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Review

ADAM proteases: ligand processing and modulation of the Notch pathway

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Abstract. ADAM metalloproteases play important roles in development and disease. One of the key functions of ADAMs is the proteolytic processing of Notch receptors and their ligands. ADAM-mediated cleavage of Notch represents the first step in regulated intramembrane proteolysis of the receptor, leading to activation of the Notch pathway. Recent reports indicate that the transmembrane Notch ligands also undergo ADAM-mediated processing in cultured cells and *in vivo*. The proteolytic processing of Notch ligands modulates the strength and duration of Notch signals, leads to generation of soluble intracellular domains of the ligands, and may support a bi-directional signaling between cells.

Keywords. Metalloprotease, disintegrin, proteolysis, signaling, Notch, Delta, ligand.

Introduction

ADAMs (containing a disintegrin and metalloprotease) are a family of modular transmembrane proteins that mediate cell surface proteolysis and modulate cell-cell and cell-matrix interactions [1–3]. To date, 23 different ADAMs have been identified in the human genome. A prototypical ADAM contains a secretion signal sequence at its N terminus, followed by a pro-domain, metalloprotease, disintegrin, cysteine-rich, epidermal-growth factor (EGF)-like, transmembrane, and cytoplasmic domains. As a result of alternative mRNA splicing, soluble isoforms of several ADAMs are also produced. A related group of 19 ADAMTS and 3 ADAMTS-like proteins represent secreted soluble metalloproteases in which the transmembrane and cytoplasmic domains are replaced with a variable number of thrombospondin motifs [4, 5]. Recent progress in structure determination of several ADAM domains has brought us closer to understanding the molecular mechanism of ADAM-mediated proteolytic reactions or protein-protein interactions [6–10]. Genetic and biochemical studies have generated a wealth of information on the role of ADAMs and ADAMTSs in modulating various signaling pathways and controlling cell behavior [11–14]. Proteolytic processing of Notch receptors and their ligands emerges as one of the key functions of ADAMs with important implications in development and disease.

The Notch pathway is an evolutionarly conserved signaling mechanism that plays a critical role in cell fate decisions and pattern formation [15–18]. In mammals, Notch provides key signals during neural, cardiovascular, immune, liver, and kidney development. In adult organisms, the Notch pathway has been implicated in tissue regeneration and the function of stem cells [19–21]. Not surprisingly, aberrant Notch signaling has been linked to various human diseases [22–24].

The Notch pathway is activated when one of the DSL ligands (Delta and Serrate in *Drosophila*, Lag2 in

Caenorhabtitis elegans), a transmembrane protein present at the surface of a signal-sending cell, binds to a Notch receptor in a signal-receiving cell. In mammals, there are five DSL ligands (Delta-like 1, 3, and 4, and Jagged 1 and 2) and four Notch receptors (Notch 1–4). During intracellular maturation, mammalian Notch receptors are cleaved at the S1 site in the extracellular domain by a furin-like protease [25]. The resulting two fragments, the extracellular domain (NECD) and the transmembrane domain (NTM), are held together by a heterodimerization (HD) domain [26]. The ligand-receptor interaction is followed by a sequential cleavage of the receptor by an ADAM protease at the S2 site in the juxtamembrane region of the extracellular domain and by presenilin-dependent γ-secretase at the S3/S4 sites in the transmembrane domain [27–30]. Cleavage by γ-secretase leads to a release of the intracellular domain of Notch (NICD) and its translocation to the nucleus. Inside the nucleus, NICD forms a complex with the CSL DNA-binding protein (CBF1 in mammals, also known as RBP-J in mouse, Suppressor of Hairless in *Drosophila*, and Lag1 in *C. elegans*) and the Mastermind/Lag3 coactivator, and activates target gene expression.

The Notch pathway is regulated by several mechanisms, including feedback regulation of receptor and ligand transcription, glycosylation, and receptor and ligand ubiquitination and endocytosis [31–34]. This review will focus on the role of ADAM proteases in modulating Notch signaling via the proteolytic processing of the Notch receptors and the DSL ligands. ADAM-mediated cleavage of Notch, an indispensable step in turning the Notch signal on, has been discussed in detail in other reviews [15–18]. Here, I will summarize the most recent advances in understanding of this reaction. Proteolytic processing of the DSL ligands and its physiological role in modulating the Notch pathway has received considerably less attention than cleavage of Notch itself. In this review, I will discuss the following questions: Which DSL ligands are substrates for ADAM-mediated cleavage? Does the cleavage occur *in vivo?* Which ADAMs mediate the processing? How is the processing regulated? Finally, what are the physiological consequences of ligand processing?

ADAM-mediated cleavage of Notch

Although Notch receptors are transmembrane proteins residing at the cell surface, the active form of Notch is represented by the soluble intracellular domain of the receptor, NICD. ADAM-mediated cleavage of Notch is a necessary prerequisite for the subsequent processing by γ-secretase and the generation of NICD (Fig. 1A). Two ADAMs have been implicated in the S2 cleavage of Notch. In *Drosophila*, ADAM10 ortholog Kuzbanian is the main protease mediating Notch processing [35–38]. In mouse cells *in vitro*, ADAM17, and not ADAM10, appears to be a protease responsible for Notch cleavage [30, 39]. ADAM17-deficient mice do not show, however, a 'Notch phenotype' [40]. In contrast, ADAM10 deficiency leads to embryonic lethality at E9.5 and multiple malformations [41] resembling those observed in Notch1 knockout mice, in mice homozygous for a γ-secretase processing-deficient allele of Notch1, or in presenilin1/presenilin2 double-knockout mice [42–44]. Thus, as proposed by Hartmann et al. [41], different ADAMs may contribute to the S2 cleavage in a tissue-specific manner, with ADAM10 playing the major role in this process *in vivo*.

In flies and vertebrates, an efficient processing by ADAMs requires that Notch present in a signal-receiving cell binds *in trans* to a DSL ligand present in a signal-sending cell and that the ligand undergoes endocytosis in the signal-sending cell. Recent structural analysis of human Notch2 provides an insight into the mechanism by which ligand binding may facilitate Notch proteolysis by ADAMs [45]. Before ligand-induced activation, Notch is maintained in a metalloprotease-resistant conformation by a conserved negative regulatory region (NRR) composed of three Lin12/Notch repeats (LNRs) and the HD domain, which contains the S2 cleavage site. Extensive interactions between LNRs and the HD domain stabilize the NRR in the autoinhibited conformation and bury the S2 site, making it inaccessible for ADAM cleavage [45]. It is clear that exposure of the S2 site requires a substantial conformational change in the NRR. Such a change could result from ligand binding followed by its endocytosis, generating a mechanical force to 'peel' the protective LNR domains from the HD domain. Indeed, deletion of all three LNR modules produces a constitutively active form of Notch [45, 46].

Upon ligand endocytosis, the NECD portion of Notch is internalized into the ligand-expressing cell, and it has been postulated that this transendocytosis of NECD depends on ADAM cleavage [47]. Interestingly, a recent study has demonstrated that treatment of mammalian cells with a metalloprotease inhibitor BB94 does not perturb NECD separation and transendocytosis. The BB94 treatment does inhibit, however, the generation of NICD and diminishes Notch reporter activity [48]. Thus, in the case of a heterodimeric mammalian Notch, NECD release and transendocytosis precede and facilitate Notch cleavage by ADAMs, rather than being a consequence of such cleavage. Dissociation of the heterodimeric Notch by

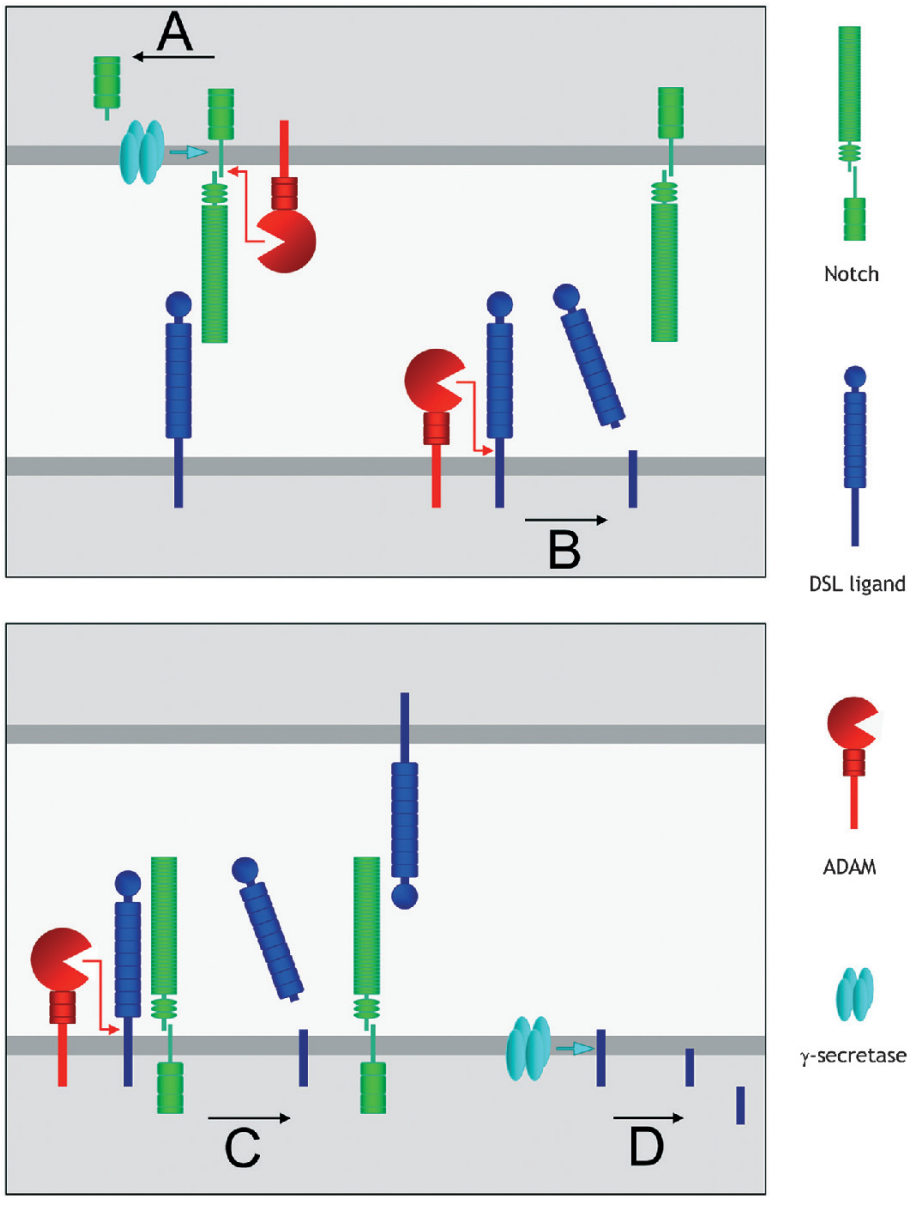

Figure 1. Different modes of ADAM modulation of the Notch pathway. (*A*) Ligand binding induces Notch cleavage by an ADAM protease at the S2 site, followed by γ-secretase cleavage at the S3/S4 site and release of the NICD from the membrane in a signal-receiving cell. (*B*) Ligand shedding by an ADAM in a signal-sending cell down-regulates Notch signaling in a signal-receiving cell. (*C*) Ligand shedding by an ADAM relieves *cis* inhibition of Notch and makes Notch available for interactions with ligands *in trans*. (*D*) After ADAM cleavage, the transmembrane fragment of the ligand is processed by γ-secretase, and the intracellular domain released from the membrane may act as a signaling molecule.

ligand-expressing cells may thus disengage the LNR domains from the HD domain, uncover the S2 cleavage site, and convert Notch from an ADAM-insensitive to an ADAM-sensitive substrate. Such "preparation" of Notch for ADAM cleavage is obviously possible only when Notch is a heterodimeric protein, processed at the S1 site. In flies, however, Notch is not cleaved at the S1 site, but is still efficiently cleaved by ADAMs upon ligand binding [49], indicating that the dissociation of the NECD prior to ADAM cleavage is not always an obligatory step in receptor activation. On the other hand, the fact that ADAM activity in mammalian cells is not required for removal of NECD but is still necessary for subsequent generation of NICD points to an important role for ADAMs in clipping the extracellular sequences of the transmembrane NTM fragment and preparing it for the γ-secretase cleavage.

The classical model of Notch activation described above implies that ADAM-mediated cleavage is an obligatory step prior to the cleavage by γ-secretase. A recent report suggests, however, that under certain conditions Notch activation may follow alternate activation mechanisms. During pancreatic acinar transdifferentiation into duct-like epithelia, a process associated with pancreatic tumorigenesis and controlled by the Notch pathway [50], Notch appears to be activated by matrix metalloproteinase 7 (MMP-7) rather than an ADAM. The MMP-7 activity is both required and sufficient for Notch-mediated transdifferentiation [51]. In COS-7 cells transfected with the full-length Notch, recombinant MMP-7 induces

the cleavage of Notch by γ-secretase, nuclear translocation of NICD, and target gene expression [51]. The authors hypothesized that the secreted MMP-7, which is overexpressed in pathological conditions, could cleave Notch at the S2 site in a ligand-independent manner, but the exact cleavage site of Notch by MMP-7 has not been determined. Given the fact, however, that in the absence of a ligand the S2 site is masked by the NRR, cleavage at this site by MMP-7 seems unlikely and the mechanism of Notch activation by MMP-7 remains unclear.

ADAM-mediated cleavage of DSL ligands

Proteolytic processing of a Notch ligand (Fig. 1B, C) was reported for the first time for *Drosophila* Delta (Dl) [52, 53]. These ground-breaking experiments have established that Dl is cleaved both in cultured cells and *in vivo* and that Kuz represents the main ADAM responsible for the cleavage [52, 53]. In S2 cells transfected with Dl, the extracellular fragment of Dl, Dl-EC, is released to culture medium. Cotransfection of the wild-type *Drosophila* ADAM10 (Kuzbanian, or Kuz) increases the abundance of Dl-EC, whereas cotransfection of a dominant-negative Kuz lacking pro- and metalloprotease domains has an inhibitory effect [52]. Coculture of Dl-transfected cells with Kuz-transfected cells does not increase the cleavage of Dl, suggesting that the cleavage occurs in *cis* orientation [54]. Dl-EC is detected in $kuz^{+/-}$ *Drosophila* embryos, but not in $kuz^{-/-}$ embryos [52]. Simultaneous detection of the extracellular and the intracellular domains of Dl in early stages of embryogenesis using two different antibodies indicates that the two domains frequently distribute to different endocytic vesicles and thus must be separated during Dl cleavage *in vivo* [53].

In S2 cell cotransfection experiments, two forms of Dl-EC have been identified, with molecular masses of 65 and 63 kDa. Based on the C-terminal sequences of these two species, it has been proposed that the cleavage occurs after Ala581 or Ala593, 14 or 2 amino acids, respectively, from the transmembrane domain [54]. Mutation of one or both of these two residues, however, does not prevent the cleavage [54]. More recently, a membrane-associated cleavage product of Dl has been described whose N-terminal sequence begins at His577 [55]. Treatment of cells with metalloprotease inhibitors GM6001 and TAPI-1 or with Kuz RNAi [56] efficiently blocks accumulation of this product, indicating that Kuz is responsible for the cleavage of the Ala576-His577 bond. Thus, it is possible that Kuz cleaves Dl at several distinct sites located in the juxtamembrane region (Fig. 2).

The *Drosophila* genome harbors five ADAM metalloproteases: two homologs for ADAM10, Kuz and Kuzbanian-like (Kul), two homologs for ADAM12, DMeltrin and Mmd, and a single homolog for ADAM17, DTACE [57]. Cotransfection experiments in S2 cells have established that Kuz, Kul, and DTACE exhibit similar potencies of cleaving Dl, whereas DMeltrin is inactive. Serrate (Ser), the second ligand of Notch in *Drosophila*, is also cleaved by Kuz, Kul, and DTACE, but not by DMeltrin [57]. The ability of Mmd to process Dl or Ser has not been tested.

Although the amino acid sequence of the membrane-proximal region of Dl is not conserved between flies and mammals, several of the mammalian DSL ligands also undergo ADAM-mediated cleavage at the juxtamembrane region of the extracellular domain (Fig. 2). Mouse Delta-like 1 (Dll1) overexpressed in N2a neuroblastoma cells, HEK293T, or COS-7 cells is constitutively cleaved by endogenous ADAMs to generate Dll1-TMIC, a transmembrane and intracellular domain fragment [58–60] and Dll1-EC, an extracellular fragment released to the medium [59, 60]. Similar processing has been observed for rat Dll1 and Jagged1 transfected into COS7 or CHO cells [61] and for human Jagged2 in NIH3T3 cells [58]. Furthermore, the endogenous Dll1 expressed in cultured primary mouse muscle cells (myoblasts) appears to be partially cleaved and a C-terminal fragment corresponding to Dll1-TMIC is detected when cells are stimulated to differentiate [60]. Microsomal membranes isolated from rat embryos at day E13, during a peak of Jagged1 expression, contain both the full-length and the C-terminal fragment of Jagged1 [61]. Thus, not only is the cleavage of Jagged1 detected in cultured cells, it may also occur *in vivo*.

To date, at least four different ADAMs have been implicated in the processing of DSL ligands in mammalian cells, namely ADAM17, ADAM10, ADAM12, and ADAM9. The cleavage of Jagged1 transfected into CHO cells is efficiently inhibited by the ADAM17 inhibitor batimistat, suggesting that ADAM17 may process Jagged1 [61]. The extent of Dll1 cleavage is decreased by ~50% when Dll1 is overexpressed in $ADAM10^{-/-}$ mouse embryonic fibroblasts (MEFs) compared to wild-type MEFs [59]. Although this result supports a role of ADAM10 in the processing of Dll1, it also suggests that other ADAMs may catalyze the Dll1 cleavage as well. Indeed, cotransfection of mouse ADAM17 or ADAM12 together with mouse Dll1 into COS-7 cells significantly enhances the cleavage of Dll1, demonstrating that these ADAMs are capable of recognizing Dll1 as a substrate [60]. Transfection of ADAM9 has a more modest effect, whereas ADAM15 is not able to process Dll1 at all. The extent

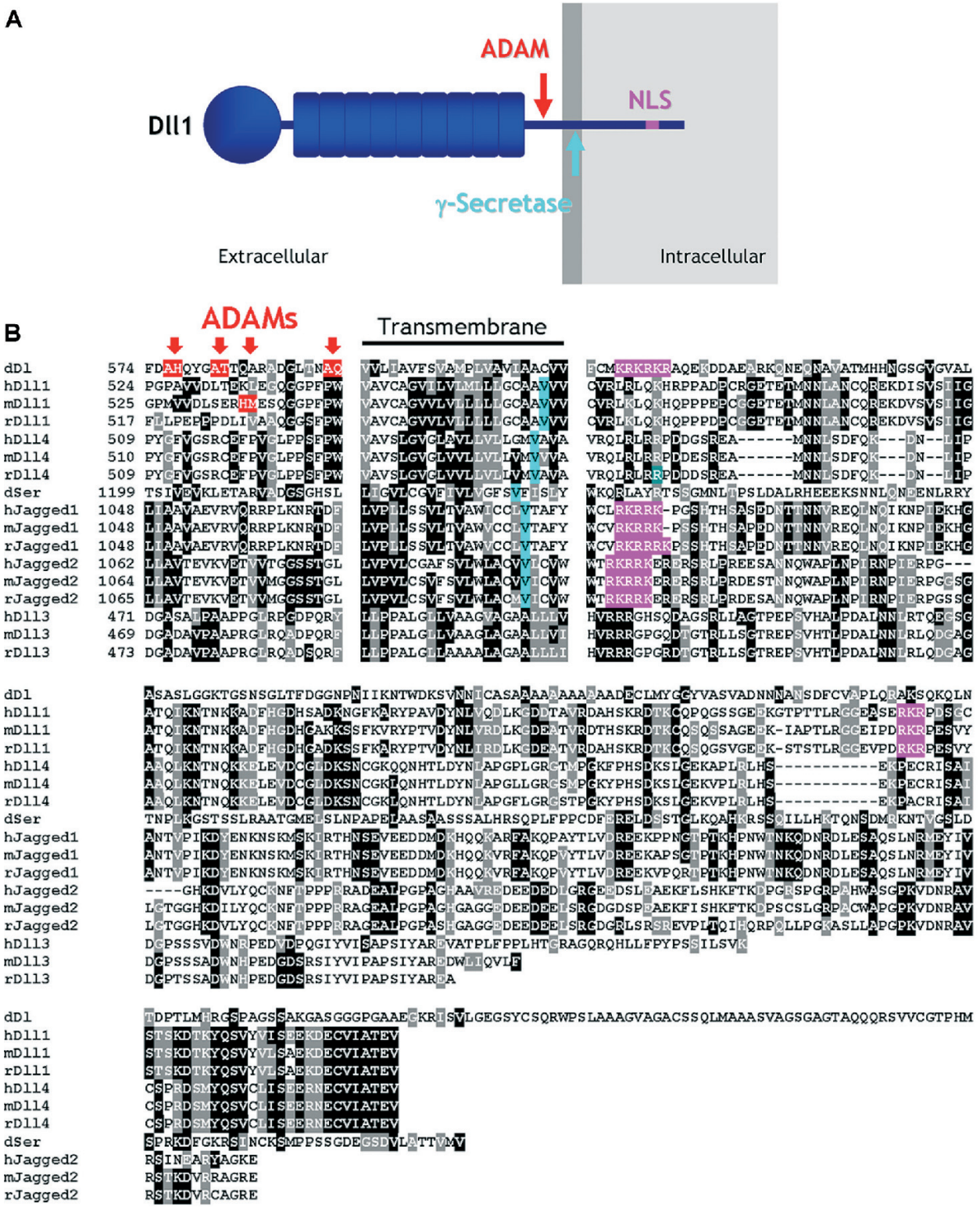

Figure 2. (*A*) Schematic diagram of Dll1, one of the mammalian DSL ligands. The DSL Notch-interacting domain (the globular part) and nine EGF-like repeats are indicated in the extracellular domain; a potential NLS motif is shown in the intracellular domain. The sites of processing by ADAM proteases and by γ-secretase are indicated by red and cyan arrows, respectively. (*B*) Comparison of the amino acid sequences of the juxtamembrane, transmembrane, and intracellular regions of *Drosophila* and mammalian DSL ligands. dDl and dSer are Delta and Serrate; human, mouse, and rat Dll1, Dll3, Dll4, Jagged1, and Jagged2 are also shown. The reported ADAM cleavage sites in dDl and in mDll1 are indicated in red. Val residues in the transmembrane domains close to the border with the intracellular domains and representing potential cleavage sites by presenilin-dependent γ-secretase are shown in cyan. Notice that the ligands known to be processed by ADAMs, dDl, Dll1, Jagged1 and Jagged2, contain putative NLS sites, shown in purple.

of Dll1 processing is diminished by ~50 % in *ADAM9/12/15*$^{-/-}$ MEFs and in primary myoblasts treated with ADAM12 small interfering RNAs, suggesting that the endogenous ADAM12 and/or ADAM9 contribute to the Dll1 processing. Hence, ADAM12- and ADAM9-mediated processing of mammalian Dll1 represent two novel proteolytic events, not previously seen in flies.

The cleavage site in mouse Dll1 has been identified between His535 and Met536, 10 amino acids away from the transmembrane domain [59] (Fig. 2B). Point mutations at or around the cleavage site do not have, however, any effect on the processing of Dll1, and more extensive changes in the Dll1 sequences are required to abolish the cleavage. For example, in two non-cleavable Dll1 mutants, 8 amino acids surrounding the cleavage site were replaced with Asp residues or 16 residues covering the cleavage site were deleted, respectively [59]. The insensitivity of the Dll1 processing to point mutations around the cleavage site and the lack of similarity between *Drosophila* Dl, mammalian Dll1, Jagged1, and Jagged2, all of which are processed at the same juxtamembrane region, are consistent with the fact that ADAMs tend to recognize a specific structural motif in their substrates rather than a primary sequence.

Since the activation status of the Notch pathway is tightly regulated in space and time, one may expect that the proteolytic processing of Notch ligands should be subjected to regulation as well. Indeed, although our understanding of the precise regulation of ADAM-mediated ligand cleavage is still very limited, it is obvious that the cleavage is responsive to various stimuli or manipulations. The catalytic activity of ADAM17 toward a variety of protein substrates in mammalian cells is augmented by phorbol esters [14, 62], whereas activity of ADAM10 is up-regulated by calcium influx and organomercurial compounds [63]. Treatment of Jagged1-transfected CHO cells with phorbol myristate acetate increases the amount of cleaved Jagged1, consistent with ADAM17-mediated processing of Jagged1 [61]. The regulation of ADAM10-mediated cleavage of mammalian Dll1 has not been examined, but Kuz-mediated processing of *Drosophila* Dl is strongly enhanced in the presence of 50 μM *p*-aminophenylmercuric acetate [56]. Dll1 cleavage mediated by ADAM12 is not affected by phorbol esters or calcium ionophores, but it requires high cell density and is very inefficient when few cell-cell contacts are allowed [60].

Cell-density dependence of Dll1 cleavage by ADAM12 is reminiscent of ADAM-mediated processing of Notch, which takes place only after Notch binds to a ligand on neighboring cells. Whether or not a similar scenario is true for DSL ligands and whether the ligands need to engage in the intercellular interactions with Notch in order to be processed by an ADAM is not clear. The fact that the extent of Jagged1 processing in CHO cells or Dl in S2 cells is increased when cells are cocultured with Notch-transfected cells suggests such a possibility [55, 61]. It is also feasible, however, that proteins other than Notch bind to DSL ligands, or even to ADAMs, *in trans* and facilitate the proteolytic reaction. Homotypic interaction between Dl molecules on opposing cell surfaces has been detected using S2 cell aggregation assays [64], and similar interactions have been reported for mammalian Dll1 [65, 66]. Thus, cell density dependence of ligand cleavage can be achieved through homotypic ligand-ligand interactions. Furthermore, it has been recently demonstrated that interactions between DSL ligands and extracellular matrix proteins can also stimulate ligand cleavage. For example, microfibril-associated glycoprotein-2 (MAGP-2) interacts with Jagged1 and facilitates the shedding of Jagged1 from transfected cells [67]. The shedding is inhibited by hydroxamate inhibitors BB3103 and BB94, possibly implicating an ADAM protease in the cleavage reaction. The extracellular domain of Jagged1 shed from cells is found complexed with MAGP-2, inviting speculation that MAGP-2 may play a role in stabilizing the Jagged1 fragment after release from the cell surface. The mechanism of up-regulation of DSL ligand cleavage by neighboring cells or by extracellular matrix proteins clearly needs to be examined in more detail.

During the past several years, ubiquitination, endocytosis, and endosomal sorting of DSL ligands have emerged as key regulatory mechanisms of Notch signaling. Neuralized and Mind bomb, two E3 ubiquitin ligases that act upon DSL ligands in a signal-sending cell and promote ligand endocytosis, are indispensable for efficient Notch signaling in a signal-receiving cell [32, 33]. Since ligand proteolysis segregates the Notch-binding extracellular domain from the cytoplasmic domain that interacts with the endocytic machinery, a possible link between ligand proteolysis and endocytosis is a question of great importance. Wang and Struhl have shown that epsin, an adaptor protein that targets mono-ubiquitinated surface proteins for clathrin-mediated endocytosis, is vital for generating functional DSL ligands [68]. *Drosophila* cells devoid of epsin cannot send DSL signals to neighbors. Surprisingly, epsin-deficient cells have been also reported to be unable to proteolyze Dl, prompting speculations that Dl cleavage might occur in endocytic vesicles [68]. It has to be pointed out, however, that the engineered form of Dl used in the epsin experiments contained six tandem copies of the myc epitope that were inserted after Ala576, exactly at

a Kuz cleavage site. Thus, it is not clear whether the processing of the myc-tagged Dl observed in epsin-positive cells was mediated by Kuz, especially since the C-terminal cleavage product was significantly larger than expected (50 vs 30 kDa). Most importantly, the loss of Dl proteolysis in epsin-deficient cells was observed only when Neuralized, a ubiquitin ligase for Dl, was overexpressed and the majority of Dl was cleared from the cell surface via clathrin-dependent and clathrin-independent endocytosis. In a different study, S2 cells were cotransfected with Neuralized and Dl lacking any foreign insertions in the juxtamembrane region. In this case, Neuralized increased the extent of Dl endocytosis, but a significant portion of Dl still remained at the cell surface and Dl processing was not changed [55]. Furthermore, when Dl was transfected into a cell line that carried a temperature-sensitive mutant allele of dynamin, Dl proteolysis was not changed at restrictive temperatures at which there was no endocytosis [55]. Based on these results, it has been concluded that proteolysis of Dl proceeds independently of endocytosis [55]. A possibility that seems consistent with the two studies discussed above is that Dl proteolysis takes place both at the cell surface and in a subset of epsin-dependent endocytic vesicles, but not in other types of endocytic vesicle.

Consequences of DSL ligand cleavage

Down-regulation of Notch activity *in trans*

Following the cleavage by ADAMs in the juxtamembrane region, the extracellular domains of DSL ligands are released to the external compartment as soluble proteins. In worms, engineered soluble forms of DSL ligands APX-1 and LAG-2 are capable of activating Notch [69] and some naturally occurring, functional ligands (for example, DSL-1) are secreted [70]. In flies and mammals, however, soluble forms of DSL ligands either do not activate Notch or have antagonistic activities. For example, soluble forms of *Drosophila* Dl bind to Notch with very weak affinities and are inactive *in vivo* [54]. In cultured mammalian cells, soluble extracellular domains of Dll1 [71–73], Dll4 [74–76], or Jagged1 [77, 78] block Notch activity, as judged by Notch target gene expression. The inability of soluble DSL ligands to elicit Notch signals is consistent with the current model of Notch activation, in which membrane-tethered ligands undergoing endocytosis exert a pulling force on Notch, facilitate the cleavage at the S2 site, and make the signaling more potent [31–34]. Interestingly, immobilization of soluble ligands on a matrix [79] or clustering with antibodies [80, 81] also induces Notch activation, most likely by creating a rigid scaffold that allows the generation of a mechanical force when a bound Notch cell moves away from the ligand source [31].

As physiologically relevant mechanisms for immobilization of soluble DSL ligands that are released by ADAMs have not been identified in mammals or flies, an immediate consequence of ADAM-mediated proteolysis is the clearance of functional ligands from the cell surface and down-regulation of Notch signaling in adjacent cells [54]. Two reports have demonstrated the importance of this mode of regulation of the Notch pathway *in vivo*. In the first report, the level of Kuzbanian-like, a *Drosophila* ADAM10 homolog processing Dl and Ser but not Notch, was knocked down in the *Drosophila* wing. As a result, the amount of uncleaved Dl was elevated, leading to alterations in the directionality of Notch signaling and abnormal wing development [57].

The second study examined the effect of *Reck* gene deletion in mice on the developing central nervous system (CNS) [82]. RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a glycosylphosphatidylinositol-anchored cell surface protein, specifically expressed in nestin-positive neural precursor cells (NPCs) [83, 84]. Previously known to inhibit matrix metalloprotease 2 and 9 (MMP2 and MMP9) and membrane type-1 matrix metalloproteinase (MT1-MMP), RECK has emerged as a physiological inhibitor of ADAM10 [82]. In RECK-deficient embryos, Notch signaling is impaired, expression of the transcriptional targets for Notch (Hes1 and Hes5) is down-regulated, and NPCs undergo precocious differentiation [82]. This phenotype is rescued by expressing constitutively active Notch or by suppressing the endogenous ADAM10 activity with a selective ADAM10 inhibitor GI254023X or with RNA interference. In cultured cells, RECK down-regulates Dll1 shedding mediated by ADAM10, induces Notch signaling in neighboring cells, and prevents their differentiation. Overexpression of RECK in Notch-transfected cells does not appear to influence Notch signaling in a cell-autonomous manner. Thus, the defective neurogenesis in $Reck^{-/-}$ mice has been attributed to excessive Dll1 shedding by ADAM10 and down-regulation of Notch activity *in trans* [82]. RECK also shows a certain degree of specificity in its inhibition of ADAMs, as it does not inhibit ADAM12-mediated Dll1 shedding [82]. Although the structural basis of the inhibition of ADAM10 activity towards Dll1 by RECK is not clear, it appears that a tight spatiotemporal control of Dll1 shedding is necessary for CNS development.

Relief of *cis* inhibition of Notch?

DSL ligands expressed at high levels act as antagonists of Notch present in the same cell, a cell-autonomous

effect known as *cis* inhibition [85]. This mode of action of the DSL ligands is best characterized in flies. In the developing *Drosophila* wing margins, clones of cells expressing high levels of Dl or Ser are insensitive to the Dl or Ser signals from neighboring cells. In contrast, elevated Notch signaling is detected in cells that lack both Dl and Ser [86]. Ectopic expression of Dl or Ser in neurons within developing bristle organs or in the wing interfere in a cell-autonomous manner with the ability of cells to receive Notch signals [87, 88].

Recent studies suggest a model of *cis* inhibition of Notch in flies in which the extracellular domain of a ligand binds to the extracellular domain of a receptor in a cell-autonomous manner, the binding takes place when both the ligand and the receptor are present at the cell surface, and the *cis*-inhibitory interactions reduce the amount of receptor available for *trans*-activation. First, *cis* inhibition by Ser requires the intact DSL domain, as mutation of five critical residues in the DSL domain to alanines abolishes the ability of Ser to *cis*-inhibit Notch [89]. Second, two mutations that severely reduce Ser endocytosis (substitution of a dileucine motif at position 1352–1353 with alanines or deletion of amino acids 1269–1285 in the cytotail), cause accumulation of both Ser and Notch at the cell surface and have an inhibitory effect on Notch signaling *in vivo*. In contrast, adding a C-terminal endoplasmic reticulum retention signal (KKYL) to Ser reduces *cis* inhibition [89]. Furthermore, ectopic expression of the wild-type Neuralized, a DSL ligand ubiquitin ligase, compromises *cis* inhibition of Notch mediated by Ser, and expression of dominant-negative Neuralized (Neur ΔRING) increases *cis* inhibition [89]. Collectively, these results strongly suggest that *cis* inhibitory interactions between Ser and Notch in flies involve their extracellular domains and take place at the cell surface.

Cis inhibition of Notch by DSL ligands has also been observed in vertebrate systems. Expression of a truncated Dll1 lacking all but 13 amino acids in the intracellular domain in the embryonic chick retinal neuroepithelium renders cells irresponsive to signals from their neighbors [90]. Overexpression of Dll1 in cultured N2a neuroblastoma cells stimulates neurite extension, increases the number of primary neurites, and decreases the rate of proliferation. Dll1-transfected N2a cells resemble cells treated with dominant-negative extracellular domain of Notch, Notch-EC [91]. Furthermore, chicken Dll1 or Ser1 cells associate in a cell-autonomous manner with mouse Notch1 when cotransfected into COS-7 or HEK293, and this interaction, similar to the *cis* interaction in *Drosophila*, is mediated by the extracellular domains of Notch1 and Ser1 [92]. Coexpression of Dll1 or Ser1 with Notch1 in signal-receiving cells and coculture with Dll1-transfected signal-sending cells reduces Notch-mediated HES-5 promoter activity, demonstrating that cell-autonomous ligand-receptor complexes decrease cell receptivity to Notch signals [92]. Intriguingly, Dll1/Notch1 and Ser1/Notch1 complexes that are immunoprecipitated from transfected cells do not appear to be cell surface biotinylated [92]. It has been suggested that these complexes, unlike complexes of *Drosophila* Ser and Notch, may form and reside inside the cell [92]. Consistent with this possibility is the finding that ubiquitination of *Xenopus* Delta, Xdelta1, by Mind Bomb expressed in C2C12 myogenic cells does not reduce *cis* inhibition of Notch mediated by Xdelta1 [93]. However, if the inhibitory ligand-receptor interactions take place inside the cell, ligand overexpression and the resulting *cis* inhibition of the receptor should be accompanied by diminished amounts of the active Notch at the cell surface. In contrast, overexpression of ligand and receptor in the same cell does not change the level of receptor at the cell surface, arguing against the intracellular localization of the ligand-receptor complexes [92, 94]. The relief of *cis* inhibition following ADAM-mediated cleavage of Dll1 (see below) further suggests that the inhibitory ligand-receptor complexes in mammalian cells, similar to the complexes in *Drosophila* cells, most likely reside at the cell surface, rather than in the intracellular compartments.

Direct *cis* interactions between rat Dll1 or Dll3 and Notch1 are also detected using transfected NIH3T3 cells and coimmunoprecipitation assays [94]. It is particularly interesting that Dll3 does not bind to Notch1 *in trans* and is not able to activate Notch in neighboring cells but, when overexpressed in NIH3T3 cells, it efficiently inhibits Notch signaling in a cell-autonomous manner [94]. However, the endogenous Dll3 has been recently found to reside in the Golgi apparatus and its role in repression of Notch activation *in vivo* has been challenged [95].

Studies utilizing human primary keratinocytes cultured *in vitro* or mice lacking Dll1expression in the epidermis have demonstrated an important role of Dll1 and Dll1/Notch *cis* interactions in epidermal homeostasis [96, 97]. In human epidermis, the highest level of Dll1 expression is observed in the basal layer, in the clusters of stem cells [96]. It appears that the high level of Dll1 has three effects: (i) blocking Notch signaling within the cluster (via *cis* inhibition) to maintain the stem cell character, (ii) signaling to neighboring cells at the boundaries of the clusters to induce their differentiation into transit-amplifying cells that later give rise to terminally differentiated cells, and (iii) enhancing the cohesiveness of stem cell clusters and preventing intermingling with neighbor-

ing cells [65, 98]. Human primary keratinocytes expressing high levels of Dll1 fail to respond to Dll1 signals from neighboring cells, consistent with their *cis*-inhibitory effect. Deletion of Dll1 in mouse embryonic or adult epidermis results in increased proliferation of keratinocytes and disturbs differentiation, a result that can be partially attributed to the abolishment of *cis* inhibition *in vivo* [97].

If *cis* interactions between the extracellular domains of DSL ligands and Notch receptors render the receptor non-responsive to the activation by ligands *in trans*, then what is the cell-autonomous effect of the ligand shedding by ADAMs? Intuitively, one might expect that the cleavage of a ligand should relieve *cis* inhibition. This hypothesis has been recently tested in the following experiment. NIH3T3 cells transfected with mouse Notch1 (signal-receiving cells) were cocultured with Dll1-transfected CHO cells (signal-sending cells) and the activity of a Notch reporter was measured in signal-receiving cells. Cotransfection of Dll1 into signal-receiving cells decreased the Notch activity due to *cis* inhibition by Dll1. Further cotransfection of Dll1-processing ADAM12 resulted in reactivation of a Notch reporter, whereas overexpression of the catalytically inactive mutant of ADAM12, E349Q, did not have any effect [60]. These results demonstrate that, in transfected cells, ADAM-mediated processing of Dll1 indeed increases Notch signaling in a cell-autonomous manner (Fig. 1C). Whether the proteolytic processing of the endogenous DSL ligands by ADAMs *in vivo* causes a similar relief of *cis*-inhibition of Notch remains to be determined.

Sequential DSL ligand cleavage by γ-secretase

ADAM-mediated processing represents the first step in the regulated intramembrane proteolysis (RIP) of mammalian DSL ligands. Cleavage by ADAMs is often followed by the processing of the TMIC fragment by a presenilin-dependent γ-secretase. Although the localization of the γ-secretase cleavage site has not been determined, it has been proposed to involve a conserved Val residue located near the end of the transmembrane domain of several DSL ligands [59, 61] (Fig. 2B). Since this position is analogous to the Val recognized by γ-secretase during the cleavage of Notch as well as several other transmembrane substrates, it seems feasible that it also represents a recognition site in the DSL ligands. Surprisingly, a transmembrane cleavage site located outside the region targeted by γ-secretase has been recently identified in *Drosophila* Dl. The processing of Dl at that site seems to be presenilin-independent and does not require a prior cleavage by ADAMs [55]. While it is not clear whether the transmembrane processing of *Drosophila* Dl occurs exclusively in this presenilin-independent manner or whether it involves, at least under certain conditions, presenilin-dependent cleavage, the processing of mammalian DSL ligands conforms to the classical sequential RIP mechanism.

The main product of the cleavage by γ-secretase is the intracellular fragment (IC) of a DSL ligand (Fig. 1D). The sequential processing by an ADAM protease and then by γ-secretase has been observed for mouse Dll1 expressed in neuroblastoma N2a cells and for human Jagged2 expressed in NIH3T3 cells [58]. The generation of the Dll1-IC or Jagged2-IC fragments is abolished when cells are treated with a γ-secretase inhibitor L-685,458 or when ligands are transfected into cells expressing a dominant-negative form of human presenilin 1 (PS1), D385A [58]. Similarly, mouse Dll1 is sequentially processed to generate Dll1-IC in $PS1^{+/+}$ MEFs, but not in $PS1^{-/-}$ MEFs [59]. Treatment of Dll1-expressing $PS1^{+/+}$ MEFs with a γ-secretase inhibitor MW167 also abolishes formation of Dll1-IC and stabilizes the Dll1-TMIC form. By gel filtration and coimmunoprecipitation analyses, it has been demonstrated that both Dll1 and PS1 are parts of the same multimolecular complexes [59]. Two mutants of Dll1 that are resistant to ADAM-mediated processing, Dll1-Apa and Dll1-D8, do not generate the Dll1-IC fragment, demonstrating that the generation of Dll1-TMIC by ADAMs is a preliminary requirement for γ-secretase cleavage [59]. Finally, rat Jagged1 transfected into CHO cells is subject to sequential cleavage by ADAMs and γ-secretase, and expression of the dominant-negative PS1 and PS2, or treatment of cells with γ-secretase inhibitors DAPT or Compound E, results in accumulation of Jagged1-TMIC [61].

The intracellular domains of transmembrane proteins processed via RIP are frequently released from the membrane and, if they contain nuclear localization signals (NLSs), translocate to the nucleus. The NLSs fall into two distinct classes termed monopartite NLSs, containing a single cluster of basic amino acids K(R/K)X(R/K), and bipartite NLSs, comprising two basic clusters separated by a spacer, $KRX_{10-12}KRXK$ [99]. All DSL ligands contain positively charged residues following the transmembrane domain that may function as stop transfer signals within the full-length proteins but, once cleaved from the membrane, may play a role in the transport to the nucleus. These membrane-proximal basic clusters in human, mouse, and rat Jagged1 and Jagged2, as well as in *Drosophila* Dl, fully conform to the consensus sequence of the monopartite NLSs (Fig. 2B). The IC domain of Dll1 does not contain a typical NLS but it harbors the sequence RKRP that is conserved between species (Fig. 2B). These clusters of basic residues indeed seem to target DSL ligands to the nucleus. For example,

when CHO cells are transfected with ΔEJagged1, a form of Jagged1 lacking a major part of the extracellular domain and mimicking an ADAM-cleaved protein, the Jagged1-IC fragment is readily produced by γ-secretase and it is retrieved in the nuclear fraction [61]. Deletion of the putative NLS in Jagged1 abolishes the nuclear localization of Jagged1-IC [61]. Similar cell fractionation studies suggest that Dll1-IC, generated from the full-length Dll1, is present in soluble fractions (cytosol and nucleus) of transfected HEK293 cells [59]. Immunofluorescence analysis of S2 cells transfected with HA epitope-tagged Dl-IC or HeLa cells transfected with V5 epitope-tagged Dll1-IC has shown that the recombinant proteins are localized to the nucleus [56, 59]. It has to be pointed out, however, that the immunofluorescence-based assays mentioned above utilized truncated DSL ligands and additional studies are needed to verify whether the full-length proteins, which are the physiological substrates of ADAMs and γ-secretase, give rise to similar intracellular species that translocate to the nucleus.

The nuclear localization of the soluble IC domains of DSL ligands suggests that they may regulate transcription of specific target genes. Indeed, evidence is accumulating that the soluble IC domains serve as transcriptional coactivators. Cotransfection of HEK293 cells with mouse Dll1-IC-Gal4VP16 fusion protein and the Gal4VP16 reporter plasmid stimulates transcription ~70-fold [58]. The soluble IC domain of rat Jagged1 activates gene expression in CHO, COS7 and HEK293 cells via the transcription factor AP1 [61]. More recently, a systematic search for transcription factors capable of binding to the soluble IC domain of Dll1 has identified Smad2, Smad3, and Smad4 as Dll1-binding partners [100]. Most importantly, using a promoter-reporter plasmid that responds specifically to Smad3, it has been demonstrated that Dll1-IC enhances the transcriptional activity of Smad3 in response to stimulation of cells with transforming growth factor β [100]. Collectively, these findings imply bi-directional signaling between Notch-expressing and ligand-expressing cells. In the classical view of the Notch pathway, signaling occurs from ligand-expressing to Notch-expressing cells. However, sequential proteolysis of the ligands which is initiated by the interactions with Notch-expressing cells and which results in the release of the ligand's intracellular domain and its translocation to the nucleus suggests that, at least in some systems, signaling may also occur in the opposite direction. ADAM-mediated cleavage of DSL ligands, the first and obligatory step in the sequential ligand processing, may thus represent a rate-limiting step in the activation of signaling in ligand-expressing cells.

Finally, it is also possible that signaling by certain DSL ligands requires cleavage by ADAMs, but it does not require processing by γ-secretase. Dll1 and Jagged1, two ligands that are processed by ADAMs, as well as Dll4, contain a consensus PDZ-binding motif, X-T/S/Y-X-V/L/I, at their C termini (Fig. 2B). The PDZ-binding motif of Dll1 interacts with Activin receptor-interacting protein 1 (Acvrinp1) [101], disc large homolog 1 (Dlg1) [66], and MAGI1 [102], members of the membrane-associated guanylate kinase (MAGUK) family. In zebrafish, DeltaC and DeltaD interact with MAGI1, MAGI2, and MAGI3 [103]. Furthermore, Dll1 binds through its PDZ-binding domain to an adaptor protein syntenin and this interaction is important in Dll1-induced cell cohesiveness [98]. The identities of proteins interacting with the PDZ-binding motif of Jagged1 are not known, but this motif is necessary for Jagged1 to induce transformation of rat kidney epithelial cells and to change the profile of gene expression [104]. Interestingly, Jagged1 TMIC, lacking the extracellular domain and resembling the product of ADAM-mediated cleavage of Jagged1, does not cause cell transformation, despite the presence of the intact PDZ-binding motif [104]. Therefore, one may speculate that shedding of the extracellular domain of the DSL ligands by ADAMs could abolish signaling events that are mediated by PDZ-binding motifs present in the ligand cytotails.

Conclusions

While the proteolytic processing of Notch receptors by ADAMs has been extensively studied and its role in activation of the Notch pathway is becoming more clear, the role and mechanism of ADAM-mediated ligand proteolysis are less understood. Although ligand cleavage is without any doubt less prevalent than the cleavage of the receptors, the proteolytic processing of DSL ligands modulates the strength and duration of Notch signals and leads to the generation of soluble ligand intracellular domains in ligand-expressing cells. Therefore, the proteolytic processing of DSL ligands as a mechanism of modulation of the Notch pathway deserves more attention. Future biochemical and genetic approaches should elucidate the mechanism and regulation of ADAM-mediated ligand cleavage. For example, one of the invaluable tools for determination of the physiological significance of ligand cleavage would be the generation of transgenic mouse models in which wild-type DSL ligands are replaced with their non-cleavable mutant forms. Since ADAM proteases are frequently deregulated in many pathological conditions including cancer, an altered processing of Notch receptors and

their ligands should be taken into consideration when studying ADAM-related diseases.

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