CHAPTER FIVE

Endocytosis and Intracellular Trafficking of Notch and Its Ligands

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Abstract

Notch signaling occurs through direct interaction between Notch, the receptor, and its ligands, presented on the surface of neighboring cells. Endocytosis has been shown to be essential for Notch signal activation in both signal-sending and signal-receiving cells, and numerous genes involved in vesicle trafficking have recently been shown to act as key regulators of the pathway. Defects in vesicle trafficking can lead to gain- or loss-of-function defects in a context-dependent manner. Here, we discuss how endocytosis and vesicle trafficking regulate Notch signaling in both signal-sending and signal-receiving cells. We will introduce the key players in different trafficking steps, and further illustrate how they impact the signal outcome. Some of these players act as general factors and modulate Notch signaling in all contexts, whereas others modulate signaling in a context-specific fashion. We also discuss Notch signaling during mechanosensory organ development in the fly to exemplify how endocytosis and vesicle trafficking are effectively used to determine correct cell fates. In summary, endocytosis plays an essential role in Notch signaling, whereas intracellular vesicle trafficking often plays a context-dependent or regulatory role, leading to divergent outcomes in different developmental contexts.

1. Notch Signaling and its Regulation by Endocytosis and Vesicle Trafficking

1.1. Introduction

Notch signaling is an evolutionally conserved signaling pathway which takes place between neighboring cells. When Notch receptors are activated by DSL (Delta/Serrate/LAG-2) ligands, Notch undergoes a set of serial proteolytic cleavages resulting in the release of the Notch intracellular domain (NICD). NICD translocates into the nucleus to form a positive transcriptional complex with a key transcription factor CSL for CBF-1/Su(H)/LAG-1 (C-promoter binding factor-1/Suppressor of Hairless/Lin-12-and-GLP-1) and a coactivator, Mastermind (Kopan and Ilagan, 2009). This CSL-dependent process is referred to as canonical Notch

signaling. It has also been shown that in certain contexts, Notch signaling activity can be mediated through a CSL-independent pathway, which is usually referred to as noncanonical Notch signaling (Ligoxygakis *et al.*, 1998; Ordentlich *et al.*, 1998; Ramain *et al.*, 2001; Zecchini *et al.*, 1999). Since both ligands and receptors are transmembrane proteins, endocytosis and vesicle trafficking play a critical role in the regulation of this signaling pathway.

1.2. Intracellular trafficking of Notch and DSL ligands

Notch receptors and DSL ligands are produced in the endoplasmic reticulum (ER) and traffic through the Golgi apparatus to reach the plasma membrane (Fig. 5.1). From the cell surface, they re-enter the cell via endocytosis, a process by which vesicles invaginate from the plasma membrane into the cytoplasm. These endocytic vesicles typically fuse with an early endosome, a sorting center of the endocytic pathway, often referred to as the "sorting endosome." From this early/sorting endosomes, proteins can be recycled back to the plasma membrane, transported to the Golgi apparatus, or transported to the late endosome, which eventually fuses with the lysosome for protein degradation (Doherty and McMahon, 2009). In the past, endocytosis was considered to only play a negative role in signaling pathways by removing receptors from the membrane. However, more and more evidence suggests that endocytosis also plays a positive role. Signaling may occur not only at the cell membrane but also in endocytosed vesicles or endosomes. Indeed, numerous signaling pathways, including Notch signaling, have been shown to depend on endocytosis for their full activation (Sorkin and von Zastrow, 2009).

1.3. Endocytosis is essential for Notch signaling

Endocytosis and endosomal trafficking have been shown to play an important role in the activation and regulation of Notch signaling. The first hint came from the phenotype associated with the *Drosophila shibire (shi)* mutant. *shi* was initially identified as a temperature-sensitive mutation that leads to embryonic lethality at restrictive temperatures (Poodry *et al.*, 1973). The gene was later shown to encode dynamin, a GTPase essential for most, if not all, forms of endocytosis (Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991). Interestingly, shi^{ts1} embryos, raised at the restrictive temperature during neuroblasts segregation, contain excessive neuroblasts and neurons (Poodry, 1990), a neurogenic phenotype that resembles the loss of Notch phenotype (Poulson, 1937). Further studies based on clonal analysis and genetic interaction assays provided the first evidence that endocytosis is required for ligand-dependent Notch activation in both signal-sending and signal-receiving cells (Seugnet *et al.*, 1997).

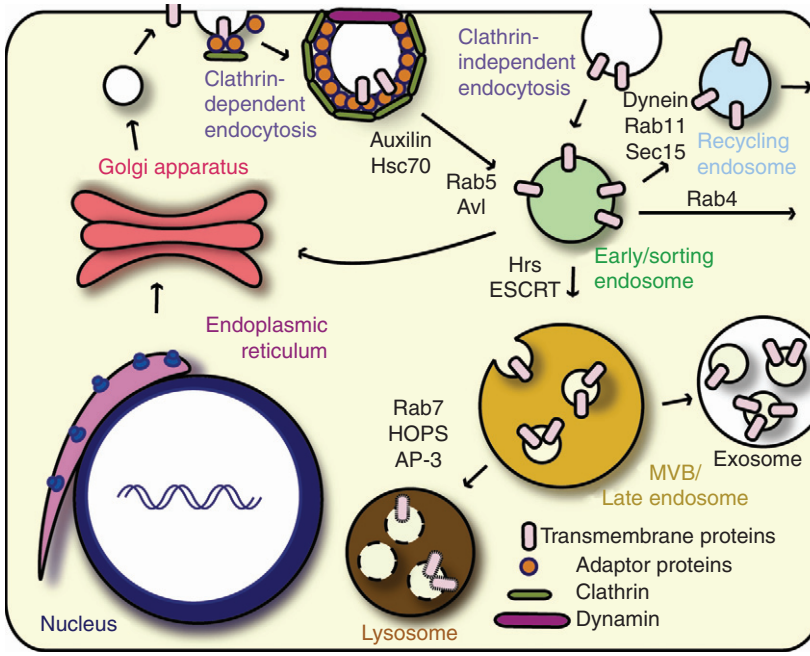

Figure 5.1 Overview of endocytosis and vesicle trafficking. Transmembrane proteins are made in the ER and traffic through the Golgi apparatus to reach the plasma membrane. From the cell surface, these proteins can re-enter the cell via various endocytosis pathways. Clathrin-dependent endocytosis is usually referred to as "canonical endocytosis." Clathrin adaptor proteins, such as the AP-2 complex, recruit clathrin and cargo transmembrane proteins to the site of endocytosis. The clathrin-coated endocytic vesicle is pinched off by the action of dynamin GTPase, and the clathrin coat is then removed by molecular chaperone Hsc70 via the assistance of auxilin. On the other hand, endocytosis can also occur without clathrin and is referred to as "noncanonical endocytosis" or "clathrin-independent endocytosis." After endocytosis, small GTPase Rab5 and SNARE protein Avalanche (Avl) mediate the fusion of endocytic vesicles with the early/sorting endosome. From the early endosome, endocytosed proteins can recycle back to the plasma membrane directly in a Rab4-dependent manner or indirectly through the recycling endosome in a Rab11-dependent manner. Alternatively, they can return back to the Golgi or travel to the late endosome and lysosome for degradation. Proteins destined for degradation are sorted into Rab7-positive late endosome or multivesicular bodies (MVB). Packaging of transmembrane proteins into intraluminal vesicles is mediated by the ESCRT complexes. In certain cell contexts, MVB can secrete their contents to extracellular regions. These secreted MVBs are referred to as exosomes. Finally, through HOPS and AP3 complexes, MVB/late endosomes fuse with the lysosome and transmembrane proteins are degraded by proteases and acid hydrolases. (See Color Insert.)

Based on these pioneering studies, various labs have focused on understanding how endocytosis regulates Notch signaling through forward and reverse genetic approaches. First, we will briefly review key steps in endocytosis and the molecular players that have been shown to affect

Notch signaling. Specific players that seemingly only affect endocytosis of Notch signaling components in a cell context-dependent manner will be discussed later.

1.4. Proteins and molecules involved in endocytosis

Canonical endocytosis requires the assembly of a clathrin lattice to form a clathrin-coated pit, which is then pinched off by the action of a GTPase, dynamin (Seugnet *et al.*, 1997; Traub, 2009). Clathrin is composed of heavy and light chains which form a triskelion upon multimerization. Clathrin is recruited to the site of endocytosis in the membrane through adaptor proteins, including the assembly protein-2 (AP-2) complex (Berdnik *et al.*, 2002). These and other adaptor proteins bind to transmembrane proteins that are targeted for endocytosis and recruited into clathrin-coated pits. The lipid composition of the plasma membrane also plays an important role in endocytosis. For example, phosphatidylinositol (4,5) diphosphate (PI(4,5) P2) is enriched in the plasma membrane at sites where endocytosis occurs, and the recruitment of many adaptor proteins depends on their binding to this lipid (Di Paolo and De Camilli, 2006; Poccia and Larijani, 2009).

A key signal to promote endocytosis of transmembrane proteins relies on the monoubiquitination of intracellular lysine residues by E3 ubiquitin ligases. The ubiquitin tag can promote the interaction with adaptor proteins and lead to recruitment and enrichment into clathrin-coated pits (d'Azzo *et al.*, 2005). Ubiquitinated proteins can be recognized by proteins that contain ubiquitin interaction motifs. Upon invagination and pinching off, vesicles are stripped of their clathrin coat by molecular chaperones such as Hsc70 with the assistance of auxilin (Eisenberg and Greene, 2007; Eun *et al.*, 2008; Hagedorn *et al.*, 2006).

Alternatively, endocytosis can also occur without the assembly of clathrin-coated pits, a process often referred to as noncanonical endocytosis or clathrin-independent endocytosis (Doherty and McMahon, 2009; Hansen and Nichols, 2009). However, compared to the well-established role of clathrin-dependent endocytosis in signaling pathways, its involvement in signal regulation is poorly understood.

1.5. Proteins involved in endocytic trafficking, sorting, recycling, and degradation

Upon the uncoating of internalized vesicles, the small GTPase Rab5 and the SNARE (Soluble N-Ethylmaleimide-Sensitive Factor Adaptor Protein Receptor) protein syntaxin 7 mediate fusion of the endocytosed vesicles with the early endosome (Lu and Bilder, 2005; Vaccari *et al.*, 2008). From the early endosome, endocytosed proteins can either be recycled to the