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Review article

Reversible and bidirectional signaling of notch ligands

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Abstract

The Notch signaling pathway is a direct cell-cell communication system involved in a wide variety of biological processes, and its disruption is observed in several pathologies. The pathway is comprised of a ligand-expressing (sender) cell and a receptor-expressing (receiver) cell. The canonical ligands are members of the Delta/Serrate/Lag-1 (DSL) family of proteins. Their binding to a Notch receptor in a neighboring cell induces a conformational change in the receptor, which will undergo regulated intramembrane proteolysis (RIP), liberating the Notch intracellular domain (NICD). The NICD is translocated to the nucleus and promotes gene transcription. It has been demonstrated that the ligands can also undergo RIP and nuclear translocation, suggesting a function for the ligands in the sender cell and possible bidirectionality of the Notch pathway. Although the complete mechanism of ligand processing is not entirely understood, and its dependence on Notch receptors has not been ruled out. Also, ligands have autonomous functions beyond Notch activation. Here we review the concepts of reverse and bidirectional signalization of DSL proteins and discuss the characteristics that make them more than just ligands of the Notch pathway.

Article history

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Keywords

Notch pathway, DSL ligands, reversible signaling, bidirectional; Regulated Intramembrane Proteolysis (RIP), ADAM, γ-secretase, PDZ

Introduction

Every cellular pathway begins with a ligand recognized by a receptor that converts the input signal to an output response. In the field of life science, the concept of a ligand-receptor complex is widely used to describe the ability of a molecule (protein, ion, or drug) to instigate an effect through its interaction with a receptor that will produce a signaling response. The concept of what is now regarded as a ligand comes from Paul Ehrlich's Latin phrase "*Corpora non agunti nisi fixata*" (agents only work when they are bound), which he coined to describe how therapeutic agents will have an effect just when bound to a receptor (Klotz 2004). This early definition of ligand and receptor intuitively sets a direction where the key (ligand) modifies the lock (receptor), which is adequate to describe the effect of a drug that binds to cell receptors but is certainly not sufficient to describe cell-cell communication based on protein-protein interactions. For example, in juxtacrine cell signaling, membrane-tethered ligands interact with receptors on neighboring cells (Yaron and Sprinzak 2012; Antebi et al. 2017); in this context, protein ligands can behave as receptors and send the signal reversibly (Yu and Rao 2009). Furthermore, bidirectional signaling is only possible when the ligand has the ability to respond in reverse.

The Notch signaling pathway has been classically described as a signal receiving and sending system, triggered by trans-interactions of receptors and ligands from adjacent cells (Kopan and Ilagan 2009; Bray 2016). However, variations to this canonical direction of signaling are less recognized. This review focuses on the reverse signaling of Notch ligands, the activities of ligand beyond Notch receptor activation and the evidence of bidirectionality of the pathway. This emerging concept pictures an even more complex classic Notch signaling.

Canonical Notch signaling

The Notch pathway consists of a few core elements and no amplification steps throughout the signal cascade (Andersson et al. 2011). In mammals, four Notch receptors and five Notch ligands have been characterized. The ligands and the receptors are types I transmembrane proteins with extracellular N-terminal and cytoplasmic C-terminal domains. Any of the four Notch receptors (Notch 1-4) can interact with any of the five DSL (Delta-like/Serrate/Lag-1) ligands: DLL1, DLL3, DLL4, JAG1, and JAG2, (Andersson et al. 2011; Bray 2016). Following ligand-receptor coupling, the Notch receptor undergoes a two-step activation mechanism known as regulated intramembrane proteolysis (RIP) (van Tetering and Vooijs 2011).

During Notch RIP activation, the juxtamembrane cleavage site S2, within the Notch receptor, is exposed due to a conformational change provoked by the interaction with a ligand in a neighboring cell. The S2 site is cleaved by members of the disintegrin and metalloprotease (ADAM) enzymes, which promote the receptors' ectodomain shedding, the proteolytic release of the Notch extracellular domain (NECD) (van Tetering and Vooijs 2011). The NECD is then trans-endocytosed along with the ligand into the signal-sending cell. In the receiving cell, the cleaved receptor, known as Notch extracellular truncation (NEXT), remains anchored to the membrane. This transient protein is rapidly cleaved by the γ-secretase complex, particularly by presenilin, at the Notchs' S3 cleaving site within the transmembrane domain (Yamamoto et al. 2010; van Tetering and Vooijs 2011). This second cleavage liberates the Notch intracellular domain (NICD), which translocates to the cell nucleus to modulate gene transcription.

The NICD is a small fragment of the receptor characterized by the presence of an RBPJ-associated module (RAM), ankyrin repeats (ANK), two nuclear localization signaling motifs (NLS), a transactivation domain, and a C-terminal PEST domain which mediates proteolytic degradation (Oberg et al. 2001; Bray 2016; Sjoqvist and Andersson 2019). In the nucleus, the NICDs RAM and ANK domains bind to the

transcriptional factor CSL (CBF1/RBPJ, Su(H), Lag1), the coactivator mastermind (MAM), and the histone acetylase p300/CBP (CREB binding protein) to form an active transcriptional complex (Kopan and Ilagan 2009; Bray 2016). The signal pathway ends with the phosphorylation of the PEST domain of NICD and subsequent targeting for proteasomal degradation by the E3 ligase FBW7 (Bray 2016) (Figure 1).

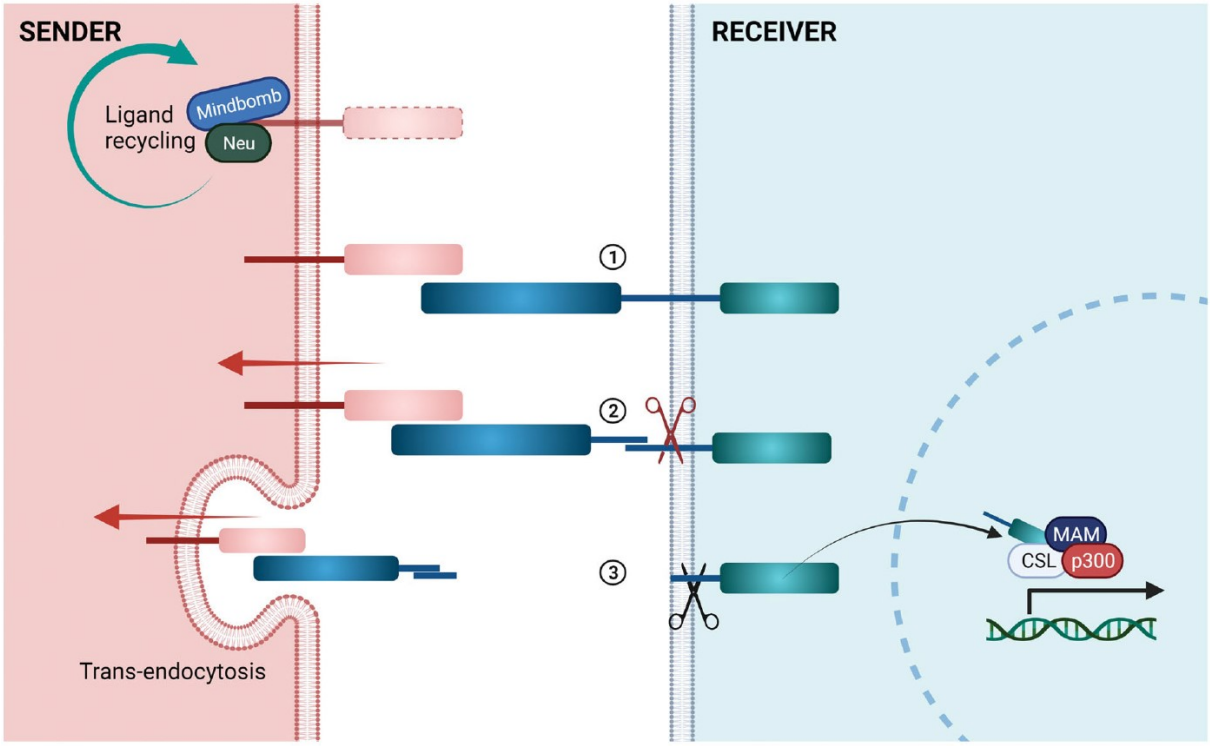

Figure 1. Canonical Notch signaling. Step 1. DSL ligand (red) in the sender cell interacts with the Notch receptor (blue) in the receiver cell to start the RIP process. Step 2. The ligand pulls the receptor (pulling force), causing a conformational change in the NECD, which exposes the cleavage site for ADAM metalloproteases (red scissors). The sender cell trans-endocytoses the ligand with the NECD. Step 3. The NEXT is sequentially cleaved in the receiver cell by the γ-secretase complex (black scissors), liberating the NICD. Once in the nucleus, NICD forms part of the transcriptional complex with CSL, MAM, and p300, to transcribe the Notch pathway target genes. Before Notch activation, the DSL ligands in the sender cells (top of the panel) undergo a maturation step driven by ligand endocytosis and recycling mediated by the ubiquitin ligases Mindbomb and Neuralized. A color version of the figure is available online.

Endocytosis plays a significant role in the activity of ligands and receptors in Notch signaling. In the sending cell, the ligand's endocytosis is a prerequisite for ligand activation. Mono-ubiquitination of the intracellular domain of the ligands by the E3 ligases Mindbomb and Neuralized promotes ligand endocytosis, sorting, and recycling to the membrane as prior "maturation" steps for Notch trans-activation. (Chitnis 2006; Yamamoto et al. 2010). On the other hand, in the receiving cell, endocytosis of the Notch receptors limits the availability of Notch at the membrane (Kopan and Ilagan 2009; Bray 2016).

An alternative Notch activation model suggests that the receptor is cleaved in late endosomes (Steinbuck and Winandy 2018). The NEXT protein generated by the S2 cleavage needs to be endocytosed to be a substrate for the γ-secretase complex, even though this is still a matter of debate (Yamamoto et al. 2010; Andersson et al. 2011). (Chapman et al. 2016) showed that cells expressing Notch receptors can trans-endocytose the ligands from the sender cell, allowing Notch activation in intracellular vesicles in mammalian cells. Contrary to the pulling-force model, this alternative

mechanism happens when ligand endocytosis is compromised, for example, due to the lack of Mindbomb or Neuralized at the sending cell. Thus, receptor endocytosis would provide the separation force for Notch activation (Chapman et al. 2016).

Directionality of the Notch pathway

Cell-cell communication can be roughly divided into juxtacrine and paracrine signaling. In juxtacrine signaling, also known as contact-dependent signaling, the ligands and receptors are in direct contact, i.e. through gap junctions or interactions from membrane-bound ligands and receptors. In this way, the signal is transmitted from the sender to the receiver cell. However, several questions remain. How is the directionality established? How does a cell determine its fate as the signal sender or receiver? In Notch signaling, cells with high expression of DSL ligands and low presence of Notch receptors at the cell surface will give rise to the sender cell type, and vice versa is true for the receiver cell (Boareto et al. 2015). These "signaling states" can be achieved by *cis*-inhibition, a cell-autonomous process based on protein interactions, or by lateral inhibition, where a feedback loop between neighboring cells allows the establishment of the sending and receiving cell throughout genetic regulation (Sprinzak et al. 2011; Yaron and Sprinzak 2012; Antebi et al. 2017). In some cases, a cell can express DSL ligands and Notch receptors simultaneously. The cell can be both a sending and receiving cell, attaining similar fates to what is known as lateral induction. These types of cells are coined hybrid sender/receiver cells (Bocci et al. 2020).

Lateral inhibition ensures proper pattern formation during development

Lateral inhibition was first used in neuroscience to describe the ability of an excited neuron to reduce the action potential in the neighboring cell and enhancing signal contrasts, thus creating a response pattern (Bakshi and Ghosh 2017; Burton et al. 2020). In 1972, Gierer and Meinhardt adopted this concept and postulated a nonlinear model for lateral inhibition based on properties such as activator and inhibitor density, the direction of the signal gradient, and the process of auto- and cross catalysis (Gierer and Meinhardt 1972; Meinhardt and Gierer 1974; 2000). The many factors behind this inhibition pattern have also led to many modern mathematical models of different developmental processes in metazoans (Meinhardt and Gierer 1974; Petrovic et al. 2014; Boareto et al. 2015; Matsuda et al. 2015; Sato et al. 2016; Seirin Lee 2016; Guisoni et al. 2017).

The Notch pathway is known to play an essential part in regulating the feedback loop for pattern formation during development. Correct pattern formation is fundamental in determining cellular position and function (Holder 1999). Lateral inhibition ensures that a cell can influence its neighboring cells to adopt a different fate than itself (Chitnis 2009). The core mechanism behind lateral inhibition is mediated by the expression of Notch and Delta ligands on the cell surface. Following the canonical Notch pathway, Delta ligands activate Notch, thus activating the transcription of target genes *Hes* and *Hey* (or the *Hairy and Enhancer-of-split* [*E(spl)*] genes in *Drosophila*) in the receptor cell. These genes encode transcriptional regulators, further repressing the expression of Delta in the receiving cell. In *Drosophila*, *Hes* negatively regulates the expression of Delta through repressing the expression of Achaete- Scute (AcSc) (Fischer and Gessler 2007). Negative feedback in the receptor cells down-regulates the expression of the ligand, thus preventing such cell from signaling back (del Álamo and Schweisguth 2009). The sending and receiving cell regulates cellular differentiation, accordingly leading to the opposite fates between neighboring cells. On the molecular level, lateral inhibition can be divided into two steps: first, cell fate determination through Notch interaction; second, inhibition pattern refinement through other factors. Lateral inhibition gives rise to the classical salt-and-pepper patterning (Figure 2).

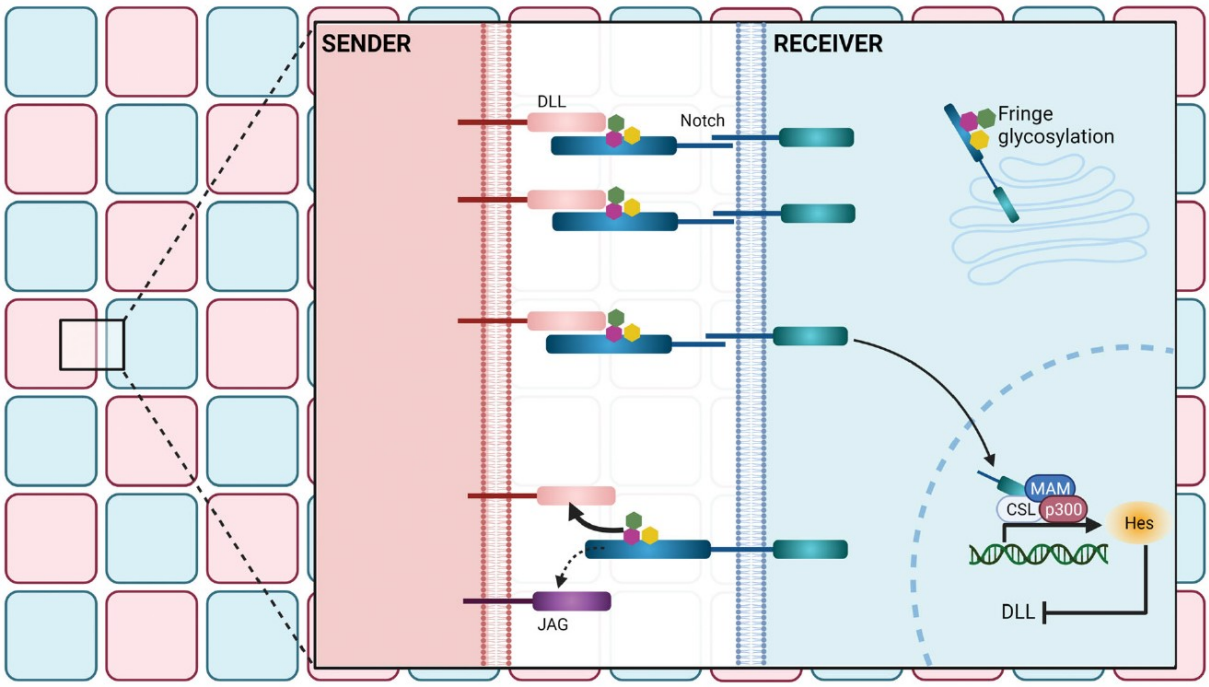

Figure 2. Notch pathway lateral inhibition. A salt-and-pepper pattern (red-blue cells) is achieved by lateral inhibition. The sender cell (red) expresses a high concentration of the Delta ligand while the receiver cell (blue) expresses a high concentration of the Notch receptor. Delta-Notch engagement triggers Notch signaling in the receiver cell. Downstream genes like Hes will further downregulate the expression of Delta in the receiving cell. Glycosylation of the Notch receptors by the Fringe family of glycosyltransferases enhances Delta over Jagged signaling. A color version of the figure is available online.

Lateral inhibition is observed in epithelial sheet development, such as lung endoderm, *Xenopus* epidermis, zebrafish pronephros, and inner ear pseudostratified sensory epithelial and mechanosensitive hair (Chrysostomou et al. 2012). In the inner ear hair development, Hes acts as a transcriptional repressor of Atoh1 (atonement in *Drosophila*), which further downregulates Delta ligand synthesis (Chrysostomou et al. 2012) (Figure 2). Nevertheless, as hair cells mature, the cyclic expression (in short waves of restricted activation) of DLL1, DLL3, and JAG2 allows for changes in the hair cell fate and furthers epidermal pattern refinement (Hartman et al. 2010). Besides pattern formation, lateral inhibition also provides cues to the timing of cell division, particularly for the sensory organ precursor (SOP) in the epithelial cells (Hunter et al. 2016).

In refining the inhibition pattern, post-translational modification (PTM) through enzymes of the Fringe family reinforces the Delta-Notch interaction to further sustained the negative feedback loop (Bocci et al. 2020). The Fringe family of glycosyl*trans*ferases reside in Golgi, where they add *N*-acetylglucosamine (GlcNAc) to previously *O*-fucose-modified EGF repeats of the Notch receptors (LeBon et al. 2014). In *Drosophila*, it has been documented that Fringe modifications of Notch promote Delta-Notch interaction and decrease the affinity of the Serrate-Notch binding (LeBon et al. 2014; Pandey et al. 2020). Mammalian cells express three Fringe enzymes: Lunatic (Lfrg), Manic (Mfrg), and Radical (Rfrg). They generally enhance the binding of Notch to Delta and decrease the binding to Jagged, except for Rfrg, which also increases Jagged binding (LeBon et al. 2014) (Figure 2). Lfng has been shown to act as either a positive or negative modulator of the Delta-Notch signaling. It impacts the Notch receptor trans-binding positively but negatively affects the *cis*-binding of Delta ligands. This dual effect was illustrated via synthetic circuitry mimicking the lateral inhibition feedback intracellularly (Matsuda et al. 2015). In short, Fringe activities finetune the sending and receiving signaling state of the Notch pathway, possibly giving rise to the hybrid phenotype where Notch has an

equal affinity toward both Delta and Jagged and one where Notch strongly prefers Delta (Boareto et al. 2015). Besides PTMs, there are still many factors affecting lateral inhibition. Some of these factors are, but not limited to, signal center and direction of the signal gradient (Matsuda et al. 2015), the distance between cells and filopodia (Cohen et al. 2010; Milán and Cohen 2010), size of cells, and area of cell-cell contact (Seirin Lee 2016; Falo-Sanjuan and Bray 2021), the polarity of ligand distribution (Jacobo et al. 2019), wave propagation of epidermal growth factor receptor (EGFR) and proneural factor bias (Chitnis 2009; Sato et al. 2016), and oscillatory activation by Delta ligands (Meinhardt and Gierer 1974; Chitnis 2009). Most of these occur during cell growth and differentiation, as other pathways crosstalk with the Notch signaling pathway, especially those involved in morphogenesis.

Cis-inhibition as a mechanism to establish directionality

Another critical mechanism responsible for cell fate determination is *cis*-inhibition, where receptors and ligands are inhibited by their respective ligands and receptors (del Álamo et al. 2011). This cell-autonomous inhibition prevents autocrine activation. In addition, in juxtacrine signaling, *cis*-inhibition impedes the cell from sending and receiving Notch signals simultaneously, thereby ensuring the signaling direction (LeBon et al. 2014).

The complete mechanism behind *cis*-inhibition is not yet fully understood. Therefore, two modes of inhibition have been proposed: receptor titration and mutual degradation. In the first case, the ligand binding to a receptor in the same cell can sequester the functional receptor into an inactive complex, enabling a "standby" state (Buchler and Louis 2008; Formosa-Jordan and Ibañes 2014). In addition, degradation or ligand cleavage would further contribute to the negative regulation of the receptors (Figure 3, panel 1). Evidence supporting this notion comes from studies on Drosophila developing wing, wherein overexpression of Serrate ligands has a dominant- negative effect on Notch activity (Klein et al. 1997). Interestingly, co-expression of Delta or Serrate ligands with Notch receptors does not affect the presence of Notch at the cell surface and prevents Notch ligand-independent activation. Notch shedding, the removal of the NECD, can be promoted by chelating agents such as EDTA (Rand et al. 2000). However, cis ligand-receptor interaction protects the receptor from ectodomain shedding induced by EDTA. Thus, it has been suggested that *cis*-inhibition stabilizes the Notch membrane pool by preventing unintentional stochastic activation (Fiuza et al. 2010; Palmer et al. 2014). In the second mode of inhibition, the ligand-receptor interaction in the same cell promotes mutual degradation; thus, the cell "turns off" the pathway until new components are available (Sprinzak et al. 2010) (Figure 3, panel 2).

Immunoprecipitation and pull-down assay experiments have shown that *cis*-inhibition occurs through direct interaction between the extracellular domains of the ligand and the receptor in the same cell (Yaron and Sprinzak 2012). Intracellular interactions between ligands and receptors can prevent Notch from reaching the cell membrane. For example, in *Drosophila*, cellautonomous receptor-ligand heterodimers do not reach the cell membrane, decreasing Notch signaling (Sakamoto et al. 2002).

The strength of *cis*-inhibition is ligand-specific. In *Drosophila*, where just two ligands exist (Delta and Serrate), studies have shown that Serrate exhibits a stronger *cis*-inhibitory effect than Delta (Li and Baker 2004). The opposite was observed in mammalian cells; wherein it takes twice the amount of JAG1 to accomplish the same *cis*-inhibition level by DLL1 (Sprinzak et al. 2010). DLL3 is peculiar in this regard, being a dedicated inhibitor of Notch (Ladi et al. 2005). This ligand cannot bind to any of the Notch receptors in *trans*, but within the same cell, DLL3 can coimmunoprecipitate with Notch (Ladi et al. 2005). *Cis*-inhibition exerted by DLL3 is crucial for the correct vertebral segmentation, and mutations provoke spondylocostal dysostosis (SCD). Studies in presomitic mesoderm showed that DLL3 is present at the Golgi apparatus and in cytoplasmic vesicles rather than on the cell membrane as the rest of the DSL ligands. DLL3 and Notch interaction occur most possibly in late endosomes and

lysosomes, preventing receptors from reaching the membrane (Chapman et al. 2011) (Figure 3, panel 3). DLL3 is the shortest among DSL ligands; its intracellular domain lacks lysines (Figure 6), the potential residue for ubiquitination, which could explain why the ligand cannot activate the Notch pathway (Ladi et al. 2005).

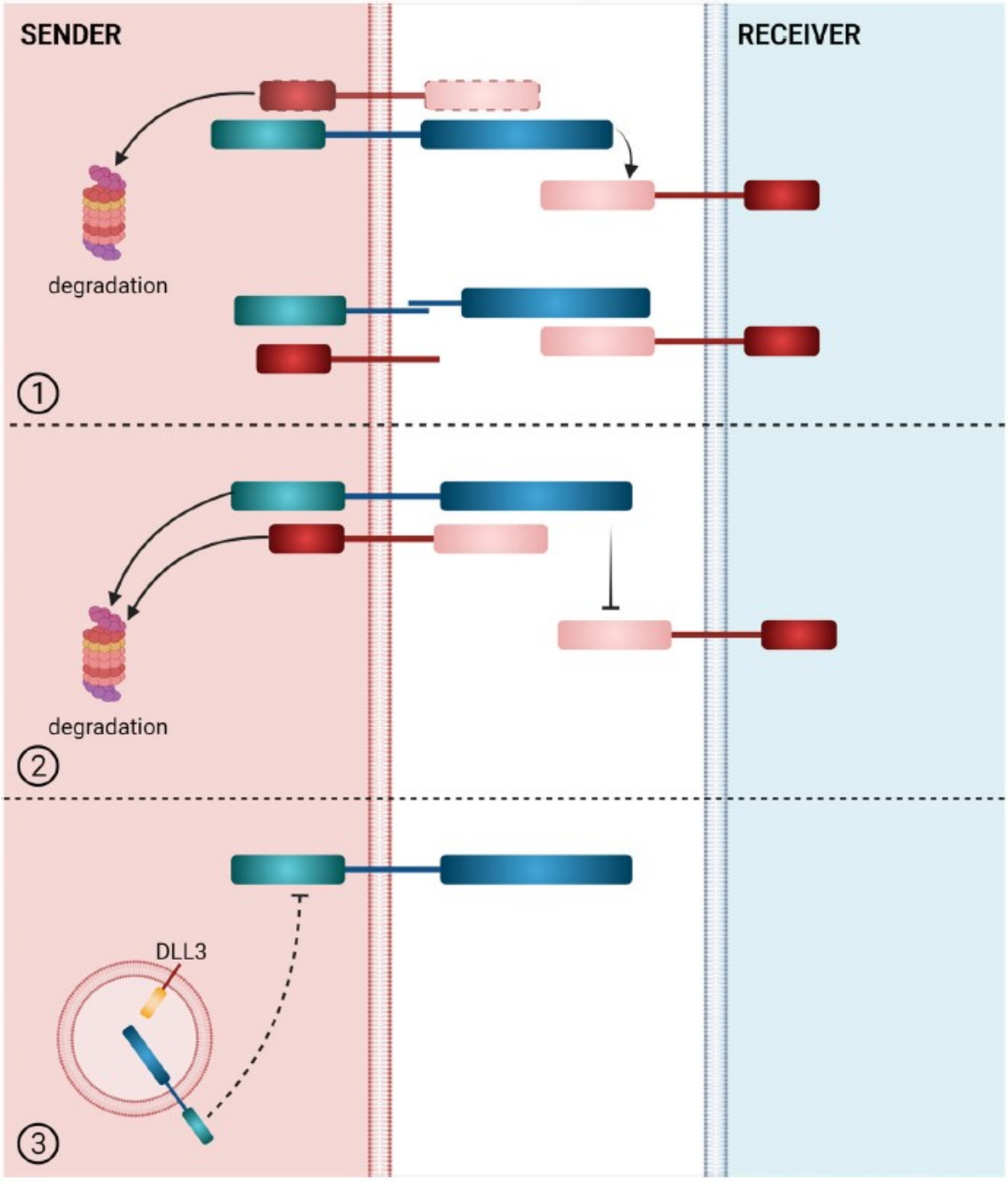

Figure 3. Cis- inhibition in the Notch pathway. The interaction of ligands and receptors in the same cell induces inhibition of signaling. Panel 1. Transient binding of the ligand to the receptor in cis stabilizes the receptor at the membrane, which can later interact with a ligand in the neighbor cell. Degradation or cleavage of the DSL ligands can terminate the cis-inhibition of the Notch receptor. Panel 2. The mutual degradation of interacting ligands and receptors in cis would permanently shut down the

signal cascade until new components are available. Panel 3. The ligand DLL3 is a dedicated cis-inhibitor of Notch. Cytoplasmic interactions of DLL3 with Notch receptors prevent the receptor from reaching the cell membrane. A color version of the figure is available online.

Notably, the expression of receptors and ligands within the same cell does not necessarily mean *cis*-inhibition (Yaron and Sprinzak 2012). Ligand and receptors can be segregated asymmetrically between cells and within the cell. The most studied example of this phenomenon is the sensory organ precursor (SOP) cells from the neuroepithelium of Drosophila. SOP cells divide asymmetrically to give rise to pIIa Notch-containing and pIIb Notch-free cells. Neuralized (Neur) is specifically inherited by the pIIb cell, which further activates Delta through endocytosis, converting pIIb cells into sender (active ligand) cells.

Moreover, Delta and Notch do not coexist in the same membrane domains. In polarized MDCK cells, Notch localizes at the apical membrane while DLL1 localizes at the basolateral membrane. Although, DLL1 can be relocated to the apical membrane by transcytosis mediated by Neur (Benhra et al. 2010). Similarly, during the formation of the immunological synapse (IS) in lymphocytes, individual components of the Notch pathway are differentially segregated in IS microdomains to ensure signaling directionality and avoid cis-inhibition (Luty et al. 2007). Recent work from Kwak et al. (2020) showed that protein sizes act as a spatial switch. In the case of Notch, the Notch protein is relocated to cadherin-based adherent junctions (cadAJs), where γ-secretase cleavage occurs after ADAM cleavage and removal of the extracellular domain (Kwak et al. 2020). This evidence suggests that *cis*-interactions prevent the Notch receptor from shedding in a different membrane microdomain than at the adherent junctions.

Cis- and trans- inhibition, competitive or complementary?

At the protein level, cis and *trans* interactions seem to be competitive (del Álamo et al. 2011). All Notch receptors are heterodimers of about 300 kDa, with an extracellular domain of multiple tandemly arranged epidermal grow factor-like repeats (EGFR); in mammalian homologs, they vary from 29 to 36 copies (Sakamoto et al. 2005). The ligand-binding site for *Drosophila* Notch and human Notch 1 rely on the EGFR 11-12, the same repeats involved in *cis*-inhibition (del Álamo and Schweisguth 2009; Chillakuri et al. 2012). Notch ligands interact and activate Notch through their DSL domain, followed by several EGFRs that vary in number between the Delta and Serrate (Jagged) ligand-families (Chillakuri et al. 2012). Loss of the intracellular or transmembrane domains of the ligands does not abrogate their Notch inhibitory effect, indicating dependence on the ECD. For example, the EGFR 4-6 of Serrate are indispensable for *cis*-inhibition but not for Notch activation (Fleming et al. 2013). Cordle et al. (2008) showed that besides the DSL domain, EGFR 1-3 are also essential for the interaction between ligand and receptor for *trans*-activation and *cis*-inhibition. However, two different orientations are possible: antiparallel complex results in Notch activation, and parallel conformation results in *cis*-inhibition (Cordle et al. 2008). The usage of the same domains for the trans and cis interactions intuitively suggests the interaction is mutually exclusive. When the ligand and receptor engage in *cis* or *trans*, they are blocked from exerting the opposite interaction (Yaron and Sprinzak 2012).

Aside from binding site competition, affinity competition between the DSL ligands also contributes to the interaction, as observed with JAG1 and DLL1 during the development of otic sensory progenitor cells. JAG1 facilitates lateral inhibition by competitively antagonizing the overall expression of Notch, even though Notch already has an intrinsic affinity toward DLL1 (Petrovic et al. 2014). Just as the Notch pathway is complex, it comes as no surprise when time-lapse microscopy revealed that Notch responds differently to *cis* and *trans* bound Delta, where the response to *cis* is sharp, while the response to *trans* is gradual. These varying responses can amplify the subtle difference within neighboring cells, even before the downstream modification adds another layer of mechanism to determining the signaling state of the cell (Sprinzak et al. 2010).

Notch pathway bidirectionality: two roads of signaling

Reverse signaling vs. Bidirectionality

The canonical direction of Notch signaling from ligand to receptor is known as forward signaling. On the contrary, when a ligand can act as a receptor in its expressing cell, it is reverse signaling (Murai and Pasquale 2003; Yu and Rao 2009; Battistini and Tamagnone 2016). Ligand reversibility has been widely recognized in juxtracrine signaling pathways such as Ephrin, Semaphorin (Murai and Pasquale 2003; Battistini and Tamagnone 2016), TNF signaling (Eissner et al. 2004), and on DSL ligands of the Notch pathway (Ascano et al. 2003). The fact that ligands can also behave as receptors give rise to two possibilities: (1) *trans*-activation by the receptor provoking the ligand to rearrange and signal in a bidirectional manner; or (2) the ligand has independent signaling activity (Figure 4). In the first case, the receptor sends a signal to the *trans*-located ligand, and the latter becomes a signaling receptor. An example of this kind of signaling was described by the pioneering works of Henkemeyer and colleagues in 1996 when they tried to elucidate Nuk/EphB2 receptor's participation in neural structure development (Henkemeyer et al. 1996; Dravis 2010). They generated a Nuk/EphB2 receptor devoid of its C-terminal tyrosine kinase region and showed that homozygous mice of this mutation exhibit normal anterior commissure development. They found that ephrin-B2 ligand expressed in this area may have a complementary function, suggesting that ligands and their functions may depend on the interaction with the receptor extracellular domain (Henkemeyer et al. 1996; Dravis 2010). Decades later, using a similar approach, they demonstrated that the intracellular domain of the ephrin-B2 ligands was crucial for proper nervous system development (Xu and Henkemeyer 2012). *Trans*-interaction of Ephrin receptors provoke the clustering of ephrins in the ligand expressing cell, and this in turn promotes the colocalization of Src-family kinases. The Src kinases phosphorylate other membrane proteins, or in the case of ephrin-B, the phosphorylation of its cytoplasmic tail converting the intracellular region onto a scaffold for other molecular binding (Dravis 2010).

Alternatively, ligands can have independent receptor activities that do not directly implicate the cognate pathway. These activities rely purely on the structure of the ligands' intracellular domains and are not linked to the receptor engagement (Figure 4). They are not dependent on the conformational changes due to interaction. For example, B ephrins encode C-terminal PDZ (PSD95/Dlg/ZO1) binding motifs (PBM) to bind to PDZ-containing proteins. This type of association can be observed between the regulator of G protein signaling (RGS) protein PDZ-RGS3 and B ephrin in inhibiting G-protein-coupled chemoattraction for cerebellar granular cells in an EphB independent phosphorylation fashion (Lu et al. 2001). The former evidence reflects the ability of ligands to act as signaling molecules beyond their interactions with their receptors.

The terms "reverse" and "bidirectional" signaling are often used indistinctively. The classic definition of a ligand being a molecule that binds to a receptor to provoke a response does not leave room for juxtacrine (non-soluble) ligands, which can undergo rearrangements due to the interaction with the receptor (Klotz 2004; Yu and Rao 2009). In addition, some ligands can act as signaling molecules within the cell presenting the ligand. However, this concept does not necessarily imply that the ligand has to respond to its cognate receptor to signal bidirectionally. Also, it is necessary to recognize that a bidirectional signal does not necessarily mean that it takes place simultaneously (Figure 4, panel 2).

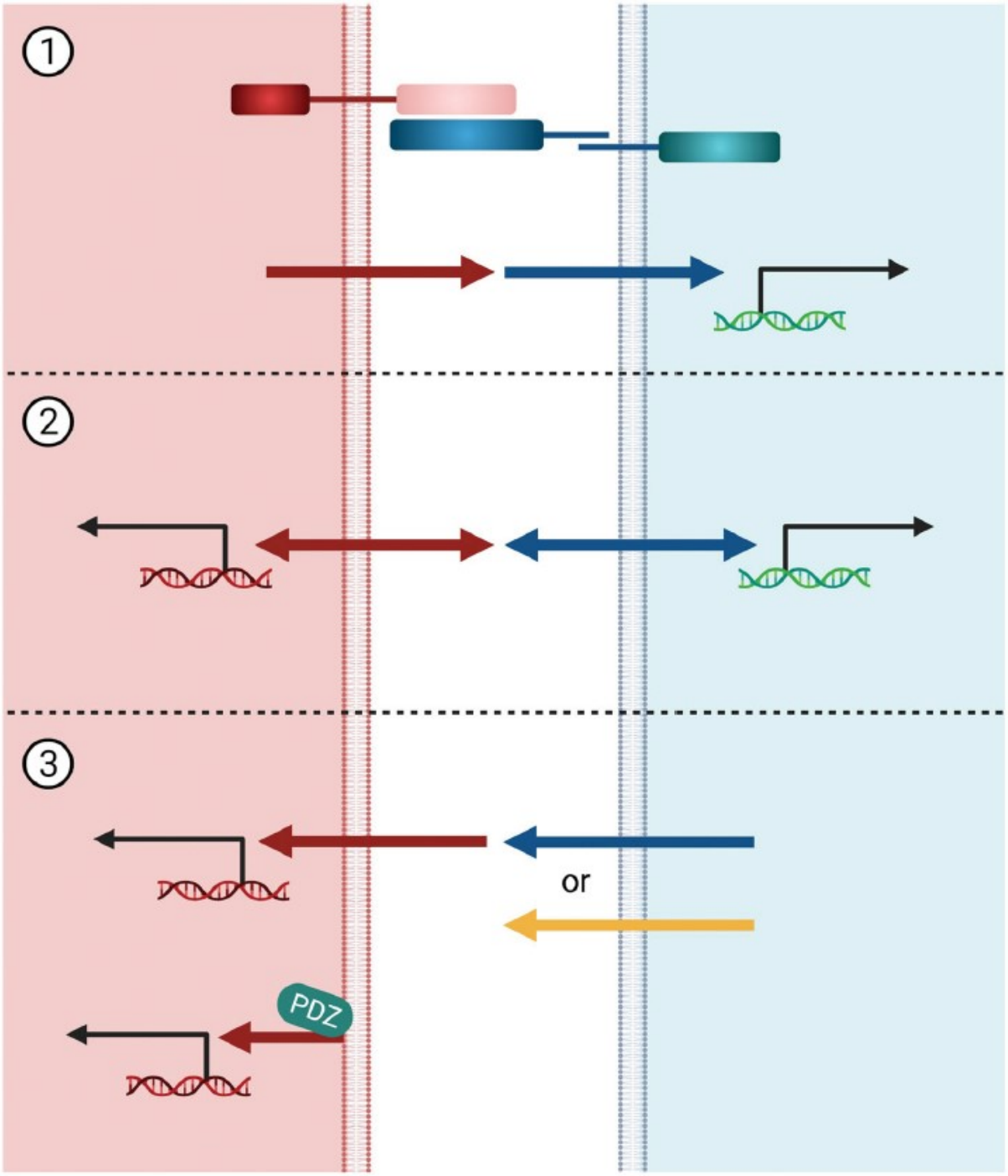

Figure 4. Reverse and bi-directional signaling of Notch ligands. DSL ligands can signal in a forward, reverse, or bidirectional manner. Panel 1. Canonical Notch activation by DSL ligands. The ligand induces a conformational change on the receptor, which ultimately sends the signal to the receiver cell. Panel 2. The engagement between the ligand and the receptor in neighboring cells provokes responses in both cells. The DSL ligands also undergo RIP liberating a signaling fragment within its cell. Panel 3. The DSL ligands activation can be independent of the activation of Notch or even not be driven by Notch receptors. Interactions within cytoplasmic proteins like PDZ-containing proteins can also trigger cellular responses in DSL ligands. A color version of the figure is available online.

Notch ligands as receptors: the reversibility of the pathway

There are three characteristics of DSL ligands that support the notion of them having activities beyond Notch activation: (1) processing of the DSL proteins through the RIP mechanism liberates extracellular

and intracellular domains (LaVoie and Selkoe 2003); (2) the presence of NLS motifs and nuclear localization of ICDs after the processing step (LaVoie and Selkoe 2003; Zolkiewska 2008); and (3) C-terminal PBM interacts with scaffolds and signals PDZ-containing proteins (Pintar et al. 2007; Zolkiewska 2008).

Regulated intramembrane proteolysis (RIP) of DSL ligands

RIP tightly regulates many membrane proteins by a series of proteolytic cleavages generating protein fragments with unique signaling properties (Lal and Caplan 2011; van Tetering and Vooijs 2011). RIP requires the protein's initial priming, or shedding, through early ectodomain cleavage by ADAM (Qi et al. 1999; van Tetering and Vooijs 2011). Sheddase cleavage can occur constitutively or as a response to specific stimuli, liberating the ECD of the protein. The transmembrane fragment is then cleaved by intramembrane cleaving proteases (iCLiPs), which release the ICD to the cytoplasm (Lal and Caplan 2011; Ye 2020). Notch and other receptors have an extensive description of this mechanism, although less is known about their DSL ligands.

Early evidence in Drosophila has demonstrated that Delta is a substrate of the ADAM metalloprotease Kuzbanian. The juxtamembrane cleavage releases Delta ECD (DECD), which activates Notch (Qi et al. 1999). Klueg et al. (1998) identified four Drosophila Delta isoforms, including a full-length (FL) Delta, two intermediate forms (I1 and I2), and a short (S) isoform that corresponds to the DECD, which is found in the cytoplasm (Klueg et al. 1998). Later on, Bland et al. (2003) showed that Delta undergoes three proteolytic cleavages, and only one is Kuzbanian dependent. After the cleavage, Delta ICD (DICD) is translocated to the nucleus, suggesting a novel function of DICD (Bland et al. 2003). The ADAM enzyme Kul (Kuzbanian-like) and DTACE (*Drosophila* TNF-a converting enzyme) were also described to mediate Delta and Serrate cleavage (Sapir et al. 2005) in *Drosophila*.

The second proteolysis occurs at the transmembrane domain (TMD) via γ-secretases. Similar to Notch RIP processing, γ-secretase is expected to cleave DSL ligands. However, in some cases, the second cleavage is not mediated by the γ-secretase complex. E.g. *Drosophila* Delta TM cleavage is facilitated by a thiolsensitive aspartyl protease and is not presenilin dependent. Also, the TM proteolysis is not a subsequent activity of Kuzbanian. Indeed, the products of Kuzbanians cleavage can exist stably in the membrane (Delwig et al. 2006). The membrane stability of truncated Delta (also known as C-terminal fragments (CTF) suggests that DICD translocation to the nucleus is not necessarily a result of the signaling process involving the ligand. Indeed, this could represent a negative regulation of Notch activation based on disassociation of the ligand (Mishra-Gorur et al. 2002; Delwig et al. 2006), or the CTFs could be competing with the NEXT at the cell membrane for the cleavage by the γ-secretase complex (LaVoie and Selkoe 2003). Thus, juxtamembrane cleavage of the DSL ligands could represent a negative feedback loop of the Notch pathway (Figure 5).

A critical question arose from previous observations: how could a cleaved ligand activate Notch according to the pulling-force model? Delwig et al. (2006) showed that endogenous Delta is cleaved into EC and IC domains prior to and independently from endocytosis in *Drosophila* embryo-derived cells. Thus, Delta ligand processing is independent of its activation, which suggests other functions involved (Delwig et al. 2006). Delta ligands are sorted, based on their activities, into different pools. Only ligands that activate Notch are monoubiquitinated and endocytosed into Epsin-specific recycling compartments (Jafar-Nejad et al. 2005). Accordingly, the ubiquitin ligase Mindbomb recognizes two different epitopes of the cytoplasmic side of JAG1. Thus, endocytosis and ligand activation may be modulated differently depending on the interacting site of the enzyme to the ligand (McMillan et al. 2015). During SOP cell differentiation, Rab11 is distributed asymmetrically and as Rab11 regulates Delta endocytosis, only cells inheriting Rab11 can recycle Delta as an active ligand into the membrane (Emery et al. 2005). It has been shown that Delta needs to be apically located for proper Notch

activation in SOP cells, possibly due to the exocyst component Sec15; Sec15 positive vesicles target Delta to membrane areas that correlate to Notch activation (Jafar-Nejad et al. 2005). Collectively, this evidence suggests that ligand-related activities of DSL proteins largely depend on endocytosis. Meanwhile, a fraction of ligands can be proteolytically regulated, conferring them properties beyond Notch activation (Figure 5).

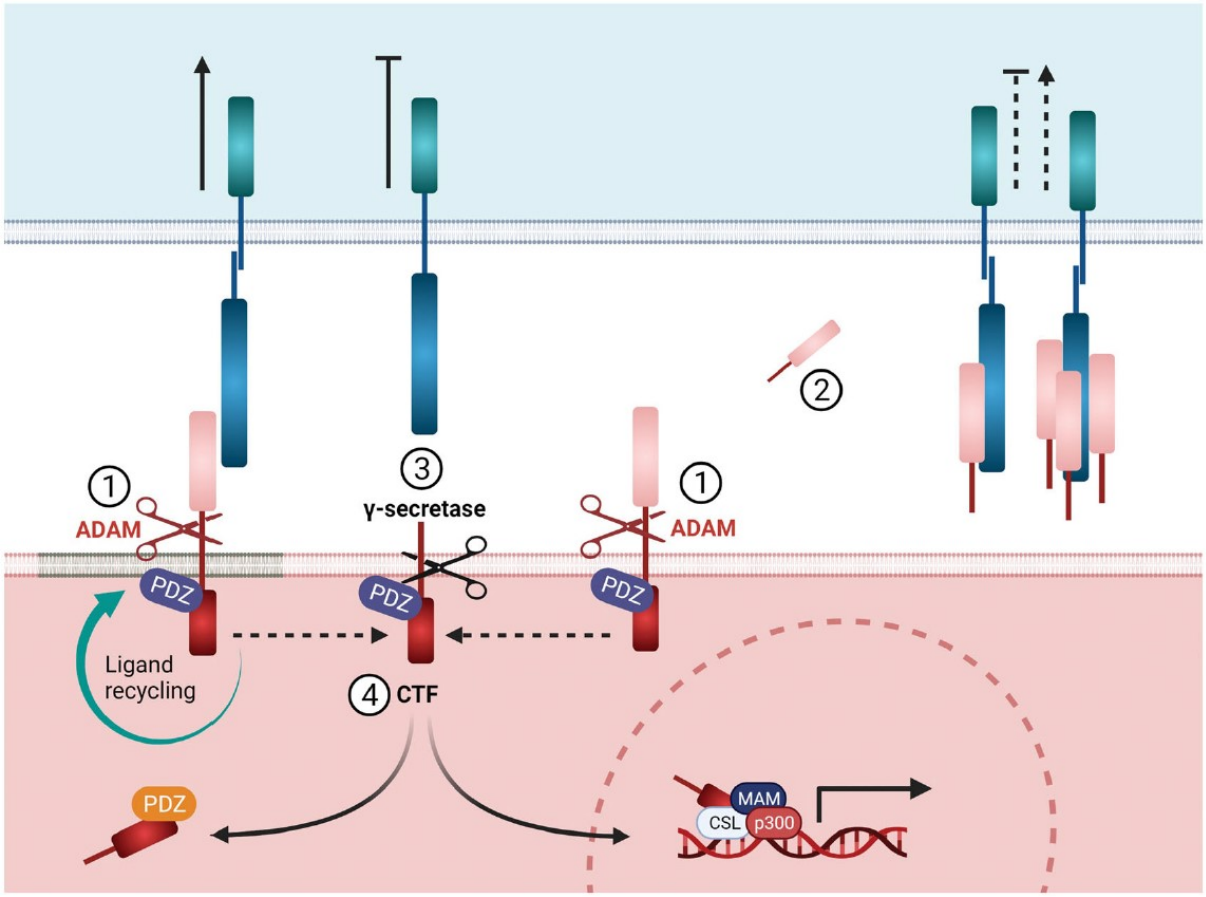

Figure 5. Regulated intramembrane processing (RIP) of Notch ligands. DSL ligands undergo proteolysis to liberate their extracellular and intracellular domains. 1 (from the left). DSL ligands can exist in different membrane compartments. Ligands activating Notch undergo ligand recycling and are shuttled to specific membrane compartments (depicted by green lipid bilayer). The ligand-receptor engagement can trigger ligand shedding, although it is unclear if γ-secretase cleavage is consequently happening. Alternatively (1, on the right), ligands can be constitutively processed by RIP. Interactions of DSL ligands with PDZ proteins at the membrane can participate in the modulation of ligand processing. 2. DSL ligand ECD liberated by ADAM (red scissors), which mediates paracrine signaling. The binding of a single or multiple ECDs to the Notch receptor can activate or inactivate Notch according to the cell type. 3. Membrane-tethered CTF is cleaved by the γ-secretase complex (black scissors), liberating ICD to the cytoplasm. 4. Interaction of PDZ proteins can be with membrane-tethered CTF or cytoplasmic ICD. The ligands ICD can also translocate to the nucleus modulating gene expression. Interactions between DSL ICDs and NICDs have also been reported; however, the effects over NICD may vary. A color version of the figure is available online.

In mammals, the DSL ligands DLL1, DLL4, JAG1, and JAG2 have been reported to be RIP processed (LaVoie and Selkoe 2003; Zolkiewska 2008). Delta proteins have been reported to be shed by ADAM 10 (Kuz homolog) (LaVoie and Selkoe 2003), ADAM17 (TACE), ADAM12, and ADAM9 (Dyczynska et al. 2007). Meanwhile, Jagged ligands are processed by ADAM17 (LaVoie and Selkoe 2003) or ADAM10 (Azimi and Brown 2019). The γ-secretase complex then cleaves the CTFs accumulated at the inner cell membrane, and the resulting ICDs are translocated to the nucleus (LaVoie and Selkoe 2003). Like Notch

processing, S3 cleavage by the γ-secretase complex in JAG2 and DLL1 requires a valine (V) in the transmembrane domain (Ikeuchi and Sisodia 2003) (Figure 6). Efforts have been made to determine the membrane microdomain of DSL ligands cleavage, albeit with contradicting results. In N3-232T lymphoma cells, JAG1 and its CTF localize at lipid rafts; disruption of the lipid raft by cholesterol removal hampers JAG1 cleavage (Pelullo et al. 2014). On the contrary, JAG1 overexpressing HEK cells showed no enrichment of either JAG1 or ADAM17 at the lipid rafts (Parr-Sturgess et al. 2010).

Figure 6. Human DSL ligands ICD alignment. Transmembrane domain marked in green with valine (V) residues marked in red, showing potential γ-secretase cleavage sites. The reported NLS sequence for each ligand is depicted in blue. Note that no NLS sequence has been recognized in DLL3 or DLL4, although DLL4 has a stretch of positively charged amino acids with the sequence RLRR representing a potential NLS sequence. The PBM of JAG1, DLL1, and DLL4 is marked in yellow. Known phosphorylation sites for human JAG1 ICD sequence are presented in magenta: T1197, S1207, S1210, and Y1216. A color version of the figure is available online.

Compared to Notch receptors, RIP processing of DLS ligands has two main differences. First, the ligand is processed to release a soluble ECD to the extracellular milieu. Second, processing of DSL ligands can be constitutive rather than induced (Figure 5). When DSL ligands are cleaved, their ECDs are found in culture-conditioned medium (CCM) modulating Notch (Small et al. 2001; Hicks et al. 2002; Urs et al. 2008). As a general observation, soluble ligands in *Drosophila* provokes phenotypes resembling the loss of Notch signaling; meanwhile, in mammals, it seems to activate Notch, although the change depends on the ECD clustering (Hicks et al. 2002). Cell aggregation assays with S2 cells showed that DECD specifically binds to Notch and competes with membrane-bound Delta (Qi et al. 1999). Mammalian N3-232 T cells have also been reported to shed Jagged ECD (JECD) into the surrounding media. Placing SCB29 lymphoid cells on N3-232 T cells-CMM provokes Notch1 and Notch3 activation (Pelullo et al. 2014). On the contrary, soluble ECDs of DLL1 and JAG1 inhibit Notch signaling in NIH 3T3 mouse fibroblast cells but displays disparate cell responses beyond Notch modulation (Trifonova et al. 2004). Competition of soluble JAG1 with its TM form has also been observed in mouse fibroblasts (Small et al. 2001). JAG1-Notch signaling induces EMT by repressing E-cadherin, and soluble JAG1 reduces E-cadherin expression in a way resembling the FL ligand (Delury et al. 2013).

DSL ligands have been reported to be processed in response to Notch receptors. Co-culture of S2 cells overexpressing Delta1 with cells overexpressing Notch caused an accumulation of Delta CTF and DICD (Bland et al. 2003). Other works suggest that DSL cleavage happens constitutively to ensure proper Notch activation (Figure 5). During *Drosophila* wing margin formation, lateral inhibition reduced Delta expression, although some Delta can escape this. Thus, constitutive processing of Delta by Kul in the sending and receiving cell complements lateral inhibition and relieves *cis*-inhibition at the receiving cell (Sapir et al. 2005). Increasing ADAM12 activity with ionomycin or phorbol myristate acetate (PMA) does not affect mouse DLL1 cleavage, indicating a constitutive rather than stimulated processing of DLL1 (Dyczynska et al. 2007).

Other proteins too can modulate DSL cleavage, as suggested by the glycoprotein MAGP-2 (microfibrilassociated glycoproteins 2) binding to the ECDs of JAG1, JAG2, and DLL1. The association between MAGP-2 and JAG1 provokes the shedding of the ligand in a metalloprotease-dependent manner (Nehring et al. 2005). Of note, MAGP-1 and MAGP-2 can also bind and activate Notch receptors and are considered noncanonical Notch ligands (D'souza et al. 2010). The b-site amyloid precursor protein cleaving enzyme 1 (BACE1) can shed JAG1 but not JAG2, and the cleavage site is different from the reported for ADAM10 and 17. The research group of He et al. (2014) has shown that CTF is a substrate for γ-secretase. Forghany et al. (2018) showed that DLL4 harbors a caspase cleavage site in its ICD. The treatment with Z-VAD, a caspase inhibitor, prevents the appearance of DLL4ICD. Moreover, DAPT treatment does not affect the appearance of DLL4ICD. The authors recognized the possibility of multiple cleavage events over DLL4, but its nature is still unknown and needs further investigation (Forghany et al. 2018).

DSL ligand processing is involved in different pathologies, e.g. the Alagille syndrome, an autosomal genetic disorder majorly caused by mutations in JAG1 ECD (Andersson et al. 2018). The mutant JAG1 in Alagille syndrome undergoes shedding via the mechanism previously described. The shed ECD competes with membrane-tethered JAG1 and induces a chord-like (spindle) morphology of NIH3T3 cells, proving the participation of JECD in Alagille syndrome (Boyer-Di Ponio et al. 2007). In T-cell acute lymphoblastic leukemia (TALL), JECD release has a paracrine effect on the surrounding cells. JECD reinforces Notch3 activity, which supports lymphoma cells' survival, proliferation, and invasion (Pelullo et al. 2014). Environmental pollutants, such as copper, can provoke disturbance in ADAM processing. Enhanced JAG1 shedding was observed after copper treatment, which correlates with an increased invasion of PC3 cells (Parr-Sturgess et al. 2010). The modulation of the Notch pathway by FL or partial ECDs of DSL ligands has been shown to have promising therapeutic applications (for an extensive review, see (Goruganthu et al. 2020).

Nuclear localization of DSL ligands

After DSL ligands undergo the complete RIP process,the ICDs can be translocated to the nucleus, perhaps to modulate gene transcription (Bland et al. 2003; LaVoie and Selkoe 2003). There is no evidence of DNA binding motifs in the DSL ICDs, but DSL ICDs must interact with transcriptional complexes to modulate gene expression (Pintar et al. 2007). All the DSL ligands-ICDs are small molecules beneath 50 kDa (Figure 6); thus, passive diffusion to the cell nucleus is possible (Timney et al. 2016). However, reports on ICDs interactions with cytoplasmic and nuclear proteins and the presence of nuclear localization sequences suggest that DSL ICD's nuclear translocation is controlled (Pintar et al. 2007; D'Souza et al. 2008; 2010; Kim et al. 2021). *Drosophila* and human DSL ligands have conserved NLS (Ikeuchi and Sisodia 2003; Pintar et al. 2007). Early analysis of sequence alignments has shown that all the DSL ligands, except Delta 3, potentially contain NLS (Pintar et al. 2007). Later, it was revealed that human DLL1 has two NLS within its ICD (^{575}KHRPP^{579} and ^{689}RKRPP^{692}) (Kolev et al. 2005). J1ICD has one NLS starting at the residue R1094 with the conserved sequence RKRRK (Delury et al.

2016). Meanwhile, J2ICD has two NLS between the residues 1107-1110 and 1108-1111 (RKRR and KRRK, respectively) (Ikeuchi and Sisodia 2003) (Figure 6).

Exogenous expression of the ICDS has shown the nuclear presence of DLS ligands (Bland et al. 2003), even though this might not reflect the in vivo situation since this model bypasses the membrane processing of ligands. Many works now focus on the endogenous expression of DLS ligands and their activity in normal and pathological conditions. The early study of LaVoie and Selkoe (2003) showed for the first time the existence of JAG1 CTF and further release of JICD in vivo, using rat embryos. They also showed that JICD promotes the activity of the transcription factor AP-1 *in vitro*, which suggests the participation of JICD in transcriptional modulation. Interestingly, JICD mutants lacking their NLS still promote AP-1 activity, indicating a cytoplasmic activity of JICD over AP-1 (LaVoie and Selkoe 2003).

DSL ligands regulate AP-1 activity by interacting with complex components. In this regard, Forghany et al. (2018) showed that DLL1 and DLL4 ICDs associated with JUN and JUNB bZIP domains to block their binding to DNA (Forghany et al. 2018). In addition, AP-1 reporter gene assays in HEK293 cells showed that treatment with ADAM or γ-secretase inhibitors counteracts AP-1 activity (Lee et al. 2015). The same group has shown that the adaptor protein Fe65 binds to JAG1 and regulates its degradation through the ubiquitin ligase Neuralized 1; Fe65 binding to J1ICD provokes the down-modulation of AP-1 activity. The authors propose a mechanism wherein J1ICD and Fe65 jointly translocate to the cell nucleus to modulate AP-1 activity. A similar mechanism has been described for amyloid precursor protein ICD, which binds to Fe65 for nuclear trafficking (Radzimanowski et al. 2008). Thus, AP-1 modulation by DSL ligand ICDs may be bimodal – direct interaction of the ICDs with AP-1 components in the cytoplasm or interaction with a third protein to form a regulatory complex in the nucleus (LaVoie and Selkoe 2003; Forghany et al. 2018).

Further clarification on the activities of DSL ICDs in the cell nucleus is also needed. Nuclear localization of the processed ligands has been shown to be dispensable in some scenarios. Kolev et al. (2005) showed that in NIH 3T3, HUVEC, and HEK293 cells overexpressing DLL1ICD, p21 expression increases and inhibits proliferation. Nonetheless, mutant variants of DLL1ICD lacking either or both of the two NLS can still induce proliferation arrest (Kolev et al. 2005). In the metastatic prostate cancer cell line PC3, authors reported that J1ICD promotes cell proliferation, even though, once again, the phenotype observed was not dependent on nuclear entry of JICD since mutants lacking NLS still behaved the same way (Delury et al. 2016). Interestingly, nuclear localization of JAG1, DLL1, and DLL4 ICDs in endothelial cells showed no effects on migration, angiogenesis sprouting, cell adhesion, or global gene expression. Thus, DSL ICDs participation in angiogenesis is dispensable (Liebler et al. 2012).

Despite the former controversy on the nuclear activity of DSL ICDs, various works have shown their involvement in transcriptional modulation. Delury et al. (2013) showed that J1ICD overexpression in HEK cells increases E-cadherin mRNA expression (Delury et al. 2013). J1ICD can also promote its FL expression, as shown in B3 cells, where JICD overexpression induced JAG1 mRNA synthesis (Azimi and Brown 2019). Interestingly, JICD expression has an inverse correlation with the transcriptional factor Nur77, wherein the overexpression of J1ICD suppresses the activation of c-AMP induced by Nur77 in the cell line MA-10. Preliminary recent online reports show that JICD behaves as a transcriptional cofactor by complexing to DDX17, TGFI2, and Smad3 to promote Sox2 transcription (Kim et al. 2021). Similarly, DLL1ICD modulates TGF-b signaling by interacting with the transcription factors Smad2, Smad3, and Smad4. The interaction occurs in the nucleus, as shown by DNA probes precipitation with nuclear extracts expressing DLL1ICD. Moreover, the expression of DLL1ICD modulates Smad3 reporter activity (Hiratochi et al. 2007). In colon cancer-derived cells, DLL1ICD augments the activity of Wnt-dependent reporters and the connective tissue growth factor (CTGF) promoter by association with Smad 2 and 3 (Bordonaro et al. 2011).

An interplay between NICD and DSL ICDs has also been reported (Figure 5). First, JICD modulation of AP-1 is counteracted by NICD (LaVoie and Selkoe 2003). Modulation of Notch activity by J1ICD is accomplished through interactions with RBPJ-k. In HEK293 cells, JICD exacerbates Notch3 activity by complexing RBPJ-k and N3ICD, inducing pTa promoter transcriptional activation, a known Notch3 target (Pelullo et al. 2014). Although JICD has an opposite role to Notch1 ICD in Hek293 cells, JICD overexpression decreases Notch1 luciferase reporter activity and disrupts the interaction of N1ICD with RBPJ-k. Furthermore, JICD promotes NICD degradation by the E3 ligase Fbw7 (Kim et al. 2011). ChIP assays demonstrated significant recruitment of J1ICD and RBPJ-k on *snail1* and *snail2* promoters in HCT-15 cells (Pelullo et al. 2019). This observation is exciting since, during vascular development, snail1 negatively regulates DLL1 expression (Wu et al. 2014). This prompts a possible mechanism for reinforcing JAG1 activity over DLL1 through genetic regulation, although this needs further research.

In cardiomyocytes, the expression of JICD also decreased Notch1 mediated transcription, leading to reduced myocyte proliferation and accelerated maturation (Metrich et al. 2015). Notably, J1ICD expression is mutually exclusive to Hes5 (Notch target) in primary glioblastoma tumor cells. The authors sustain that during Notch pathway activation, ligands and receptors ICDs are generated. In the sending cell, J1ICD interacts with NICD and negatively regulates its activity. Thus, JICD expression promotes lateral inhibition in a mixed population of different signaling states of glioblastoma cells (Lim et al. 2015). In the case of DLL1, it modulates Myod transcriptional activity both dependent and independent of NICD. DLL1 binds directly to NICD and impedes the formation of the transcriptional activator complex with RBPJ-k and MAM (Jung et al. 2011).

The expression and localization of DSL ICDS have been studied in correlation with clinicopathological patient characteristics. In oral squamous cell carcinoma, immunohistochemistry evaluation has revealed JAG1 expression at the cell cytoplasm (Hijioka et al. 2010). Others found that JAG1 staining is predominantly nuclear in oral carcinomas. Nuclear expression correlates with more prolonged disease-free survival and overall survival rates (Krikelis et al. 2014). In squamous cervical carcinoma, cytoplasmic and nuclear immunohistochemistry expression decreased from premalignant to cancerous samples. The expression of cytoplasmic and nuclear JAG1 is associated with more aggressive tumor behavior (Tripathi et al. 2018). In triple-negative breast cancer (TNBC), JAG1 localization is mainly cytoplasmic, with a low fraction of tumors positive in the nucleus. Those tumors positive for nuclear JAG1 presented higher Ki67 expression (Strati et al. 2017).

DSL ligands and PDZ proteins

Protein-protein interactions (PPI) determine the map of cell signaling. Specific structures in different proteins determine the specificity of the interactions. The PDZ domain is one of the most common PPI domains, with around 300 human proteins containing it (Christensen et al. 2019). The participation of PDZ interactions in cell signaling has been well defined for the WNT (Subbaiah et al. 2011), Hippo (Shimomura et al. 2014), and G protein-coupled receptors (GPCRs) pathways (Romero et al. 2011). Some of the DSL ligands ICDs contain motifs for PDZ recognition (Pintar et al. 2007), although not many interactors of these ligands have been described.

The PDZ domain comprises around 90 amino acids folded into six β-sheets and two α-helixes that create a binding pocket for the interacting protein's C-terminal. PDZ binding motifs (PBM) are short linear motifs (SLiMs) of around five amino acids long (Subbaiah et al. 2011; Christensen et al. 2019). The broader classification of PDZ proteins recognizes three classes. Class I PDZ domain recognizes the C-terminal sequence T/S-X-Ψ (X represents any amino acid and Ψ a hydrophobic one). Class II identifies the sequence Ψ-X-Ψ, and class III D/EX-Ψ. Considering a more extended sequence, some authors recognize up to 16 subclasses (Christensen et al. 2019).

The *Drosophila* Serrate ligand has the C-terminal sequence TVMV (Glittenberg et al. 2006), a class II PDZ sequence similar to its human homolog JAG1. *Caenobarditis elegans* APX-1 ligand harbors a C-terminal PBM class I (SSFRV) (D'Souza et al. 2010). The vertebrate DSL ligands JAG1, DLL1, and DLL4 share the presence of terminal PDZ binding motifs at their cytoplasmic tail. DLL1 and DLL4 have a class I domain with the sequence IATEV. Meanwhile, JAG1 contains a class II PBM with the sequence MEYIV (Pintar et al. 2007) (Figure 6). The PBMs on the different DSL ligands always have a terminal V, regardless of their class. The presence of terminal V seems to be preferential in many PBMs and confers superior binding over other residues (Kurakin et al. 2007). In zebrafish, the biding between DeltaC and DeltaD (homologs to DLL1 and DLL4, respectively) to the fourth PDZ domain of MAGI proteins relies on the terminal V (Wright et al. 2004).

The terminal residue may be responsible for the processing and membrane localization of DSL ligands, just as other membrane-tethered factors that undergo membrane-regulated cleavage. For example, the activated cleavage of TGFα at the cell membrane depends on the C-terminal V (Harano and Mizuno 1994). Removal of that V correlates with decreased membrane expression and endoplasmic accumulation of TGFα (Briley et al. 1997). The recognition of cytoplasmic residues for extracellular membrane cleavage suggests an inside-to-outside cell signaling. The modulation of metalloproteases by PDZ-containing proteins was recognized later. As an example, ADAM17 binds to the postsynaptic density protein 95 (PSD95/DlgA) and zonula-occludens 1 (ZO-1) (Gooz 2010). Thus, it is possible for the terminal V on DSL ICDs ligands to individually modulate the cleavage, or the whole PBM mediates PDZ interactions that bring together the cleaving enzymes and substrates. To the best of our knowledge, the role of PDZ interactions on DSL ligand cleavage has not been determined yet.

Table 1 shows known PDZ interactors of vertebrate DSL ligands. Estrach et al. (2007) made their two-hybrid assay data available, showing other putative PDZ interactors with DLL1; however, validation and characterization remain to be done. From those putative interactors, the synectin (Gipc1) PDZ-protein seems to be an interesting interactor due to its role in angiogenesis (Chittenden et al. 2006). Similarly, the activing receptor-interacting protein (Arip2), a component in the TGFβ signaling, stands interesting based on the observation that DLL1ICD can modulate the downstream effector of TGFβ/activin (see above) (Hiratochi et al. 2007).

Although various PDZ interactors have been described for DSL ligands, the case of JAG1 remains poorly defined compared to DLL proteins. The DSL ligands ICDs are intrinsically disordered; they can adopt different conformations due to environmental changes. The cytoplasmic tail of the ligands is highly plastic in their three-dimensional arrangement (Pintar et al. 2007; Popovic et al. 2007; Biasio et al. 2008). Studies conducted with DLL4ICD showed that the protein could fold reversibly into a coil, strand, or helix in response to pH, denaturing agents, or micelles (Biasio et al. 2008). Similarly, JAG1ICD gains a secondary structure when exposed to phospholipid vesicles (mimicking the plasma membrane) in a pH-dependent manner. These conditions directly affect how some residues are exposed or hidden to be post-translationally modified. It is worth noting that JAG1 and JAG2 present a slightly higher intrinsic disorder than DLL1 or DLL4 (Popovic et al. 2007).

Table 1. DSL ligands and their PDZ interactors.

DSL ligand (species)	**PDZ interactor**	**Function**	**Reference**
DLL1 (human) DeltaC (Zebrafish)	Dlg1	Mouse DLL1 14 last C-terminal residues were us to retain Hela cells crude membrane preparations. The identity of Dlg was confirmed by mass spectrometry. The interaction modulates 3T3 cells migration. Disruption of the interaction has no effects on Notch driven differentiation of B or T cells	(Six et al. 2003)

	MAGI1, MAGI2 and MAGI3	Synthetic peptide from human DLL1 was used to purify binding patterns from mouse brain or human neuroblastoma cell line NB100. A similar approach was used to determine the interaction for Zebrafish homologs. Specific function for DLL1 was not assessed	(Wright et al. 2004)
		DLL1 ICD was engineered for yeast two-hybrid assay and screened to cDNA library of embryonic mouse neural tube, founding that DLL1 binds equally to MAGI1a and MAGI1c isoforms. The interaction provokes the recruitment of DLL1 at adherent junctions (AJ) and stabilizes the ligand at the membrane	(Mizuhara et al. 2005)
		Yeast two-hybrid assay determines the interaction with ACVRINP1 (MAGI2), and the interaction with the PDZ4 was confirmed by GSTpulldown assay. During mouse embryogenesis, DLL4 and ACVRINP1 are co-expressed in the neural crest suggesting a role in neural migration and differentiation.	(Pfister et al. 2003)
	MUPP1 (MPDZ)	The interaction was determined by co-immunoprecipitation in HEK293 and HUVEC cells and whole murine kidney lysates. MPDZ recruits the ligands at AJ by forming a complex with Nectin-2. The interaction stabilizes the membrane presentation of the ligands to activate Notch in endothelial cells.	(Tetzlaff et al. 2018)
	Syntenin	To determinate the interaction, the mouse DLL1 C-terminal sequence was bait as bait in yeast two-hybrid assay. GST-pulldown further determined the interaction and PDZ specificity. The interaction provokes the cohesion of cultured epidermal stem cells. Syntenin regulates the retention of DLL1 at the cell membrane, thus increases Notch activation.	(Estrach et al. 2007)
	SYNJBP2 (ARIP2)	The interaction was determined by yeast-two hybrid assay and in silico analysis. SYNJBP increases the half-life of DLL1 by blocking lysosomal degradation.	(Adam et al. 2013)
DLL4 (human) DeltaD (Zebrafish)	Dlg1	Immunoprecipitations of overexpressed HA-tagged protein confirmed the interaction. The functionality was not determined.	(Six et al. 2003)
	MAGI1, MAGI2 (ACVRINP1), and MAGI3	The interaction of DeltaD with MAGI1 modulates neuronal migration in zebrafish. Disruption of the interaction has no effect on the Notch pathway	(Wright et al. 2004)
	MUPP1 (MPDZ)	Similar to the observations for DLL1. Loss of MPDZ promotes angiogenesis during brain development and alters tumor angiogenesis. Both mechanisms are associated with disrupted Notch activation.	(Tetzlaff et al. 2018)
	SYNJBP2 (ARIP2)	The interaction increases DLL4 half-life, promotes membrane protein accumulation, and increases Notch signaling due to ligand availability.	(Adam et al. 2013)
JAG1	Afadin	The C-terminal PBM from JAG1 was used to titrate the Afadin PDZ domain and be analyzed by heteronuclear NMR spectroscopy. The interaction regulates JAG1 localization. Increase affinity of the interaction due to mutations is associated with extrahepatic biliary atresia	(Popovic et al. 2011 Mar-Apr)
	SITAC	Binding was shown by yeast-2-hybrid assay, although functionality was not studied.	(Borrell-Pagés et al. 2000)

Based on the previous observations, three essential aspects should be considered when studying DSL ICD interactions. First, the interactions of membrane-tethered ligands could be different than the soluble forms. The FL ligands and the CTF forms could have similar interactors because the cytoplasmic tail exists close to the membrane. Although protein segregates with size, as previously mentioned for Notch (see above) (Kwak et al. 2020), along with lateral crowding and membrane fluctuations (Schmid et al. 2016), could determine different affinities for FL or CTF proteins (Figure 5). In this regard, studying PPI in intact cells or membrane models seems essential to understand DSL ICDs interactors. Recently, supported cell membrane sheets have been used to determine the assembly of PDZ scaffolds (Erlendsson et al. 2019). Second, besides the proximity to the plasma membrane, environmental conditions can also modulate the PPI of the DSL ligands, meaning that events like hypoxia, inflammation, osmotic stress, or metabolism could induce different interactions. Third, PTMs indeed regulate PPI. The best-characterized DSL ligands PTM is ubiquitinylation due to its central role in ligand activity (Chitnis 2006; Yamamoto et al. 2010). Phosphorylation of DSL ICDs has also been shown. Mouse DLL1ICD is phosphorylated at the S693 residue when associated with the plasma membrane and independently of ECD (Braune et al. 2014). In JAG1ICD, the residues T1197, S1207, S1210, and Y1216 are potential targets of phosphorylation (Figure 6). The phosphorylation of Y1216 indeed relies on the PBM and modulates the binding with afadin (Popovic et al. 2011 Mar-Apr).

Even though DSL ligands interactomes are not fully defined, the importance of the PBM has been well established and characterized in some cases (Table 1). Ascano et al. (2003) demonstrated that the expression of JAG1 with an intact PBM is essential for epithelial cell transformation, prompting the idea of intrinsic signaling based on PDZ interactions. It is also important to point out that they have shown ECD and ICD to be necessary for promoting transformation. Other authors have also shown that JAG1 lacking PBM could normally transactivate Notch but not promote gene transcription (Ascano et al. 2003). The deletion of PBM from the *Drosophila* Serrate ligand showed similar results. The mutant ligand retained activity and cis-inhibitory functions (Glittenberg et al. 2006). Thus, PBM is dispensable for Notch activation. In the case of DLL1, deletion of the PBM has no effect on Notch activation in a hematopoietic co-culture system (Six et al. 2003). However, others have shown that the intracellular deletion of Delta or Serrate negatively affects Notch activity, representing dominant-negative forms of the ligands (Sun and Artavanis-Tsakonas 1996). Notably, the truncated forms in the previous report were ligands that retained just 10-25 amino acids downstream of the TMD. Later reports showed that the ICD of Delta and Serrate have a short motif conserved with vertebrates where the deletion abrogates the ligands' ability to transactivate (Glittenberg et al. 2006). This motif is farther than 25 amino acids from the TMD; hence this observed dominant- negative behavior cannot be attributed to the PBM.

Still, the role of the PBM on the ligands' ability to transactivate Notch is inconclusive; PDZ proteins may have a direct effect on the activity of the ligand pictured by vimentin's regulation of JAG1 activity. The interaction of JAG1ICD binding to vimentin contributes to the signal strength required for ligand-receptor *trans*-endocytosis in Notch activation (Antfolk et al. 2017). Vimentin interacts with PDZ proteins, like Scribble, which encodes 4 PDZ domains (Phua et al. 2009). JAG1ICD and vimentin may have a common PDZ protein hub. Another observation supporting the participation of PDZ interactions in the ability of DSL ligands to modulate Notch comes from the central role of PDZ proteins in regulating protein localization. As described for DLL1 and DLL4, the interaction of these ligands with membrane-associated guanylate kinase family proteins (MAGUK) like MAGI or MUPP1 proteins provokes the stabilization of the ligands at the cell membrane where they are available for Notch activation (Table 1).

PDZ proteins have a central role in organizing signaling pathways. Thus, the disruption of these interactions is common in several diseases ranging from cancer to viral infections (Christensen et al. 2019). An example is the modulation of PDZ proteins by oncogenic viruses. Some viral oncoproteins can trigger PDZ-protein degradation and mislocalization to promote cancer through regulating the apico-basal polarity of epithelial cells, a feature lost during the epithelial to mesenchymal transition (EMT), and a hallmark of invasion and metastasis (Gandalovi_cov_a et al. 2016). The disruption of PDZ proteins is well recognized during carcinogenesis; however, its effects over DLS ligands hadn't been extensively researched. We are currently investigating the hypothesis that viral oncoproteins can modulate DSL ligands activities through the disruption of PDZ interactions. Ultimately, this can help prove the importance of PDZDSL ligands interactions as a drug target alternative in modulating the Notch pathway.

DSL ligands mediate Notch bidirectionality

The early reports of DSL ligands processing precluded the possibility of Notch pathway bidirectionality (Bland et al. 2003). Based on current data, the reversibility of Notch ligands is undeniable. The ligands can modulate transcription, cell adhesion, and transformation, to mention a few. Thus, the DSL ligands have activities beyond simply activating Notch receptors. But are those DSL activities triggered by Notch receptors? As mentioned earlier, some works have shown that Notch indeed promotes the cleavage of DSL ligands. Bland et al. (2003) showed that co-culture of Notch S2 or Kc167 overexpressing cells with Delta overexpressing cells caused an accumulation of the Delta-CTF which later becomes DICD with a corresponding decrease in Delta FL (Bland et al. 2003). It has been shown that IL-4 enhances the expression of JAG1 and its cleaved forms in CLL cells. IL-4 directly promotes JAG1 mRNA expression, and the increase in JAG1 cleaved forms accounts for the rise in FL JAG1 expression. Blocking JAG1-Notch interaction in the cells reduces JAG1 cleavage (De Falco et al. 2018).

Likewise, co-culturing of COS cells transiently expressing Notch with DLL expressing cells has led to a significant increase in DLL-CTF expression. The authors also mentioned that co-transfection of DLL and Notch in the same cell had a more dramatic effect on DLL processing (LaVoie and Selkoe 2003). In contrast to the report on *Drosophila* cells by Bland et al. and the results with CLL cells, these results with COS cells showed the accumulation of the DLL-CTF but not of the DLL ICD. We can speculate that Notch receptors indeed promote the shedding of the ligands ECD, but not necessarily provoking the liberation of the ICD, an effect which seems to be cell-dependent (Figure 5).

Moreover, in prenatal mouse lenses and B3 lens epithelial cells, the expression of endogenous J1ICD is undetectable even when a high expression of JAG1-CTF is observed. Treatment with epoxomicin to inhibit proteasome degradation failed to increase J1ICD detection but significantly increased JAG1-CTF. Transfections with constructs of FL-JAG1 and JAG1-CTF both promote the appearance of J1ICD. However, these same constructs showed JAG1 nuclear localization just when JAG1-CTF was overexpressed but not JAG1-FL. Additionally, blots of cells overexpressing the JAG1-CTF construct presented additional bands compared to JAG1-FL, meaning either extra cleavages or exclusive post-translational modification of the JAG1-CTF protein isoform (Azimi and Brown 2019). The authors mentioned the possible existence of different pools of JAG1-CTF, which are differentially processed by the γ-secretase complex. Although the authors did not extend their observations to determine if ligand processing depends on the Notch receptor, it is imaginable that different stimuli generate different CTF isoforms, and they would have different fates. Like in the Notch receptor, the length of the N-terminus determines the efficiency of the γ-secretase complex after shedding. The tethered NEXT is less effectively cleaved when it has a longer N-terminus (Kopan and Ilagan 2009). The absence of JAG1 nuclear staining after FL-JAG1 overexpression supports the hypothesis of differential cleavage affecting nuclear translocation. It is crucial then to carry out experiments, including the different ICDs generated

by alternative cleavage of various valine in the transmembrane domain (Figure 6) (Ikeuchi and Sisodia 2003). Also, other ICDs are generated by different enzymes (Forghany et al. 2018) since other cleavage products might account for different activities.

The reports by Gravano et al. (2010), Liebler et al. (2012), and Redeker et al. (2013) concluded that there is no clear evidence to claim bidirectionality of the Notch pathway. Experiments with lymphocytes showed that sender cells expressing a non-cleavable DLL1 (NC-DLL1) mutant, in which the ADAM proteolytical cleavage site was removed, do not affect lymphocyte cell fate determination in co-culture experiments. Moreover, NC-DLL1 can induce Notch targets at the receiving cells, although with different effects, exemplified by the stronger induction of Hes5 compared to the wild-type ligand. Inhibition of DLL1 proteolysis does not affect T-cell developmental potential (Gravano and Manilay 2010). In another study, the authors showed that exogenously expressed JAG1, DLL1, and DLL4 are cleaved in HUVEC cells, although they failed to show this for endogenous proteins. Forced expression of JAG1ICD, DLL1ICD, and DLL4ICD reduce HUVEC cell proliferation, and DSL ICDs could not block NICD activity. Also, ICDs did not affect migration, adhesion, angiogenesis, or gene expression. The authors concluded that ligand cleavage limits membrane ligands' availability (Liebler et al. 2012).

Finally, embryonic stem cells (ESC) were engineered to carry different recombinant DLL1 cleaved forms, and the proliferation, expression of p21, and differentiation of the cells were similar to control cells. Transgenic mice carrying DLL1ICD develop normally, and Notch target genes were not affected. Also, the authors showed that mouse DLL1ICD was not translocated to the nucleus, but instead underwent an extra cleavage at the cytoplasm. However, this last result needs further clarification. In conclusion, DLL1ICD overexpression had no measurable effect during development and supported the notion that ligand cleavage is a means to regulate them negatively (Redeker et al. 2013).

It is important to stress that although the idea of Notch pathway bidirectionality has been suggested several times, the evidence is still scarce, and many questions remain open. The triggering of DSL ligands cleavage is not well described, and concomitant activation has not been entirely determined. The presence of of DSL ligands ICDs in the nucleus is not enough evidence to claim bidirectional Notch signaling. However, it is clear that DSL proteins are more than just Notch ligands, and more functions of these proteins await to be unraveled, changing the paradigm of Notch ligands.

Conclusions and further directions

Signals sent from a ligand-expressing cell to a receptorexpressing cell have long been recognized as the way information flow in the Notch signaling pathway. Meanwhile, other events occurring in the signal-sending cells add to the complexity of the pathway. DSL ligands have been shown to undergo multistep processing before the ICDs are released; however, it is necessary to determine the mechanisms regulating their processing to confirm the bidirectionality of the Notch pathway. Determination of ligand interactomes will give further insight into the activity of DSL proteins. The intrinsically disordered nature of DSL protein ICDs has been a challenge for resolving their interactions. Computational molecular dynamics have been explicitly designed to study these unstable structure proteins, such as the recently developed IDP-LZerD, the first program capable of docking long-fragments of disordered proteins (Christoffer and Kihara 2019). A tool like this would greatly facilitate the modeling of DSL interactions; moreover, experimentally, the mass spectrometry- based approach seems promising. Cross-link of proteins followed by proteomic analysis could also contribute to interactomes determination. Finally, DSL ligands' intrinsic activity has been demonstrated, although it is relatively unexplored. The central role of these proteins in different pathologies like Alagille syndrome and various cancers makes them promising therapeutic targets. Also, several strategies in targeting Notch signaling, including ligand/receptor-specific decoys, antibodies against the ligands, and

post-translational modifications to influence ligand-receptor interactions (Goruganthu et al. 2020), need further studies while taking into consideration the ligands reversibility and bidirectionality to strengthen the understanding of their mechanisms.

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