Unidirectional Notch signaling depends on continuous cleavage of Delta

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Summary

Unidirectional signaling from cells expressing Delta (Dl) to cells expressing Notch is a key feature of many developmental processes. We demonstrate that the Drosophila ADAM metalloprotease Kuzbanian-like (Kul) plays a key role in promoting this asymmetry. Kul cleaves Dl efficiently both in cell culture and in flies, and has previously been shown not to be necessary for Notch processing during signaling. In the absence of Kul in the developing wing, the level of Dl in cells that normally receive the signal is elevated, and subsequent alterations in the directionality of Notch signaling lead to prominent phenotypic defects. Proteolytic cleavage of Dl by Kul represents a general mechanism for refining and maintaining the asymmetric distribution of Dl, in cases where transcriptional repression of Dl expression does not suffice to eliminate Dl protein.

Key words: Notch, Delta, Serrate, ADAM metalloproteases, Drosophila, Wing development, Kuzbanian-like

Introduction

The Notch signaling pathway serves as one of the cardinal means by which distinct fates are induced in adjacent cells during development. This is achieved by presentation of the ligands Delta (Dl) or Serrate (Ser) on the cell surface of the sending cell, and activation of the Notch receptor in the receiving cells. Given the transmembrane nature of the ligands, activation is executed only in cases of direct contact between the cells. Depending upon the context of the receiving cell and the convergence of other signaling pathways, the final outcome may be inhibition or induction of differentiation, proliferation or apoptosis (Artavanis-Tsakonas et al., 1999; Lai, 2004; Schweisguth, 2004).

A sharp distinction between the sending and receiving cells is essential, because signaling is carried out only between neighboring cells. In some cases the Notch pathway itself provides the means for initiating the primary distinction between cells, in a seemingly homogeneous population of cells. It is thought that random fluctuations elevating the levels of Dl in a given cell embedded within a group of equivalent cells (e.g. a pro-neural field) will trigger a further elevation in Dl and a concomitant reduction in Notch levels. In parallel, activation of Notch in the adjacent (signal-receiving) cells leads to a reduction in Dl levels (Heitzler et al., 1996).

In other instances the Notch pathway is dedicated to the refinement of an already established asymmetry between adjacent cell populations. A case in point is the definition of vein borders in the pupal wing. The epidermal growth factor receptor pathway is activated in the future veins, leading to induction of Dl expression. Localized expression of Dl activates Notch signaling in the adjacent cells, to inhibit the formation of veins in this territory (de Celis et al., 1997; Huppert et al., 1997). In this system Notch signaling also relies upon the simultaneous increase in Dl and decrease of Notch in the sending cells, and the elimination of Dl in the receiving cells. These responses are at the heart of maintaining a stable, unidirectional signaling by the Notch pathway.

Which mechanisms contribute to changes in levels of Dl and Notch as a result of Notch signaling? Transcriptional repression of Dl, mediated by the Enhancer of split [E(Spl)] complex induced following Notch activation is a general and direct mechanism contributing to the reduction in Dl levels (Heitzler et al., 1996; Hinz et al., 1994; Kunisch et al., 1994). In other cases, induction of specific transcriptional repressors, such as Cut in the wing margin, by Notch activation leads to repression of Dl transcription (de Celis and Bray, 1997; Micchelli et al., 1997).

Mechanisms for Notch protein modification also play a role in maintaining an asymmetric distribution or activity of Notch. In specific areas, such as the wing margin, modification of Notch by Fringe, a glycosyltransferase, renders it refractive to signaling by ligands such as Ser, or conversely more responsive to Dl expressed by the adjacent cells (Bruckner et al., 2000; Okajima and Irvine, 2002; Panin et al., 1997). In sensory-organ precursor cells, enhanced endocytosis of Notch by asymmetric segregation of Numb/α-Adaptin was shown to reduce Notch signaling (Berdnik et al., 2002; Frise et al., 1996). Enhanced endocytosis of Notch was also observed in Caenorhabditis elegans vulval development, in the cell that is induced to become the primary source for the Notch ligand (Shaye and Greenwald, 2002). In specific areas, such as the wing margin, high levels of Dl were shown to confer refractivity to Notch signaling, through an ill-defined dominant-negative effect of Dl (de Celis and Bray, 1997; Micchelli et al., 1997).
Proteolysis plays an integral part in the Notch signaling pathway, particularly in sequential cleavages of Notch itself (Baron, 2003; Mumm and Kopan, 2000). The first cleavage (termed S1), carried out by Furins, bisects Notch at the extracellular domain, leaving the resulting two polypeptides noncovalently associated. S1 is a constitutive event. The second cleavage (termed S2) is a regulated event that takes place only once Notch is bound to DI presented by the adjacent cell. This cleavage is thought to be carried out by the Kuzbanian (Kuz) ADAM metalloprotease (Lieber et al., 2002). Initiation of endocytosis of the DI/Notch complex into the DI-presenting cell facilitates this S2 cleavage, and internalizes the extracellular domain of Notch (Le Borgne and Schweisguth, 2003; Parks et al., 2000). Finally, the truncated portion of Notch, containing the transmembrane domain, becomes a target for the Presenilin intra-membrane protease (S3 cleavage), releasing the cytoplasmic domain of Notch, which is targeted to the nucleus to induce transcriptional responses (Struhl and Adachi, 1998).

Could proteolytic events also contribute to generation or maintenance of differences in the levels of DI between sending and receiving cells? The Kuz metalloprotease was shown to cleave DI in cell culture and in flies, and to release a secreted form of DI (Mishra-Gorur et al., 2002; Qi et al., 1999). However the role of this cleavage in promoting or suppressing Notch signaling could not be tested, due to the essential role of Kuz in the regulated S2 cleavage, which is necessary for activation of Notch (Lieber et al., 2002). We identified an ADAM metalloprotease (termed Kul) dedicated to cleavage of DI, and demonstrated the necessity of removing DI in the cells receiving the signal, in order to maintain unidirectional Notch signaling.

Materials and methods

Fly lines and clonal misexpression

The following lines were used: scabrous-GAL4 (sd-GAL4) (from A. Garcia-Bellido), MS1096-GAL4; spalt-GAL4 (sal-GAL4), ptc-GAL4, Gbe+Su(H)sd-lacZ (from S. Bray), and hs-flp; actin>CD2c-GAL4, UAS-gfp (from K. Basler), mato4-GALVP16, UAS-DI, and UAS-kuz, neur[GAL4-A101] (used in conjunction with GAL80 to drive expression in sensory bristles) were obtained from the Bloomington Stock Center. Clones of cells expressing different UAS constructs were generated by FLP-mediated mitotic recombination. Early or late second instar larvae were subjected to a 35°C heat shock in a water bath for 30 minutes. Transgenic lines carrying the UAS constructs for the ADAM proteins and the ds-RNA (described below) were generated.

To identify flip-out clones in the wing disc expressing only DI or DI and Kul, the following cross was carried out. Males carrying UAS-DI on the X chromosome and UAS-kul/+ on the third chromosome were crossed to females carrying the flipout cassette. Female larvae were selected for dissection. Thus, all larvae expressed DI in the flipout clones, and half also expressed Kul in the same clones, as identified by Kul antibodies.

Molecular biology

To reconstitute the Kul ORF, RNA from embryos at all stages was isolated by the Tri reagent (Molecular Research) and was reverse transcribed into a pool of first strand cDNAs using SuperScript (Invitrogen). This pool served as a template for PCR, using primers designed according to the BDGP ORF prediction. The GenBank accession number for the complete ORF (CG1964) is AY525767. For structure-function studies, different derivatives of Kul protein were made, using a site-directed mutagenesis kit (Promega). Catalytically inactive Kul was constructed by generating the E643A mutation in the protease catalytic site. Kul lacking the intracellular domain (Ex-Kul) was generated by truncating the protein after residue 991, resulting in a short cytoplasmic tail of 42 amino acids. The Kul precursor in which pro-domain cleavage was blocked (Pro-Kul) was made by changing RK at positions 219-220 in the putative Furin-cleavage site into AG. All Kul constructs (except EX-Kul) were HA tagged at their C-terminus.

Kul ds-RNA constructs were generated by PCR amplification of antisense 794-1705 followed by sense 340-1705 (where A in the ATG codon is defined as 1). This sequence covers the pro-domain of Kul, and shows no similarity to other Drosophila DNA sequences. The same procedure was carried out with sequences from the Kul cytoplasmic tail (antisense 3381-4425, sense 2911-4425) to form another ds-kul construct.

Full-length DTACE sequence (CG7908, GenBank accession number AY525768) from expressed sequence tag (EST) GH06244 was tagged with HA at the C-terminus. Full length DMeltrin cDNA (CG31314, CG31385; GenBank accession number AY525769) was constructed from EST SD34743. The cDNA was HA-tagged at the C-terminus. Kul dsRNA was generated by antisense 2626-3515, sense 2247-3515, DTACE and DMeltrin dsRNA constructs were generated from the pro-domain region in a similar way to ds-kul. All constructs were cloned into pUAST. UAS-Kuz plasmid (from D. Pan) was tagged at the C-terminus with HA.

Additional UAS lines used in cell culture assays were UAS-Myc-Del (from K. Klueg), and UAS-Myc-Serrate (from R. J. Fleming).

Cell culture

Drosophila S2 cells were grown in Schneider’s medium (Beit-Haemek) with 10% heat-inactivated fetal calf serum. The calcium phosphate method was used for transfection. To express the various constructs, UAS vectors were co-transfected with the actin5c-GAL4 plasmid. Following transfection the cells were grown in Schneider’s medium without serum, and medium was collected after 2-3 days. Cells were lysed in RIPA buffer. Protein extraction and Western-blot analysis was performed as in Tsruya et al. (Tsruya et al., 2002). For Delta detection, Laemmli sample buffer without β-mercaptoethanol was used. UAS-GFP plasmid was used to calibrate the number of transfected cells. Western blotting was carried out with anti-HA (Babco), anti-GFP (Roche), and additional antibodies described below.

Immunohistochemistry and in-situ hybridization

Immunohistochemistry of larval discs was performed according to Tomlinson and Ready (Tomlinson and Ready, 1987). Papu wing staining was performed as in Axelrod (Axelrod, 2001). The following primary antibodies were used: monoclonal C594.9B anti-Delta (1:100) (from S. Artavanis-Tsakonas), rat anti-Serrate (1:1000) (from K. Irvine), and rabbit anti-β-Gal (1:2000) (Cappel). The monoclonal antibodies 4D4 anti-Wg, (1:10) and 2B10 anti-Cut, (1:10) were obtained from Developmental Studies Hybridoma Bank. Secondary antibodies were obtained from Jackson ImmunoResearch. In-situ hybridization on embryos and wing discs was performed according to www-biology.ucsd.edu/~davek/.

To generate antibodies against Kul, a kul cDNA fragment encoding amino acids 1215-1529 from the cytoplasmic tail was cloned into pRSETA. The resulting protein was injected into rats.

Sequence analysis

Phylogenetic analysis was performed using the extracellular region of the ADAM proteins in the MEGA packages using the neighbor-joining method.
Results

Five Drosophila ADAM metalloproteases

ADAM proteins have a characteristic domain signature, including a signal peptide followed by a pro-domain, a metalloprotease domain possessing a zinc-binding catalytic pocket, and a disintegrin domain. A cysteine-rich region is followed by a transmembrane domain and cytoplasmic tail (Primakoff and Myles, 2000). Sequence similarity searches determined that the Drosophila genome harbors five ADAM-family metalloproteases. The full open reading frames of these metalloproteases were obtained from a combination of cDNA clones, reverse-transcription based on gene prediction, and 5' RACE in cases where the cDNAs did not cover the entire coding region (see Materials and methods).

Among the five Drosophila metalloproteases, a single homolog was identified for TNF-α converting enzyme (TACE), two homologs were found for Meltrin-α, and two for ADAM10. Analysis of the ADAM family phylogenetic tree identified gene duplication events that took place most probably after the divergence of the ancestors of nematodes and insects (Fig. 1A). While Kuz shows a high degree of similarity to human ADAM10, another Drosophila protein, which we term Kuzbanian-like (Kul), exhibits an even higher degree of similarity to ADAM10, especially in the disintegrin domain (which facilitates substrate recognition), as well as in the metalloprotease catalytic domain (Fig. 1B).

Several Drosophila ADAMs can cleave Delta

In view of the ability of Kuz to cleave Dl, three other Drosophila ADAM proteins were examined for this activity. Dl was co-expressed with each of the ADAM proteins in Drosophila S2 cells, and the levels of Dl were monitored in the cells and in the medium, by probing with an antibody directed against the Dl extracellular domain. When Dl alone was expressed in the cells, basal cleavage by endogenous proteases was detected by an accumulation of the cleaved form of Dl in the medium. Co-expression of Kuz led to a marked elevation of Dl in the medium, concomitant with a reduction in the levels of the membrane-bound Dl in the cells (Fig. 1C) (Qi et al., 1999). Two additional ADAM proteins, Kul and DTACE, exhibited a similar potency of cleaving and releasing Dl to the medium. By contrast, the expression of DMeltrin had no effect (Fig. 1C).

Serrate (Ser) is a second ligand of Notch in Drosophila and is employed in more restricted biological settings. A similar profile of cleavage was also observed for Ser, which was cleaved by Kuz, Kul and DTACE, but only marginally by DMeltrin (Fig. 1C). Detection of efficient cleavage in S2 cells, which are grown in suspension, supports the notion of cell-autonomous cleavage. Thus, cleavage of DI or Ser by ADAM proteins is likely to take place within the same cell, rather than between adjacent cells.
Which ADAM proteins affect Notch signaling in the wing?

To examine the biological roles of the ADAM proteins that can cleave Dl, it was necessary to compromise their activity in flies. Mutations or P-element insertions in DMeltrin, DTACE and kul are not currently available. We therefore generated double-stranded RNA (dsRNA) ‘knock-down’ constructs for each of these genes, directed against a region of minimal similarity with the other family members.

Activation of the Notch pathway is required many times during normal wing development. Especially notable is the role of Notch activation in restricting the width of the wing veins within a pro-vein territory (de Celis, 2003; Huppert et al., 1997). Utilizing the UAS-GAL4 system, dsRNA constructs of Drosophila ADAM metalloproteases were expressed in the wing. Expression of ds-DMeltrin or ds-DTACE did not lead to any detectable wing phenotypes, even when expressed under the regulation of the potent wing driver MS1096-GAL4 (not shown). By contrast, induction of ds-kul expression by the same driver gave rise to distorted wings (not shown), and loss of the wing margin following induction by sd-GAL4 (Fig. 7J).

Expression of ds-kul by the weaker driver, sal-GAL4, resulted in two distinct adult wing phenotypes in the spalt-expression domain, which encompasses the region between veins L2-L4; formation of multiple wing hairs (A.S. and B.-Z.S., unpublished), and partial loss of veins (Fig. 2B, arrow). The first phenotype is Notch-independent, and will not be further addressed in this work.

The opposite phenotype with respect to vein loss, i.e. vein thickening, was observed when full-length Kul was overexpressed by sal-GAL4 (Fig. 2C, arrow). These phenotypes suggest a functional link between Kul and Notch signaling in patterning the wing veins. Additional defects in the morphology of the wing were observed, and may stem from effects of Kul on other substrates independent of the Notch pathway, in accordance with the additional defects observed following ds-kul misexpression. Compromising the levels of Kul by a ds-kuz construct resulted in vein thickening, representing a Notch loss-of-function (Fig. 2H). This vein phenotype of ds-kuz is similar to the reported kuz loss-of-function wing phenotype (Sotillos et al., 1997), and is also consistent with observations in cell culture, which have demonstrated that ds-kuz RNA abolished the S2 step of Notch cleavage, while ds-kul RNA had no effect on Notch (Lieber et al., 2002).

Characterization of Kul

In light of the high sequence similarity between Kul and Kuz, the specificity and activity of ds-kul was verified in cultured cells. When Kul was expressed in S2 cells, both a high molecular weight precursor form, and a cleaved, mature form were detected. Expression of ds-kul eliminated both forms of Kul, but did not affect Kuz protein levels, demonstrating the specificity of ds-kul (Fig. 2I).

The activity of ds-kul was also examined in vivo. A broad distribution of kul transcripts was detected during all embryonic stages (not shown) and in the wing imaginal discs (Fig. 2D). ds-kul expression in the wing reduced endogenous kul mRNA levels, illustrating the potency of ds-kul in vivo (Fig. 2E).

We further characterized the structural requirements for Kul function, by examining in S2 cells protein maturation and the role of the different domains in promoting Dl cleavage (Fig. 2F-H).

Fig. 2. Reduction of Kul levels leads to loss of wing veins. (A) Wild-type wing. (B) Expression of ds-kul in the central part of the wing by sal-GAL4 led to loss of veins (arrow). This phenotype suggests that Kul may normally be required for proper signaling by the Notch pathway. (C) Overexpression of Kul by sal-GAL4 led to an expansion of veins (arrow). (D) kul RNA is broadly distributed in the wing imaginal disc. (E) Expression of ds-kul at the anterior-posterior boundary of the wing disc by ptc-GAL4 led to elimination of endogenous kul RNA within this domain (arrowhead). (F) Expression of kul by the ptc-GAL4 driver exhibited a marked elevation in kul RNA. (G) Overexpression of Kuz by sd-GAL4 led to an expansion of veins (arrow), similar to Kul. (H) Expression of ds-kuz by MS1096-GAL4 led to thickening of the veins, similar to a kuz loss-of-function phenotype. (I) The specificity of ds-kul was monitored in S2 cells. HA-tagged Kul (Kul-HA) is detected as a high molecular precursor, and as a mature protein lacking the pro-domain. Co-expression of ds-kul eliminated expression of the protein. By contrast, ds-kuz had no effect on the expression of Kul-HA. However, ds-kul had no effect on the expression of Kuz-HA, while ds-kuz eliminated the expression of the Kuz-HA protein. We conclude that ds-kul is specific. Expression of UAS-GFP was used to demonstrate similar transfection levels.
Fig. 3. Structure-function analysis of Kul. The activity of Kul was monitored in S2 cells, by its capacity to release DI to the medium and reduce the levels of the protein in the cells. Following expression of DI alone, some cleavage can be detected, presumably by endogenous ADAM proteins. The levels of cleaved DI are markedly elevated following co-expression of full-length Kul. A mutation leading to the elimination of the catalytic activity (Kul E-A), removal of the cytoplasmic tail (Kul Ex), or inactivating the cleavage site of the pro-domain (Pro-Kul), abolished the capacity of Kul to cleave DI. Furthermore, in all three cases the activity of the endogenous ADAMs was also compromised.

Fig. 4. Kul is required for unidirectional Notch signaling in the pupal wing veins

In the pupal wing, activation of the Notch pathway by DI contributes to refinement and restriction of the veins. DI, expressed by the central pro-vein cells, activates Notch in the lateral pro-vein domain, forcing these cells to adopt an inter-vein fate, while the DI-expressing cells themselves differentiate as veins (de Celis, 2003) (Fig. 4E). Reduction of Notch signaling causes lateral pro-vein cells to adopt a vein cell fate, leading to vein thickening in the adult wing. By contrast, ectopic expression of DI by the inter-vein cells results in Notch activation in the central pro-vein cells, leading to vein loss (Huppert et al., 1997).

Kul regulates Notch signaling in the pupal wing veins

In the pupal wing, activation of the Notch pathway by DI was enhanced by co-expression of full-length Kul. By contrast, a Kul variant bearing an E-to-A substitution within the metalloprotease catalytic domain (E-A Kul), which abolishes catalytic activity, failed to cleave DI. This demonstrates the need for an active protease domain in Kul. A similar mutation abolished the catalytic activity of Kuz (Pan and Rubin, 1997).

Interestingly, E-A Kul reduced DI cleavage below the basal level, probably due to formation of a complex between E-A Kul and DI that is refractive to cleavage by the endogenous proteases. Expression of this construct in the wing gave rise to broadened veins (not shown), again probably due to the sequestration of DI. A similar inhibitory effect in cell culture was detected when expressing a Kul protein lacking the intracellular domain. This domain is important for correct trafficking and sorting of ADAM proteins (Cao et al., 2002).

Finally, we analyzed the role of pro-domain removal, by expressing a form of Kul in which cleavage was blocked by mutating two conserved amino acids at the putative pro-domain cleavage site. This variant full-length form of Kul again failed to carry out DI cleavage, demonstrating the need of pro-domain removal in order to convert Kul to an active protease.
ds-kul expression gave rise to loss of veins (Fig. 2B), which is consistent with an effect on Notch signaling. To verify that this is indeed the case, the effects of compromising Kul levels in the pupal wing were monitored by a transcriptional reporter of Notch activation termed Gbe+Su[H]ms (Furriols and Bray, 2001), and by following the expression of DI. In a wild-type pupal wing 30 hours after pupariation, DI protein is restricted to the future veins, while reporter expression is prominent in the lateral pro-vein cells and excluded from the vein cells (Fig. 4A,C). It is interesting to note that reporter expression is detected even several cell rows away from the vein cells, possibly resulting from the capacity of DI-expressing cells to form far-reaching cellular extensions and protrusions (De Joussineau et al., 2003).

Expression of ds-kul by sal-GAL4 had a marked effect on the expression of DI, as well as on the Notch reporter (Fig. 4B,D). Irregular expansion of DI into the lateral pro-vein territory was observed, while in other parts of the wing DI protein disappeared from the veins. Notch-reporter expression expanded into the vein cells. Elevation in DI levels in the lateral pro-vein cells endowed them with the capacity to activate Notch signaling within the vein, demonstrating a role for Kul in maintaining unidirectional signaling by the Notch pathway. These patterns account for the loss of veins seen in the adult wing following expression of ds-kul (Fig. 2B). The irregular patterns observed in the pupal wing may represent snapshots of a dynamic sequence, which is initiated by expansion of DI to the lateral pro-vein cells, followed by expansion of Notch activation and loss of DI expression in the vein cells.

**Kul cleaves Delta in vivo in a cell-autonomous manner**

The expansion of DI-protein distribution in the pupal wing following ds-kul expression implied that Kul normally contributes to the restricted distribution of DI in this tissue. To examine in more detail the capacity of Kul to cleave DI in vivo, we monitored the larval wing imaginal disc. DI protein, detected by an antibody recognizing the extracellular domain, is normally observed as a membrane-associated protein that is elevated in the vein and juxta-margin cells and excluded from the wing margin (Fig. 5A) (Kooh et al., 1993). We monitored changes in DI distribution in discs where Kul was overexpressed by the MS1096 driver. DI membrane-associated staining in the wing pouch was diminished, and a residual punctate staining appeared, possibly reflecting endocytosis of secreted DI (Fig. 5B). Normal Delta distribution was retained in the notum, where Kul was not overexpressed. Similarly, expression of Kul by sal-GAL4 eliminated DI in the sal domain (Fig. 5C,D). To verify that Kul directly affects the cleavage of DI, rather than DI expression, we generated clones

![Fig. 5. Kul cleaves Delta but not Serrate in the wing disc.](image)

(A) DI expression (red) in a wild-type wing disc.
(B) Overexpression of Kul in the wing pouch eliminated the normal DI protein in the wing margin and future veins. Note that in the notum, where MS1096-GAL4 is not expressed, normal levels of DI were retained (arrow).
(C,D) Overexpression of Kul by sal-GAL4 eliminated DI in the sal domain. (E-G) Expression of DI (red) in flipout clones (marked by GFP expression). (H-J) Co-expression of Kul (blue) and DI in flipout clones eliminated the DI protein. Thus, Kul mediates direct cleavage of DI, rather than affecting the expression of DI. (K-M) Clones of cells expressing Kul (green) showed elimination of DI but not Ser. The loss of DI was confined to the clone, demonstrating the cell-autonomous activity of Kul. (N-P) Overexpression of Kuz eliminated both DI and Ser within the clones.
overexpressing Dl under the regulation of actin-GAL4, in the absence or presence of ectopic Kul. Indeed, the prominent appearance of Dl was completely abolished when Kul was co-expressed in the same clone (Fig. 5E-J).

We demonstrated previously the capacity of Kul to cleave Ser in S2 cells. To check if Kul can also cleave Ser in vivo, the distribution of Ser was analyzed following overexpression of Kul, using an antibody recognizing the Ser extracellular domain. Ser is normally expressed in the wing disc at this phase in a similar pattern to that of Dl, with a more pronounced appearance in the dorsal side of the pouch (Fig. 6A). The effects of Kul overexpression on Ser were distinct from the effect on Dl. Ser protein did not disappear, but instead displayed an altered, punctate localization within the cells. Kul overexpression also led to a uniform expansion of Ser expression, especially in the dorsal part of the pouch, where the expression of MS1096-GAL4 is more pronounced (Fig. 6B,C). We do not know in which compartment(s) Ser accumulated, nor the mechanism by which Kul overexpression leads to this sequestration.

To test if Kul cleaves Dl cells autonomously, we generated cell clones overexpressing Kul. Dl staining was diminished only in the clone cells, implying a cell autonomous activity of Kul (Fig. 5K). There was no detectable change in Ser distribution within these small clones (Fig. 5L). The same cleavage assay carried out with Kuz overexpression revealed a different substrate specificity. Kuz cleaved both Dl and Ser within the overexpression clones (Fig. 5N,O). Like Kul, the activity of Kuz was cell autonomous, i.e. restricted to the cells overexpressing the protease.

In the wing disc, Notch signaling plays a key role in defining and maintaining the margin, in two distinct signaling phases. First, the asymmetry between the dorsal and ventral compartments defines the margin and induces the expression of Wg by the future margin cells. The process is dictated by expression of Fringe only in the dorsal compartment, facilitating Notch signaling in the two cell rows comprising the border between the two compartments (Fleming et al., 1997; Neumann and Cohen, 1996). The wing margin fate is subsequently maintained by complementary unidirectional signals between the margin and juxta-margin cells. In the margin, Notch signaling leads to the expression of Wg and Cut (Fleming et al., 1997). The latter operates as a transcriptional repressor of Dl. In parallel, Wg activates expression of Dl and Serrate in the juxta-margin cells. High levels of Dl and Serrate prevent Notch activation in these cells (de Celis and Bray, 1997; Micchelli et al., 1997). Thus a stable loop of two reinforcing signals is generated (Fig. 7K).

It was interesting to examine the biological consequences of the response to Kul overexpression. Genetic removal of Dl or Ser alone was not sufficient to alleviate the dominant-negative effect of the remaining ligand on Notch signaling in the juxta-margin cells (de Celis and Bray, 1997; Micchelli et al., 1997). However, in the case of Kul overexpression, both ligands were affected, i.e. Dl was efficiently cleaved and Ser was predominantly sequestered within the cells. We observed an expansion in the expression of the Notch-target genes wg and cut. In addition, the expression of Ser was broader (Fig. 6C-G). We assume that effective removal of DI in conjunction with sequestration of Ser, gave rise to alleviation of their dominant-negative effect on Notch signaling in the juxta-margin cells expressing these ligands. Thus, a residual level of Dl or Ser on the cell surface could trigger Notch signaling within these cells. Activation of Notch subsequently leads to ectopic production of Wg, which in turn spreads to neighboring cells to trigger ectopic expression of Ser.

Kul regulates Notch signaling in the wing margin

Does endogenous Kul in the wing disc have a role in maintaining the asymmetric distribution of DI? Expression of ds-kul by the potent sd-GAL4 driver gave rise to loss of Cut and Wg expression in the margin, and a reduction in the size

![Fig. 6. Effects of Kul overexpression in the wing imaginal disc.](image)
of the wing pouch (Fig. 7B,D). The adult wings that developed were significantly reduced in size and showed no indication for veins, and only rudimentary margin bristles in very restricted domains (Fig. 7J). These results demonstrate that Kul is essential for maintaining the spatial balance of Notch signaling in the wing margin.

Induction of ds-kul by the sal-GAL4 driver did not affect the adult wing margin (Fig. 2B) and resulted only in a reduction in Wg levels in the wing margin (Fig. 7E,F), without a pronounced effect on Cut levels (not shown). In view of the role Kul plays in Dl cleavage, we wanted to test whether the changes in Notch target-gene expression in the wing margin resulted from elevation in Dl levels within the margin cells. Indeed, higher levels of Dl could be detected in the margin within the sal domain where ds-kul was expressed (Fig. 7I). By contrast, no effects of ds-kul on the distribution of Ser were observed (see Fig. S1 in the supplementary material).

Discussion
Kul cleaves Delta
This study demonstrates that the Kul ADAM metalloprotease cleaves Dl in vivo. In the context of the Notch pathway, the function of Kul is restricted to Dl cleavage. Thus, the biological significance of this cleavage could be examined in isolation. In the absence of Dl cleavage by Kul, the unidirectional feature of Notch signaling is disrupted. In cell culture several ADAM metalloproteases can cleave Dl and Ser, including Kul, Kuz and DTACE. Yet, in the wing disc the spectrum of functional ADAM proteins is more restricted. Only overexpression of Kul and Kuz resulted in cleavage of Dl, and only Kuz was able to cleave and remove Ser efficiently. Other players, which bias the capacity to cleave the ligands, may be operating. The catalytic domain of ADAM metalloproteases does not have restricted substrate specificity. In fact, in several instances mutagenesis of the cleavage site of the ADAM substrate did not abolish cleavage (Hattori et al., 2000). Specificity is governed mainly by the association between the ADAM protein and its substrate, which is mediated in many cases by the disintegrin domain. It is possible that specific proteins may modulate this interaction, by facilitating or blocking it. Thus, the profile of cleavage in vivo is more restricted than the spectrum observed upon overexpression of the ADAM metalloproteases and the putative substrates in cultured cells.

It is interesting to note that, with respect to Dl cleavage, there is no redundancy between the ADAM proteins that are able to cleave Dl in cell culture. Removal of kul RNA alone was sufficient to give rise to dramatic phenotypes. This feature is especially noteworthy with respect to Kul and Kuz, which are both capable of cleaving Dl upon overexpression in the wing disc. The requirement for Kul may reflect a quantitative aspect,
i.e. the activity of Kuz may not suffice to remove excess Dl. Alternatively, there may be specific qualitative features to the removal of Dl by Kul. ADAM10 was also shown to cleave Dl in cell culture (Six et al., 2003). It is not clear what is the molecular basis for the Notch-mutant phenotype ADAM10 knockout mice display (Hartmann et al., 2002), because ADAM10 does not carry out the S2 cleavage of Notch (Mumm et al., 2000).

While naturally secreted versions of Notch ligands were recently identified in *C. elegans* (Chen and Greenwald, 2004), it seems that in *Drosophila* cleavage of Dl does not generate a biologically active form. Overexpression of cleaved DI had no detectable phenotypic consequences when expressed either in the eye or the wing, two tissues in which DI was shown to play a crucial role during many phases (Mishra-Gorur et al., 2002).

**Kul maintains unidirectional Notch signaling**

We have demonstrated that the biological activity of Kul in removing DI is essential and non-redundant with other ADAM metalloproteases. In the absence of this activity, the level of Dl in cells receiving the Notch signal is elevated. As a result, the unidirectional signaling of Notch is skewed, because the cells that normally receive the signal are converted to signal-generating cells, and fail to respond to the normal cues presented by DI-expressing cells.

How does Kul activity impinge on the distribution of DI? Overexpression of Kul in the wing disc resulted in a dramatic diminution of the levels of Dl. This effect is cell autonomous, i.e. Kul can only eliminate DI within the cells in which it is expressed. It is not known if cleavage takes place once both proteins are localized to the cell surface, or if removal of DI occurs during trafficking to the cell surface. Since no accumulation of DI was observed within the cells following Kul overexpression, we favor the first possibility. Kul activity appears to be constitutive (see below), implying that there is no preferential cleavage of DI by Kul in the receiving cells. Rather, the final outcome is likely to result from the activity of Kul in both cell types. In the receiving cells, where the levels of DI are low, the proteolytic activity of Kul effectively eliminates the DI protein. By contrast, in the sending cells expressing high levels of DI, while Kul may cleave some of the ligand, sufficient levels of DI remain to allow efficient signaling.

Disruption of Notch unidirectional signaling following removal of Kul highlights the necessity of continuously removing the DI protein, in order to generate a setting in which it would be hard for the DI protein to accumulate. Transcriptional repression of DI expression is not sufficient. For example, in the wing margin, activation of the Notch pathway specifically leads to the induction of E(spl) and Cut, which are transcriptional repressors of DI expression (de Celis and Bray, 1997; Micchelli et al., 1997). Yet, in the absence of Kul, some DI protein is produced by the margin cells (Fig. 7I). Similarly, in the pupal wing, activation of Notch in the lateral pro-vein cells induces E(spl) expression (de Celis et al., 1997). Nevertheless, DI is produced by these cells when Kul is eliminated (Fig. 4D). These observations underscore an inherent difficulty in shutting down DI transcription efficiently. They also imply that even residual levels of DI have detrimental biological consequences. The constitutive cleavage of DI by Kul is therefore a crucial safeguard, continuously removing low levels of DI that have escaped transcriptional repression.

The biological role of Kul was demonstrated in this work in two stages in which Notch signaling refines a pre-existing asymmetry between adjacent cells: the wing margin and the wing veins. In other instances, Notch signaling actually generates the asymmetry between cells. Notch defines the correct number and spacing of differentiated cells within a field of equipotent cells, e.g. in the embryonic neuroectoderm or among pupal sensory organs. In these cases, it is thought that stochastic fluctuations in the levels of DI, coupled to mechanisms that amplify these changes, lead to differentiation of some cells and concomitant repression of differentiation in the neighboring cells (Heitzler et al., 1996). Kul does not seem to impinge on these process. No effects on the number and organization of neuroblasts were observed following induction of *ds-kul* by broad maternal and early zygotic drivers (not shown). Another avenue of Notch signaling is triggered by asymmetric cell divisions in the sensory neuron precursors (Schweisguth, 2004). Again, induction of *ds-kul* by *neu-GAL4* did not give rise to any Notch-related phenotypes in the sensory bristles (not shown). We therefore conclude that the activity of Kul appears to be essential for Notch signaling specifically in cases where a pre-existing spatial asymmetry is used to guide the directionality of Notch signaling.

**Is Kul activity regulated?**

In view of the central role of Kul in Notch signaling, it was important to examine the different junctions in which Kul activity may be regulated. At the transcriptional level, Kul appeared to be broadly expressed, in embryos and in imaginal discs. This broad expression is also reflected in the Notch-independent multiple-wing hair phenotype that was observed in all wing cells where *ds-kul* was induced. It is still possible that the basal level of Kul expression may be elevated in cells where Notch signaling takes place, to reduce the levels of DI in these cells more efficiently.

At the post-transcriptional level, however, there are several steps in the generation of an active Kul protein, which could be regulated. The protein must be correctly targeted to the plasma membrane, a process that may rely on the cytoplasmic domain of Kul and its interaction with the intracellular trafficking machinery. The precursor form of Kul undergoes processing by Furins, to remove the pro-domain. In the absence of this processing, Kul cannot cleave DI. Finally, association of Kul with its substrates is mediated by the disintegrin domain, and possibly also by additional proteins that could bias this interaction.

In spite of the sequential processes necessary for the formation of a mature, active Kul protein, there is no evidence that any of these steps is regulated in time or space. The data so far support the notion of a constitutive maturation and processing of Kul. In every cell where Kul was misexpressed, an outcome was observed, as monitored by removal of DI. In the wing, removal of Kul activity also gave rise to additional phenotypes that are not related to Notch, e.g. the appearance of multiple wing hairs (not shown). This phenotype was observed in all cells where *ds-kul* was expressed, again supporting the notion that Kul is normally expressed and activated uniformly.

In conclusion, while Kul may be broadly active, it enhances and maintains the asymmetrical activation of Notch, by relying on the initial differences in the levels of DI. Kul effectively
removes the ligand from the cells expressing DI at low levels, while retaining sufficient levels of DI in the cells that will activate Notch. Thus, a uniform activity of Kul can amplify a bias in the levels of DI expression, and lead to a strict unidirectional activation of Notch, that is central to patterning the organism at multiple stages of development.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/1/123/DC1

References


