. **A functional model for Delta cleavage.** Notch and Delta are initially expressed equivalently on the cell surface. Delta (Dl) is cleaved and consequently inactivated either directly or indirectly by Kuzbanian (Kuz). The ligand is down-regulated so as to no longer interact with the Notch receptor in the same or adjacent cells; Delta removal is reinforcing the "signal receiving status" of that cell. Thus, the Notch receptor (N) can interact with the ligands in the adjacent, "signaling" cell. Although the model implies that Kuz is differentially regulated between critical neighbors, this hypothesis is yet to be tested. Furthermore, the model allows for the possibility of a positive interaction between Kuz and Notch, which has been postulated by some studies.

Notch ligands. Delta can influence Notch through two modes of action: in trans, where Notch and Delta are presented on adjacent cells and Delta can act as agonist (Jarriault et al., 1998, Heitzler and Simpson, 1991), or in cis, where Notch and Delta are presented on the same cell and Delta (and Serrate) can act as a dominant-negative antagonist (de Celis and Bray, 1997, Klein et al., 1997, Micchelli et al., 1997; Jacobsen et al., 1998). It is well established that cells in tissues undergoing Notch signaling can express Notch and Delta simultaneously. For instance, in the early *Drosophila* embryo, all cells in the proneural clusters, the group of cells which will eventually segregate into epidermal and neuronal lineages via Notch–Delta signaling, express both Notch and Delta (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995; Kimble and Simpson, 1997; Weinmaster, 1997). However, in order for proper signaling to occur, there must be a distinction between a signaling versus a receiving cell. The accumulated studies to date suggest that the critical parameter for a cell to be a receiving or signaling cell is the ratio rather than the absolute expression levels of Delta and Notch (Simpson, 1990, 1997a,b; Kimble and Simpson, 1997; Greenwald, 1998). Moreover, feedback loops may be responsible for consolidating and amplifying a given state (ratio) (Muskavitch, 1994; Huppert et al., 1997; Kimble and Simpson, 1997; Greenwald, 1998). Thus, a mechanism that inactivates Delta in a given cell may contribute to the feedbacks that are necessary to establish a critical expression differential between two neighbors.

Mosaic analysis in *Drosophila* during cell fate acquisition in the neuroectoderm demonstrated that Kuz is required in cells to receive signals that inhibit the neural fate (Rooke et al., 1996; Rooke and Xu, 1998). These signals are known to be transmitted through the Notch receptor and this cell autonomous effect of Kuz is consistent with studies in nematodes (Wen et al., 1997). Similarly, using a cell-culture system, we find that dominant-negative or WT forms of Kuzbanian can affect Delta only when cotransfected in the same cell, and have no effect when transfected into adjacent cells (unpublished data). Hence, we suggest that Kuz acts on Delta in the same cell, although it is not clear whether the cleavage occurs at the cell surface or inside the cell. In either case, we propose that this proteolysis renders Delta incapable of interacting with Notch either on an adjacent cell or on the same cell.

Therefore, we favor a model (Fig. 9) whereby proteolytic processing of Delta on a Notch/Delta-expressing cell has the overall effect of rendering that cell the signal receiving cell by (a) alleviating the dominant-negative activity of Delta toward Notch on that cell; and (b) down-regulating the Delta available to signal Notch on adjacent cells. Consistent with this model, Lai et al. (2001) propose a similar role for the neuralized ubiquitin ligase in Delta down-regulation (Pavlopoulus et al., 2001). Interestingly, because neuralized is not required in all Notch dependent developmental contexts, these authors emphasize that multiple mechanisms must exist to clear Delta from the plasma membrane. The above model is also compatible with the possibility that Kuzbanian may play more than one role in Notch signaling. For example, if Kuz is somehow involved in the activation of the Notch receptor (Pan and Rubin, 1997; Lieber et al., 2002), the existence of an activity such as Kuzbanian in a particular cell, which is able to simultaneously enhance receptor function and inactivate the ligand, is a hypothesis worth testing. One of the predictions of the proposed scheme is that Kuzbanian activity must be differentially regulated between critical neighbors. More experimentation will be necessary to confirm or discount this hypothesis and indeed this model.

Materials and methods

Preparation of Delta conditioned media and purification of DlEC

Medium concentrates from *Drosophila* Schneider 2 cells (S2) and Delta-expressing S2 cells (S2-Dl) were prepared, and the extracellular domain of Delta was purified as described previously (Qi et al., 1999). For the present study, cultures of S2-Dl cells (2–4 $\times$ 10^6cells/ml) were scaled up to 2–4 liter. The cells were centrifuged at 700 *g* for 10 min and then resuspended in Sang's M3 medium without serum or bactopeptone or yeastolate supplement at $\sim 10^7$cells/ml. Delta expression was induced with the addition of $CuSO_4$ to 0.7 mM and cultured for an additional 48 h. The conditioned medium was collected after centrifugation at 2000 *g* and clarified by centrifugation at 10,000 *g* for 10 min. Proteins were precipitated with 70% saturation of $(NH_4)_2SO_4$ and centrifuged at 20,000 *g* for 40 min. The pellet was resuspended in 20 ml of 20 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4 (HBS, Ca^{2+}), at which point it was dialyzed 3$\times$ against 2 l of HBS, Ca^{2+}. The dialyzed sample containing DlEC was used for immunoaffinity purification and size exclusion chromatography.

Immunopurification of DlEC was performed by passing the S2-Dl cell medium protein concentrate over a 1.5-ml anti-Delta (9B; Qi, Rand et al., 1999) Sepharose column, followed by washing with 10 column volumes each of HBS, Ca^{2+}, and HBS, 1 M NaCl, Ca^{2+}. The DlEC was eluted with 0.1 M glycine, pH 2.8, and neutralized immediately with 1 M Tris HCl, pH 8.0. The peak fractions were pooled and dialyzed extensively against HBS, Ca^{2+} and stored at 4°C. The flowthrough fraction was collected as a pool and was stored at 4°C or frozen at –20°C. Fractions were analyzed by SDS-PAGE and Western blotting with anti-Delta (9B) monoclonal antibody and with the aggregation assay described below.

Size exclusion chromatography was performed in HBS, Ca^{2+} on a Superdex HR200 column using an AKTA FPLC system (Amersham Biosciences). Fractions were collected and analyzed by SDS-PAGE and Western blotting with anti-Delta (9B) monoclonal antibody and with the aggregation assay described below.

COOH-terminal amino acid sequencing and mass spectrometry

COOH-terminal amino acid sequencing and mass spectrometry was performed at the Harvard Microchemistry Facility (Boston, MA). Samples were prepared from Coomassie blue–stained SDS-PAGE gel slices of immunopurified DlEC. COOH-terminal sequencing was performed with an HP G1005A protein sequencer with online 1090HPLC. MS/MS peptide sequencing was performed on tryptic digests of DlEC followed by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer. The spectra were then correlated with known sequences using the algorithm Sequest (Eng et al., 1994; Yates, 2000) and programs developed at the Harvard Microchemistry Facility (Chittum et al., 1998)

Delta mutant construction

Various Delta mutant forms were generated using the Stratagene site-directed mutagenesis kit and primers with the specific amino acid codons altered. For $DLEC^{581}$ and $DLEC^{593}$ a stop codon was added after the GCG at position 1885 or after GCC at position 1921, respectively. The ~2-kb EcoR1 fragment encoding the soluble Delta was then cloned into the pMT vector (Invitrogen). For the point mutations, the GCG at position 1881 (encoding Ala581) was changed to CGA for serine and the GCC at position 1918 (encoding Ala593) changed to CCT for serine. The mutant forms were cloned into the EcoRI site of either pRmHa-3 (Bunch et al., 1988) or pUAST (Brand and Perrimon, 1993) vectors.

Cell culture

Drosophila S2 cells were routinely cultured in Sangs M3 medium with 10% fetal bovine serum and bactopeptone (2.5 g/L) and yeastolate (1 g/L) supplement (Difco). Transient transfections were done using 10 μg of DNA and the Cellfectin reagent (GIBCO BRL). Stable lines were generated by cotransfection with pGHCO (Bourouis and Jarry, 1983) and methotrexate selection. Protein expression in the cells was routinely induced with 0.7 mM CuSO4 in M3 in the absence of serum. The medium and cells were harvested 24 h later. Cells were lysed with a Tris-based lysis buffer (50 mM Tris, 1% NP40, 150 mM NaCl) containing protease inhibitor PMSF, aprotinin, leupeptin (Calbiochem). Protein expression was analyzed by Western blot analysis using the anti–Delta 9B antibody by standard procedures.

Notch–Delta cell aggregation assay

The interaction of DlEC with Notch expressing S2 cells (S2-N) was assayed in a competitive Notch–Delta cell aggregation assay as previously described (Qi et al., 1999). Samples of S2-Dl cell medium protein concentrate, or immunoaffinity or size exclusion column fractions were preincubated with S2-N cells for 15 min before mixing with S2-Dl cells and subsequent measurement of aggregation. The initial rate of aggregation was determined from the change in transmitted light (320 nm) over the first 3-min interval of aggregation. Rates of aggregation are expressed as a percent of the rate observed with control buffer (HBS, Ca^{2+}) preincubation.

Cortical neuron neurite retraction assay

Low-density primary cultures of cortical neurons were made as described before (Qi et al., 1999; Sestan et al., 1999). The neurons were either cocultured with Jagged expressing cells or treated overnight with 5× concentrated medium from S2 cells or S2 cells expressing Delta, $DlEC^{581}$, or $DlEC^{593}$ induced overnight with 0.7 mM $CuSO_4$. The assay was also performed with affinity purified DlEC. The medium was harvested by spinning at 1,000 *g* for 10 min. The supernatant medium was further spun at 14,000 *g* for 15 min followed by concentrating with a 30-kD cutoff Centricon filter. The neurons were then stained with anti-Tuj1 antibody (Covance) and visualized using secondary antibody conjugated to Cy2.

Neurite outgrowth assay

The Neuro 2a (N2a) cell line (ATCC CCL-131) was used to assay neurite outgrowth. This cell line is well characterized for its ability to differentiate upon withdrawal of serum. This differentiation to produce extensive neurite outgrowth, has been previously documented to be associated with Notch activity modulation (Franklin et al., 1999). N2a cells were grown in DME with 10% fetal calf serum and penicillin and streptomycin (D10). Confluent cultures were split 1:30 and plated for 1 to 2 h in D10 to allow the cells to adhere. Differentiation was induced by switching the cells to serum free DME. After 16–20 h of culture the degree of neurite outgrowth was quantified by counting the number of cells expressing a neurite of greater than one cell body diameter in length. The effect of DlEC containing medium and purified DlEC was assayed by the addition of a 20 μl sample to the 1 ml of culture medium in each well. Assays were performed in quadruplicate.

Detection of *m3* expression

S2-N and S2-Dl cells were induced overnight with 0.03 mM $CuSO_4$, and then washed once with serum-free M3. Cells were quantified using a Coulter Counter (Beckman Coulter) and equal number of S2-N and S2-Dl cells were mixed and allowed to aggregate for various time intervals. The cells were spun down, washed 2× with PBS, and the RNA was harvested with the TriZol reagent (GIBCO BRL). For analyzing the juxtacrine effects or the role of full-length Delta, S2-Dl cells were washed 2× with PBS and then fixed with 5% formalin in PBS for 5 min (Nakagawa et al., 1996). The cells were then washed 2× with PBS and 2× with serum-free M3. The fixed cells were then mixed with equal number of S2-N cells and allowed to aggregate for various time intervals and the RNA harvested. For analyzing the effect of soluble Delta, the six-well 0.45 μ transwell plates (Costar) were used where S2-N were plated in serum-free M3 in the lower chamber and S2-Dl cells in the upper chamber. Twice the amount of S2-N cells were plated as compared with the other aggregation assays to compensate for the RNA from Delta expressing cells. The plates were incubated for various time intervals and then RNA was harvested from the S2-N cells. As a control, RNA was harvested from S2-N and S2-Dl cells incubated alone. 750 ng of RNA was used for the RT reaction using a poly-dT24 primer. The same RT was then used to perform PCR for mβ, mγ, m3, and rp49. The following primers were used for the PCR reactions: rp49, AGT ATCTGATGCCCAACATCG and TTCCGACCAGGTTACAAGAAC; m3, AACAGCAACAACACCAGCAG and GGACTCCTGCGAGCTAACC; mβ, CTACGTTCATGCTGCCAATG and ATTCAGAGGGTGGTGGAGTG; and mγ, GTCAATGAGGTCTCCCGTTC and GGTCAACAGGGAATGACTGG.

Fly strains

All transgenic flies were generated in a w^{1118} background. The fly strains used in genetic interaction studies were Dl^{9p39}/*TM3*. The *gal4* lines used were: *A9-gal4*, a gift from Dr. K. Wharton (Brown University, Providence, RI); *Vg-gal4*, a gift from Dr. K. Vijayraghavan (TIFR, Bangalore, India); and *GMR-gal4*, a gift from Dr. G.M. Rubin (Howard Hughes Medical Institute, Chevy Chase, MD).

Construction of transgenic flies, scanning EM, and sectioning of adult eyes

Germline transformation was performed using standard procedures described by Spradling (1986). Each construct was injected with the Δ2-3 helper plasmid into w^{1118} embryos. For scanning EM, adult flies were dehydrated sequentially in 25, 50, 75, and 100% ethanol, for at least 12 h in each step. The 100% dehydration was repeated three times. The scanning EM was performed at the Electron Microscopy facility at Northeastern University (Boston, MA). Plastic sections were prepared and observed as described previously (Carthew and Rubin, 1990).

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References

Al-Atia, G.R., P. Fruscoloni, M. Jacobs-Lorena. 1985. Translational regulation of mRNAs for ribosomal proteins during early *Drosophila* development. *Biochemistry.* 24:5798–5803.

Artavanis-Tsakonas, S., K. Matsuno, and M.E. Fortini. 1995. Notch signaling. *Science.* 268:225–232.

Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science*. 284:770–776.

Bourouis, M., and B. Jarry. 1983. Vectors containing a prokaryotic dihydrofolate reductase gene transform *Drosophila* cells to methotrexate-resistance. *EMBO J.* 2:1099–1104.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 118:401–415.

Brou, C., F. Logeat, N. Gupta, C. Bessia, O. LeBail, J.R. Doedens, A. Cumano, P. Roux, R.A. Black, and A. Israel. 2000. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Cell.* 5:207–216.

Bunch, T.A., Y. Grinblat, and L.S. Goldstein. 1988. Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila* melanogaster cells. *Nucleic Acids Res.* 16:1043–1061.

Carthew, R.W., and G.M. Rubin. 1990. Seven in absentia, a gene required for specification of R7 cell fate in the *Drosophila* eye. *Cell.* 63:561–577.

Chittum, H.S., W.S. Lane, B.A. Carlson, P.P. Roller, F.D. Lung, B.J. Lee, and D.L. Hatfield. 1998. Rabbit B-globin is extended beyond its UGA stop codon by multiple suppressions and translational reading gaps. *Biochemistry.* 37:10866–10870.

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

Egan, S.E., B. St-Pierre, and C.C. Leow. 1998. Notch receptors, partners and regulators: from conserved domains to powerful functions. *Curr. Top. Microbiol. Immunol.* 228:273–324.

Eng, J.K., A.L. McCormick, and J.R. Yates. 1994. An approach to compare tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass. Spectrom.* 5:976–989.

Fambrough, D., D. Pan, G.M. Rubin, and C.S. Goodman. 1996. The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 93:13233–13238.

Fehon, R.G., P.J. Kooh, I. Rebay, C.L. Regan, T. Xu, M.A. Muskavitch, and S. Artavanis-Tsakonas. 1990. Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in *Drosophila*. *Cell.* 61:523–534.

Fitzgerald, K., and I. Greenwald. 1995. Interchangeability of *Caenorhabditis elegans* DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. *Development.* 121:4275–4282.

Fleming, R.J., K. Purcell, and S. Artavanis-Tsakonas. 1998. The Notch receptor and its ligands. *Trends Cell Biol.* 7:437–441.

Franklin, J.L., B.E. Berechid, F.B. Cutting, A. Presente, C.B. Chambers, D.R. Foltz, A. Ferreira, and J.S. Nye. 1999. Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1. *Curr. Biol.* 9:1448–1457.

Greco V., and S. Eaton. 2001. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell*. 106:633–645.

Greenwald, I. 1998. LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev.* 12:1751–1762.

Heitzler, P., and P. Simpson. 1991. The choice of cell fate in the epidermis of *Drosophilia*. *Cell*. 64:1083–1092.

Hukriede, N.A., Y. Gu, and R.J. Fleming. 1997. A dominant-negative form of Serrate acts as a general antagonist of Notch activation. *Development.* 124: 3427–3437.

Huppert, S.S., T.L. Jacobsen, and M.A. Muskavitch. 1997. Feedback regulation is central to Delta-Notch signalling required for *Drosophila* wing vein morphogenesis. *Development.* 124:3283–3291.

Jacobsen, T.L., K. Brennan, A.M. Arias, and M.A. Muskavitch. 1998. Cis-interactions between Delta and Notch modulate neurogenic signalling in *Drosophila. Development.* 125:4531–4540.

Jarriault, S., O. Le Bail, E. Hirsinger, O. Pourquie, F. Logeat, C.F. Strong, C. Brou, N.G. Seidah, and A. Israel. 1998. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Mol. Cell. Biol.* 18:7423–7431.

Kimble, J., and P. Simpson. 1997. The LIN-12/Notch signaling pathway and its regulation. *Annu. Rev. Cell Dev. Biol.* 13:333–361.

Klein, T. 2002. Kuzbanian is required cell autonomously during Notch signalling in the *Drosophila* wing. *Dev. Genes Evol.* 212:251–255.

Klein, T., K. Brennan, and A.M. Arias. 1997. An intrinsic negative activity of Serrate that is modulated during wing development in *Drosophila. Dev. Biol.* 189:123–134.

Klueg, K.M., and M.A. Muskavitch. 1999. Ligand-receptor interactions and trans-endocytosis of Delta, Serrate and Notch: members of the Notch signalling pathway in *Drosophila. J. Cell Sci.* 112:289–3297.

Klueg, K.M., T.R. Parody, and M.A. Muskavitch. 1998. Complex proteolytic processing acts on Delta, a transmembrane ligand for Notch, during *Drosophila* development. *Mol. Biol. Cell.* 9:1709–1723.

Kopczynski, C.C., K. Fechtel, P.J. Kooh, S.B. Shepard, K.A. Bauer, and M.A. Muskavitch. 1990. Molecular and genetic interactions of the function od Delta, an egg-like gene required for ectodermal differentiation in *Drosophila*. *Mol. Reprod. Dev.* 27:28–36.

Kramer, H. 2001. Neuralized regulating Notch by putting away Delta. *Dev. Cell.* 1:725–726.

Lai, E.C., and G.M. Rubin. 2001a. Neuralized functions cell-autonomously to regulate a subset of notch- dependent processes during adult *Drosophila* development. *Dev. Biol.* 231:217–233.

Lai, E.C., and G.M. Rubin. 2001b. Neuralized is essential for a subset of Notch pathway-dependent cell fate decisions during *Drosophila* eye development. *Proc. Natl. Acad. Sci. USA.* 98:5637–5642.

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

Lieber, T., S. Kidd, and M.W. Young. 2002. Kuzbanian-mediated cleavage of *Drosophila* Notch. *Genes Dev.* 16:209–221.

Micchelli, C.A., E.J. Rulipson, and S.S. Blair. 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.

Mumm, J., E.H. Schroeter, T.S. Meera, A. Griesemer, T. Xiaolin, D.J. Pan, W.J. Ray, and R. Kopan. 2000. A lighand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Cell.* 5:197–206.

Muskavitch, M.A. 1994. Delta-notch signaling and *Drosophila* cell fate choice. *Dev. Biol.* 166:415–430.

Nakagawa, T., S. Higashiyama, T. Mitamura, E. Mekada, and N. Taniguchi. 1996. Amino-terminal processing of cell surface heparin-binding epidermal growth factor-like growth factor up-regulates its juxtacrine but not its paracrine growth factor activity. *J. Biol. Chem.* 271:30858–30863.

Pan, D., and G.M. Rubin. 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during *Drosophila* and vertebrate neurogenesis. *Cell*. 90:271–280.

Parks, A.L., K.M. Klueg, J.R. Stout, and M.A. Muskavitch. 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development.* 127:1373–1385.

Pavlopoulus, E., C. Pitsouli, K.M. Klueg, M.A. Muskavitch, N.K. Moschonas, and C. Delidakis. 2001. Neuralized encodes a peripheral membrane protein involved in delta signaling and endocytosis. *Dev. Cell.* 1:807–816.

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

Rebay, I., R.J. Fleming, R.G. Fehon, L. Cherbas, P. Cherbas, and S. Artavanis-Tsakonas. 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell*. 67: 687–699.

Rebay, I., R.G. Fehon, S. Artavanis-Tsakonas. 1993a. Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. *Cell*. 74:319–329.

Rebay, I., M.E. Fortini, and S. Artavanis-Tsakonas. 1993b. Analysis of phenotypic abnormalities and cell fate changes caused by dominant activated and dominant negative forms of the Notch receptor in *Drosophila* development. *C. R. Acad. Sci. III.* 316:1097–1123.

Rooke, J.E., and T. Xu. 1998. Positive and negative signals between interacting cells for establishing neural fate. *Bioessays.* 20:209–214.

Rooke, J.E., D. Pan, T. Xu, and G.M Rubin. 1996. KUZ, a conserved metalloprotease-disintegrin protein with two roles in *Drosophila* neurogenesis. *Science.* 273:1227–1231.

Sestan, N., S. Artavanis-Tsakonas, et al. 1999. Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science.* 286:741–746.

Shimizu, K., S. Chiba, K. Kumano, N. Hosoya, T. Takahashi, Y. Kanda, Y. Hamada, Y. Yazaki, and H. Hirai. 1999. Mouse Jagged1 physically interacts with Notch2 and other Notch receptors. *J. Biol. Chem.* 274:32961–32969.

Shimizu, K., S. Chiba, T. Saito, K. Kumano, and H. Hirai. 2000. Physical interaction of Delta1, Jagged1, and Jagged2 with Notch1 and Notch3 receptors. *Biochem. Biophys. Res. Commun.* 276:385–389.

Simpson, P. 1990. Notch and the choice of cell fate in *Drosophila* neuroepithelium. *Trends Genet.* 6:343–345.

Simpson, P. 1997a. Notch signaling in development. *Perspect. Dev. Neurobiol.*

4:297–304.

Simpson, P. 1997b. Notch signalling in development: on equivalence groups and asymmetric developmental potential. *Curr. Opin. Genet. Dev.* 7:537–542.

Simpson, P. 1998. Introduction: Notch signalling and choice of cell fates in development. *Semin. Cell Dev. Biol.* 9:581–582.

Sotillos, S., F. Roch, and S. Campuzano. 1997. The metalloprotease-disintegrin Kuzbanian participates in Notch activation during growth and patterning of *Drosophila* imaginal discs. *Development.* 124:4769–4779.

Spradling, A.C. 1986. P element–mediated transformation. *In Drosophila* Practical Approach. IRL Press, Oxford, England. 175–197.

Sun, X., and S. Artavanis-Tsakonas. 1996. The intracellular deletions of Delta and Serrate define dominant negative forms of the *Drosophila* Notch ligands. *Development.* 122:2465–2474.

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

Varnum-Finney, B., L. Wu, M. Yu, C. Brashem-Stein, D. Flowers, S. Staats, K.A. Moore, I. Le Roux, R. Mann, G. Gray, S. Artavanis-Tsakonas, and I.D. Bernstein. 2000. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J. Cell Sci.* 113:4313–4318.

Wang, S., A.D. Sdrulla, G. diSibio, G. Bush, D. Nofziger, C. Hicks, G. Weinmaster, and B.A. Barres. 1998. Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron.* 21:63–75.

Weinmaster, G. 1997. The ins and outs of notch signaling. *Mol. Cell. Neurosci.* 9:91–102.

Weinmaster, G. 1998. Notch signaling: direct or what? *Curr. Opin. Genet. Dev.* 8:436–442.

Wen, C., M.M. Metzstein, and I. Greenwald. 1997. SUP-17, a *Caenorhabditis elegans* ADAM protein related to *Drosophila* KUZBANIAN, and its role in LIN-12/NOTCH signalling. *Development.* 124:4759–4767.

Yates, J.R., III. 2000. Mass spectrometry. From genomics to proteomics. *Trends Genet.* 16:5–8.

Ye, Y., and M.E. Fortini. 1999. Apoptotic activities of WT and Alzheimer's disease-related mutant presenilins in *Drosophila* melanogaster. *J. Cell Biol.* 146:1351–1364.