The many facets of Notch ligands

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Abstract

The Notch signaling pathway regulates a diverse array of cell types and cellular processes and is tightly regulated by ligand binding. Both canonical and noncanonical Notch ligands have been identified that may account for some of the pleiotropic nature associated with Notch signaling. This review focuses on the molecular mechanisms by which Notch ligands function as signaling agonists and antagonists, and discusses different modes of activating ligands as well as findings that support intrinsic ligand signaling activity independent of Notch. Post-translational modification, proteolytic processing, endocytosis and membrane trafficking, as well as interactions with the actin cytoskeleton may contribute to the recently appreciated multi-functionality of Notch ligands. The regulation of Notch ligand expression by other signaling pathways provides a mechanism to coordinate Notch signaling with multiple cellular and developmental cues. The association of Notch ligands with inherited human disorders and cancer highlights the importance of understanding the molecular nature and activities intrinsic to Notch ligands.

Keywords

Notch ligands; Notch signaling; endocytosis; proteolysis; actin cytoskeleton

Introduction

The Notch pathway is an evolutionary conserved signaling system that is absolutely required for normal embryonic development and also functions to regulate tissue homeostasis and maintenance of stem cells in adults (Artavanis-Tsakonas *et al.*, 1999; Gridley, 1997; Gridley, 2003). Ligand-induced Notch signaling regulates a variety of cell types during specification, patterning, and morphogenesis through effects on differentiation, proliferation, survival and apoptosis (Bray, 2006; Fiuza and Arias, 2007). Given the large repertoire of cellular processes dependent on Notch signaling, it is not surprising that defects in the Notch ligands are associated with hereditary diseases such as Alagille syndrome and spondylocostal dysostosis and several cancers display aberrant ligand expression (Koch and Radtke, 2007; Leong and Karsan, 2006; Piccoli and Spinner, 2001; Turnpenny *et al.*, 2007).

The canonical DSL (D̲elta, S̲errate, L̲ag2) ligands are responsible for the majority Notch signaling effects; however, a growing number of non-canonical ligands have also been shown to activate Notch. The canonical DSL ligands are type1 cell surface proteins, that like Notch

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have multiple tandem Epidermal Growth Factor (EGF) repeats in their extracellular domains (Figure 1). The DSL domain together with the flanking N-terminal (NT) domain and first two EGF repeats are required for DSL ligands to bind Notch (Parks *et al.*, 2006;Shimizu *et al.*, 1999). Based on structural homology to the two Drosophila ligands, Delta and Serrate, the mammalian canonical ligands are designated as either Delta-like (Dll1, Dll3 and Dll4) or Serrate-like (Bray, 2006;Fiuza and Arias, 2007). There are two distinct Serrate-like ligands, known as Jagged1 and Jagged2 in vertebrates that have almost twice the number of EGF repeats as Delta-like ligands, some of which contain conserved insertions of unknown function (Weinmaster, 1997). Jagged1 and Jagged2 have an additional cysteine-rich region (CR) not found in Delta-like ligands, which has partial homology to the von Willebrand factor type C domain (VWFC), but lacks the terminal CCX8C spacing found in almost all other VWFC domains (Vitt *et al.*, 2001). The intracellular regions of DSL ligands lack obvious sequence homology except that most, but not all, contain multiple lysine residues and a C-terminal PDZ (PSD-95/Dlg/ZO-1)-ligand motif (Pintar *et al.*, 2007), which are required for ligand signaling activity and interactions with the cytoskeleton, respectively.

Activation of Notch signaling requires interactions between a DSL ligand expressed on the surface of one cell (signal-sending cell) and a Notch receptor (Notch1-4) expressed on the surface of an apposing cell (signal-receiving cell). Notch is presented to ligand as a heterodimer produced as a result of processing by a furin-like protease during transit to the plasma membrane (reviewed in, (Nichols *et al.*, 2007b). Ligand binding triggers additional proteolytic cleavages of Notch, first by A-Disintegrin-And-Metalloproteases (ADAM) within the juxtamembrane region followed by γ-secretase within the transmembrane domain resulting in the release of the Notch intracellular domain (NICD) from the membrane. NICD translocates to the nucleus where it directly interacts with the CSL (C̲BF1, S̲u(H), L̲AG1) transcription factor and recruits coactivators including Mastermind to turn on expression of Notch target genes such as hairy and enhancer of split (HES) family.

DSL ligands as inhibitors of Notch signaling

In addition to the well-characterized role of activating Notch signaling through cell-cell interactions (trans-interactions), DSL ligands can also affect Notch signaling through interactions with Notch within the same cell (cis-interactions) (Fiuza and Arias, 2007; Zolkiewska, 2008). Compared with the activating trans-interactions, cis-interactions between DSL ligands and Notch inhibit Notch signaling (Glittenberg *et al.*, 2006; Jacobsen *et al.*, 1998; Klein and Arias, 1998; Klein *et al.*, 1997; Ladi *et al.*, 2005; Micchelli *et al.*, 1997; Sakamoto *et al.*, 2002b); however, the molecular basis of cis-interactions and their effects on Notch are not well understood. Nonetheless, cis-inhibition by DSL ligands appears to play an important role in a subset of Notch-dependent development events (de Celis and Bray, 1997; Jacobsen *et al.*, 1998; Klein and Arias, 1998; Klein *et al.*, 1997). While these studies have relied on overexpression of DSL ligands, cis-inhibition of Notch signaling has also been demonstrated by loss of ligand expression, suggesting that endogenous ligands also exert inhibitory effects (Micchelli *et al.*, 1997). Compared to invertebrates, the physiological relevance of cis-inhibition in vertebrate systems is not as well established. However, overexpression of truncated ligands lacking most of the intracellular domain function cell autonomously to block Notch signaling and promote retinal neurogenesis and neurite outgrowth as well as inhibit keratinocyte differentiation within the epidermal stem cell niche (Dorsky *et al.*, 1997; Franklin *et al.*, 1999; Henrique *et al.*, 1997; Lowell *et al.*, 2000; Lowell and Watt, 2001).

The mechanism underlying *cis*-inhibition of Notch signaling is unknown, but may involve sequestration of cell surface Notch that precludes its availability for interactions with ligands on neighboring cells. Cis-interactions could compete out trans ligand interactions with Notch

if the cis and trans Notch binding sites overlap. In support of this mechanism, cells coexpressing Dll1 and Notch1 are unable to bind soluble DSL ligands (J. Nichols and G. W., unpublished data). Inhibitory cis-interactions formed in the secretory pathway could prevent Notch receptors from reaching the cell surface (Sakamoto *et al.*, 2002a); however, other studies have indicated that ligand cell surface expression is required for the cis-inhibitory effects on Notch signaling (Glittenberg *et al.*, 2006; Ladi *et al.*, 2005). Although it is not clear how cell surface ligand could prevent Notch signaling, it could stimulate Notch endocytosis; however, cis-inhibition is not associated with losses in cell surface Notch (Glittenberg *et al.*, 2006; Ladi *et al.*, 2005). Additionally, intercellular ligand-ligand interactions could decrease trans ligand available for Notch activation; however, ligand-ligand interactions are predicted to be weaker than ligand-Notch interactions (Fehon *et al.*, 1990; Klueg and Muskavitch, 1999; Parks *et al.*, 2006), making this scenario less likely.

Regulation of DSL ligand activity by glycosylation

Glycosylation of Notch plays an important role in regulating ligand activity through modulating ligand-binding properties and these effects have been extensively reviewed elsewhere (Irvine, 2008; Okajima *et al.*, 2008a; Rampal *et al.*, 2007; Stanley, 2007). Both DSL ligands and Notch receptors have conserved sequences within specific EGF repeats that can be modified by O- and N-linked glycans; however, only O-fucose and O-glucose additions have so far been shown to modulate Notch signaling. In contrast, N-glycan-modification of Notch appears dispensable for Notch-dependent development in mice (Haltiwanger and Lowe, 2004). Although DSL ligands are also glycosylated (Panin *et al.*, 2002), it is unclear whether these modifications affect ligand activity.

In Drosophila, the glycosyltransferase O-fucosyltransferase-1 (OFUT1) is absolutely required for Notch signaling, and both enzymatic and chaperone activities for OFUT1 have been proposed (Irvine, 2008; Rampal *et al.*, 2007; Stanley, 2007). While the addition of O-fucose is a pre-requisite for fringe modification of Notch that modulates ligand binding, the chaperone activity of OFUT1 facilitates proper folding and trafficking of Notch from the endoplasmic reticulum to the cell surface (Okajima *et al.*, 2008b). In contrast to OFUT1, the mammalian O-fucosyl transferase-1, Pofut1, is not required for Notch cell surface expression; however, its fucosyltransferase activity is proposed to regulate proper Notch folding to achieve optimal ligand binding and Notch signaling (Stahl *et al.*, 2008). The apparent lack of a chaperone activity for Pofut1 in mammalian cells may be due to the presence of a functionally redundant protein, perhaps a glucosyltransferase similar to the recently identified Drosophila Rumi (Acar *et al.*, 2008). Functional studies in flies have suggested that the addition of O-glucose to Notch by Rumi is required for signaling in a temperature-sensitive manner, suggesting that this modification may affect the folding, stability and/or conformation of Notch without affecting ligand binding (Acar *et al.*, 2008; Irvine, 2008); however, a role for O-glucosylation of mammalian Notch has yet to be reported.

Following Notch O-fucosylation, some O-fucose moieties are further elongated by fringe, a β 1,3-N-acetylglucosaminyltransferase that catalyzes addition of N-acetylglucosamine and is required for a subset of Notch-dependent developmental events (Fiuza and Arias, 2007; Okajima *et al.*, 2008a; Rampal *et al.*, 2007; Stanley, 2007; Visan *et al.*, 2006b). Specifically, fringe modification of Notch potentiates signaling by Delta-like ligands and this could function to stimulate Notch activation when ligand is limiting (Koch *et al.*, 2001; Visan *et al.*, 2006a; Visan *et al.*, 2006b). In contrast, Serrate-like ligands are unable to activate fringe-modified Notch. The molecular basis of these differences appears to be at the level of ligand binding, such that fringe modification increases binding of Delta-like ligands to Notch, while Serrate/Jagged binding is perturbed. Interestingly, in vitro binding assays with beads or non-adherent Drosophila and mammalian cells indicate a complete loss of Serrate/Jagged binding to fringe-

modified Notch (Bruckner *et al.*, 2000; Lei *et al.*, 2003; Okajima *et al.*, 2003; Shimizu *et al.*, 2001; Xu *et al.*, 2007), while Jagged binding to adherent cells is similar in the presence and absence of fringe (Hicks *et al.*, 2000; Visan *et al.*, 2006a; Yang *et al.*, 2005). These differences may reflect differences in cytoskeletal structure, which could facilitate stronger ligand binding to Notch expressed in adherent cells, precluding detection of any changes in ligand-Notch interactions mediated by fringe. Given these considerations, it seems likely that fringe glycosylation of Notch differentially modulates ligand-induced Notch signaling by affecting the strength of ligand-Notch interactions (Yang *et al.*, 2005).

Regulation of DSL ligand activity by ubiquitination

Modification of DSL ligands by ubiquitination regulates ligand signaling activity and cell surface expression (Chitnis, 2006; Le Borgne, 2006; Le Borgne and Schweisguth, 2003a; Nichols *et al.*, 2007b). As found for Drosophila Delta and Serrate, the intracellular domains of Dll1, Dll4, Jagged1 and Jagged2 contain multiple lysine residues that can serve as potential sites for the addition of ubiquitin by E3 ligases. Two structurally distinct RING-containing E3 ligases, Neuralized (Neur) and Mind bomb (Mib), influence Notch signaling through interacting with and ubiquitinating DSL ligands to enhance their endocytosis. Neur was originally isolated in screens for zygotic lethal mutations that produce the classic Notch neurogenic phenotype in flies (Lehmann *et al.*, 1983); however, nearly two decades passed before the biochemical basis of Neur activity in Notch signaling was realized. Initial studies in Drosophila and Xenopus reported that Neur had intrinsic ubiquitin ligase activity and interacted with Delta to promote its internalization and degradation through ubiquitination (Deblandre *et al.*, 2001; Lai *et al.*, 2001; Pavlopoulos *et al.*, 2001; Yeh *et al.*, 2001). Given that Neur is required for Notch signaling these findings are difficult to reconcile; however, based on the cell autonomous activity identified for Neur (Lai and Rubin, 2001a; Lai and Rubin, 2001b; Yeh *et al.*, 2000) a model was suggested in which the loss of cell surface Delta induced by Neur might indirectly enhance Notch signaling through relieving cis-inhibition imposed by Delta (Deblandre *et al.*, 2001). However, subsequent analyses indicated that both the expression and localization of Neur are enhanced in signal-sending cells (Bardin and Schweisguth, 2006; Le Borgne and Schweisguth, 2003b; Morel *et al.*, 2003) and that Neur functions nonautonomously in cell fate decisions regulated by Notch signaling (Pavlopoulos *et al.*, 2001), providing support for the idea that Neur-induced endocytosis functions directly to stimulate ligand signaling activity. Although studies in flies and frogs support a role for Neur in generating a productive signal and/or regulating cell surface levels, gene targeting of the mammalian Neur homolog yields viable mice lacking obvious Notch developmental defects (Ruan *et al.*, 2001; Vollrath *et al.*, 2001). This surprising finding suggested that mammalian Neur might not be an essential component of the Notch signaling pathway or alternatively, additional E3 ubiquitin ligases exist to modify DSL ligands and facilitate Notch activation. Indeed, a structurally distinct E3 ligase was subsequently identified as the target of the Mind bomb neurogenic mutant in zebrafish (Chen and Casey Corliss, 2004; Itoh *et al.*, 2003). Like Neur, Mib binds and ubiquitinates Delta and upregulates Delta endocytosis; however, in contrast to Neur, Mib functions exclusively in the ligand cell to activate Notch signaling and is unable to reverse the cis-inhibitory effects of Delta on Notch reception (Koo *et al.*, 2005a).

Neur and Mib homologs have been isolated from a number of different species and despite being conserved throughout evolution and having similar molecular activities, Neur and Mib genes may have evolved to serve different roles in vertebrate Notch signaling. Drosophila has a single Neur gene (dNeur) and two related Mib genes (dMib1 and dMib2) that regulate distinct Notch-dependent developmental events (Lai *et al.*, 2005; Le Borgne *et al.*, 2005; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005), apparently due to differential expression. Neur and Mib ubiquitinate both Delta and Serrate to stimulate ligand endocytosis and signaling activities, and gene rescue experiments indicate that for the most part these structurally distinct E3 ligases

are functionally redundant. Genetic evidence in mice indicate that the mammalian Neur1 and Neur2 genes are dispensable for normal development and animals defective in Neur1, Neur2 and Mib2 gene expression do not display any Notch-dependent phenotypes; however, additional removal of Mib1 produces a Notch embryonic lethality (Koo *et al.*, 2007). Importantly, disruption of Mib1 alone produces the known constellation of Notch mutant phenotypes in developing mouse embyros (Barsi *et al.*, 2005; Koo *et al.*, 2005a). Although Mib1 and Mib2 appear functionally redundant (Zhang *et al.*, 2007a; Zhang *et al.*, 2007b), Mib2 is not strongly expressed during embryonic development accounting for the absolute requirement for Mib1 in Notch-dependent developmental processes (Koo *et al.*, 2007). In contrast to findings reported for the functionally redundant E3 ligases in flies, Mib2 but neither Neur1 nor Neur2 can rescue the Mib1 mutant neurogenic phenotype in zebrafish (Koo *et al.*, 2005b). Moreover, while both Neur1 and Neur2 are dispensable for normal neurogenesis in mice, Mib1 mutant embryos display strong neurogenic phenotypes in the developing brain and neural tube (Koo *et al.*, 2005b; Koo *et al.*, 2007). Therefore, while Neur and Mib appear to perform similar roles in Notch signaling in flies, the vertebrate Neur and Mib proteins do not seem to be functionally equivalent.

Findings from mammalian cells have suggested that Mib, not Neur is the E3 ligase responsible for DSL ligand endocytosis that activates Notch signaling, while Neur functions downstream of Mib to direct lysosomal degradation of internalized ligands and regulate the level of ligand available for Notch activation (Song *et al.*, 2006). Consistent with this idea, overexpression of Neur1 monoubiquitinates Jagged1 leading to degradation and attenuation of Jagged1-induced Notch signaling (Koutelou *et al.*, 2008); however, Mib2 (skeletrophin) ubiquitination of Jagged2 is associated with activation of Notch signaling (Takeuchi *et al.*, 2005). The different functional roles for Neur and Mib ligases in Notch signaling might reflect different ubiquitin states of DSL ligands mediated by these structurally distinct E3 ligases. DSL ligands have been reported to be mono- and/or polyubiquitinated; however, the functional consequences of these types of ubiquitination to Notch signaling are not well documented. In this regard, it will be important to determine if DSL ligands are ubiquitinated at the same or distinct sites by Neur and Mib since this might influence ligand activity and trafficking. Polyubiquitination is associated with proteasome degradation, while both mono and multi-mono ubiqutination can signal endocytosis of membrane proteins from the cell surface and further influence intracellular trafficking (Staub and Rotin, 2006). In particular, interactions of ubiquitinated proteins with ubiquitin-binding proteins can direct intracellular trafficking to allow either sorting to the lysosome for degradation or recycling back to the plasma membrane. Trafficking events that degrade internalized DSL ligands could function to downregulate Notch signaling, while recognition of ubiquitinated ligands by specific adaptor/sorting molecules might promote signaling.

Regulation of DSL ligands by endocytosis

Although activating proteases have been identified, it is still unclear how ligand binding induces Notch proteolysis required for downstream signaling. A unique aspect of DSL ligands in Notch activation is their strict requirement for endocytosis. In the absence of endocytosis, DSL ligands accumulate at the cell surface where they are unable to activate Notch (Itoh *et al.*, 2003; Nichols *et al.*, 2007a; Parks *et al.*, 2000). That ligand on the surface of a signal-sending cell must be internalized to activate Notch on the signal-receiving cell has contributed to an intense interest, as well as controversy, in understanding the roles that DSL ligand endocytosis and trafficking play in Notch signaling.

Genetic and cellular studies have implicated a large number of proteins associated with endocytosis that are required for DSL ligand activity (reviewed in (Le Borgne, 2006; Nichols *et al.*, 2007b)). DSL ligands appear to be internalized by multiple, but poorly characterized

endocytic pathways; however, only ubiquitinated DSL ligands internalized in an epsin-dependent manner are competent to signal (Chen and Casey Corliss, 2004; Deblandre *et al.*, 2001; Glittenberg *et al.*, 2006; Itoh *et al.*, 2003; Koo *et al.*, 2005a; Lai *et al.*, 2001; Overstreet *et al.*, 2004; Pavlopoulos *et al.*, 2001; Wang and Struhl, 2004; Wang and Struhl, 2005; Yeh *et al.*, 2001). Signal-sending cells also require additional proteins that function in endocytosis such as clathrin (Eun *et al.*, 2006; Nichols *et al.*, 2007a), dynamin (Nichols *et al.*, 2007a; Parks *et al.*, 2000; Seugnet *et al.*, 1997), and auxilin (Eun *et al.*, 2006; Hagedorn *et al.*, 2006) for DSL ligands to signal effectively. Epsin participates in endocytosis through interactions with the plasma membrane, clathrin endocytic vesicles, as well as ubiquitinated cargo (Horvath *et al.*, 2007). Together these properties could allow epsin to recruit ubiquitinated DSL ligands into a endocytic pathway to obtain signaling activity; however, it is still unclear how these events contribute to Notch activation.

Models have been proposed to address roles for DSL ligand endocytosis both before and after binding to Notch (reviewed in, (Chitnis, 2006; Le Borgne, 2006; Nichols *et al.*, 2007b)). In the absence of Notch, DSL ligands may undergo constitutive endocytosis and recycling to and from the plasma membrane to produce active ligands (Wang and Struhl, 2004). In support of this idea, following asymmetric cell division during Drosophila sensory cell fate determinations, Delta is concentrated in recycling endosomes enriched to signal-sending cells (Emery *et al.*, 2005). Moreover, losses in Rab11 or Sec15, that function together to recycle proteins to the cell surface, produce cell fate transformations indicative of losses in DSL ligand activity (Emery *et al.*, 2005; Jafar-Nejad *et al.*, 2005; Langevin *et al.*, 2005; Wu *et al.*, 2005). However, not all Notch-dependent signaling events require Sec 15 (Jafar-Nejad *et al.*, 2005), as one might expect if recycling is an absolute requirement for signaling activity. Asymmetric enrichment of recycling endosomes may be necessary only in specific cellular contexts, to concentrate ligand at the plasma membrane and ensure strong signaling potential. It is important to note that even though Delta and Rab11 colocalize in endocytic vesicles, direct evidence that DSL ligands actually recycle and that recycling positively affects either Notch binding or activation is lacking.

A second model, initially proposed by Muskavitch and colleagues, involves a more "active" role for endocytosis beyond presentation of an active cell surface ligand (Parks *et al.*, 1997). Based on the presence of Delta-Notch vesicular structures within ligand signaling cells in Drosophila, the authors suggested that ligands might undergo endocytosis while bound to Notch. The uptake of Notch from adjacent cells was termed "transendocytosis" and this process was proposed to induce a "mechanical strain" in Notch to expose the ADAM cleavage site and allow proteolytic activation for downstream signaling. Subsequent studies in mammalian cell culture confirmed transfer of Notch to DSL ligand cells and linked this event to activation of Notch signaling (Nichols *et al.*, 2007a). Surprisingly, broad-spectrum metalloprotease inhibitors did not diminish Notch transendocytosis, suggesting that ADAM proteolysis was not responsible for the removal of Notch by DSL ligand endocytosis. Importantly, Notch heterodimer formation is required for Notch transendocytosis, suggesting that destabilization of the non-covalent bonds that maintain the heterodimer structure is a prerequisite for Notch dissociation. Structural analysis of the Notch heterodimer has suggested that considerable force would be required to access the ADAM cleavage site (Gordon *et al.*, 2007). Given the importance of ligand endocytosis in Notch signaling, it is a good "force producing" candidate, however, it is not known if any force is generated during endocytosis, or if such a force can dissociate the Notch heterodimer. In this regard, both the actin cytoskeleton and dynamin have been implicated in inducing membrane constriction and tension during the process of endocytosis (Itoh *et al.*, 2005; Roux *et al.*, 2006). Nonetheless, heterodimer dissociation would expose the ADAM cleavage site and allow for proteolytic activation of Notch. The non-enzymatic dissociation of Notch has identified a mechanical event important in Notch signaling not previously considered by other proteolytic cleavage models (Nichols *et al.*, 2007b).

How could bound Notch alter ligand endocytosis and why is there an absolute dependence on ubiquitination and epsin for ligand signaling activity? Notch binding may induce ubiquitination and/or clustering of DSL ligands to generate multiple ubiquitin-binding sites for epsin. By assembling multiple low affinity ubiquitin interactions, strong epsin-DSL ligand interactions could be formed (Barriere *et al.*, 2006; Hawryluk *et al.*, 2006), which could anchor the ligand within endocytic vesicles during internalization of bound Notch. This is especially important since the proposed "pulling" force needed to dissociate the heterodimer is predicted to be very strong. Implicit in the force/dissociation model is the need for even stronger ligand-Notch interactions, and in this regard, it is tempting to speculate that Jagged binding to fringe-modified Notch might not be strong enough to survive the endocytic "pulling" force. If this were the case, disruption of Jagged binding to fringe-modified Notch would preclude heterodimeric dissociation and thus proteolytic activation of Notch, accounting for the loss in signaling induced by Jagged in the presence of fringe.

Recent studies in flies indicate that Neur plays additional roles in DSL ligand endocytosis to enhance signaling activity beyond ubiquitination (Pitsouli and Delidakis, 2005; Skwarek *et al.*, 2007). A Neur phosphoinositide-binding domain localizes Neur to the plasma membrane and although membrane localization is not required for interactions with or ubiquitination of Delta, it is required for Delta endocytosis and thus Notch signaling (Skwarek *et al.*, 2007). Epsin also binds phosphoinositides, an activity proposed to function in membrane curvature during endocytic vesicle formation (Horvath *et al.*, 2007); however, epsin-phosphoinositide interactions also function in endosomal sorting and trafficking of internalized proteins (Traub and Lukacs, 2007). Therefore, both epsin and Neur could perform multiple functions during DSL ligand endocytosis and membrane trafficking. Since both Neur and epsin bind Delta and the plasma membrane, it seems possible that they could work together to recruit and/or stabilize Delta-Notch complexes within endocytic vesicles and contribute to a physical force for mechanical dissociation of Notch to allow proteolytic activation for downstream signaling.

Regulation of DSL ligand activity by proteolysis

As described for Notch, DSL ligands undergo proteolytic cleavage in the juxtamembrane and transmembrane regions by ADAMs and γ-secretase, respectively. Although it is clear that ligand proteolysis will affect Notch signaling by decreasing cell surface expression, it is less clear if the proteolytic cleavage products have intrinsic activity. A detailed review covering the proteases that cleave DSL ligands has recently been published (Zolkiewska, 2008); here we highlight possible mechanisms by which ligand proteolysis could affect Notch signaling (outlined in Figure 2). A number of ADAMs (ADAM9, ADAM10, ADAM12, ADAM17) have been reported to cleave mammalian DSL ligands, while the ADAM10 (Kuzbanian/Kuz and Kuzbanian-like/Kul) and ADAM17 homologs (DTACE) are implicated in cleavage of Drosophila ligands. These proteases may cleave at multiple sites and some appear to be functionally redundant. ADAM cleavage of DSL ligands results in shedding of the extracellular domain (ECD) and the effects on Notch signaling are different depending on whether the cleavage occurs in the ligand signal-sending cell or the Notch signal-receiving cell.

ADAM proteolysis in the signal-sending cell would reduce the amount of ligand available for Notch activation. In support of this idea, Kul overexpression increases ectodomain shedding of Delta and produces wing vein defects characteristic of loss of Notch (Sapir *et al.*, 2005). Moreover, Kul specifically cleaves ligands and not Notch, identifying Kul as a regulator of Notch signaling through ligand shedding (Lieber *et al.*, 2002; Sapir *et al.*, 2005). As a positive regulator of Notch signaling, Kul functions to maintain low levels of ligand to ensure efficient Notch reception, which is necessary for normal wing margin formation (Sapir *et al.*, 2005). In mammalian cell culture, ectopic expression of ADAM12 causes ectodomain shedding of DSL ligands and enhances Notch signal reception, presumably due to the relief of cis-inhibition

(Dyczynska *et al.*, 2007); however, the biological relevance of ADAM12 to Notch signaling remains to be demonstrated. The level of ligand available for Notch activation, can be indirectly regulated by the glycosylphosphatidyl-anchored cell-surface protein, RECK (reversion-inducing cysteine-rich protein with kazal motifs), which specifically inhibits ADAM10 activity (Muraguchi *et al.*, 2007). By preventing ADAM10-dependent ectodomain shedding of DSL ligands, RECK functions as a positive regulator of Notch signaling. Consistent with this idea, mouse embryos deficient in RECK have a loss in Notch target gene expression and display some Notch-dependent developmental defects, presumably due to loss of cell surface ligand (Muraguchi *et al.*, 2007). Even though RECK inhibits DSL ligand proteolysis, it is less clear if RECK also regulates ADAM10 cleavage of Notch.

ADAM proteolysis produces several cleavage products that could potentially affect Notch signaling (Figure 2). The activity of the ADAM shed ECDs is highly controversial, and in some cases they appear to be inactive, while several studies have suggested that they can either activate or inhibit Notch signaling depending on the cellular context. Interestingly, naturally occurring soluble ligands have been identified in C. elegans and mammalian cells where they appear to function as Notch agonists (Aho, 2004;Chen and Greenwald, 2004). The signaling activity of soluble ligands is difficult to reconcile given the strict requirement for ligand endocytosis in Notch activation. However, pre-fixed Delta cells that are presumably endocytosis-defective activate Notch signaling (Mishra-Gorur *et al.*, 2002), suggesting that under certain conditions the requirement for ligand-mediated endocytosis may be dispensable for Notch activation, and that other mechanisms facilitate Notch heterodimer dissociation. Perhaps soluble ligands immobilized by the extracellular matrix or cell surfaces allow interactions with Notch cells, and that either movement of Notch cells away from the ligand source and/or endocytosis of Notch itself generates a mechanical force sufficient to pull the heterodimer apart and activate Notch signaling. Consistent with this idea, recombinant soluble ligands usually require clustering or immobilization to activate Notch signaling and induce biological responses (Hicks *et al.*, 2002;Karanu *et al.*, 2000;Morrison *et al.*, 2000;Shimizu *et al.*, 2002;Varnum-Finney *et al.*, 2000;Vas *et al.*, 2004). Furthermore, while unclustered soluble ligands can bind Notch, they are unable to activate signaling but rather appear to block signaling induced by trans ligands (Hicks *et al.*, 2002;Shimizu *et al.*, 2002;Varnum-Finney *et al.*, 2000;Vas *et al.*, 2004). In these cases, soluble ligands may compete with membrane-bound ligands for binding to Notch, providing a mechanistic basis for the antagonistic activities identified for soluble engineered forms of Drosophila (Hukriede *et al.*, 1997;Sun and Artavanis-Tsakonas, 1997) and mammalian DSL ligands (Li *et al.*, 2007;Lobov *et al.*, 2007;Noguera-Troise *et al.*, 2006;Small *et al.*, 2001;Trifonova *et al.*, 2004).

Ligand ectodomain shedding leaves behind the membrane-tethered fragment containing the intracellular domain (TMICD), which in mammalian cells undergoes further cleavage by γ-secretase (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six *et al.*, 2003) (Figure 2). There is evidence to support that the released ICD translocates to the nucleus (Hiratochi *et al.*, 2007; Ikeuchi and Sisodia, 2003; Kolev *et al.*, 2005; LaVoie and Selkoe, 2003; Six *et al.*, 2003), similar to that identified for activation of Notch signaling. Moreover, ligand ICDs have been shown to activate transcription of various gene reporters (Hiratochi *et al.*, 2007; Kolev *et al.*, 2005; LaVoie and Selkoe, 2003), and in one case transcription of an endogenous gene was upregulated (Kolev *et al.*, 2005). Interestingly, the Dll1 ICD has been reported to enhance TGFβ-induced Smad3 transcriptional activation (Hiratochi *et al.*, 2007), reminiscent of Smad-enhanced NICD transcriptional activation (Dahlqvist *et al.*, 2003; Itoh *et al.*, 2004). Important for these effects on gene expression, the ICDs contain positively charged amino acids that could function as nuclear localization signals (NLSs) that when mutated prevent nuclear translocation (Kolev *et al.*, 2005; LaVoie and Selkoe, 2003), suggesting that cleaved ICDs are actively transported. Together these studies have provided some support for the idea that DSL ligands undergo reverse signaling; however, this has remained highly controversial.

Furthermore, it is important to note that the demonstration of ICDs moving to the nucleus and participating in gene activation has mostly relied on the use of engineered fragments, rather than physiological proteolytic cleavage of full-length ligands. Although the nuclear translocation and transcriptional activation of DSL ligand ICDs is highly suggestive of bi-directional signaling, the published data are not as convincing as those reported for the EphB/ephrinB signaling system (Aoto and Chen, 2007; Dravis *et al.*, 2004; Holland *et al.*, 1996) that also involves signaling induced by integral membrane ligands and receptors. Nonetheless, the existence of bi-directional signaling for the DSL ligand-Notch pathway remains an intriguing possibility, awaiting a clear demonstration of the occurrence of signaling events in both DSL ligand and Notch cells following ligand-Notch interactions.

Compared to the mammalian DSL ligands, the fate and functional significance of the proteolytic cleavage products of Drosophila DSL ligands are less clear. Soluble forms of Delta are detected in Drosophila embryos (Klueg *et al.*, 1998; Qi *et al.*, 1999) and while in vivo studies have suggested that soluble engineered forms of Delta and Serrate act as Notch antagonists (Hukriede *et al.*, 1997; Sun and Artavanis-Tsakonas, 1997), in vitro studies have not produced clear results (Mishra-Gorur *et al.*, 2002; Qi *et al.*, 1999). Unlike mammals, the TMICD fragment generated by ADAM cleavage of Drosophila Delta (dDelta) does not appear to be further processed (Bland *et al.*, 2003; Delwig *et al.*, 2006) (Figure 2). Although this fragment lacks a Notch binding domain, it could potentially antagonize Notch signaling through competing with full-length ligands for the ubiquitination and/or endocytic machinery.

The intramembrane cleavage of mammalian DSL ligands is triggered by γ-secretase and requires prior ADAM cleavage (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six *et al.*, 2003; Yang *et al.*, 2005). However in Drosophila cells, cleavage of Delta within the membrane-spanning region is ADAM-independent and does not involve γ-secretase (Delwig *et al.*, 2006) (Figure 2). Rather, this cleavage is induced by a thiol-sensitive activity that occurs close to the extracellular face of the membrane, and thus it is unclear whether the ICD would be readily released as found for ligand ICDs generated by γ-secretase (Delwig *et al.*, 2006). If the ECD containing fragment (ECDTM) remains membrane-tethered, it could function similarly to ICD truncated ligands, which are endocytosis-defective and unable to send signals but are efficient cis-inhibitors (Chitnis *et al.*, 1995; Henrique *et al.*, 1997; Nichols *et al.*, 2007a; Shimizu *et al.*, 2002). However if the ECDTM is released, it may function as proposed for soluble DSL ligands. The corresponding ICD-containing intramembrane cleavage product (TMICD^{TSA}) would be expected to function similarly to the Drosophila Delta TMICD if it remained membrane-bound; however, if released it might move to the nucleus and activate gene transcription. Since nuclear staining of dDelta has only been detected using engineered ICD forms (Bland *et al.*, 2003; Sun and Artavanis-Tsakonas, 1996), it is unclear whether the ICD is released from full-length Delta and moves to the nucleus. Like dDelta, Serrate also undergoes ADAM cleavage (Sapir *et al.*, 2005); however, intramembrane cleavage of Serrate has not been reported as yet.

In contrast to the highly regulated proteolytic activation of Notch, it is less clear if or how ligand proteolysis is induced or regulated. In cell culture, DSL ligands are actively cleaved (Bland *et al.*, 2003; Delwig *et al.*, 2006; Dyczynska *et al.*, 2007; LaVoie and Selkoe, 2003; Six *et al.*, 2003; Yang *et al.*, 2005); however, this proteolysis could be induced by serum activation of signaling pathways (Seals and Courtneidge, 2003). In fact, phorbol esters are known to activate intracellular signaling as well as ADAMs, both of which could contribute to DSL ligand proteolysis (Seals and Courtneidge, 2003). The extracellular matrix protein MAGP2 has been reported to regulate DSL ligand proteolysis (Nehring *et al.*, 2005). Interestingly, MAGP2 interacts with different DSL ligands, yet only the Jagged1 ectodomain is shed in a metalloprotease-dependent manner. Direct cell-cell interactions may also enhance ADAM cleavage of DSL ligands and both homotypic ligand-ligand and ligand-Notch interactions have

been implicated (Bland *et al.*, 2003; Delwig *et al.*, 2006; Dyczynska *et al.*, 2007; Hiratochi *et al.*, 2007; LaVoie and Selkoe, 2003). Finally, gains and losses in neuralized activity have been found associated with Delta proteolytic processing in flies (Delwig *et al.*, 2006; Pavlopoulos *et al.*, 2001; Wang and Struhl, 2004), raising the possibility that ligand cleavage may occur within the cell and involve endocytosis.

DSL ligand interactions with PDZ-domain proteins

With the exception of Dll3 and Jagged2, vertebrate DSL ligands have PDZ-binding motifs at their extreme carboxy termini (Pintar *et al.*, 2007), which facilitate interactions with PDZ-containing scaffold/adaptor proteins (Ascano *et al.*, 2003; Estrach *et al.*, 2007; Mizuhara *et al.*, 2005; Pfister *et al.*, 2003; Six *et al.*, 2004; Wright *et al.*, 2004). Although the PDZ-binding sequences are dispensable for ligand activation (Ascano *et al.*, 2003; Mizuhara *et al.*, 2005; Six *et al.*, 2004; Wright *et al.*, 2004) and inhibition of Notch signaling (Glittenberg *et al.*, 2006), they are required for ligands to effect cell adhesion (Estrach *et al.*, 2007; Mizuhara *et al.*, 2005), migration (Six *et al.*, 2004; Wright *et al.*, 2004), and oncogenic transformation (Ascano *et al.*, 2003). There are some sequence differences in the DSL ligand PDZ-binding motifs (Pintar *et al.*, 2007), which likely account for their interactions with different PDZ-containing proteins. For example, Jagged is unable to bind the PDZ domain partners, MAGI-1 (membrane-associated guanylate kinase with inverted domain arrangement-1) and Dlg1 (human homolog of Drosophila discs large 1) identified for Delta-like ligands (Mizuhara *et al.*, 2005; Six *et al.*, 2004), while the closely related Dll1 and Dll4 proteins both bind Dlg1 (Six *et al.*, 2004). Even though PDZ interactions are not required for activation of Notch signaling, Delta lacking its PDZ motif has enhanced signaling potential (Estrach *et al.*, 2007). These findings raise the intriguing possibility that PDZ-based interactions indirectly influence ligand activity by restricting their access to specific endocytic pathways necessary for signaling competent ligands.

PDZ-containing proteins are important for the organization of specialized sites of cell-cell contact at adherens junctions as well as facilitating anchoring of membrane proteins to the cytoskeleton (Brone and Eggermont, 2005; Harris and Lim, 2001; Jelen *et al.*, 2003). DSL ligands co-localize with actin (Lowell and Watt, 2001) and their specific PDZ-domain partners at regions of cell-cell contact (Estrach *et al.*, 2007; Mizuhara *et al.*, 2005; Six *et al.*, 2004; Wright *et al.*, 2004), consistent with the proposed role for DSL ligands in promoting cell adhesion and inhibiting cell motility. In addition to effecting changes in cellular morphology and movement through interactions with the cytoskeleton, Jagged1-PDZ interactions may effect changes in gene expression required for oncogenic transformation (Ascano *et al.*, 2003). How these interactions at the cell surface could allow for activity in the nucleus is unknown, but PDZ-domain proteins such as CASK, Bridge-1 or GRIPtau act as transcriptional activators (Hsueh *et al.*, 2000; Lee *et al.*, 2005; Nakata *et al.*, 2004). Whether the DSL ligand PDZ interactions affect gene expression either indirectly from the plasma membrane or directly through translocation to the nucleus is currently unknown. Release of PDZ-bound proteins from cell surface DSL ligands or proteolytic release of the DSL ICD could allow for nuclear activity. Additionally, DSL ligands could indirectly effect gene transcription while still remaining at the cell surface by binding PDZ proteins that interact with signal transducers that effect changes in gene expression. For example, the PDZ protein Acvrinp1 that binds to Dll1 (Pfister *et al.*, 2003) is also known to interact with Smad3 and inhibit Smad3-dependent transcription (Shoji *et al.*, 2000). Moreover, Jagged1 binds to the PDZ-domain containing protein afadin/AF6, which in turn can interact with RAS (Ascano *et al.*, 2003; Quilliam *et al.*, 1999) that activates signaling to the nucleus to promote changes in gene expression. Finally, that the cellular effects associated with DSL-PDZ interactions require both the extracellular and intracellular domains of DSL ligands suggests that homotypic ligand-ligand interactions could activate ligand signaling (Lowell *et al.*, 2000; Lowell and Watt, 2001), while ligand-

Notch interactions could induce bi-directional signaling (Ascano *et al.*, 2003). Interestingly, a model in which fringe could block Jagged1-induced Notch1 signaling yet allow Jagged1 to mediate PDZ-dependent intracellular signaling has been proposed (Ascano *et al.*, 2003).

Regulation of DSL ligand expression

Notch mediated lateral inhibition and inductive signaling negatively and positively regulate DSL ligand expression, respectively. In fact, increased Dll1 (Barrantes *et al.*, 1999; de la Pompa *et al.*, 1997) or Dll4 (Suchting *et al.*, 2007) expression has been used as a reliable indicator of defects in Notch signaling. In contrast, Notch inductive signals upregulate DSL ligand expression, which is necessary for proper wing margin formation in flies (Doherty *et al.*, 1996) as well as somite formation and patterning in vertebrates (Barrantes *et al.*, 1999; de la Pompa *et al.*, 1997; Doherty *et al.*, 1996; Takahashi *et al.*, 2003). The Notch signaling pathway also interacts with a number of different signaling systems and many of these also affect DSL ligand expression (Hurlbut *et al.*, 2007). In particular, fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), Hedgehog (Hh) and Wnt have been found to modulate ligand expression and produce specific cellular responses (Table 1). The majority of these signaling pathways increase ligand expression, such as VEGF induced expression of Dll4 in endothelial cells that promotes tip cell selection during polarized angiogenic sprouting (Roca and Adams, 2007; Sainson and Harris, 2008; Thurston *et al.*, 2007; Yan and Plowman, 2007), and canonical Wnt signaling that drives Dll1 transcription in the tail bud and presomitic mesoderm during somitogenesis (Hofmann *et al.*, 2004). In contrast, FGF downregulates Dll1 expression in neuroepithelial precursors to maintain the progenitor state by preventing neuronal differentiation (Faux *et al.*, 2001). In the immune system, specific inflammatory responses upregulate expression of either Delta-like or Jagged1 ligands in dendritic cells to direct activated CD4+ T cells towards either a T-helper (Th)-1 or Th-2 response, respectively (Cheng and Gabrilovich, 2007; Osborne and Minter, 2007; Raymond *et al.*, 2007). Importantly, regulation of DSL ligand expression by other signaling pathways allows for Notch signaling to be integrated into a highly ordered and complex molecular network (Hurlbut *et al.*, 2007), which could regulate embryonic development as well as the induction of immune and vascular responses in the adult.

Long-range signaling by DSL ligands

A hallmark of ligand-induced Notch signaling is the requirement for direct cell-cell interactions; however, studies in flies have indicated that Delta can activate Notch on cells positioned several cell diameters away from where it is produced (de Joussineau *et al.*, 2003). That soluble ligands are released from the cell surface through proteolysis raises the possibility that soluble ligands could diffuse from the ligand-producing cell to activate Notch at distant sites. Alternatively, actin-based cellular projections extending from Delta cells have been imaged in Drosophila and proposed to function in long-range activation of Notch (de Joussineau *et al.*, 2003). Delta is concentrated in filopodia-like cellular projections and appears to either induce or stabilize these structures (de Joussineau *et al.*, 2003; Renaud and Simpson, 2001). Importantly, disruption of Delta-containing filopodia, produce developmental defects consistent with losses in lateral inhibition mediated by Notch signaling. Interestingly Scabrous (Sca), that is also enriched in actin-based cellular extensions has been proposed to participate in Delta long-range signaling, possibly through stabilizing Delta-Notch interactions (Chou and Chien, 2002; Renaud and Simpson, 2001); however, the molecular basis by which Sca enhances Delta signaling over a long range is unclear.

Cellular extensions, known as cytonemes or cytoneme-like filopodia have been implicated in regulating the release or reception of a number of different signals over long distances during

Drosophila development (Hsiung *et al.*, 2005). In addition, the C. elegans distal tip cell has long cellular processes that contain the DSL ligand Lag2, which appear to extend all the way to the mitotic/meiotic border where they may regulate proliferation of the germ line through activation of the Notch homolog, Glp1 (Fitzgerald and Greenwald, 1995). In mammalian cells, Dll1 is also concentrated in actin-rich cellular projections that appear to reach out and make contact with cocultured Notch cells (J. Nichols and G. W., unpublished data). Whether these Dll1-rich projections reflect long-range signaling in mammalian cells and/or function in intact animals as proposed for DSL ligand activation of Notch in invertebrates remains to be determined.

The DSL family outlier

Dll3 is a structurally divergent DSL family member (Dunwoodie *et al.*, 1997) that is expressed in the developing brain, thymus and paraxial mesoderm; yet losses in Dll3 are associated with vertebral-segmentation and rib defects in patients with spondylocostal dysostosis (Bulman *et al.*, 2000; Turnpenny *et al.*, 2003) and the pudgy mouse (Kusumi *et al.*, 2004; Kusumi *et al.*, 1998). Somites contain vertebral precursors and are rhythmically generated from the presomitic mesoderm through coordinated interactions between the Wnt, FGF and Notch signaling pathways (Dequeant *et al.*, 2006). Since Dll3 is expressed in the presomitic mesoderm, and losses in Dll3 produce defects in somite formation and patterning, it seems likely that Dll3 functions in Notch signaling during somitogenesis. In addition to Dll3, Dll1 is also expressed in the presomitic mesoderm where it functions in somitogenesis; however, Dll1 and Dll3 mutant mice display very different somite defects (Dunwoodie *et al.*, 2002; Kusumi *et al.*, 2004; Zhang *et al.*, 2002). Importantly, Dll3 is unable to rescue the Dll1 mutant somite phenotype in developing mouse embryos, indicating that these related DSL ligands are not functionally equivalent (Geffers *et al.*, 2007). Consistent with this idea, Dll1 is a potent activating Notch ligand, while Dll3 lacks structural characteristics important for DSL ligands to bind to Notch in trans and thereby activate Notch signaling (Geffers *et al.*, 2007; Ladi *et al.*, 2005).

Overexpression of Dll3 in mammalian cells blocks Notch signaling and in Xenopus embyros produces phenotypes indicative of loss of Notch signaling, supporting the notion that Dll3 is a Notch antagonist (Ladi *et al.*, 2005). Although it is unclear how Dll3 inhibits Notch signaling in these cellular contexts, Dll3 coexpressed with Notch is detected at the cell surface and binds Notch, suggesting a role for Dll3 in cis-inhibition. However, endogenous Dll3 is detected in the Golgi and shows little if any cell surface localization (Geffers *et al.*, 2007), suggesting that overexpression may override the Dll3 Golgi retention mechanism and allow Dll3 to traffic to the cell surface. Together these findings suggest that Dll3 surface expression is highly regulated; however, the Golgi localization of Dll3 is difficult to reconcile with a role for this DSL ligand in Notch signaling. Perhaps Dll3 functions as a modulator of Notch signaling by regulating the transit of Notch and its activating proteases as they traffic through the Golgi to their appropriate cellular locales required for efficient Notch activation. In support of this notion, Dll3 interacts with Notch and is cleaved by metalloproteases and γ-secretase (E. Ladi, E. Cagavi, G. W.; unpublished data).

Although there is a consensus that Dll3 in unable to activate Notch (Geffers *et al.*, 2007; Ladi *et al.*, 2005), its Golgi localization is inconsistent with cis-inhibition by DSL ligands requiring cell surface expression. These findings and inconsistencies for Dll3 raise the intriguing question of whether Dll3 actually functions in Notch signaling to regulate somitogenesis. Indeed, genetic interactions between Dll3 and Notch1 in mice yield only mild heterozygous mutant phenotypes compared to the strong synergistic interactions reported for known Notch pathway genes (Loomes *et al.*, 2007). Given that during somitogenesis, Wnt and FGF signaling are coordinated with Notch signaling to regulate the periodic expression of a large network of

genes (Dequeant *et al.*, 2006), it is tempting to speculate that Dll3 trafficking between the Golgi and plasma membrane might also be regulated during somitogensis. However, at this point, how changes in levels or subcellular localization of Dll3 would affect Notch signaling or other signaling pathways required for somitogenesis is completely unknown.

Non-canonical Notch ligands

The diverse and frequent uses of Notch signaling are at odds with the small number of canonical DSL ligands and receptors encoded in metazoan genomes. One molecular explanation for the pleiotropic nature of Notch signaling is the presence of non-canonical Notch ligands. Unlike the canonical ligands that share many features (Figure 1), non-canonical ligands are structurally diverse and include integral membrane, GPI-linked, and even secreted proteins (Figure 3).

Membrane-tethered non-canonical ligands

One of the earliest described non-canonical ligands for Notch is Delta-like 1 (Dlk-1), also known as Pref-1, or FA-1 (Bachmann *et al.*, 1996; Laborda *et al.*, 1993; Smas and Sul, 1993), whose predominant role is inhibiting adipogenesis (Wang *et al.*, 2006). Other than the lack of a DSL domain, Dlk-1 is otherwise quite similar in structure to other Delta-like proteins, as it is an integral membrane protein containing tandem EGF repeats in its extracellular domain (Figure 3). Moreover, like Delta, Dlk-1 can be cleaved by ADAMs and is negatively regulated at the transcriptional level by Notch signaling (Ross *et al.*, 2004; Wang and Sul, 2006). The preponderance of evidence support only cis-interactions between Dlk-1 and Notch, and in fact, Dlk-1 overexpression phenotypes are consistent with Dlk-1 functioning only in cis-inhibition and not trans-activation of Notch signaling (Baladron *et al.*, 2005; Bray *et al.*, 2008). Dlk-1 cis-inhibition may depend on the amount of ADAM proteolysis, since an ADAM-resistant, membrane-bound form of Dlk-1 is more potent than wild-type or soluble forms at blocking Notch signaling. This suggests that Dlk-1-mediated Notch antagonism may require low cellular ADAM activity that favors membrane-bound Dlk-1. High levels of Dlk-1 are also associated with loss of Notch target gene expression such as Hes-1 and E(spl)mβ in mammals and flies, respectively (Baladron *et al.*, 2005; Bray *et al.*, 2008; Nueda *et al.*, 2007). The molecular basis of this antagonism is unclear, but it is possible that Dlk-1 binding to Notch EGF 10-11 or EGF 12-13 may compete with activating trans-DSL ligand that requires Notch EGF 11-12 to block binding and signaling. However, direct binding of full-length Dlk-1 and Notch, either endogenously or ectopically expressed, has not been reported. Moreover, there is conflicting data on whether Dlk-1-induced loss of Hes-1 expression directly involves Notch since Hes-1 is regulated by more than one signaling pathway (Hatakeyama *et al.*, 2004; Kluppel and Wrana, 2005; Ross *et al.*, 2004).

Another Delta-like protein is Delta/Notch-like EGF-related receptor (DNER) that is an integral membrane protein containing extracellular tandem EGF repeats but lacking a DSL domain (Eiraku *et al.*, 2002). Despite the absence of a DSL domain, DNER binds Notch when presented in trans and can activate a CSL reporter in cells co-cultured with DNER-expressing cells (Eiraku *et al.*, 2005). Both in vitro and in vivo studies support DNER's function as a trans-ligand to effect glial morphological changes through activation of Notch. DNER does not affect the number of glial cells present in vivo, suggesting that its effect is limited to later stages of differentiation and not early cell fate decisions. DNER is expressed in Purkinje cells where it is available to activate Notch in the adjacent Bergmann glia, and indeed DNER mutant mice show morphological defects in Bergmann glia (Eiraku *et al.*, 2005). Soluble DNER (DNER-Fc) can also affect Bergmann glia morphology *in vitro* in a γ-secretase-dependent but CSL-independent manner, suggesting that Notch proteolysis plays a role in this process, but not to generate a transcriptional co-activator for CSL proteins. Instead of CSL, the E3 ubiquitin ligase Deltex has been implicated as an alternative downstream effector of Notch through in vitro studies in which a dominant-negative form of Deltex blocked the DNER-induced

morphological changes. Deltex can bind directly to the Notch intracellular domain, and mediate a trimeric complex between itself, full-length Notch, and β-arrestin, making it possible that Notch could activate signaling through β-arrestin that would require Deltex but not CSL (Mukherjee *et al.*, 2005). One caveat of DNER function as a non-canonical ligand is that that its effects have not been formally shown to require Notch receptor expression in Bergmann glia.

Recently, a putative DSL ligand-like protein called Jagged and Delta protein (Jedi) was reported based on sequence data (Krivtsov *et al.*, 2007). However, upon closer examination, the putative DSL and EGF repeats of Jedi do not contain the conserved cysteine spacing common to either the signature motif of canonical ligands or EGF repeats that are also present in DNER and Dlk-1. Instead, the Jedi extracellular domain contains an N-terminal emilin domain followed by multiple tandem repeats of an 8-cysteine variation of the EGF domain interspersed with two single 6-cysteine EGF repeats (Krivtsov *et al.*, 2007; Nanda *et al.*, 2005). In fact, Jedi has neither trans-activating nor cis-inhibitory activity, and has not been reported to interact with any of the Notch receptors. Although soluble Jedi added to Notch-expressing cells weakly inhibits a Notch reporter, there is currently no strong evidence linking Jedi to Notch signaling.

Structurally distinct from the integral membrane non-canonical ligands are F3/contactin1 and NB3/contactin6 that encode GPI-linked neural cell adhesion molecules. Both contactins have been reported to activate Notch signaling to induce oligodendrocyte (OL) differentiation (Cui *et al.*, 2004; Hu *et al.*, 2003). Binding and fractionation studies indicated that either contactin could interact with Notch in trans, although cis interactions cannot be ruled out since both endogenous F3 and NB3 co-immunoprecipitate with Notch (and vice versa). Both contactins interact with Notch EGF repeats distal to the DSL binding site, while only F3 can interact with Notch EGF repeats 1-13 that contain the DSL ligand-binding site at EGF 11-12. While this interaction makes it possible that F3 competes for the DSL ligand-binding site, further studies will be required to determine whether the F3 and DSL binding sites actually overlap.

Similar to DSL ligand treatment, adding soluble forms of either contactin to OL cells produces NICD in a γ-secretase-dependent fashion that can translocate to the nucleus for signaling. However, downstream of NICD generation, contactin-based signaling does not appear to involve CSL. F3-Notch signaling does not activate Hes-1 transcription, and there are no reports on the ability of NB3 to activate canonical CSL-induced Notch signaling (Hu *et al.*, 2003; Lu *et al.*, 2008). Instead of CSL, the contactins both induce Notch signaling that involves Deltex to induce glial maturation. An interesting dichotomy is raised in these in vitro assays in which the same cells (and presumably the same Notch receptors) differentiate in response to contactins and remain progenitors in response to DSL ligand or NICD expression. It is thought that temporal regulation of DSL ligand and contactin expression may regulate in vivo which effect takes precedent as DSL ligands are expressed early in embryonic development while contactins are highly expressed only after birth. Therefore, like DNER, the contactins appear to utilize Notch to effect changes late in differentiation as opposed to DSL ligands that can impact early cell fate decisions (Hu *et al.*, 2003).

Secreted non-canonical ligands

Despite the fact that DSL ligands require membrane tethering and endocytosis mediated by their ICDs to be active Notch ligands, soluble forms of DSL ligands can activate Notch signaling. Similarly, there are secreted, non-DSL proteins reported to be non-canonical Notch ligands.

In Drosophila, Scabrous (Sca) plays a role in Notch-dependent patterning of eye ommatidia and sensory bristles (Baker *et al.*, 1990; Mlodzik *et al.*, 1990). Sca is a secreted protein with

no vertebrate homolog based on sequence similarity that binds to Notch in trans to activate transcription of the Notch target gene E(spl)C m3 (Mok *et al.*, 2005; Powell *et al.*, 2001). However, it is not known whether the Sca-induced E(spl)C m3 expression requires γ-secretase proteolysis, the Notch downstream effector Su(H), or indeed activation of some other signaling pathway. Another reported Drosophila secreted non-DSL ligand for Notch is Wingless (Wg), the fly ortholog of mammalian Wnt proteins. Screening of a phage display library expressing Drosophila embryo transcripts identified Wg as a Notch-binding protein, and immunoprecipitation of endogenous Notch and Wg in fly embryos supports such an interaction in vivo (Wesley, 1999). In cell culture, the gene shaggy can be transcriptionally activated in a Wg- and Notch-dependent manner, indicative of a productive signaling interaction between Wg and Notch. However, it is not clear if binding of Wg to Notch is required for shaggy transcription, or what Notch downstream effector is required. While many vertebrate Wnt proteins exist, none has been shown to bind Notch as reported for Drosophila Wg.

In vertebrates, two secreted, non-DSL proteins have also been identified as putative Notch ligands. The first is a member of the Connective Tissue Growth Factor/cysteine-rich 61/ Nephroblastoma Overexpressed Gene (CCN) family of proteins. CCN3, also known as NOV, is required for proper development of the vertebrate heart and skeleton, and its expression has been correlated with both positive and negative regulatory roles in carcinogenesis (Heath *et al.*, 2008; Leask and Abraham, 2006). CCN3 has a number of protein-protein interaction modules that can interact with BMPs, integrins, as well as Notch, suggesting that CCN3 is a potential integrator of these signaling systems. Direct binding of CCN3 in trans to Notch has not been reported, but when co-expressed CCN3 can interact with Notch via the CCN3 C-terminal cysteine knot (CTCK); CCN3's CTCK may be a general tandem EGF repeat-binding domain, as it also interacts with six tandem EGF repeats of fibulin-1 (Thibout *et al.*, 2003). While endogenous Notch and CCN3 have not been reported to interact, endogenous levels of soluble CCN3 can interact with fibulin-1 in a sandwich ELISA assay. Unlike other non-canonical ligands that interact with Notch only when co-expressed in the same cell, CCN3 does not appear to have cis-inhibitory activity, but rather promotes Notch signaling. While it has not been formally shown that CCN3 generates NICD in a γ-secretase manner, co-expression of CCN3 can potentiate endogenous CSL-dependent Notch signaling in reporter assays. Additionally, both gains and losses in CCN3 lead to corresponding changes in Hes-1 expression, suggesting that CCN3 may be activating Notch in an autocrine fashion (Gupta *et al.*, 2007; Minamizato *et al.*, 2007; Sakamoto *et al.*, 2002b). Whether CCN3 activates Notch in an autocrine manner in vivo is unresolved, but it is tempting to speculate that for cells that require Notch signaling and cannot undergo canonical juxtacrine signaling via DSL ligand, autocrine signaling may allow for Notch signaling to occur. Cells such as chondrocytes or vascular smooth muscle cells that are isolated by the extracellular matrix they secrete would be likely candidates, and in fact chondrocytes do express CCN3.

A role for CCN3 as an activating co-factor for canonical ligand-induced signaling has also been suggested, as losses in CCN3 also reduce the ability of a cell to activate a reporter construct in response to trans-DSL ligand (Gupta *et al.*, 2007). Moreover, exogenously added CCN3 can potentiate Jagged-1 induced colony forming activity of hematopoietic precursor cells in vitro (Gupta *et al.*, 2007). It is not known whether the effect of secreted CCN3 in this assay requires direct Notch binding in trans.

The second type of soluble, non-DSL vertebrate protein found to have Notch signaling activity is the microfibril associated glycoprotein family, MAGP-1 and MAGP-2 (Gibson *et al.*, 1996; Gibson *et al.*, 1991). MAGP-Notch interactions induce γ-secretase-dependent NICD generation and CSL-dependent activation of reporter constructs (Miyamoto *et al.*, 2006). Similar to CCN3, MAGP-2 only activates Notch when expressed in the same cell as the receptor, suggestive of autocrine signaling, and is expressed in a cell type that might be limited

to such signaling, vascular smooth muscle cells (Albig *et al.*, 2008; Miyamoto *et al.*, 2006). Like DSL ligand, MAGP-2 can induce ADAM-independent dissociation of the Notch heterodimer that is required for proteolytic activation and downstream signaling. To date, MAGP-2 is the only non-canonical ligand that has been shown to mediate non-enzymatic dissociation of Notch. Although the biological relevance of MAGP-2-induced Notch signaling is unclear, endogenous Notch1 and MAGP-2 can interact in co-immunoprecipitation studies. Additionally, it now appears that depending on the cell type MAGP-2 can also have inhibitory effects on Notch signaling although the molecular basis for these cell-type differences are not understood (Albig *et al.*, 2008).

In summary, the notion that non-enzymatic dissociation of Notch leads to signaling raises the interesting possibility that any protein that can bind and destabilize the heterodimeric structure might activate signaling. Indeed, non-canonical ligands are a structurally diverse group of proteins that all lack a DSL motif; yet most appear to activate signaling. Interestingly, all the type-1 transmembrane non-canonical ligands do contain lysines in their intracellular domains that could serve as ubiquitination sites to facilitate transendocytosis as proposed for DSL ligands; however, no current studies have determined whether endocytosis is required for activity of these non-canonical ligands. It is less obvious how Notch binding to secreted non-canonical ligands could provide enough force to cause heterodimer dissociation, but perhaps tethering to the extracellular matrix allows these proteins to induce a pulling force on the Notch receptor, as suggested for soluble DSL ligands. While non-canonical ligands may be a partial answer to the question of the pleiotrophic nature of Notch, many of the studies discussed above used only in vitro assays and await confirmation in vivo. In this regard, it is interesting to note that in terms of survival and viability in the mouse, DSL ligands are required for embryonic development and viability, while none of the reported non-canonical ligands are similarly necessary. Whether this is due to the ability of non-canonical ligands to interact with multiple Notch receptors or other signaling systems to effect cellular changes is unknown, but it does imply that non-canonical ligands may be important modulators of Notch function in the adult animal.

Future directions

Although unique ligand-receptor combinations have been identified that induce specific cellular responses, the molecular mechanisms underlying ligand-specific signaling remains an outstanding question in the field. Moreover, given the direct and somewhat simple signaling mechanism ascribed to Notch it is unclear how different Notch ligands could induced distinct signaling responses. It will be important to determine if different ligand-Notch complexes recruit unique signaling effectors and whether the distinct responses involve activation of cytoplasmic and/or nuclear signaling pathways. That ligands have intrinsic signaling activity independent of Notch as well as their potential to participate in bi-directional signaling, are exciting but relatively unexplored areas of ligand biology that warrant further investigation. The importance of Notch ligands in cancer and other pathological states involving aberrant angiogenesis have identified Notch ligands as potential and promising therapeutic targets (Roca and Adams, 2007; Sainson and Harris, 2008; Thurston *et al.*, 2007; Yan and Plowman, 2007). Finally, the use of Notch ligands in the expansion and maintenance of stem cells for tissue regeneration/replacement underscores their fundamental biological importance (Dallas *et al.*, 2005; Delaney *et al.*, 2005).

Acknowledgments

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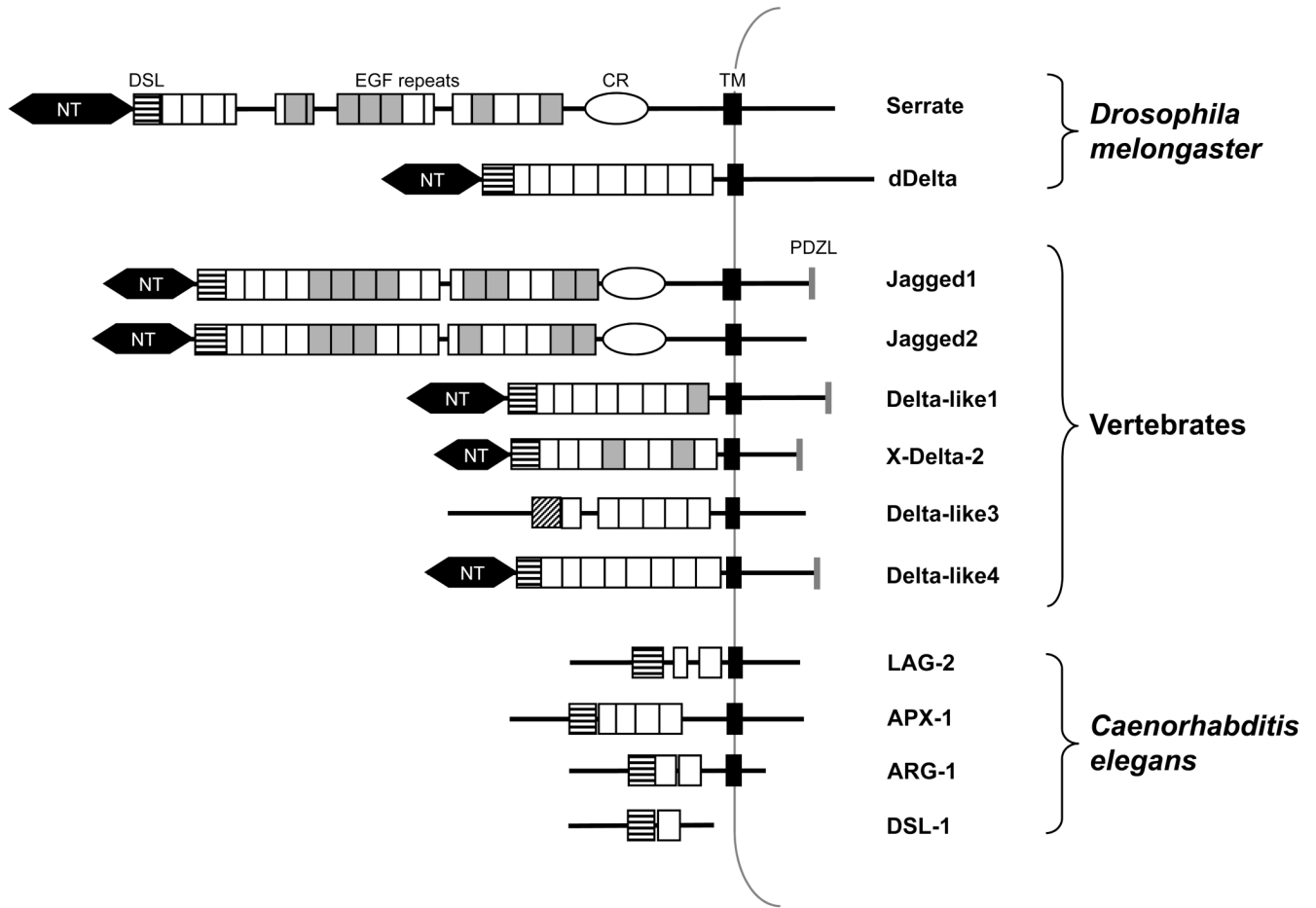
Figure 1.
Protein structure of the DSL family of ligands. Red boxes, DSL domain; white boxes, EGF repeat; grey boxes, calcium-binding EGF repeat; red box with diagonal lines, DSL with non-conserved cysteine spacing. See text for details. All DSL ligands contain a N-terminal signal sequence (not shown). Structures are based on the following protein sequences from GenBank: Drosophila Serrate, P18168; Drosophila Delta, P10041; human Jagged1, XP056118; human Jagged2, Q9Y219; human Delta-like1, O00548; Xenopus X-Delta-2, AAB37131; human Delta-like3, Q9NYJ7; human Delta-like4, Q9NR61; C. elegans LAG-2, P45442; C. elegans APX-1, P41990; C. elegans ARG-1, T16213; C. elegans DSL-1, AAC04450. The drawing is approximately to scale. NT, N-terminal domain; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor-like; CR, cysteine-rich region; TM, transmembrane domain; PDZL; PDZ ligand motif.
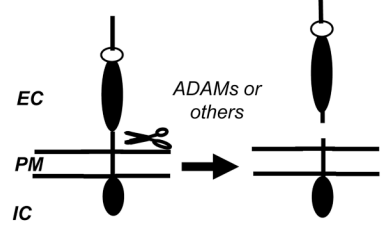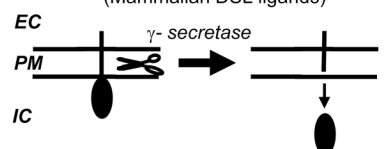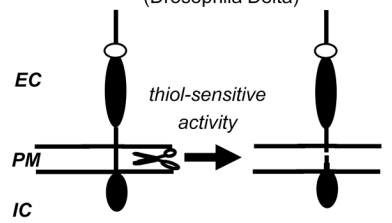
Proteolytic cleavage event	Proteolytic cleavage fragment	Soluble / membrane-tethered	Effect on Notch signaling
Juxtamembrane cleavage (Mammalian and Drosophila DSL ligands) EC — ADAMs or others — PM — IC	**ECD**	Soluble (unclustered)	Inactive
			Antagonist (competes with trans ligand for Notch binding
		Soluble (clustered or immobilized)	Agonist
	TMICD	Membrane-tethered	Substrate for γ-secretase cleavage in mammals
			Antagonist in trans for Drosophila Delta (competes with co-expressed full-length ligand for ubiquitination and/or endocytic machinery)?
Sequential intramembrane cleavage (Mammalian DSL ligands) EC — γ-secretase — PM — IC	**ICD**	Soluble	Gene transcription?
			Bi-directional signaling?
Independent intramembrane cleavage (Drosophila Delta) EC — thiol-sensitive activity — PM — IC	**ECDTM**	Membrane-tethered ?	Inactive (endocytosis-defective) ?
			Antagonist in trans (competes with cis ligand for Notch binding)?
			Antagonist in cis (competes with trans ligand for Notch binding)?
		Soluble ECD?	Same as for ECD above?
	TMICD^{TSA}	Membrane-tethered?	Same as for TM-ICD generated from Drosophila Delta above?
		Soluble?	Same as for ICD above?

Figure 2.

Effects of proteolytic cleavage of DSL ligands on Notch signaling. Mammalian and Drosophila DSL ligands undergo juxtamembrane and intramembrane cleavages. Juxtamembrane cleavage of mammalian and Drosophila DSL ligands by A-Disintegrin-And-Metalloproteases (ADAMs) results in shedding of the extracellular domain (ECD). The shed/soluble ECD may be inactive or can act as either an agonist or antagonist of Notch signaling depending on its state of clustering. In mammalian cells, the membrane-tethered fragment containing the intracellular domain (TMICD) undergoes sequential intramembrane γ-secretase, releasing the intracellular domain (ICD) from its membrane tether. The released ICD can translocate to the nucleus and activate gene transcription suggesting that ligand-Notch interactions can trigger bi-directional signaling. Unlike TMICD generated from mammalian DSL ligands, the Drosophila Delta TMICD fragment is not further processed and could antagonize Notch signaling in trans. A thiol-sensitive activity (TSA) catalyzes ADAM-independent intramembrane cleavage of Drosophila Delta resulting in cleavage products that may or may not remain membrane-tethered. If the ECD containing fragment (ECDTM) remains membrane-tethered, it could act as a Notch signaling antagonist either in cis or in trans. If ECDTM is released from the membrane it could act as proposed for soluble ECD. If the ICD containing intramembrane cleavage product TMICD^{TSA} remains membrane-tethered, it could act as a Notch signaling antagonist in trans. Alternatively, the ICD may be released from the membrane, translocate to the nucleus and activate gene transcription. EC = extracellular; PM = plasma membrane; IC = intracellular.

Ligand	Ligand Structure	Notch-binding domain of ligand	Ligand-binding domain of Notch	Effect on Notch signaling	Proposed Notch signaling effector(s)
Jagged1		NT/DSL/EGF1+2	EGF11-12	*trans*-activation *cis*-inhibition	CSL
Delta-like1		NT/DSL/EGF1-2	EGF11-12	*trans*-activation *cis*-inhibition	CSL
Dlk-1/Pref-1		EGF1-2 or EGF5-6	EGF10-11 or EGF12-13	*cis*-inhibition *trans*-activation?	CSL
DNER		DNER EGF1-2	Full-length*	*trans*-activation	CSL or Deltex
Jedi		Not tested	Not tested	inhibition (as secreted protein)	CSL
F3/Contactin1		Full-length*	EGF1-13, EGF 22-34	*trans*-activation	Deltex
NB3/Contactin6		Full-length*	EGF22-34	*trans*-activation	Deltex
scabrous		Full-length*	Full-length*	*trans*-activation	CSL
wingless		Full-length*	EGF19-36	*trans*-activation	Unknown
CCN3/NOV		C-terminal cysteine knot	EGF repeats	*cis*-activation/ modulator?	CSL
MAGP-2		Matrix binding domain	EGF repeats	*cis*-activation/ modulator?	CSL
MAGP-1		Full-length*	Full-length*	*cis*-activation/ modulator?	CSL

Figure 3.

Non-canonical ligands reported to affect Notch signaling. Accession numbers for human proteins: Jagged1, XP056118; Delta-like1, O00548; DNER, Q8NFT8; DLK-1, P80370; Jedi, Q5VY43; F3/Contactin, Q12860; NB-3, Q9UQ52; MAGP-1, P55001; MAGP-2, Q13361; CCN3/NOV, P48745. See text for details. All non-canonical ligands contain a N-terminal signal sequence (not shown). The drawings are approximately to scale. NT, conserved N-terminal domain found in DSL ligands; DSL, Delta/Serrate/LAG-2 domain; EGF, 6-cysteine epidermal growth factor repeat; cys, cysteine; CR, cysteine-rich domain; TM, transmembrane domain; PDZL, PDZ ligand; aa, amino acids; EMI, emilin-like domain; EGF-like, EGF-like motif with 8 cysteines that is not laminin-like; Ig-CAM, immunoglobulin-containing cell adhesion molecule domain; FNIII, fibronectin type III domain; GPI, glycosylphosphatidylinositol; Q, glutamine-rich region; FReD, fibrinogen-related domain; MBD, matrix binding domain; RGD, integrin binding motif; IGFBP, insulin-like growth factor-binding protein-like domain; VWF-C, von Willebrand factor type C-like domain; TSP-1, thrombospondin type 1-like domain; CTCK, C-terminal cysteine knot domain. *Only full-length constructs were tested for binding.

Table 1

Regulation of DSL ligand expression by other signaling pathways

Effector of DSL ligand expression	DSL ligand	Effect on ligand expression: Upregulation (+) Downregulation (-)	Cell type	Biological effect	References
VEGF	Dll4	+	Endothelial	Selection of endothelial tip cells for angiogenic sprouting; arterial specification	(Hellstrom et al., 2007; Liu et al., 2003; Lobov et al., 2007; Patel et al., 2005; Seo et al., 2006; Williams et al., 2006)
FGF	Dll1	+	Neural stem cells	Maintenance of spinal cord stem cells	(Akai et al., 2005)
Lipopolysaccharide	Dll4	+	Dendritic cells	CD4$^+$ Th1 polarization	(Amsen et al., 2004)
Lipopolysaccharide	Jagged1	+	Dendritic cells	CD4$^+$ Th2 polarization	(Amsen et al., 2004)
Prostaglandin E2	Jagged1	+	Dendritic cells	CD4$^+$ Th2 polarization	(Amsen et al., 2004)
Hedgehog	Jagged1	+	Mesenchymal cells	Limb development	(McGlinn et al., 2005)
VEGF+ FGF2	Dll1	+	Endothelial cells	Postnatal Arteriogenesis	(Limbourg et al., 2007)
Wnt	Jagged1	+	Hair follicle precortex	Hair follicle differentiation	(Estrach et al., 2006)
Wnt	Dll1	+	Presomitic mesoderm	Somitogenesis	(Hofmann et al., 2004)
DER and/or Heartless	Drosophila Delta	+	Embryonic mesoderm	Specification of muscle and heart progenitors as well as photoreceptor and non-neuronal cone cells	(Carmena et al., 2002; Tsuda et al., 2002)
TGF-β	Jagged1	+	Epithelial cells	Epithelial - mesenchymal transformation	(Zavadil et al., 2004)
FGF1/FGF2	Dll1	-	Neuroepithelium	Maintenance of neuroepithelial precursors	(Faux et al., 2001)
PDGF/angiotensin II	Jagged1	-	Vascular smooth muscle cells	Growth retardation	(Campos et al., 2002)
Lipopolysaccharide	Jagged1	-	Bone-marrow mesenchymal stem cells	Proliferation of CD4$^+$ T cells	(Liotta et al., 2008)

Abbreviations: Th, T helper cell; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; TGF, transforming growth factor; DER: Drosophila epidermal growth factor receptor, PDGF: platelet-derived growth factor