Seminars in Cell & Developmental Biology 23 (2012) 429–436

Contents lists available at SciVerse ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Notch ligand endocytosis: Mechanistic basis of signaling activity

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ARTICLE INFO

Article history:
Available online 24 January 2012

Keywords:
Notch
DSL ligands
ADAM10
Endocytosis
Mechanical force
Signaling

ABSTRACT

Regulation of Notch signaling is critical to development and maintenance of most eukaryotic organisms. The Notch receptors and ligands are integral membrane proteins and direct cell–cell interactions are needed to activate signaling. Ligand-expressing cells activate Notch signaling through an unusual mechanism involving Notch proteolysis to release the intracellular domain from the membrane, allowing the Notch receptor to function directly as the downstream signal transducer. In the absence of ligand, the Notch receptor is maintained in an autoinhibited, protease resistant state. Genetic studies suggest that Notch ligands require ubiquitylation, epsin endocytic adaptors and dynamin-dependent endocytosis for signaling activity. Here we discuss potential models and supporting evidence to account for the absolute requirement for ligand endocytosis to activate signaling in Notch cells. Specifically, we focus on a role for ligand-mediated endocytic force to unfold Notch, override the autoinhibited state, and activate proteolysis to direct Notch-specific cellular responses.

Contents

1. Introduction

The Notch pathway is a highly conserved signaling system used extensively throughout embryonic development that continues to function in the maintenance of tissues and stem cells in adults [1,2]. Notch signaling is linked to genetic disorders and cancer and recent studies have identified Notch as a potential therapeutic target [3]. Defining the mechanistic basis of ligand-induced Notch signaling is critical to the design of successful therapies. The integral membrane nature of Notch pathway receptors and canonical ligands provides a mechanism for cells to directly interact and communicate with each other. The ligand transmembrane structure also facilitates endocytosis, which is absolutely required for ligand cells to activate signaling in contacted Notch cells [4–6]. The proposal that ligands on the surface of a signal-sending cell must be internalized to activate a receptor on the signal-receiving cell represents a novel role for endocytosis in activation of intercellular signaling.

Notch signaling is mechanistically remarkable in its reliance on ligand endocytosis to promote receptor proteolysis and release

Abbreviations: ADAM, a disintegrin and metalloprotease; AFM, atomic force microscopy; CSL, CBF1, Su(H), LAG-1; Dll, Delta-like; EDTA, ethylenediaminetetraacetic acid; GSI, γ-secretase inhibitor; HD, heterodimerization domain; LNRs, Lin12/Notch repeats; NECD, Notch extracellular domain; NICD, Notch intracellular domain; NRR, negative regulatory region; SOP, sensory organ precursor; T-ALL, T-cell acute lymphoblastic leukemia.

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doi:10.1016/j.semcdb.2012.01.011

the Notch intracellular domain (NICD) that directly participates in downstream signaling [7]. In the majority of cellular responses, NICD released from the membrane moves to the nucleus and interacts with the major downstream effector, CSL pre-bound to Notch target genes, to recruit co-factors for transcriptional activation [8]. NICD generation is dependent on an initial activating proteolytic event within the Notch extracellular domain (NECD) at a designated S2 cleavage site close to the extracellular face of the plasma membrane. Two members of the ADAM family of metalloproteases, ADAM10 and ADAM17, are implicated in S2 cleavage of Notch [9–13]. S2 cleavage is considered the rate-determining step in Notch activation because ADAM-mediated removal of inhibitory extracellular sequences is necessary for efficient intramembrane γ-secretase cleavage at the S3 site [14] to release the active NICD signaling fragment.

In addition to these ligand-induced proteolytic cleavage events, Notch is cleaved independent of ligand by a furin-like protease during trafficking to the cell surface [15,16]. Cleavage at the S1 site by furin produces N- and C-terminal fragments that remain stably associated through multiple non-covalent interactions to form the mature heterodimeric receptor [17–19]. Although furin cleavage is highly conserved among the four mammalian Notch receptors [20], *Drosophila* Notch does not appear to undergo furin processing [21]. In contrast to the convincing biochemical data for furin processing of mammalian Notch, the data supporting Notch heterodimeric formation in flies do not agree [21–24], and thus, this aspect of Notch signaling has remained somewhat unsettled. In fact, the importance of furin cleavage in general has received considerable debate with some arguing that Notch furin processing is a pre-requisite for efficient signaling [15,25], while others purport only a regulatory role in trafficking Notch to the cell surface [16,26].

Despite extensive evidence implicating ligand endocytosis in Notch signaling, the mechanistic basis of this requirement has also remained poorly understood and controversial. Genetic, biochemical, cell biological and structural studies have provided insight into how ligand cells might activate signaling in Notch cells. Here we briefly review the evidence supporting a critical role for ligand endocytosis in activation of Notch signaling. We then discuss proposed models for ligand endocytosis in Notch signaling with a focus on the mechanistic aspects of Notch activation gleaned from high resolution structural studies and recent computational simulations and modeling.

2. Current models for ligand endocytosis in activation of Notch signaling

Early studies with shibire, the *Drosophila* homolog of dynamin identified the classic Notch neurogenic phenotype [27]. Since dynamin is best known for its role in releasing endocytic vesicles from the plasma membrane [28], this report provided the first evidence that Notch signaling requires endocytosis. Subsequent genetic mosaic analysis with shibire indicated a critical role for dynamin in signal reception by the Notch cell [29], as found for other cellular signaling receptors. What was surprising, however, was the requirement for dynamin by cells expressing the Notch ligand Delta to activate signaling in contacted Notch cells. Additional findings for dynamin-dependent endocytosis by Delta cells to internalize the NECD from cells producing active NICD, offered the first, yet novel, role for ligand endocytosis in regulating Notch proteolysis [30]. Further findings that ligands must be ubiquitylated [31–34] and require epsin endocytic adaptors [35–37] known to direct trafficking of ubiquitylated cargo [38], suggested functional and mechanistic links for ligand endocytosis and recycling in Notch signaling (reviewed in Ref. [39]).

Although paradoxical in nature, a requirement for endocytosis by the signal-sending cell represents an aspect of cellular signaling unique to the Notch pathway. Nonetheless, the exact function ligand endocytosis serves to activate Notch signaling has remained a mystery. Two popular models for ligand endocytosis are (1) prior to Notch binding, endocytosis enables ligand processing and recycling of an active ligand back to the cell surface [6,40] and (2) following ligand binding to Notch, endocytosis by the ligand cell produces mechanical force to pull on Notch and induce structural changes that permit activating proteolysis to release the NICD [4,7]. It is possible, however, that both models account for the absolute requirement for ligand endocytosis, especially if recycling produces a high affinity ligand [41] to secure ligand–Notch interactions and sustain ligand-mediated pulling for activating proteolysis.

3. Ligand recycling in Notch signaling is context dependent and not a general requirement

Studies in *Drosophila* and mammalian cells have suggested signaling activity requires endocytosis for ligand trafficking through the recycling endosome prior to engagement with Notch [36,41–45]. This "recycling" model proposes that ligand delivered to the cell surface is not competent to signal and must be internalized and recycled back to the cell surface, perhaps to a specific microdomain (Fig. 1a), where it can activate Notch on adjacent cells. The major support for this model comes from studies with *Drosophila* sensory organ precursors (SOPs) and polarized mammalian cells, both of which display complex ligand trafficking proposed to regulate signaling activity [42–45]. Specifically, basal-to-apical ligand trafficking in SOPs requires ubiquitylation, recycling components and actin polymerization to activate signaling and direct cell fate choices regulated by Notch. More recent genetic studies in *Drosophila*, however, provide evidence that the ligand recycling requirement may be related to a particular cell context. Although, SOP cell fates require the recycling components Rab11 and Sec15 for ligand signaling activity [43,44], neither Rab11 nor Rab5 (which directs access to the Rab11 recycling endosome, Fig. 1a), are required for ligand cells to activate Notch signaling in the germline or developing eye [46,47]. Based on these studies, ligand trafficking through the recycling pathway does not appear to be essential for all Notch-dependent developmental events. These findings provide support for the view that despite being absolutely required for SOP cell fates, ligand recycling is not a general, core requirement for ligand-induced Notch signaling.

The alternative "pulling force" model incorporates the critical role for ligand endocytosis with Notch structural findings (Fig. 1b). Specifically, mechanical force produced during transendocytosis of the NECD by the ligand cell is proposed to drive proteolytic activation of Notch [30,48]. Here, interactions between ligand and Notch cells are predicted to produce resistance to endocytosis of bound Notch by ligand cells. Based on reported ligand signaling requirements, resistance to ligand endocytosis may stimulate ligand ubiquitylation and allow recruitment of ubiquitin-binding epsin endocytic adaptors [39]. Along with clathrin, dynamin and actin, mechanical force produced by ligand endocytosis could be used to unfold Notch and expose it to activating proteolysis for NICD to direct downstream signaling events. Important to this model, ligand cells defective in endocytosis bind and cluster Notch, yet fail to dissociate or activate Notch [48]. These findings underscore the critical role for ligand endocytosis and indicate that ligand binding alone is not sufficient to activate Notch. Furthermore, dynamin, epsins and actin associated with mechanical force for membrane bending during invagination [49] are also required for ligand signaling activity.