Unidirectional Notch signaling depends on continuous cleavage of Delta

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Accepted 26 October 2004

Development 132, 123-132
Published by The Company of Biologists 2005
doi:10.1242/dev.01546

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 Dl 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 Dl/Notch complex into the Dl-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 Dl between sending and receiving cells? The Kuz metalloprotease was shown to cleave Dl in cell culture and in flies, and to release a secreted form of Dl (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 Dl, and demonstrated the necessity of removing Dl 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: *scallopd-GAL4* (*sd-GAL4*) (from A. Garcia-Bellido), MS1096-*GAL4*; *spalt-GAL4* (*sal-GAL4*), *ptc-GAL4*, *Gbe+Su(H)$_{m8}$-lacZ* (from S. Bray), and *hs-flp*; *actin*>CDC2>GAL4, UAS-*gfp* (from K. Basler). matα4-GALVP16, UAS-*Dl*, 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 Dl or Dl and Kul, the following cross was carried out. Males carrying *UAS-Dl* 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 Dl 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 BGDP 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. Kuz 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-Delta (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). Pupal 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 Dl or Ser by ADAM proteins is likely to take place within the same cell, rather than between adjacent cells.

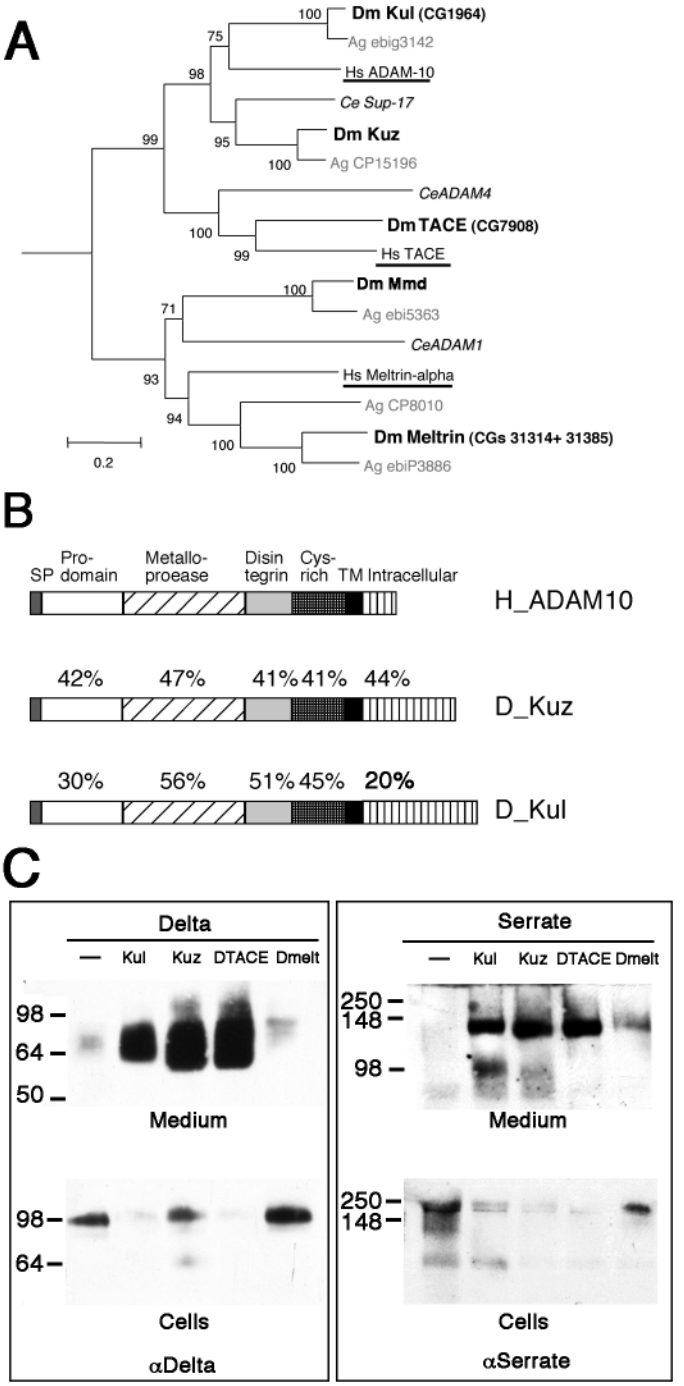


Fig. 1. Several *Drosophila* ADAM metalloproteases cleave Delta and Serrate in S2 cells. (A) Phylogenetic tree of ADAM metalloproteases. *Drosophila* in bold, *Anopheles* in gray, *C. elegans* in italics and human underlined. The different human ADAMs have distinct homologs in each of the species. Note that ADAM10 has two homologs in *Drosophila* (Kuz and Kul) and in *Anopheles*. Similarly, Meltrin-α has two homologs in insects. (B) Kuz and Kul have all the signature domains of ADAM metalloproteases. In the protease and disintegrin domains, Kul shows a higher degree of similarity to the human ADAM10. (C) Dl or Ser were expressed in S2 cells, and their capacity to serve as substrates for co-expressed *Drosophila* ADAM metalloproteases monitored by the appearance of ligand in the medium, and the concomitant disappearance from the cells. Kul, Kuz and DTACE displayed potent cleavage of both ligands. DMeltrin induced only low levels of Ser cleavage. The activity of the Mmd Meltrin homolog was not examined, because its expression is restricted to the central nervous system (Chase et al., 1987).

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 Kuz 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.

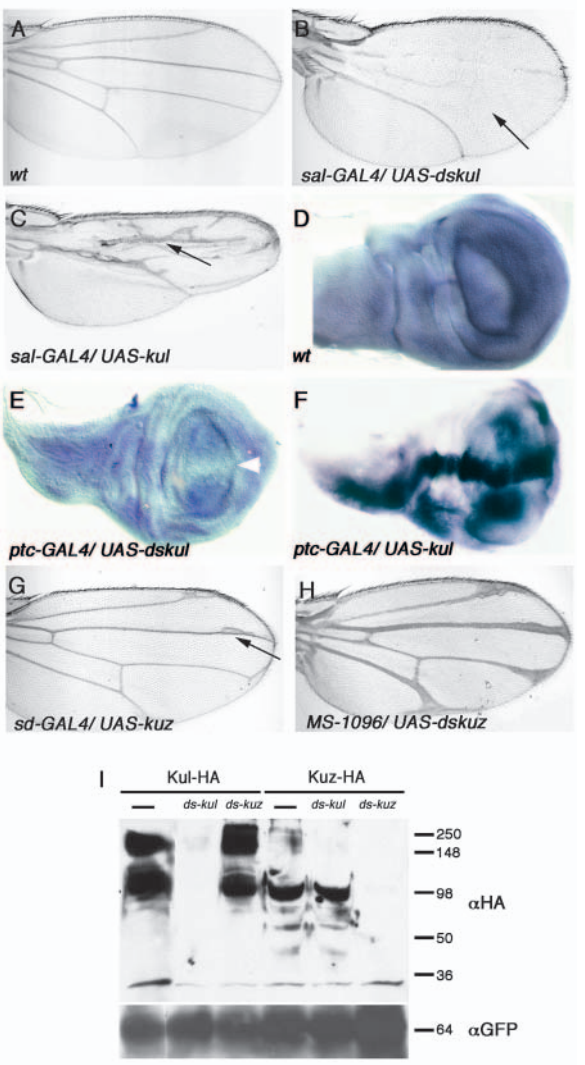


Fig. 2. Reduction of Kul levels leads to loss of wing veins. (A) Wild-type wing. (B) Expression of *ds-kul* in the central part of the wing by *sal-GAL4* led to loss of veins (arrow). This phenotype suggests that Kul may normally be required for proper signaling by the Notch pathway. (C) Overexpression 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.

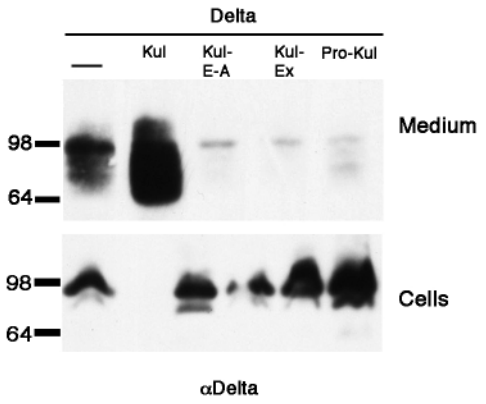


Fig. 3. Structure-function analysis of Kul. The activity of Kul was monitored in S2 cells, by its capacity to release Dl to the medium and reduce the levels of the protein in the cells. Following expression of Dl alone, some cleavage can be detected, presumably by endogenous ADAM proteins. The levels of cleaved Dl 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 Dl. Furthermore, in all three cases the activity of the endogenous ADAMs was also compromised.

3). Basal cleavage of Dl 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 Dl. 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 Dl cleavage below the basal level, probably due to formation of a complex between E-A Kul and Dl 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 Dl. 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 Dl cleavage, demonstrating the need of pro-domain removal in order to convert Kul to an active protease.

Kul regulates Notch signaling in the pupal wing veins

In the pupal wing, activation of the Notch pathway by Dl contributes to refinement and restriction of the veins. Dl, 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 Dl-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 Dl by the inter-vein cells results in Notch activation in the central pro-vein cells, leading to vein loss (Huppert et al., 1997).

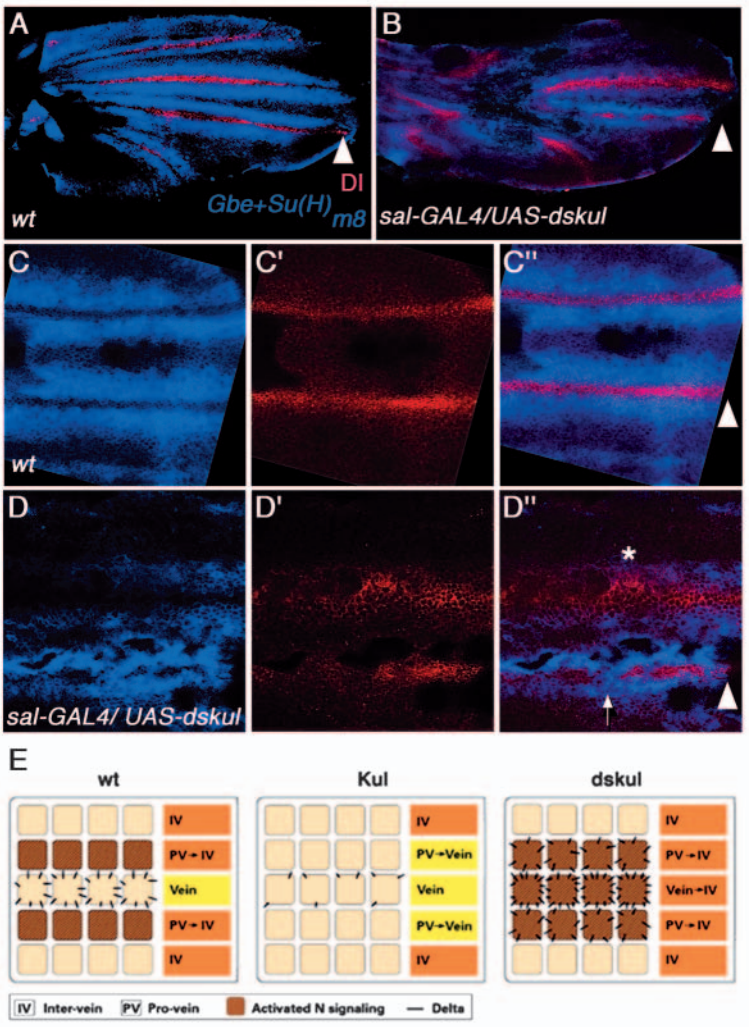
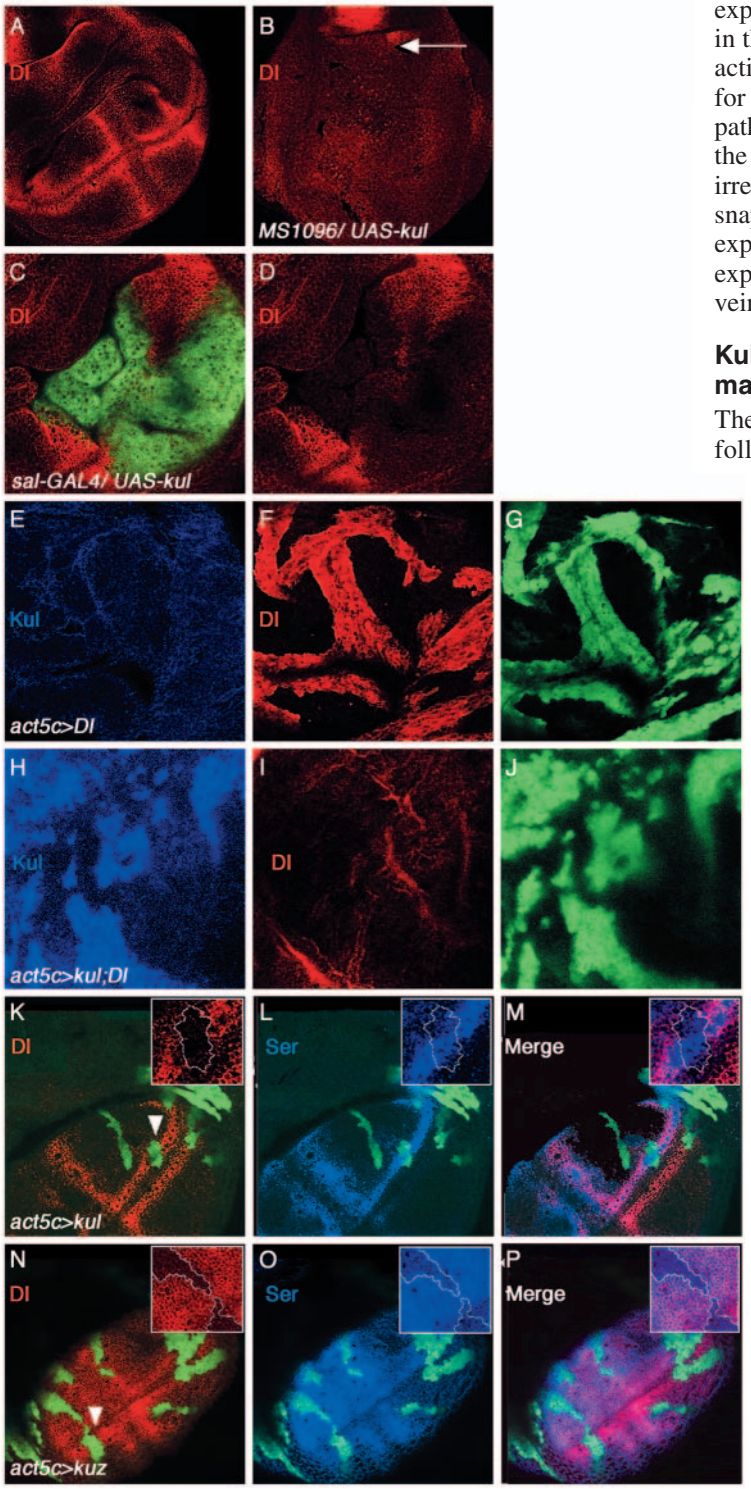


Fig. 4. Kul is required for unidirectional Notch signaling in the pupal wing. Expression of Dl in the wing veins was followed by anti-Dl (red), while the activation of Notch signaling was monitored by anti-β-Gal staining of the $Su(H)_{m8}$-*lacZ* reporter (blue). The L4 vein is indicated by an arrowhead. (A,C) In wild-type wings, Dl is expressed in 2-3 cell rows, marking the future veins. $Su(H)_{m8}$ is excluded from the veins and is induced in up to five cell rows adjacent to the vein on each side. (B,D) In wings expressing *ds-kul* in the *sal* domain, dramatic alterations in both patterns were observed. Dl is expanded beyond the normal vein region (asterisk). Consequently, the cells expressing ectopic Dl trigger activation of Notch signaling within the vein (arrow). In some regions, expression of Dl is eliminated in the vein (arrowhead), possibly as a result of ectopic Notch signaling. (E) Scheme: in wild-type wings, Dl is expressed by the vein cells, activating Notch in the adjacent cells to induce an inter-vein fate within the pro-vein territory. Following Kul overexpression, the levels of Dl are reduced and Notch signaling is compromised, leading to an expansion of vein cell fate within the pro-vein territory (Fig. 2C). By contrast, when *ds-kul* is expressed, the levels of Dl rise in the cells adjacent to the veins, leading to Notch activation within the vein, and to the ectopic induction of an inter-vein fate (Fig. 2B and Fig. 4B,D).

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)$_{m8}$* (Furriols and Bray, 2001), and by following the expression of Dl. In a wild-type pupal wing 30 hours after pupariation, Dl 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 Dl-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 Dl, as well as on the Notch reporter (Fig. 4B,D). Irregular expansion of Dl into the lateral pro-vein territory was observed, while in other parts of the wing Dl expression disappeared from the veins. Notch-reporter expression expanded into the vein cells. Elevation in Dl 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 Dl to the lateral pro-vein cells, followed by expansion of Notch activation and loss of Dl expression in the vein cells.



Kul cleaves Delta in vivo in a cell-autonomous manner

The expansion of Dl-protein distribution in the pupal wing following *ds-kul* expression implied that Kul normally contributes to the restricted distribution of Dl in this tissue. To examine in more detail the capacity of Kul to cleave Dl in vivo, we monitored the larval wing imaginal disc. Dl 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 Dl distribution in discs where Kul was overexpressed by the *MS1096* driver. Dl membrane-associated staining in the wing pouch was diminished, and a residual punctate staining appeared, possibly reflecting endocytosis of secreted Dl (Fig. 5B). Normal Delta distribution was retained in the notum, where Kul was not overexpressed. Similarly, expression of Kul by *sal-GAL4* eliminated Dl in the *sal* domain (Fig. 5C,D). To verify that Kul directly affects the cleavage of Dl, rather than Dl expression, we generated clones

Fig. 5. Kul cleaves Delta but not Serrate in the wing disc. (A) Dl expression (red) in a wild-type wing disc. (B) Overexpression of Kul in the wing pouch eliminated the normal Dl protein in the wing margin and future veins. Note that in the notum, where *MS1096-GAL4* is not expressed, normal levels of Dl were retained (arrow). (C,D) Overexpression of Kul by *sal-GAL4* eliminated Dl in the *sal* domain. (E-G) Expression of Dl (red) in flipout clones (marked by GFP expression). (H-J) Co-expression of Kul (blue) and Dl in flipout clones eliminated the Dl protein. Thus, Kul mediates directly cleavage of Dl, rather than affecting the expression of *Dl*. (K-M) Clones of cells expressing Kul (green) showed elimination of Dl but not Ser. The loss of Dl was confined to the clone, demonstrating the cell-autonomous activity of Kul. (N-P) Overexpression of Kuz eliminated both Dl 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 (Neumann and Cohen, 1996), the latter operating 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 Dl 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.

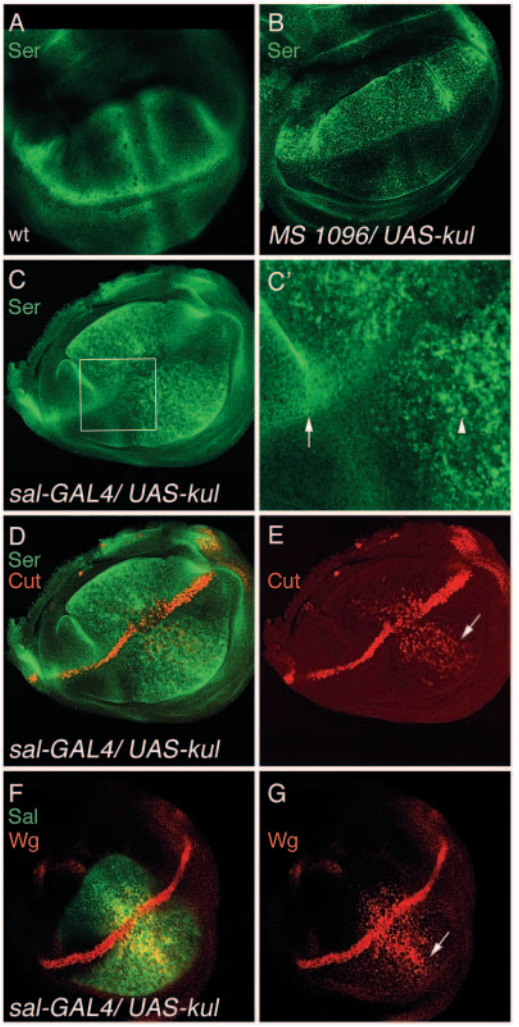


Fig. 6. Effects of Kul overexpression in the wing imaginal disc. (A) Ser expression (green) in a wild-type wing disc. (B) Overexpression of Kul by *MS1096* resulted in a uniform distribution of Ser, which is punctate. The effect is more pronounced in the dorsal part of the pouch, where expression of the driver is higher. (C-G) Kul was overexpressed in the central part of the wing disc by *sal-GAL4*. (C) Ser expression is expanded within the *sal* domain. Instead of the typical membrane-associated distribution of Ser (arrow), it is detected in a punctate intracellular pattern (arrowhead). (D) Merged image showing Ser (green) and Cut (red). (E) Cut expression is expanded throughout the domain of Kul overexpression (arrow). (F,G) Expression of Wg (red) is expanded within the same domain (arrow).

Kul regulates Notch signaling in the wing margin

Does endogenous Kul in the wing disc have a role in maintaining the asymmetric distribution of Dl? 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

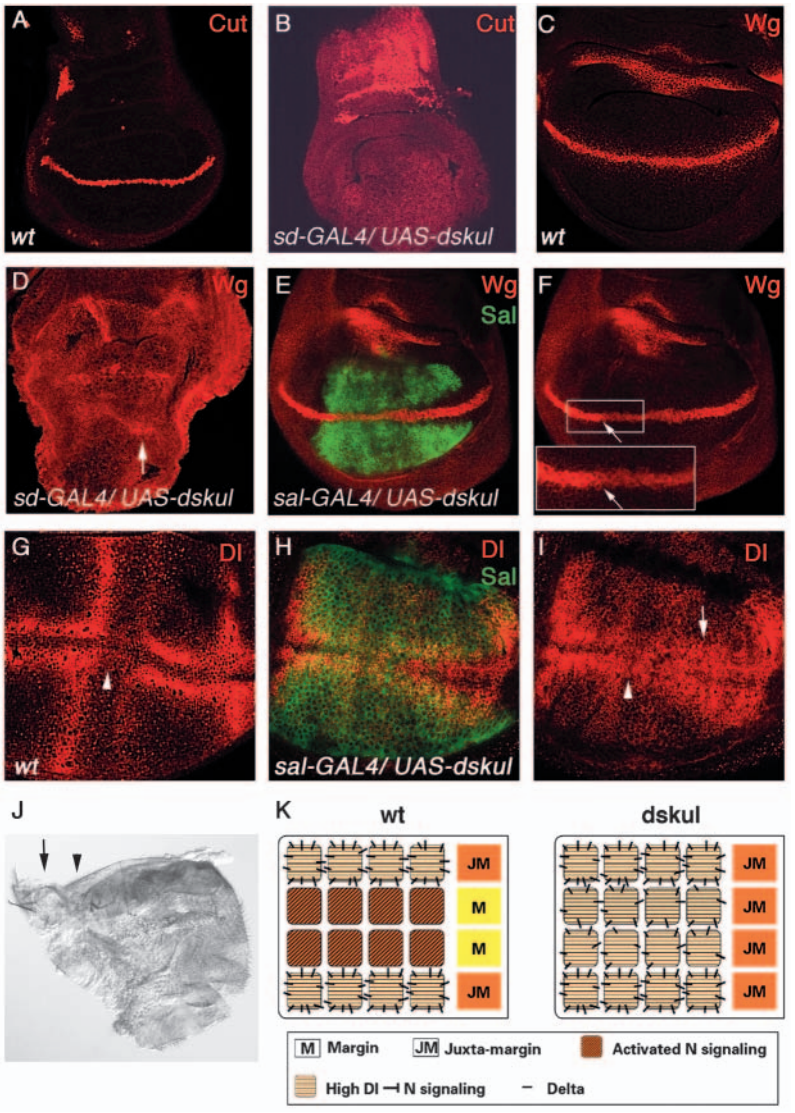


Fig. 7. Kul is required for unidirectional Notch signaling at the wing margin. (A,C) In wild-type wing discs, Cut and Wg are expressed by the two rows of wing margin cells. (B,D) Expression of *ds-kul* by *sd-GAL4*, which is broadly expressed in the wing pouch, gave rise to a reduced pouch and loss or severe reduction of Cut and Wg expression (arrow). The adult wing (J) was dramatically reduced in size and exhibited only a rudimentary wing margin in very restricted domains (arrowhead). Arrow-wing hinge. (E,F) *ds-kul* expression by *sal-GAL4* gave rise to a reduction in Wg expression. (G) In *wt* wing discs Dl is not detected at the wing margin (arrowhead). (H,I) Expression of *ds-kul* in the *sal* domain altered the distribution of Dl, which accumulated also in the wing margin (arrowhead), and expanded beyond the normal dorsal and ventral borders of Dl expression at the juxta margin (arrow). This experiment demonstrates that continuous cleavage of Dl by Kul is necessary for complete removal of the protein in the margin. In the absence of Kul, residual levels of Dl transcription give rise to accumulation of Dl, and lead to disruption of unidirectional Notch signaling.
(K) Scheme: in wild-type wing discs, the margin cells express Wg and Cut, and suppress the expression of *Dl*. Wg activates Dl and Ser expression in the juxta-margin cells. High levels of Dl and Ser in these cells have a dominant-negative effect on Notch activation, but activate Notch signaling in the margin cells. Thus, a stable signaling loop is maintained. Elimination of Kul leads to the appearance of Dl in the margin, and alleviation of Notch signaling due to the dominant-negative effect of Dl.

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 Dl had no detectable phenotypic consequences when expressed either in the eye or the wing, two tissues in which Dl 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 Dl 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 Dl-expressing cells.

How does Kul activity impinge on the distribution of Dl? 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 Dl 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 Dl occurs during trafficking to the cell surface. Since no accumulation of Dl 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 Dl 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 Dl are low, the proteolytic activity of Kul effectively eliminates the Dl protein. By contrast, in the sending cells expressing high levels of Dl, while Kul may cleave some of the ligand, sufficient levels of Dl remain to allow efficient signaling.

Disruption of Notch unidirectional signaling following removal of Kul highlights the necessity of continuously removing the Dl protein, in order to generate a setting in which it would be hard for the Dl protein to accumulate. Transcriptional repression of *Dl* 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 *Dl* expression (de Celis and Bray, 1997; Micchelli et al., 1997). Yet, in the absence of Kul, some Dl 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, Dl is produced by these cells when Kul is eliminated (Fig. 4D). These observations underscore an inherent difficulty in shutting down *Dl* transcription efficiently. They also imply that even residual levels of Dl have detrimental biological consequences. The constitutive cleavage of Dl by Kul is therefore a crucial safeguard, continuously removing low levels of Dl 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 Dl, 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 Dl 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 Dl. 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 Dl. 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 Dl. Kul effectively

removes the ligand from the cells expressing Dl at low levels, while retaining sufficient levels of Dl in the cells that will activate Notch. Thus, a uniform activity of Kul can amplify a bias in the levels of Dl expression, and lead to a strict unidirectional activation of Notch, that is central to patterning the organism at multiple stages of development.

We thank S. Artavanis-Tsakonas, K. Basler, S. Bray, R. Fleming, A. Garcia-Bellido, K. Irvine, K. Klueg, D. Pan, the Bloomington Stock Center and the Developmental Studies Hybridoma Bank for reagents. We thank T. Volk, Z. Paroush and members of the Shilo lab for discussions and critical reading of the manuscript, and I. Yanai for help in phylogenetic analysis. This work was supported by grants from the Israel Science Foundation and the Minerva Foundation to B.-Z.S., who is an incumbent of the Hilda and Cecil Lewis professorial chair in Molecular Genetics.

Supplementary material

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

References

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

Axelrod, J. D. (2001). Unipolar membrane association of Dishevelled mediates Frizzled planar cell polarity signaling. *Genes Dev.* **15**, 1182-1187.

Baron, M. (2003). An overview of the Notch signalling pathway. *Semin. Cell Dev. Biol.* **14**, 113-119.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M. and Knoblich, J. A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. *Dev. Cell* **3**, 221-231.

Bruckner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-415.

Cao, Y., Kang, Q., Zhao, Z. and Zolkiewska, A. (2002). Intracellular processing of metalloprotease disintegrin ADAM12. *J. Biol. Chem.* **277**, 26403-26411.

Chase, B. A., Holliday, J., Reese, J. H., Chun, L. L. and Hawrot, E. (1987). Monoclonal antibodies with defined specificities for Torpedo nicotinic acetylcholine receptor cross-react with *Drosophila* neural tissue. *Neuroscience* **21**, 959-976.

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

de Celis, J. F. (2003). Pattern formation in the Drosophila wing: the development of the veins. *BioEssays* **25**, 443-451.

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

de Celis, J. F., Bray, S. and Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing. *Development* **124**, 1919-1928.

De Joussineau, C., Soule, J., Martin, M., Anguille, C., Montcourrier, P. and Alexandre, D. (2003). Delta-promoted filopodia mediate long-range lateral inhibition in Drosophila. *Nature* **426**, 555-559.

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. *Development* **124**, 2973-2981.

Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1996). The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proc. Natl. Acad. Sci. USA* **93**, 11925-11932.

Furriols, M. and Bray, S. (2001). A model Notch response element detects Suppressor of Hairless-dependent molecular switch. *Curr. Biol.* **11**, 60-64.

Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lubke, T., Lena Illert, A., von Figura, K. et al. (2002). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Hum. Mol. Genet.* **11**, 2615-2624.

Hattori, M., Osterfield, M. and Flanagan, J. G. (2000). Regulated cleavage of a contact-mediated axon repellent. *Science* **289**, 1360-1365.

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

Hinz, U., Giebel, B. and Campos-Ortega, J. A. (1994). The basic-helix-loop-helix domain of Drosophila lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87.

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

Kooh, P. J., Fehon, R. G. and Muskavitch, M. A. (1993). Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. *Development* **117**, 493-507.

Kunisch, M., Haenlin, M. and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. *Proc. Natl. Acad. Sci. USA* **91**, 10139-10143.

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

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

Lieber, T., Kidd, S. and Young, M. W. (2002). kuzbanian-mediated cleavage of Drosophila Notch. *Genes Dev.* **16**, 209-221.

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

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

Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* **228**, 151-165.

Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J. and Kopan, R. (2000). A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Mol. Cell* **5**, 197-206.

Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the Drosophila wing. *Development* **122**, 3477-3485.

Okajima, T. and Irvine, K. D. (2002). Regulation of notch signaling by o-linked fucose. *Cell* **111**, 893-904.

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

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.

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

Primakoff, P. and Myles, D. G. (2000). The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet.* **16**, 83-87.

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

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

Shaye, D. D. and Greenwald, I. (2002). Endocytosis-mediated downregulation of LIN-12/Notch upon Ras activation in Caenorhabditis elegans. *Nature* **420**, 686-690.

Six, E., Ndiaye, D., Laabi, Y., Brou, C., Gupta-Rossi, N., Israel, A. and Logeat, F. (2003). The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. *Proc. Natl. Acad. Sci. USA* **100**, 7638-7643.

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

Struhl, G. and Adachi, A. (1998). Nuclear access and action of notch in vivo. *Cell* **93**, 649-660.

Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the Drosophila Ommatidium. *Dev. Biol.* **120**, 366-376.

Tsruya, R., Schlesinger, A., Reich, A., Gabay, L., Sapir, A. and Shilo, B. Z. (2002). Intracellular trafficking by Star regulates cleavage of the Drosophila EGF receptor ligand Spitz. *Genes Dev.* **16**, 222-234.