The Role of Receptor Transport in Notch Signaling

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Dedication

This thesis is dedicated to my cherished sister Abby. She is my best friend and kindred spirit. Thank you for always expressing unbridled enthusiasm to hear about my “discoveries”.

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Chapter I: Introduction

The Notch signaling is an evolutionarily conserved short-range signaling pathway critical for metozoan development and maintenance of adult self-renewing tissues (Kopan & Ilagan, 2009; Tein et al., 2009). Notch signaling can promote or suppress cell proliferation, apoptosis, cell fate specification and cellular differentiation events. This signaling pathway must be tightly regulated to prevent aberrant development, classically illustrated in *Drosophila melanogaster* or *C. elegans.* In addition, regulation of Notch signaling is critical for human disease prevention (Fortini, 2009; Tien et al., 2009).

Notch signaling in development

Notch was initially described in *Drosophila* mutants exhibiting missing or serrated tissue (notches) at the wing tip (Mohr, 1919). Since then, Notch signaling has been implicated in the development of the retina (Cagan & Ready, 1989), the wing margin, and the peripheral nervous system in *Drosophila* (Fortini, 2009; Hartenstein & Posakony, 1990). For example, loss of Notch signaling affects all the cell fate decisions, both neural and nonneural, during *Drosophila* eye development (Cagan & Ready, 1989). In contrast to flies, which have only one Notch receptor, genetic screens for developmental defects in C. *elegans* identified two Notch proteins encoded by the LIN-12 and GLP-1 genes. During development of the *C. elegans* hermaphrodite, proper LIN-12 function allows for equivalent gonadal cells to differentiate into one Anchor cell (AC), a terminally differentiated cell type, and one ventral uterine precursor cell (VU), which contributes to the ventral uterus. LIN-12 mutations cause either two AC cells (loss-of-function LIN-12) or AC cell loss (gain-of-function LIN-12) (Fares &

Greenwald, 1999; Greenwald et al., 1983). GLP-1 is essential in the germline, as loss of zygotic GLP-1 causes germ cells to prematurely enter meiosis (Austin and Kimble, 1987).

Notch signaling and disease

Since Notch signaling regulates a variety of fundamental processes during animal development, it is not surprising that Notch signaling defects cause a variety of human diseases. Genetic mutations in Notch signaling components cause inherited human diseases such as: Alagille syndrome, tetralogy of Fallot, spondylocostal dysostosis, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Gridley, 2003). Although the clinical manifestations of these diseases vary greatly from cardiac/vascular defects to vertebral defects resulting in dwarfism (Gridley, 2003), all of these conditions are characterized by abnormal cell or tissue differentiation (Fortini, 2009). Since Notch signaling is critical in cellular proliferation and differentiation decisions, aberrant Notch signaling can contribute to tumorogenesis (Allenspach et al., 2002). Notch involvement in malignancies is well characterized in T-cell acute lymphoblastic leukemia (T-ALL). Acute lymphoblastic leukemia (ALL) causes the excessive production of lymphoblasts that are committed to either the B-cell lineage or the T-cell lineage (Demarest et al., 2008). T-ALL represents 10%–15% of pediatric and 25% of adult ALL cases (Demarest et al., 2008; Ferrando et al., 2002) and recent studies indicate that more than 50% of T-ALL cases have activating mutations in the NOTCH1 gene (Weng et al., 2004).

The canonical Notch signaling pathway

The canonical Notch signaling pathway confers short-range cell-to-cell communication without the use of secondary messengers. Specifically, Notch is activated by membrane-bound ligands expressed on neighboring cells, as opposed to secreted ligands. In addition, activated Notch functions as a membrane-tethered transcription factor. Explicitly, upon signal activation, Notch undergoes a cleavage event to release the Notch cytoplasmic tail that will translocate to the nucleus and directly participate in gene transcription (Fortini, 2009; Kopan, 2002; Struhl & Adachi, 1998).

The Notch gene encodes a large type-1 transmembrane receptor that undergoes three cleavage events. A mature mammalian Notch receptor exists at the cell surface as an intramolecular heterodimer, consisting of the Notch extraceullar domain (NECD) tethered to the Notch C-terminal fragment (the transmembrane and intracellular domains of the receptor) (Fortini et al., 2009). This maturation step occurs in the secretory pathway by cleavage within the Notch extracellular domain by a furin-like convertase (Blaumueller et al., 1997; Kopan et al., 1996, Logeat et al., 1998). Canonical Notch signaling initiates when Notch binds one of several transmembrane ligands DSL, Delta and Serrate/ Jagged in *Drosophila* and mammals and LAG-2 in *C. elegans* (Figure 1).

Following ligand binding, a conformational change within the Notch extracellular domain (NECD) is thought to expose a cleavage site (Nichols et al., 2007; Gordon et al, 2007). This conformational change enables a second cleavage mediated by Adam17/TNF-α converting enzyme (TACE) (Brou et al., 2000) and/or Adam10 metalloproteinases (van Tetering et al., 2009). This cleavage event dissociates NECD from the cell surface, which, in complex with its ligand, undergoes trans-endocytosis into the signal-sending cell (Parks

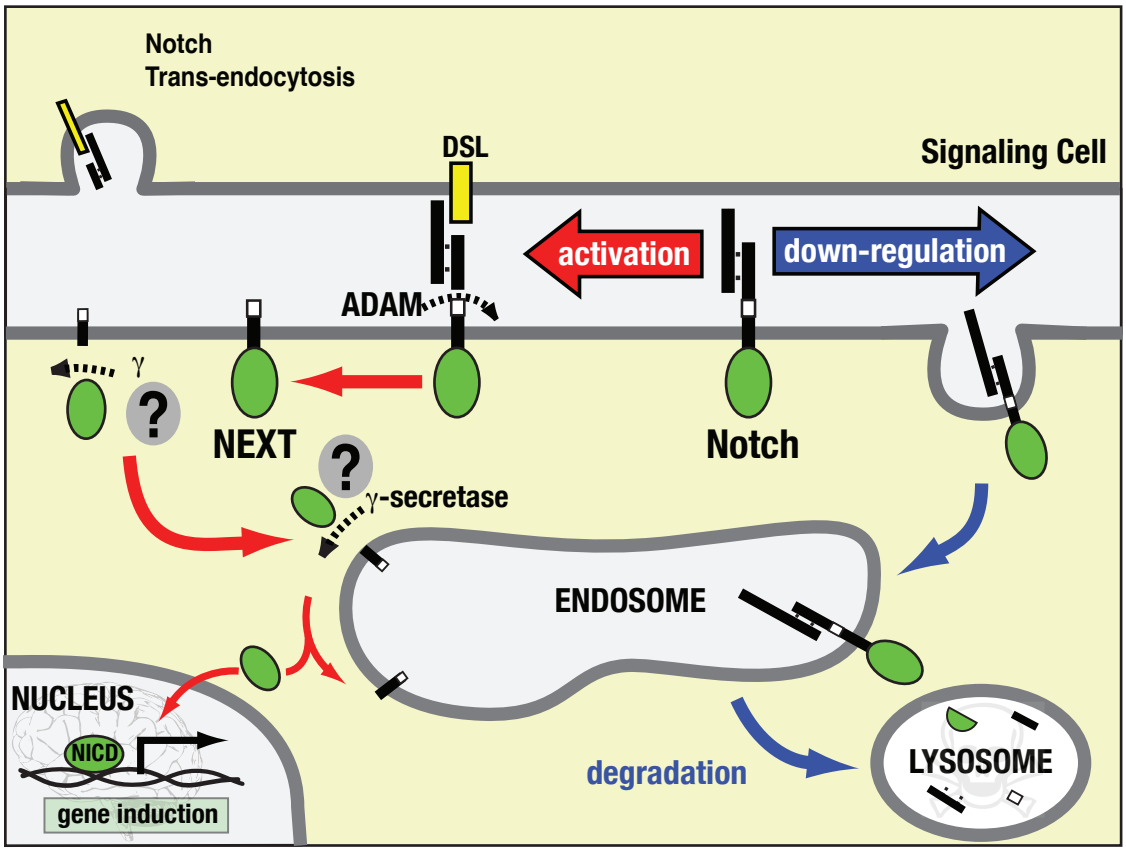

Figure 1: Notch signaling is regulated by endosomal transport. During receptor activation, the Notch receptor binds DSL ligand leading to the ADAM cleavage within the Notch extracellular doman (NECD) and trans-endocytosis of NECD into the ligand expressing cell. The activated receptor, NEXT, undergoes γ–secretase processing, an intramembrane proteolytic event. Conflicting data suggest that γ–secretase cleaves at either the plasma membrane (Shaye & Greenwald, 2002, 2005; Struhl & Adachi, 2000) or within the endosome after NEXT endocytosis (Gupta-Rossi et al., 2004; Vaccari et al., 2008). The entire Notch intracellular domain (NICD) translocates to the nucleus and influences transcription with CSL and Mastermind. Downregulation of the Notch receptor occurs in the absence of ligand binding. The receptor is internalized and targeted to the lysosome for degradation.

et al., 2000; Nichols et al., 2007) (Figure 1). The resulting membrane-bound Notch extracellular truncation fragment (NEXT) undergoes a third cleavage event mediated by γ-secretase, an intramembrane protease complex consisting of at least four subunits: presenilin, nicastrin, APH-1 and PEN-2 (Kopan et al., 1996; Steiner et al., 2008; Struhl & Greenwald, 1999). This final cleavage event releases the entire Notch intracellular domain (NICD), which translocates to the nucleus and acts as a transcriptional activator in concert with CSL proteins (CBF1/ *Dropsophila* , Su(H)/C. elegans LAG-1) (Fortini, 2009) and co-activator Mastermind (Drosophila and mammals) or LAG-3 (*C. elegans*, Figure 1, Wu et al., 2000; Petcherski and Kimble, 2000).

Receptors and Endocytosis

Given that both the Notch receptor and its ligands are integral membrane proteins, endocytosis and endosomal sorting of the Notch receptor and/or DSL ligands are critical mechanisms to regulate Notch signaling events. Endocytosis is an essential plasma membrane internalization process that delivers extracellular soluble macromolecules and membrane receptors, sometimes associated with its ligand, into the cell (Conner & Schmid, 2003[1]).

Signaling and nutrient receptors rely on endocytosis for nutrient uptake and the regulation of signal transduction. Endocytosis functions in the uptake of nutrients such as the cholesterol-bound low-density lipoprotein (LDL), which binds the LDL receptor, and iron-bound transferrin, which interacts with the transferrin receptor. These nutrient receptors are constitutively internalized via clathrin-mediated endocytosis, independent of ligand binding, and are often recycled to the plasma membrane many times for additional rounds of nutrient uptake (Conner & Schmid, 2003[1]).

In the case of signaling receptors, such as Notch, endocytosis may be critical to modulate signaling in several ways. 1) endocytosis can down-regulate signaling receptors by sorting receptors to the lysosome for degradation. Receptor degradation prevents the cell from responding to additional signaling molecules (Figure 1, Seto et al., 2002). 2) endocytosis may regulate the distribution of signaling molecules. For example, in polarized tissue culture cells, Benhra et al. (2010) observed that the Notch receptor and its ligand, Delta, are present on different regions of the cell membrane. Notch localizes to the apical membrane whereas Delta localizes basolaterally. Using a compartmentalized antibody uptake assay, Benhra et al. (2010) observe Delta antibody internalization from the basolateral membrane followed by transcytosis to the apical membrane. The authors concluded that transcytosis of Delta from the basolateral to the apical membrane may regulate Notch signaling by relocalizing Delta to a membrane where it can interact with its receptor and activate Notch signaling (Benrha et al., 2010). 3) in the case of Notch, receptor endocytosis may be required for signal activation to deliver Notch to the endosome for γ-secretase-mediated cleavage. Therefore, endocytosis allows the release of membrane-tethered Notch, which can translocate to the nucleus and influence gene expression (Figure 1, Gupta-Rossi et al., 2004; Vaccari et al., 2008).

Receptor endocytosis involves a two-part system that includes: cytosolic proteins that facilitate receptor (cargo) internalization and endocytic/sorting signals within the receptor tail that these cytosolic factors engage. Endocytic sorting signals may be either intrinsic, encoded within the receptor amino acid sequence, or added by post-translational modification (e.g. ubiquitination). There are multiple endocytic pathways for receptor endocytosis, which include: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin/caveolae-

independent endocytosis (Conner & Schmid, 2003[1]). Here, I will elaborate on the cytosolic players in the best characterized endocytic pathway: clathrin-mediated endocytosis (CME).

Three types of cytosolic factors are required for efficient CME. These include: 1) a protein that drives the process, 2) a protein capable of recognizing and engaging cargo and 3) a protein that uses energy to promote internalization and vesicle formation (Conner & Schmid, 2003[1]). The core cytosolic component that drives CME is clathrin. Clathrin is a triskelia protein capable of self-assembling into a polygonal coat. However, clathrin itself cannot interact with lipids or with receptor tails (Figure 2, Conner & Schmid, 2003[1]). Therefore, clathrin uses endocytoic adaptor molecules that can recognize and engage endocytic cargo. Therefore, adaptors link clathrin to endocytic cargo and plasma membrane lipids. Adaptors also function to form a protein scaffold at the plasma membrane as they also form interactions with each other (Maldonado-Baez & Wendland, 2006). Endocytic adaptor molecules include the heterotetrameric adaptors, such as AP2, and monomeric adaptor proteins, such as Numb, Eps15, epsin, ARH, and Dab2 among others (Figure 2, Maldonado-Baez & Wendland, 2006; Kirchhausen, 1999). Some of these adaptors recognize intrinsic tyrosine-based sorting signals within the receptor tail that conform to either NPXY (ie. Numb, ARH and Dab2) or YXXϕ (ie. AP2). Whereas other adaptors, i.e. Eps15 and epsin, contain ubiquitin-interacting motif (UIMs), which bind cargo marked by ubiquitin-modification (Maldonado-Baez & Wendland, 2006). Lastly, dynamin, which also functions in clathrin-independent pathways, utilizes GTP to drive vesicle fission from the plasma membrane. Dynamin, which is known as *shibire in Drosophila*, is a large GTPase that, when bound to GTP, assembles into collar-like structures around the neck of an invaginated coated pit. GTP hydrolysis

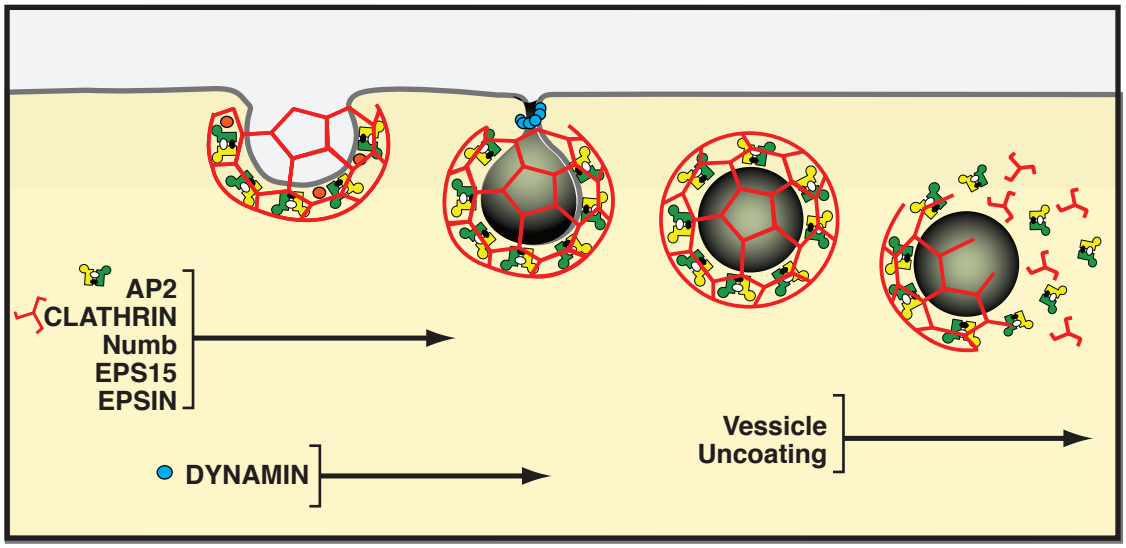

Figure 2: Core cytosolic components in Clathrin-meidated Endocytosis. CME is initiated when an adaptor molecule engages an endocytoic/soriting signal within receptor cytoplasmic tails. AP2 is used here as an example. Adaptor proteins also interact with plasma membrane lipids and each other. Clathrin, a triskelia protein, then interacts with to AP2 to form a curved latice. Dynamin GTPase assembles into a collar-like structure around the neck of a coated pit. Dynamin is responsible for membrane fission and the release of a coated vessicle into the cell. Once internalized, these cytosolic coat proteins are removed from the vesicle and recycled for reuse.

drives the “pinching off” from the plasma membrane to generate an internalized clathrin-coated vesicle (Figure 2, Damke, 1996).

Following clathrin-coated vesicle fission from the plasma membrane, the coat proteins are removed and the uncoated vesicles traffick within the cell. Coat proteins are often recycled for reuse in another round of transport (Conner & Schmid, 2003[1]). Uncoated vesicles fuse with each other or with existing early (sorting) endosomes (Maxfield and McGraw, 2004). At the early endosome, receptors undergo sorting decisions. Nutrient receptors, such as the transferrin receptor, undergo dissociation of the nutrient with its receptor within the endosome, due to the acidic pH. Nutrient receptors are often recycled back to the plasma membrane for reuse (Maxfield & McGraw, 2004). By contrast, signaling receptors may be trafficked for degradation in lysosomes, for storage or recycled to the plasma membrane (LeBorgne et al., 2005). In the case of Notch receptor endocytosis, it is unclear if internalization is required for both signal activation and signal attenuation. Specifically, it is unclear if γ-secretase-mediated Notch cleavage, which leads to downstream signaling, occurs at the plasma membrane or following receptor internalziation, within the endosome.

Ligand endocytosis promotes Notch signaling

Given that both the Notch receptor and its ligands are integral membrane proteins, endocytosis and endosomal sorting of receptor and/or ligands are critical mechanisms to regulate Notch-mediated signaling. Numerous studies suggest that DSL ligand endocytosis and endosomal sorting are essential for Notch signaling. Initial evidence for endocytic regulation of DSL ligand came from analysis of temperature sensitive (ts) alleles of *Drosophila shibire* (dynamin), a GTPase responsible for pinching off endocytic vesicles from the

plasma membrane (Damke, 1996). In *shibire*^{ts} flies shifted to the nonpermissive temperature, Seugnet et al. (1997) found an overproduction of mechanosensory bristles, which phenocopy hypomorphic Notch or Delta ts alleles. Given this observation, the authors concluded that dynamin-dependent endocytosis is required for Notch signaling; however, it was unclear if endocytosis was a requirement in the signal-sending cells (ligand expressing), signal-receiving cells(Notch expressing) or both. Additional evidence revealed that constitutively active, membrane-tethered, Notch is epistatic over *shibire* suggesting that endocytosis is required prior to Notch activation and therefore not needed in the signal-receiving cell (Seugnet et al., 1997). This data proposes that endocytosis is critical during Notch receptor interaction with its ligand (Seugnet et al., 1997).

DSL ligand endocytosis plays a critical role in Notch receptor activation. How does DSL endocytosis, removal of ligand from the cell surface where interaction with Notch can occur, promote Notch signaling? Several models have been recently proposed. 1) ligand endocytosis (complexed with NECD) into the ligand-presenting cells is critical for Notch receptor activation by pulling on NECD to expose S2 processing site for ADAM metalloproteinases (Parks et al., 2000; Nichols et al., 2007; Gordon et al., 2007). 2) ligand endocytosis and endocytic recycling are critical for converting the ligand from an inactive to an active state. For example, internalization and recycling mediated by Liquid facets (epsin, Overstreet et al., 2003; Tian et al., 2004) and the E3 ligases Neuralized (Deblandre et al., 2001; Lai et al, 2001, Pavlopoulos et al., 2001) and Mindbomb (Itoh et al., 2003; Koo et al., 2005), may activate Notch ligands by some unknown molecular mechanism (Wang & Struhl, 2004, 2005). Similarly, DSL endocytosis and endosomal sorting instead relocalizes ligand from a basolateral to an apical membrane domain where DSL can interact with Notch present at the surface

of an adjacent cell (Benhra et al., 2010). The “pulling” and “activation” models for DSL endocytosis in Notch activation are not mutually exclusive as ligand activation, via endocytosis, may proceed pulling (Benhra et al., 2010).

Endosomal transport regulates Notch signaling

Clear evidence for DSL ligand endocytosis in Notch signaling activation exists; however, it is unclear if Notch receptor internalization is required for Notch signaling. Specifically, the cellular location where γ-secretase cleaves Notch is controversial. As stated above, *Drosophila* mechanosensory development was unaffected in *shibire*^{ts} (dynamin) mutants expressing constitutively active Notch receptors (Seugnet et al., 1997). Likewise, Struhl and Adachi (2000) found that presenilin-dependent Notch cleavage and nuclear access of a constitutively active Notch receptor mimic in *Drosophila shibire*^{ts} embryos was unaffected. Moreover, analysis of LIN-12 in *C. elegans* demonstrates that internalization-defective LIN12/Notch rescues the sterility and lethality of *Lin-12/Notch* null animals (Shaye and Greenwald, 2002, 2005). These results suggest that endocytosis of the Notch receptor, itself, functions to downregulate the Notch signaling pathway (Figure 1).

In contrast, recent observations in mammalian cells and *Drosophila* reveal defects in γ-secretase-mediated Notch cleavage when cells express dominant negative constructs that disrupt endocytosis (Gupta-Rossi et al., 2004; Vaccari et al., 2008). Specifically, overexpression of dominant-negative dynamin or Eps15 in mammalian cells prevented the nuclear accumulation of NICD and caused a loss in the lower migrating Notch species by immunoblot, presumed to be γ-secretase-mediated NICD, in cells expressing a constitutively active Notch (NΔE), a NEXT mimic (Gupta-Rossi et al., 2004). Likewise, using dominant-

negative *shibire*, Vaccari et al. (2008) observed a significant reduction in the expression of hindsight, a Notch target gene, in the follicle cells of the developing *Drosophila* ovary. Vaccari et al. (2008) also observed the same loss of the faster migrating Notch species by immunoblot from mutant fly wing discs expressing dominant-negative *shibire*. Taken together, these two studies concluded that Notch internalization is required for γ-secretase-mediated cleavage and Notch activation. In this work, I set out directly test if endocytosis is required for γ-secretase-mediated Notch cleavage and subsequent downstream signaling.

The mechanisms that drive Notch internalization

Several studies propose that the adaptor protein Numb and AAK1, or its homologs, regulate Notch receptor endocytosis (Berdnik et al., 2002; Chein et al., 1998; Guo et al., 1996; Santolini et al., 2000). The role of these two proteins in Notch signaling is best described in asymmetric cell division during sensory organ precursor (SOP) development of the *Drosophila* peripheral nervous system. During *Drosophila* asymmetric cell division, Numb localizes asymmetrically into one of the two daughter cells, where it prevents Notch signaling (Berdnik et al., 2002). Numb mutant flies mimic Notch gain-of-function phenotypes. Therefore, Numb is characterized as a Notch signaling antagonist (Uemura et al., 1989). Further studies reveal that Numb is required for the asymmetric localization of endocytic adaptor AP2 (Berdnik et al., 2002), Numb can directly interact with Notch (Guo et al., 1996), and mammalian Numb can function as a general endocytic adaptor molecule (Santolini et al., 2000). Given these observations, researchers proposed that Numb antagonizes Notch signaling by targeting Notch for endocytosis and subsequent degradation in the endosomal pathway (Berdnik et al., 2002, Guo et al., 1996, Santolini et al., 2000).

The Numb binding partner NAK, the *Drosophila* AAK1 homolog, was identified in a yeast two-hybrid screen. Analysis of *Drosophila* sensory organs reveal that NAK overexpression phenocopies numb null flies (Chein et al., 1998; Uemura et al., 1989), both of which yield a gain-of-function Notch phenotype. The mechanism for Nak function during asymmetric cell division is unclear; however, Nak, a kinase, may modulate the asymmetric distribution of Numb or other Numb functions via phosphorylation (Chein et al., 1998). In addition, loss of *Sel5*, the *C. elegans* AAK1 homolgue, was identified in a genetic screen as a suppressor of constitutively active membrane-bound LIN-12/Notch but not an activated cytosolic LIN-12 (Fares & Greenwald, 1999). This positions Sel5/AAK1 to regulate LIN-12/Notch prior to γ-secretase-mediated Notch cleavage, possibly by regulating of Notch endocytic and/or trafficking events (Fares & Greenwald, 1999). Additional experiments are necessary to determine the role of AAK1 and Numb in Notch receptor transport and signaling.

The relationship between receptor endocytosis and Notch signaling

To date, the requirement for Notch receptor endocytosis in signal activation versus downregulation remains controversial with two competing models. 1) γ-secretase-mediated Notch cleavage occurs at the plasma membrane (Shaye & Greenwald, 2002, 2005; Struhl & Adachi, 2000) and 2) γ -secretase cleavage occurs after Notch receptor internalization at the level of the endosome (Gupta-Rossi et al., 2004; Vaccari et al., 2008). In addition, mammalian studies arguing for endocytic Notch activation have been limited to the use of dominant-negative dynamin overexpression. Overexpression of this construct can impair multiple internalization pathways as well as impair endosomal transport (Conner & Schmid, 2003[1]; van Dam & Stoorvogel, 2002).

Moreover, various studies testing the role of endocytic molecules in Notch signaling have relied on defective Notch signaling phenotypes in *Drosophila* (Seugnet et al. 1997; Struhl & Adachi, 2000; Vaccari et al., 2008). Given the requirement of DSL ligand endocytosis in Notch signal activation (Parks et al., 2000; Nichols et al., 2007; Gordon et al., 2007), identification of endocytic components required for Notch signaling by phenotypic analysis may not reveal if a component is required for ligand endocytosis, receptor endocytosis, or both. For example, if an endocytic molecule is also required in the ligand-expressing cell to activate the Notch signaling pathway, it may not be possible to determine if the molecule also functions in Notch receptor transport. Lastly, cytosolic components involved in Notch receptor internalization that are lethal upon complete knockout will be missed using conventional genetic null mutants.

To resolve these two competeing models for Notch activation (i.e. where γ-secretase cleavage occurs), required the development of a quantitative cell-based assay to test the Notch signaling capacity following disruption of Notch internalization. I developed a Notch internalization assay, in mammalian cells, to measure changes in Notch internalization following siRNA-mediated depletion of endocytic components. Using this loss-of-function approach, I identified the core machinery involved in Notch receptor internalization and correlated the receptor transport status with Notch γ-secretase-mediated cleavage and downstream signaling. In addition, this approach facilitated the identification of a cytosolic factor only previously thought to regulate Notch ligand endocytosis. Lastly, I tested if AAK1 regulates the function of Numb, the Notch signaling antagonist, through phosphorylation.

Chapter II: γ-secretase-dependent Notch cleavage occurs at the plasma membrane

Reprint from: Sorensen, EB and Conner, SD. gamma-secretase-dependent cleavage initiates notch signaling from the plasma membrane. Traffic 2010; 11: 1234-45. See appendix for copyright clearance.

Notch signaling is critical to animal development, and its dysregulation leads to human maladies ranging from birth defects to cancer. Although endocytosis is currently thought to promote signal activation by delivering activated Notch to endosome-localized γ-secretase, the data are controversial and the mechanisms that control Notch endocytosis remain poorly defined. Here, we investigated the relationship between Notch internalization and signaling. siRNA-mediated depletion studies reveal that Notch endocytosis is clathrin-dependent and requires epsin1, the adaptor protein complex (AP2) and Nedd4. Moreover, we show that epsin1 interaction with Notch is ubiquitin-dependent. Contrary to the current model, we show that internalization defects lead to elevated γ-secretase-mediated Notch processing and downstream signaling. These results indicate that signal activation occurs independently of Notch endocytosis and that γ-secretase cleaves Notch at the plasma membrane. These observations support a model where endocytosis serves to downregulate Notch in signal-receiving cells.

Introduction:

The highly conserved Notch signaling pathway performs critical roles in metazoan development by regulating cell proliferation, viability, cell-fate specification and differentiation (Kopan and Ilagan, 2009; Tien et al., 2009). In addition, the continued role of Notch in adults is exemplified by the fact that disrupting the signaling pathway can result in a variety of human malignancies ranging from leukemia to brain cancer (Allenspach, 2002; Lasky and Wu, 2005; Weng et al., 2004). The canonical Notch signaling pathway is initiated when Notch binds one of several transmembrane ligands belonging to the Delta, Serrate and Lag2 (DSL) family, which are expressed on neighboring cells (Kopan and Ilagan, 2009).

Following ligand binding, a conformational change within the Notch extracellular domain is thought to occur (Gordon et al., 2007; Nichols et al., 2007) that enables cleavage by either Adam17/TNF-α converting enzyme (TACE) (Brou et al., 2000) or Adam10 (van Tetering et al., 2009). This releases the Notch ectodomain from the cell surface, which in complex with its ligand, is subsequently internalized into the signaling cell (Nichols et al., 2007; Parks et al., 2000). The remaining membrane-tethered Notch extracellular truncation (NEXT) fragment undergoes cleavage by γ-secretase, an intramembrane protease complex consisting of at lease four subunits: presenilin, nicastrin, anterior pharynx-defective 1 (APH-1) and presenilin enhancer 2 (PEN-2) (Kopan et al., 1996; Mumm et al., 2000; Steiner et al., 2008; Struhl and Greenwald, 1999). This cleavage event releases the Notch intracellular domain (NICD) from the membrane, which then translocates to the nucleus to regulate gene expression in concert with CSL and Mastermind (Kitagawa, 2001).

Genetic studies in Drosophila implicate a role for endocytosis in the

Notch signaling pathway, where internalization of Notch and its ligand is thought to be critical for productive signaling (Parks et al., 2000, Seugnet et al., 1997; Vaccari et al., 2008; Wang & Struhl, 2004). However, the role of Notch internalization remains controversial. Struhl and Adachi (2000) originally reported that presenilin-dependent Notch cleavage remains unaffected in flies expressing a temperature-sensitive mutant form of shibire, the gene encoding dynamin. From this, the authors concluded that Notch cleavage by presenilin occurs independently of endocytosis (Struhl & Adachi, 2000). This conclusion is supported by two subsequent studies in Caenorhabditis elegans, where internalization-defective LIN-12/Notch rescues the sterility and lethality of Lin-12/Notch null animals (Shaye & Greenwald, 2002, 2005).

By contrast, in mammalian cell culture systems, disrupting endocytosis by overexpression of dominant-negative dynamin was found to prevent γ-secretase-denpendent cleavage and nuclear targeting of Notch (Gupta-Rossi et al., 2004). This observation suggested that endocytosis promotes Notch signaling by delivering NEXT to endosome-localized γ-secretase. In agreement, mosaic analysis in flies showed that mutant dynamin overexpression (Vaccari et al., 2008) or complete loss of dynamin or clathrin (Windler & Bilder, 2010) impairs Notch signaling in ovarian tissues.

Here, we re-evaluate the relationship between receptor internalization and Notch signaling. To do so, we developed a robust endocytosis assay to quantitatively measure the internalization of a NEXT mimic in mammalian cell culture. Using this assay, we find that Notch endocytosis occurs via a clathrin-dependent mechanism that requires the coordinated activities of two endocytic adaptors, AP2 and epsin1, as well as the E3 ubiquitin ligase Nedd4. We also provide evidence that γ-secretase mediated Notch cleavage and subsequent

activation of downstream signaling are independent of receptor internalization. Taken together, our findings support the previously proposed model where receptor internalization serves to downregulate the Notch signaling pathway (Shaye & Greenwald, 2002).

Materials and Methods:

Reagents

The monoclonal antibody (mAb) E7, 12CA5 and 9E10 were used to identify β-tubulin, hemagglutinin (HA)- and myc-tagged protein, respectively. The mouse hybridoma 51.1 (HB-230) was obtained from the American Type Culture Collection (ATCC) and rabbit antisera against CD8-α(H-160) were obtained from Santa Cruz Biotechnology. Mouse antisera against μ2 (611350) and mAb hudy1 (05-319) were obtained from BD Transduction Laboratories and Upstate, respectively. Polyclonal rabbit antisera against myc (600-401-381) was obtained from Rockland Immunochemicals. Antibodies against eps15 and epsin1 were a generous gift from Dr Sandra Schmid (The Scripps Research Institute), and the CD8-LDLR receptor construct, which was used as a backbone for generation of CD8 Notch fusion constructs, was a generous gift from Dr Margaret Robinson (University of Cambridge). The mAbs TD.1 and AP.6 were used to detect clathrin and α-adaptin, respectively. Rabbit antisera against Notch (ab27526) were obtained from Abcam and were a generous gift from Drs Neetu Gupta-Rossi and Alain Israël (Insitiut Pasteur). Nedd4 (2740) and Notch V1744 (2421 and 4147) antisera were purchased from Cell Signaling Technology. AAK1 antisera were previously described (44). ^{125}I-Protein A and ^{125}I-transferrin were purchased from Perkin Elmer (NEX146 and NEX212, respectively); γ-secretase inhibitor XXI (CE)

was purchased from Calbiochem (565790).

Constructs

WT and T102A Numb1 adenovirus are described in Chapter 3. WT and K74A AAK1 adenovirus (aa 1-863) were used as previously described (Conner & Schmid, 2003[2]). HA-K44A dynamin 1 adenovirus was a gift from the Schmid lab. Rat WT epsin1 and Ubiquitin Interacting Motif (UIM) mutant [deletion of second UIM and two-point mutations (D234A D235A) in the third UIM] in pcDNA-3myc were a generous gift from Dr Pietro De Camilli (Yale University School of Medicine). Epsin1 constructs were subcloned into the EcoRI and XhoI sites of pcDNA3.1(+). NΔE Notch (aa 1704-2532) in pCeMM-CTAP(SG)-6W was a generous gift from Dr Alain Israel (Insitiut Pasteur). TGN38-GFP plasmid was a generous gift from Dr George Banting (University of Bristol).

4myc-NΔE and CD8-NΔE chimera construction

Notch signal sequence with 4myc tags was PCR amplified from NΔE Notch in pCeMM-CTAP(SG)-6W to incorporate a 5 StuI site and a 3 EcoRI site. CD8α signal sequence and extracellular domain were PCR amplified to incorporate a 5 StuI site and a 3 EcoRI site, preceding the transmembrane domain of CD8. Both the 4myc and CD8α tags were then subcloned into pAdtet7. NΔE Notch (aa 1704-2532) was amplified from NΔE Notch in pCeMM-CTAP(SG)-6W by PCR to incorporate EcoRI sites. NΔE Notch was ligated into the EcoRI site at either the 3' end of the 4myc tags or extracellular domain coding sequence of CD8α chain in pAdtet7 creating a 4myc-NΔE or CD8-NΔE Notch chimeras. Both chimeras in pADtet7 were then used for adenovirus production as previously described (Damke et al., 1995).

siRNA-mediated depletion

siRNA depletions were performed essentially as described (Motley et al., 2003). In short, two siRNA transfections were performed. One on day 1 and another on day 2. After 50-h incubation, cells were infected with adenovirus encoding CD8-NΔE and processed for endocytosis 18 h later. The extent of protein expression knockdown was evaluated by immunoblot using the appropriate antibody. For AP2, the α siRNA target sequence was AAGAGCAUGUGCACGCUGGCCA and the μ2 target sequence was CAGCAGTCACCAAGCAGAATGTCAA. The CHC target sequence was UAAUCCAAUUCGAAGACCAAU. The epsin1 siRNA target sequence was GGAAGACGCCGGAGUCAUU. The Nedd4 siRNA target sequence was GUCCGUCGCTAAUUAUGCAUU. Eps15 siRNA target sequence was AAACGGAGCUACAGAUUAU. AP2 siRNAs, CHC, epsin1 and eps15 siRNAs were obtained from Invitrogen. Nedd4 and Silencer Negative control #1 siRNA were obtained from Ambion.

Protein production/isolation

The Notch IC (aa1759-2306) in pET28a+ was transformed into BL21 (DE3) Rosetta cells (Novagen). Expression of the fusion proteins was induced by the addition of 0.4 mM IPTG for 3 hours at 37°C. Bovine brain AP2 was isolated as previously described (Manfredi and Bazari, 1987).

***In vitro* binding and coimmunoprecipitation**

For *in vitro* interaction experiments, AP2 was incubated with either 6HIS-Notch IC immobilized on HIS-Select Ni^{2+}-agarose or just Ni^{2+}-agarose alone (Sigma) for 1hr at RT. Beads were pelleted and washed 3 times to remove unbound protein. Bead-bound proteins were then solubilized with protein sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted for AP2 using α-adaptin-specific antisera. For coimmunoprecipitation experiments, $5x10^6$ tTA HeLa cells were infected with 4myc-NΔE adenovirus alone or transfected with

WT epsin1 or UIM mutant epsin1 in pCDNA3.1. Transfections were perfomed using Lipofectamine LTX (Invitrogen), according to manufacturer's protocols. Cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM NaCl) supplemented with 1% NP-40 and 1X protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were diluted in the ratio of 1:1 in 50 mM Tris–HCl, pH 8, and then incubated for 1.5 h at room temperature with 9E10 mAb that had been prebound to protein G–Sepharose (Calbiochem). Matrices were washed, and bead-bound proteins were then processed for immunoblot analysis.

Immunolocalization

tTA HeLa cells grown on coverslips were infected with adenovirus encoding CD8-NΔE or 4myc-NΔE. Cells were fixed with ice-cold acetone for 5 min and extracted with methanol. Cells were then washed with PBS containing 0.1% Triton-X-100, incubated with primary antibody for 1 h at room temperature. Cells were washed and incubated for 1h at room temperature with the appropriate secondary antibody conjugated to either Alexa Fluor(R) 488 or Alexa Fluor(R) 555 (Invitrogen). Samples were then visualized by epifluorescence or TIRF microscopy using a Zeiss Axio Observer Z.1. Images were then imported, cropped and assembled into panels using Photoshop CS4 and Illustrator CS4 (Adobe Systems Inc.).

Endocytosis assays

tTA HeLa cells were infected with adenovirus encoding CD8-NΔE for 18 h alone, or coinfected with adenoviruses encoding the indicated dominant negative proteins. For siRNA depletion studies, cells were infected with CD8-NΔE adenovirus 60 h after initial siRNA transfection. Cells were then transferred to 1.5-mL tubes, incubated at 4°C for 45min in DMEM containing 10% FBS

supplemented with the CD8 mAb (51.1). Cells were then washed and incubated at 4°C for 45 min in DMEM containing 0.5% BSA and ^{125}I-labeled protein A (1:200 diluted; Perkin Elmer). Tubes were moved in batch to a 37°C water bath to allow endocytosis for indicated time-points and internalization was stopped by moving the cells back to 4°C. Surface-bound ligand was acid stripped (0.2 M acetic acid, 0.5 M NaCl), cells were pelleted and internalized CD8-NΔE was measured by γ counting. Each time-point is expressed as a percentage of total surface-bound γ counts. For internalization of ^{125}I-transferrin, the experiment was performed 72 h following knockdown. On the day of the experiment, cells were resuspendend in PBS^{4+} (1x PBS, 1 mM MgCl2, 1mM CaCl2, 5mM glucose, 0.2% BSA) containing 0.5 µCi ^{125}I-labeled transferrin for 45 min at 4°C. Unbound ^{125}I-labeled transferrin was removed by washing and cells were gently pelleted by centrifugation. Cells were then resuspended in DMEM/10% FBS and transferrin internalization was evaluated as described earlier.

Notch signaling assay

Signaling was evaluated using a dual-luciferase RBP-Jk reporter assay (SA Biosciences) and assayed according to manufacturer's published protocols (Promega). In each case, RBP-Jk-promoted firefly luciferase activity was normalized to renilla luciferase activity to control for transfection efficiency and minor differences in cell number. In all experiments, non-inducible background levels of firefly luciferase activity were less than 0.1%.

Results:

γ-Secretase-mediated Notch cleavage is independent of endocytosis

To directly test the idea that NEXT internalization is required for productive Notch signaling in mammalian systems, we first engineered a Notch chimera encoding four amino terminal copies of the myc epitope followed by 20 amino acids of the Notch extracellular domain through the carboxy terminal cytoplasmic tail (4myc-NΔE). To validate that 4myc-NΔE accurately reflects NEXT, we evaluated its γ-secretase-dependent processing, targeting to the nucleus, and ability to promote gene expression. To resolve if 4myc-NΔE is an effective γ-secretase substrate, we used antisera that specifically recognize γ-secretase derived NICD [^{V1744}NICD; (Schroeter et al., 1998)] to analyze protein lysates from 4myc-NΔE-expressing cells. Immunoblot lysates from 4myc-NΔE-expressing cells reveals ^{V1744}NICD production following 4myc-NΔE overexpression (Figure 3A). The ^{V1744}NICD cleavage product is specific for γ-secretase activity as its generation is inhibited by pretreating cells with a γ-secretase inhibitor, compound E (Kornilova et al., 2003). Consistent with ^{V1744}NICD targeting to the nucleus following γ-secretase-mediated cleavage, immunolocalization analysis reveals ^{V1744}NICD localized to nuclei in 4myc-NΔE-expressing cells (Figure 3B). In addition, we find that 4myc-NΔE promotes expression of endogenous c-myc (Figure 3E), a known downstream Notch target (Weng et al., 2006). From these observations, we conclude that 4myc-NΔE faithfully mimics NEXT in its post-translational processing and ability to promote downstream changes in gene expression.

We then evaluated the impact of disrupting endocytosis on 4myc-NΔE. To do so, we first overexpressed a mutant form of the dynamin 1 GTPase (K44A dyn1), which competes with endogenous endocytic machinery to

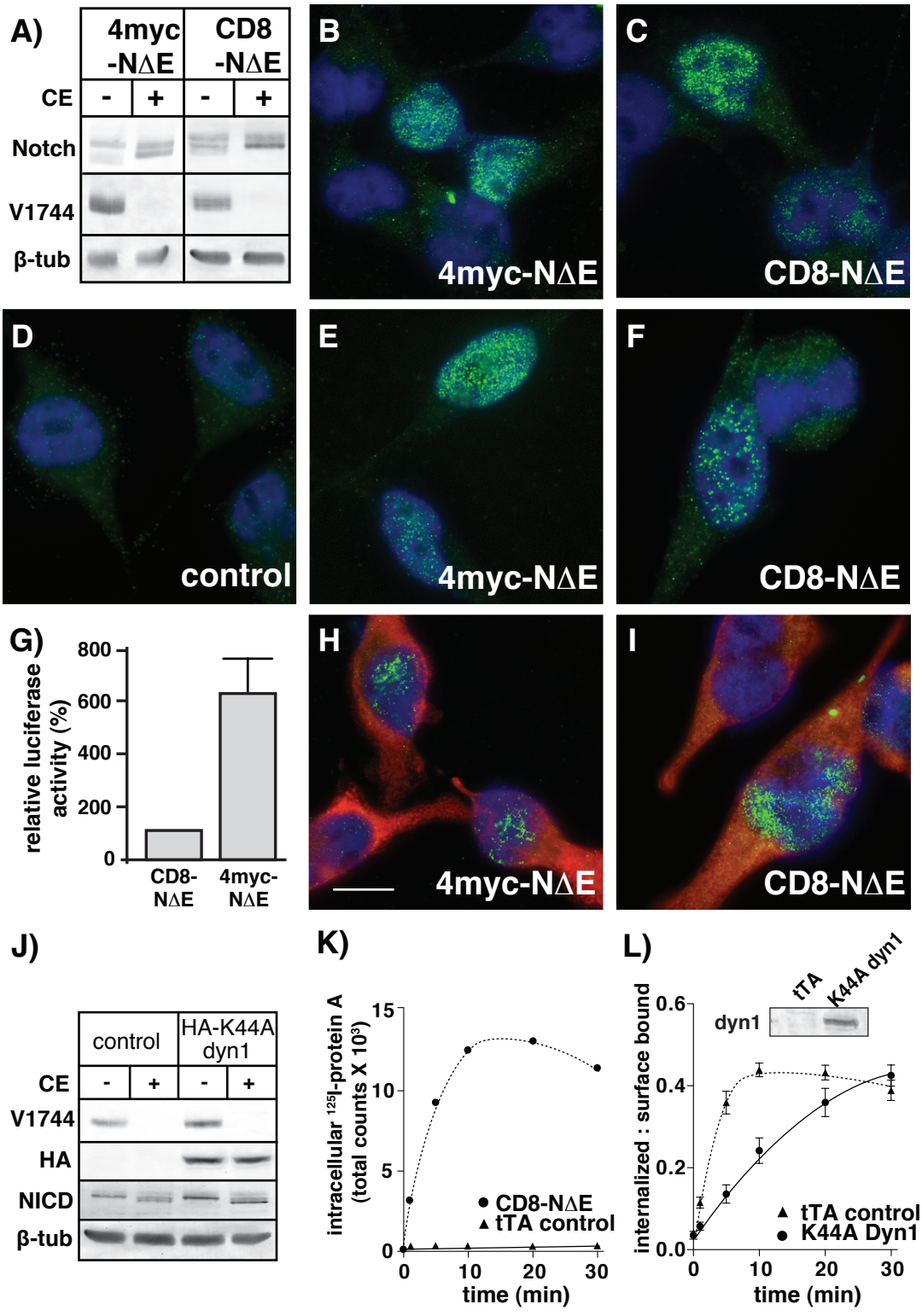

Figure 3: γ-Secretase-dependent Notch cleavage does not require endocytosis.

Figure 3: γ-Secretase-dependent Notch cleavage does not require endocytosis. A) 4myc-NΔE- or CD8-NΔE-expressing cells were pretreated with either dimethyl sulfoxide (DMSO) (control) or CE and analyzed by immunoblot for V1744NICD production. 4myc-NΔE or CD8-NΔE-expressing cells were evaluated by immunolocalization for nuclear recruitment of V1744NICD (B/C, green) or induction of endogenous c-myc gene expression using pAbs against endogenous c-myc (E/F, green), control represents tTA-infected cells (D) 4myc-ΔE- or CD8-NΔE-expressing cells were analyzed using an RBP-Jk luciferase reporter assay (G) or infected with K44A dyn1 (red)-encoded adenovirus and analyzed for V1744NICD nuclear recruitment (H/I, green) by immunoblot to evaluate V1744NICD productionlevels (J). Merged images include DAPI stain (blue) to mark nuclei. K) tTA HeLa cells were infected with adenovirus encoding tTA (control) or CD8-NΔE and incubated with the mAb 51.1 to evaluate endocytosis by measuring total internalized γ counts (see Materials and Methods). L) CD8-NΔE-expressing cells were infected with tTA control or K44A dyn1-encoded adenovirus and analyzed for CD8-NΔE uptake. Immunoblot inset shows K44A dyn1 expression levels. Error bars represent Å} SEM of five independent experiments, bar = 10 μm.

disrupt internalization (Damke et al., 1994; van der Bliek et al.,1993). Based on published reports (Gupta-Rossi et al., 2004), we anticipated that disrupting endocytosis by overexpressing K44A dyn1 would prevent 4myc-NΔE processing by γ-secretase. To the contrary, we found that K44A dyn1 overexpression, to levels that significantly impair transferrin uptake (Figure S1), did not prevent γ-secretase-dependent 4myc-NΔE cleavage, as evidenced by ^{V1744}NICD production, recruitment to nuclei (Figure 3H) and, c-myc induction in K44A dyn1-expressing cells (Figure S2). Collectively, these observations provide evidence that K44A dyn1 expression does not prevent γ-secretase-mediated NEXT cleavage.

^{V1744}NICD production following K44A dyn1 overexpression (Figure 3H) suggests that γ-secretase-mediated NEXT cleavage occurs at the plasma membrane. If this is the case, the membrane-tethered Notch cytoplasmic tail would be released into the cytosol, thereby eliminating our ability to robustly track 4myc-NΔE endocytosis. To test this idea, we replaced the 4myc tag with the extracellular domain of CD8 to generate a larger Notch chimera (CD8-NΔE), because increases in the size of the extracellular domain were reported to reduce presenilin-dependent Notch cleavage efficiency in Drosophila (Struhl & Adachi, 2000). Indeed, CD8-NΔE-expressing cells generate less ^{V1744}NICD relative to that produced in 4myc-NΔE-expressing cells (Figure 3A). Despite the reduced rate of ^{V1744}NICD production in CD8-NΔE-expressing cells, ^{V1744}NICD was still detectable within nuclei and capable of promoting endogenous c-myc expression (Figure 3C,F). To quantitatively evaluate the differences in Notch-dependent downstream signaling between 4myc-NΔE and CD8-NΔE, we employed an RBP-Jk luciferase reporter assay and found that 4myc-NΔE signaling is approximately six-fold greater than that of CD8-NΔE (Figure 3G).

However, similar to that observed for 4myc-NΔE, co-expressing CD8-NΔE with K44A dyn1 did not impact ^{V1744}NICD production or nuclear targeting (Figure 3I). Although differences in the level of nuclear-localized ^{V1744}NICD can be observed between individual cells by immunolocalization, immunoblot analysis, which evaluates the entire cell population, revealed that K44A dyn1 overexpression leads to somewhat increased ^{V1744}NICD levels relative to controls (Figure 3J). The observed increases are consistent with published reports indicating elevated Notch signaling following K44A dyn1 overexpression (Tagami et al., 2008). To evaluate CD8-NΔE endocytosis, we first infected tetracycline transactivator (tTA) HeLa cells with adenovirus encoding CD8-NΔE to maximize the number of Notch chimera-expressing cells. Cells were then incubated with 51.1, a monoclonal antibody that specifically recognizes the extracellular CD8-tag (Martin et al., 1984), to measure single-round internalization kinetics (see Materials and Methods). In contrast to 4myc-NΔE, we were able to quantitatively measure CD8-NΔE internalization, which reaches a maximum uptake within 10 min (Figure 3K). After 10 min, we observed a consistent decrease, indicating CD8-NΔE recycles back to the plasma membrane following endocytosis. By comparison, control-infected tTA HeLa cells show only background levels of 51.1 uptake when treated under identical conditions, showing that the observed CD8-NΔE uptake is specific.

Our results and those of others (Seugnet et al., 1997; Struhl & Adachi et al., 2000; Tagami et al., 2008) show that overexpression of dominant-negative dynamin forms did not prevent NEXT-mediated Notch signaling. These observations support the idea that NEXT endocytosis is not required for signaling by Notch. However, it remains possible, as previously suggested (Vaccari et al., 2008), that NEXT uptake occurs via an endocytic pathway that is

unaffected by K44A dyn1 overexpression. To test this possibility, we measured the impact of K44A dyn1 overexpression on CD8-NΔE endocytosis. We found that the CD8-NΔE internalization rate is signficiantly reduced when K44A dyn1 is overexpressed (Figure 3L). We also found that CD8-NΔE continued to accumulate in cells overexpressing K44A dyn1 over time. This suggests that K44A dyn1 also disrupts CD8-NΔE recycling, consistent with the known impact of mutant dyn1 overexpression on receptor recycling from early endosomes (van Dam et al., 2002; van Dam and Stoorvogel, 2002). Given that CD8-NΔE internalization is impaired following K44A dyn1 overexpression, yet ^{V1744}NICD is still produced and targeted to nuclei, these data provide evidence that γ-secretase-dependent NEXT cleavage does not require endocytosis.

Clathrin drives CD8-NΔE internalization

Results presented here and several published reports link Notch internalization to the clathrin-mediated endocytic pathway (Le Borgne et al., 2005). However, the requirement for clathrin in Notch endocytosis has not been directly tested in mammalian systems. To define the mechanisms that govern Notch internalization, we tested if CD8-NΔE clearance from the plasma membrane is clathrin-dependent using an siRNA depletion strategy. Following clathrin heavy chain (CHC) depletion, we observed a marked reduction in CD8-NΔE internalization and an increase in CD8-NΔE stability relative to controls (Figure 4A,B). Similar to that observed following K44A dyn1 overexpression, CHC knockdown did not impact ^{V1744}NICD production or targeting to the nucleus (Figure 4C).

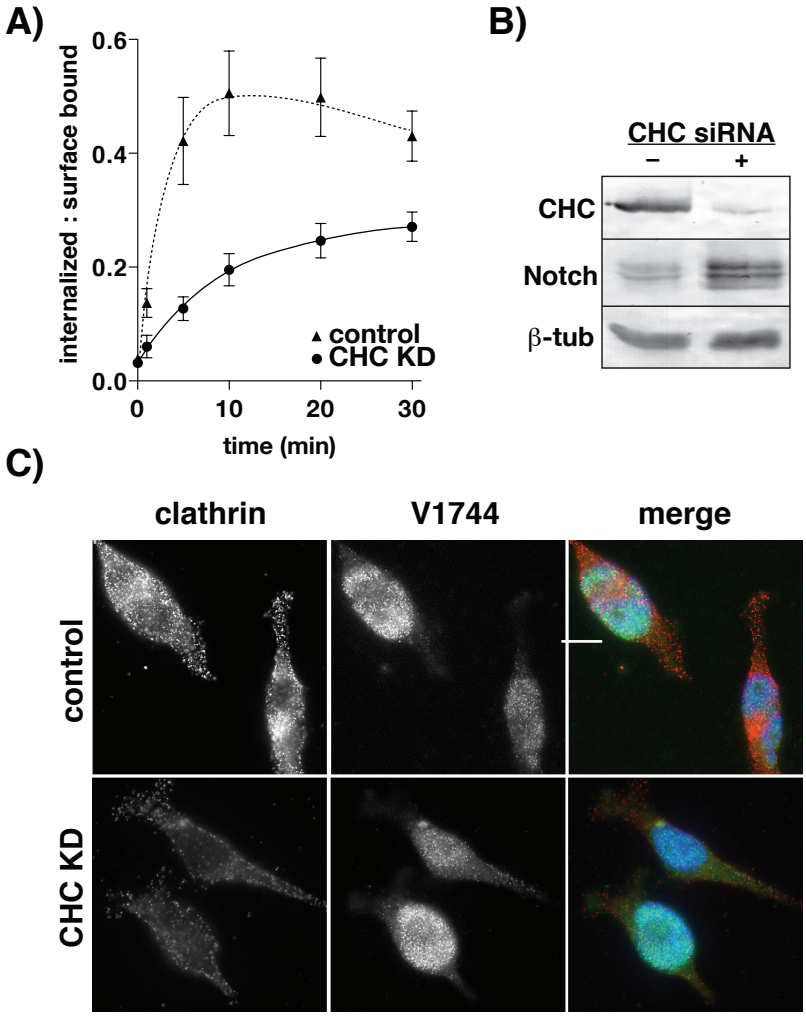

Figure 4: CD8-NΔE internalization is clathrin-dependent. tTa HeLa cells were tranfected with scrambled (control) siRNA or siRNAs directed against CHC (A–C) Cells were then analyzed for CD8-NΔE internalization (A/D), by immunoblot to score protein expression (B), or by immunofluorescence (C), as described in the Materials and Methods. Error bars represent the SEM of six independent experiments. C) V1744NICD (green) and clathrin or AP2 (red) were detected with epitope-specific antisera and merged images include DAPI (blue) to mark nuclei. bar = 10 μm.

Epsin and AP2 promote Notch internalization

Defects in CD8-NΔE internalization following CHC knockdown indicate that Notch endocytosis occurs via a clathrin-dependent mechanism. However, clathrin coupling to endocytic cargo requires additional endocytic accessory factors (Maldonado-Baez and Wendland, 2006; Traub, 2009). One such factor is the heterotetrameric adaptor protein complex AP2 (Bonifacino and Traub, 2003). We tested the possibility that AP2 promotes Notch uptake by evaluating CD8- NΔE endocytosis following adaptor complex depletion. To maximize AP2 expression knockdown, we pretreated CD8-NΔE-expressing cells with siRNAs that target the α and μ2 subunits (Motley et al., 2003) (5B). We found that the rate of CD8-NΔE endocytosis is impaired in AP2-depleted cells relative to controls (Figure 5A/B). Moreover, like that for CHC knockdown, we observed increased CD8-NΔE stability with no defect in ^{V1744}NICD nuclear localization (Figure 5B/E).

Because AP2 promotes internalization by coupling receptors to clathrin and other components of the endocytic machinery (Maldonado-Baez and Wendland, 2006; Owen et al., 2004), we hypothesized that AP2 might directly engage the Notch cytoplasmic tail to coordinate receptor uptake. To resolve this possibility, we tested whether AP2, isolated from bovine-brain, could directly bind the Notch cytoplasmic tail fused to 6HIS (IC-6HIS). We find that AP2 readily binds Ni^{2+}-agarose immobilized IC-6HIS, but not Ni^{2+} alone (5D). While these results indicate that AP2 performs an important role in Notch endocytosis, the CD8-NΔE internalization defect observed was less severe relative to clathrin loss (Figure 4A). This raised the possibility that our conditions were not sufficient to maximally disrupt AP2 activity. To test this, we measured ^{125}I-labeled-transferrin endocytosis using the same AP2-depleted cell population as that used for

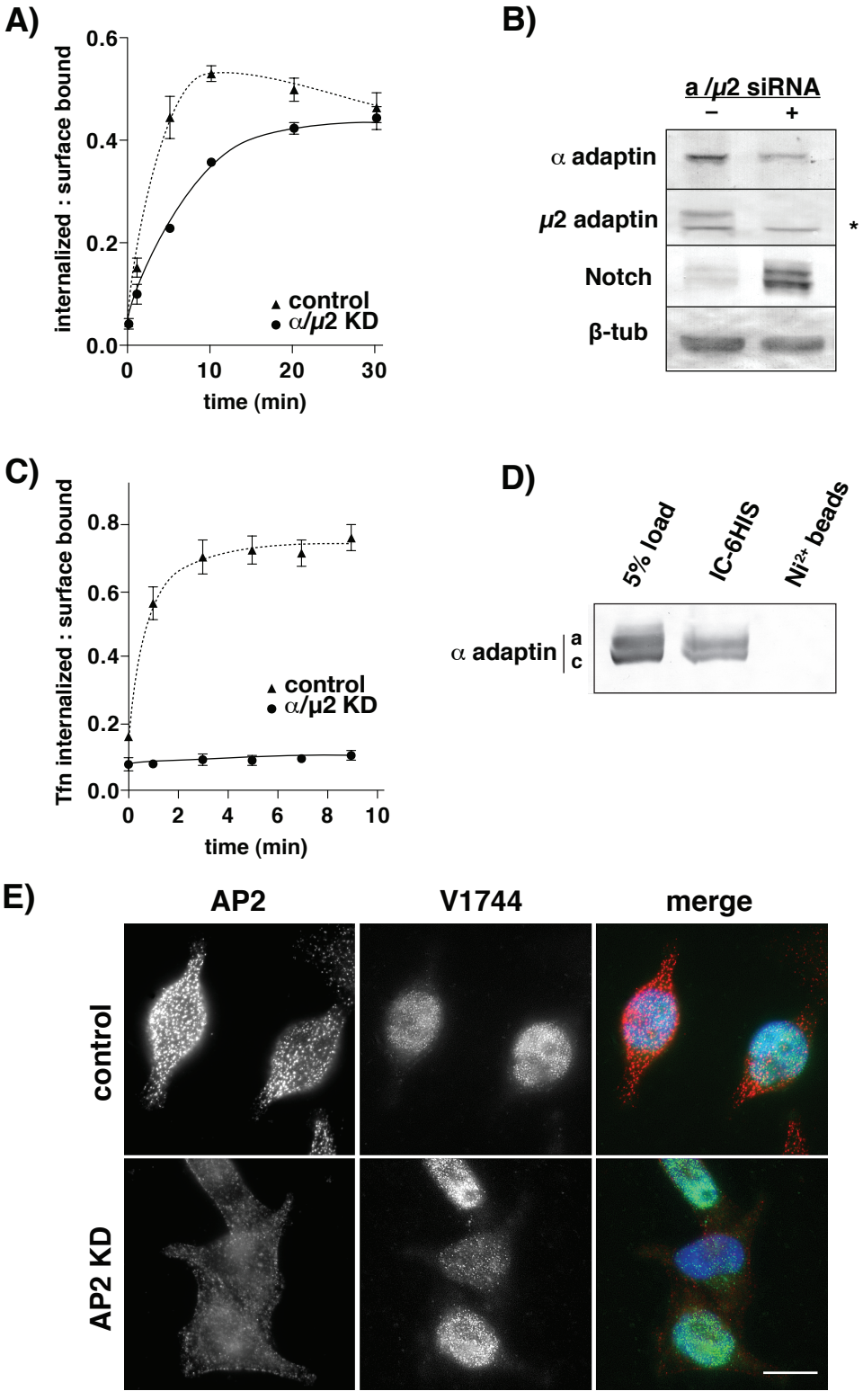

Figure 5: CD8-NΔE internalization is AP2-dependent.

Figure 5: CD8-NΔE internalization is AP2-dependent. tTa HeLa cells were tranfected with scrambled (control) siRNA or siRNAs directed against the α and μ2 subunits of AP2 (D–F). Cells were then analyzed for CD8-NΔE internalization (A), 125I-labeled transferrin internalization(C), by immunoblot to score protein expression (B), or by immunofluorescence (E), as described in the Materials and Methods. A/C) Error bars represent the SEM of five independent experiments. The asterisk (B) indicates a non-specific band. D) AP2 can directly bind the Notch cytoplasmic tail. Bovine brain-isolated AP2 was incubated with 6HIS-Notch IC immobilized on Ni2+ agarose or Ni2+ agarose alone. AP2 binding was detected by immunoblot using α-specific antisera. Both a and c isoforms of alpha adaptin are detected by immunoblot. E) V1744NICD (green) and clathrin or AP2 (red) were detected with epitope-specific antisera and merged images include DAPI (blue) to mark nuclei, bar = 10 μm.

CD8-NΔE uptake (Figure 5A). In agreement with published reports (Motley et al., 2003), we found that transferrin uptake was potently impaired (Figure 5C). This showed that our experimental conditions sufficiently disrupted AP2 activity, and suggested that additional endocytic adaptors perform more critical roles in promoting Notch endocytosis.

Studies in Drosophila reveal an important role for the E3 ubiquitin ligase, Nedd4, in Notch downregulation by endocytosis (Sakata, 2004). Although Nedd4 null mutations are embryonic lethal in flies, Sakata et al. (2004) found that mutant forms of Nedd4 disrupt ligand-independent Notch uptake when overexpressed and siRNA-mediated Nedd4 depletion in S2 cells increased Notch stability. Consistent with these results, we found that siRNAmediated Nedd4 depletion in mammalian cells disrupts CD8-NΔE endocytosis and increases CD8-NΔE stability (Figure 6A,B). These observations reinforce the idea that ubiquitination is critical for Notch endocytosis. Moreover, these results implicate an essential role for endocytic adaptors that engage ubiquitinated cargo.

The endocytic adaptor proteins Eps15 and epsin both encode functional ubiquitin-interacting motifs (Polo et al., 2002; Shih et al., 2002). Thus, we postulated that these adaptors might perform essential roles in Notch endocytosis. We tested this by measuring CD8-NΔE internalization following siRNA-mediated depletion of each adaptor (6D,F). Despite significant expression knockdown, CD8-NΔE endocytosis was not impacted by a reduction in Eps15 (Figure 6C). By contrast, epsin1 depletion revealed a marked reduction in CD8-NΔE internalization and increased stability (Figure 6E,F). This suggests that epsin1 performs a key role in Notch internalization.

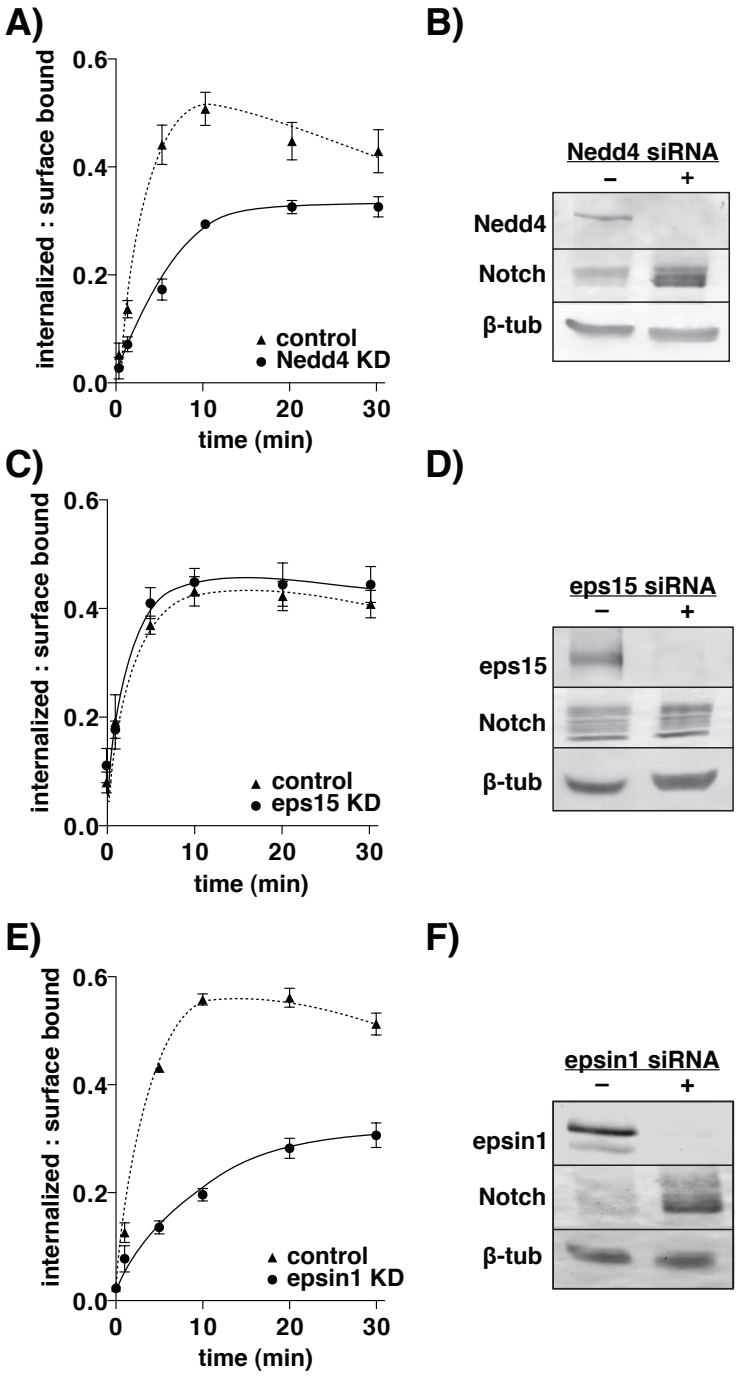

Figure 6: Robust CD8-NΔE internalization requires Nedd4 and epsin1. A) tTa HeLa cells were transfected with control siRNA or siRNAs targeting Nedd4 (A/B), eps15 (C/D) or epsin1 (E/F). CD8-NΔE internalization was then scored and immunoblot analysis performed to validate protein expression and equal loading, as described in Figure 2. Errors bars represent ± SEM of five to eight independent experiments.

γ-Secretase-dependent NEXT cleavage occurs at the plasma membrane

Our immunolocalization analyses indicated that ^{V1744}NICD production and nuclear targeting occurs independent of receptor endocytosis (Figures 3,4, and 5). However, the extent of nuclear-localized ^{V1744}NICD between cells within a given population was heterogeneous. Therefore, we used immunoblot analysis to evaluate the potential impact of impaired CD8-NΔE internalization on ^{V1744}NICD production. In each case where endocytosis is disrupted by siRNA-mediated depletion of CHC, AP2 or epsin1, we observed increased ^{V1744}NICD production relative to controls (Figure 7A). Conversely, no significant change in ^{V1744}NICD was observed in Eps15-depleted cells, which show control levels of CD8-NΔE endocytosis (Figure 6C).

To ensure that increased ^{V1744}NICD production reflects increased downstream signaling, we used an RBP-Jk luciferase reporter assay to measure Notch signaling activity. Consistent with our immunoblot analyses, we observed increases in luciferase activity relative to controls when CD8-NΔE internalization was disrupted by clathrin, AP2, epsin1 or Nedd4 depletion (Figure 7B). Together, these observations support the conclusion that elevated Notch signaling results from increased CD8-NΔE exposure to plasma membrane-localized γ-secretase. We therefore predicted that Notch should accumulate at the plasma membrane when both γ-secretase activity and CD8-NΔE internalization are disrupted. To test this idea, CD8-NΔE-expressing cells were depleted of clathrin and treated with compound E (CE). In these cells, we observed a significant increase in plasma membrane-localized CD8-NΔE that was not observed in control cells (Figure 7C).

Consistent with published reports (Struhl & Adachi, 2000), our observations indicate that 4myc-NΔE cleavage by γ-secretase is significantly more efficient than that of CD8-NΔE (Figure 3G). Moreover, we found that

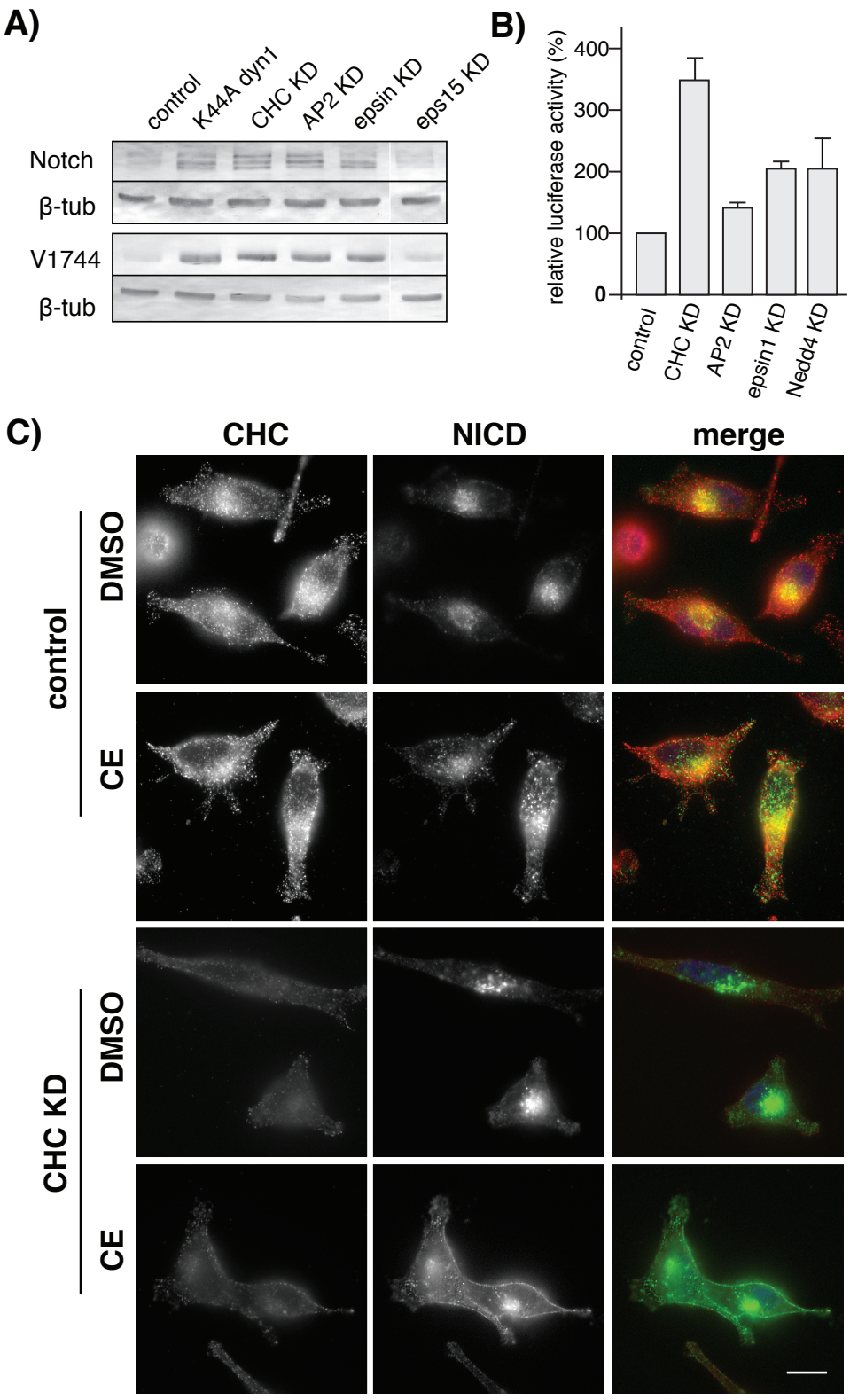

Figure 7: γ-Secretase-dependent CD8-NΔE cleavage occurs at the plasma membrane.

Figure 7: γ-Secretase-dependent CD8-NΔE cleavage occurs at the plasma membrane. A) tTa HeLa cells were transfected with control siRNA or siRNAs targeting CHC, AP2 (α and μ2 subunits), epsin1 or Eps15. Cells were then infected with CD8-NΔE-encoded adenovirus and V1744NICD production was evaluated by immunoblot. B) Notch signaling was evaluated using an RBP-Jk luciferase reporter assay in control cells or those depleted of CHC, AP2, epsin1 or Nedd4. C) Control siRNA treated or CHC-depleted cells expressing CD8-NΔE were pretreated with either DMSO or CE and analyzed by immunolocalization for clathrin (red) or the cytoplasmic Notch tail (green). Merged images include DAPI stain (blue) to mark nuclei (C). Error bars represent ± SEM of four independent experiments.

endocytosis defects did not impair γ-secretase-dependent 4myc-NΔE or CD8-NΔE cleavage (Figure 3H,I). These observations likely explain our inability to consistently measure 4myc-NΔE endocytosis using our current protocol. For example, our current method requires two pre-binding steps at 4ºC to allow antibody and ^{125}I-protein A binding and to prevent their premature endocytosis into cells expressing 4myc-NΔE or CD8-NΔE. We postulate that γ-secretase remains active during these pre-binding steps, it efficiently cleaves 4myc-NΔE and eliminates our ability to quantitatively track 4myc-NΔE uptake. To test this idea, 4myc-NΔE-expressing cells were incubated with pre-warmed media containing 9E10 for 10 min before fixation. Immunolocalization analysis reveals significant antibody binding and uptake after 10 min, showing 4myc-NΔE is expressed at the cell surface and capable of internalization (Figure S3A). From these observations, we suspect that γ-secretase cleaves 4myc-NΔE during the 4ºC pre-incubation step and prevents quantitatively measuring 4myc-NΔE uptake. This limitation is circumvented by using the CD8-NΔE chimera as its cleavage by γ-secretase is less efficient (Figure 3A,G).

However, the possibility remained that 4myc-NΔE and CD8-NΔE might be transported differently within cells and thus account for the differences in their signaling capacity (Figure 3G). To address this possibility, we tested 4myc-NΔE signaling following clathrin depletion. Consistent with that observed for CD8-NΔE, we find that siRNA-mediated clathrin depletion leads to increased signaling by 4myc-NΔE relative to siRNA controls (Figure S3B). Pretreating cells with CE abolishes signaling indicating that the signaling is γ-secretase-dependent. From this, we conclude that 4myc-NΔE and CD8-NΔE employ the same internalization mechanism. The increases in ^{V1744}NICD production and signaling when endocytosis is disrupted, combined with Notch accumulation at the plasma

membrane following clathrin depletion and γ-secretase inhibition (Figure 7), lead us to conclude that γ-secretase is active at the plasma membrane.

Clathrin is essential at multiple Notch transport steps

Immunolocalization analysis of CD8-NΔE following clathrin depletion revealed accumulation within a perinuclear compartment (Figure 7C). In addition to endocytosis, clathrin also functions at the trans Golgi network (TGN) and endosome to facilitate receptor transport within the cell. The perinuclear accumulation of CD8-NΔE suggested the possibility that clathrin might also function in Notch transport from the TGN. To test this, we transfected CD8-NΔE-expressing cells with a TGN marker, TGN38-GFP (Girotti & Banting, 1996), and evaluated Notch localization. In control cells, we find little colocalization between CD8-NΔE and TGN38-GFP. By contrast, a marked increase in colocalization was observed when clathrin is depleted (Figure 8). Despite impaired transport from the TGN, CD8-NΔE is still observed in association with cytoplasmic vesicles when clathrin expression is reduced. We interpret this to mean that efficient Notch transport from the TGN requires clathrin, although the TGN transport defect that results in clathrin-depleted cells can be overcome by protein overexpression.

Ubiquitination enhances Notch–epsin1 interaction

Internalization data presented here indicate an important role of Nedd4 and epsin1 in promoting CD8-NΔE endocytosis (Figure6). Nedd4 is known to ubiquitinate Notch (Sakata et al., 2004) and epsin1 is thought to couple receptors to the internalization machinery by engaging ubiquitinated cargo (Maldonado-

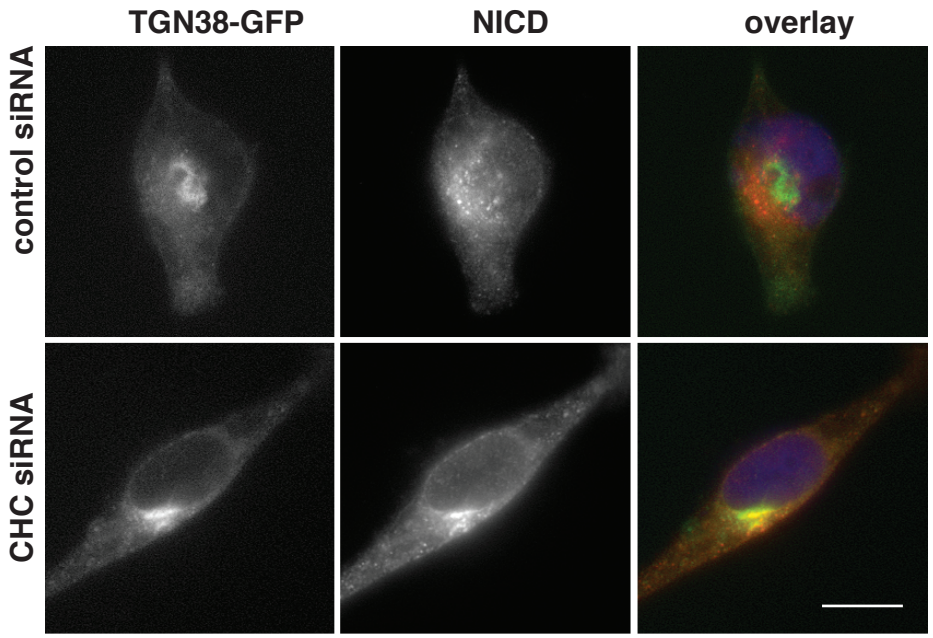

Figure 8: Clathrin functions in Notch transport from the TGN. tTA HeLa cells were treated with control or CHC-specific siRNA for 50 h. Cells were then infected with adenovirus encoding CD8- NΔE and transfected with plasmid encoding TGN38-GFP. After an additional 16-h incubation, cells were then fixed and processed for immunolocalization. NICD was detected using polyclonal anti-sera directed against the Notch cytoplasmic tail. Overlays indicate colocalization of NICD (red) with TGN38-GFP (green) in yellow. Blue marks nuclei, bar = 10 μ m.

Baez & Wendland , 2006). Given this, we postulated that ubiquitination might regulate epsin1 activity in coordinating Notch uptake. To test this idea, we first performed immunolocalization analysis to ask if endogenous epsin1 colocalized with CD8-NΔE at the plasma membrane. Using total internal reflection fluorescence (TIRF), we observed significant colocalization between endogenous epsin1 and CD8-NΔE at the cell surface (Figure 9A). To extend our analysis, we next evaluated a potential interaction between epsin1 and Notch by coimmunoprecipitation. To do so, we co-expressed wildtype (WT) epsin1 with either CD8-NΔE or 4myc-NΔE in tTA HeLa cells. Notch chimeras were then immunoprecipitated from cell lysates and analyzed for WT epsin1. We found that WT epsin1 was detected by immunoblot from cells co-expressing recombinant WT epsin1 and either CD8-NΔE (not shown) or 4myc-NΔE (Figure 9B). We next tested if the epsin1 interaction with the Notch cytoplasmic tail was ubiquitin-dependent by coimmunoprecipitation analysis of 4myc-NΔE with an epsin1 ubiquitin-binding mutant, previously reported incapable of binding ubiquitinated proteins (Kazazic et al., 2009). Immunoblot analysis revealed significantly less mutant epsin1 coimmunoprecipitation with 4myc-NΔE than that observed for WT epsin1 (Figure 9B). This result suggests that epsin1 recruitment to the Notch cytoplasmic tail is enhanced by receptor ubiquitination. To evaluate epsin1 mechanism of action, we next wanted to test if the epsin1 ubiquitin-binding mutant could rescue the CD8-NΔE internalization defect that results from endogenous epsin1 depletion. However, this was not possible as WT epsin1 overexpression was found to impair CD8-NΔE internalization (Figure 9C,D). Similar to published reports, this dominant-negative effect likely reflects competition with other endocytic factors, including clathrin and AP2, both of which are known to stably bind epsins (Rosenthal et al.,1999).

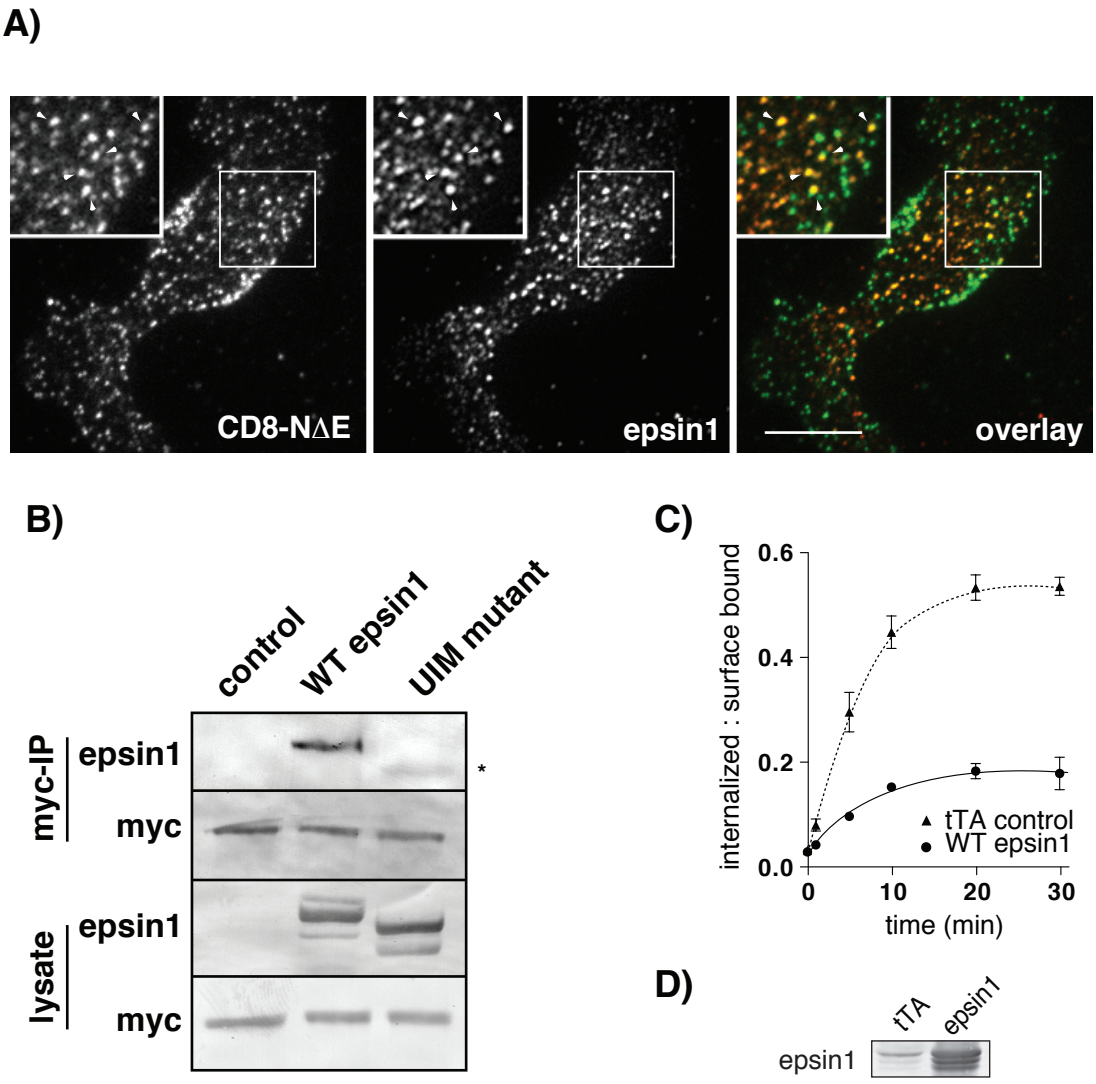

Figure 9: Ubiquitination promotes epsin1 binding to Notch. A) CD8-NΔE-expressing tTA HeLa cells were analyzed by TIRF microscopy using antibodies against the extracellular CD8 epitope (green) and pAb against endogenous epsin (red). Boxed regions indicate areas of higher magnification with arrowheads marking colocalization examples. B) 4myc-NΔE-expressing tTA HeLa cells were transfected with either WT epsin1 or UIM mutant epsin1 (see Materials and Methods). The mAb 9E10 was then used to immunoprecipitate 4myc-NΔE from cell lysates, which were then analyzed for epsin1 binding by immunoblot. The asterisk indicates a minor interaction between 4myc-NΔE and UIM mutant epsin1. C) CD8-NΔE-expressing cells were transfected with WT epsin1 or infected with tTA-expressing adenovirus (control) and analyzed for CD8-NΔE internalization. D) Epsin1 expression levels were evaluated by immunoblot. Errors bars represent ± SEM of three independent experiments, bar = 10 μm.

Discussion:

The observations we report here provide additional mechanistic insight into Notch internalization and signaling. Our results argue that clathrin-mediated receptor internalization downregulates Notch signaling. This interpretation reinforces the conclusions of previously published work (Shaye & Greenwald, 2002, 2005; Struhl & Adachi, 2000) and is supported by three main results in this study. First, we show that when the CD8-NΔE internalization rate is impaired by siRNA-mediated depletion of multiple endocytic components (i.e. clathrin, AP2 or epsin1), Notch signaling continues. Likewise, K44A dyn1 overexpression disrupts endocytosis but did not reduce Notch signaling. Second, our results show that disrupting internalization, which prolongs CD8-NΔE transit time at the plasma membrane, leads to elevated ^{V1744}NICD production and increases in downstream Notch signaling. Third, Notch accumulates at the plasma membrane when receptor uptake and γ-secretase activity are inhibited.

NEXT cleavage and signal activation occur at the plasma membrane

Endocytosis is currently modeled as a requirement for NEXT cleavage by endosome-localized γ-secretase (Fortini and Bilder, 2009; Tien et al., 2009). In support of this model, mosaic analysis of the developing Drosophila ovary shows that follicle cells, which overexpress a dominant-negative dynamin or those that lack clathrin or dynamin, are unable to activate the Notch signaling pathway (Vaccari et al., 2008; Windler & Bilder, 2010). Likewise, K44A dyn1 overexpression in mammalian cells was reported to limit γ-secretase cleavage of a truncated form of Notch that mimics NEXT (Gupta-Rossi et al., 2004). Based on our observations, we favor the originally proposed model in which

γ-secretase-mediated NEXT cleavage occurs at the plasma membrane (Struhl & Adachi, 2000). Although processing within the endosome cannot be completely excluded. Our quantitative analyses reveal that Notch signaling is independent of endocytosis and instead proportional to the level of γ-secretase activity at the plasma membrane. The model for plasma membrane-linked cleavage is supported by data from previous studies in both genetic and cell-based systems, which show productive Notch signaling despite receptor internalization defects. For example, acute disruption of endocytosis in Drosophila did not impact presenilin-dependent NEXT processing or subsequent downstream signaling when flies expressing a temperature-sensitive shibire allele were shifted to a non-permissive temperature (Struhl & Adachi, 2000). In support of this observation, Notch was found to accumulate at apical membranes in cells expressing mutant forms of nicastrin or presenilin (Guo et al., 1999; Lopez-Schier & St Johnston , 2002). The idea that NEXT processing occurs at the plasma membrane is reinforced by results in *C. elegans* where mutations in the Lin12/Notch cytoplasmic tail, which disrupt receptor internalization, remain functional for signaling and rescue the lethality caused by loss of endogenous Lin12/Notch (Shaye & Greenwald, 2002, 2005).

Consistently, the results obtained in both flies and worms are supported by findings in mammalian cell culture, where Notch signaling is unimpaired or even elevated in cells overexpressing dominant-negative K44A dyn1 (Kaether et al., 2006; Tagami et al., 2008,). Moreover, Tarassishin et al. (2004) reported that treating HEK293 cells with a membrane-impermeable γ-secretase inhibitor, MRL631, significantly disrupted Notch signaling mediated by a NEXT mimic, while γ-secretase-mediated cleavage of amyloid precursor protein within the endosome remained unperturbed. Our observations support these findings

and, collectively, they provide strong evidence for a model in which γ-secretase initiates Notch signaling by cleaving NEXT at the plasma membrane.

Endosomal Notch activation

How do we reconcile our observations with those showing a requirement for NEXT internalization in activating the Notch signaling pathway? In mammalian cells, Gupta- Rossi et al. (2004) failed to detect nuclear accumulation of NICD by immunocytochemistry following overexpression of mutant forms of dynamin 2 or Eps15. However, in this study, the changes in the kinetics of ^{V1744}NICD production were never specifically evaluated. Moreover, it is possible that endosomal transport of the truncated Notch form employed, which lacks nearly half the cytoplasmic tail, may not reflect the behavior of wildtype NEXT. By contrast, Vaccari et al. (2008) clearly show defective Notch signaling in Drosophila follicle cells overexpressing a mutant *shibire* allele. Similarly, a more recent study shows defects in Notch signaling in follicle cells lacking clathrin or dynamin (Windler & Bilder, 2010). Given the well-characterized requirement for clathrin and dynamin in endocytosis, the simple interpretation of these results is that Notch internalization is essential for signaling. However, it is possible that loss of dynamin and/or clathrin function, in addition to disrupting endocytosis, may also impact Notch transport throughout the cell. Indeed, we find that Notch accumulates at the TGN in clathrin-depleted cells. This indicates that clathrin functions at multiple Notch transport steps. Moreover, it suggests the possibility that failure to observe Notch signaling in fly ovarian cells, which completely lack clathrin or dynamin, might result from limited endogenous Notch delivery to the plasma membrane. Indeed, immunolocalization analysis reveals less Notch targeting to the plasma membrane in fly ovarian cells that lack either dynamin

or clathrin relative to controls. By contrast, the cells lacking AP2, the archetypal endocytic adaptor, reveal strong Notch accumulation at the plasma membrane, yet Notch signaling remains unaffected (Windler & Bilder, 2010). This latter observation is consistent with our findings that efficient Notch endocytosis is AP2-dependent and that signaling continues when receptor uptake is impaired.

Clathrin-dependent endocytosis downregulates Notch signaling

Our findings and those of others support a model in which endocytosis serves to downregulate, not activate, the Notch signaling pathway (Kaether et al., 2006; Shaye & Greenwald, 2002, 2005; Tagami et al., 2008; Tarassishin et al. 2004). We show that Notch internalization occurs via a clathrin-dependent mechanism. Consistent with this, we discovered that robust internalization requires AP2 and that AP2 and the Notch cytoplasmic tail can directly interact. However, the requirement for AP2 during Notch internalization appears to be less than that observed for clathrin. We suggest that AP2 performs an auxillary role in promoting Notch uptake and that receptor internalization is more heavily reliant on other endocytic factors. In contrast, we discovered that epsin1 depletion disrupts Notch endocytosis to a similar extent as that observed following clathrin loss. Given the recently described redundancy of epsin1 and epsin2 in mice (Chen et al., 2009), our observation suggests that the epsin2 isoform in HeLa cells is not sufficient to compensate for epsin1 loss. In general agreement, genome-wide expression analyses indicate that HeLa cells may express as much as sevenfold more epsin1 than epsin2 (Su et al., 2004).

Although the molecular details by which epsin1 coordinates Notch internalization remain to be resolved, two key observations implicate receptor

ubiquitination as a critical step in promoting Notch endocytosis. 1) Mammalian Nedd4 depletion impairs Notch endocytosis. This observation agrees well with similar results in *Drosophila* S2 cells, where Notch becomes trapped at the plasma membrane following siRNA-mediated Nedd4 knockdown (Sakata et al., 2004). 2) The epsin1 ubiquitin-binding mutant shows impaired association with Notch. Given that epsin1 is required for Notch endocytosis, these observations provide new information on how post-translational modification mediates the trafficking of Notch. Thus, we propose that Nedd4-mediated receptor ubiquitination promotes epsin1 interaction with Notch. Epsin1 could then deliver Notch to clathrin-coated pits through its interaction with other components of the endocytic machinery [i.e. AP2 and/or clathrin; (Chen et al., 1998)] to promote receptor uptake into the cell. In future studies, it will be important to further test this mechanism and to elucidate the regulatory pathways that control Notch receptor ubiquitination.

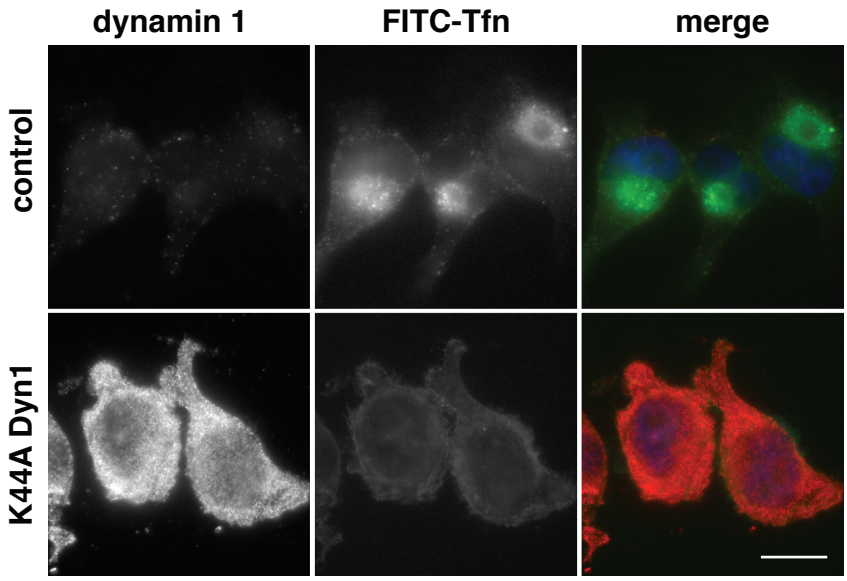

Figure S1: K44A dynamin 1 overexpression disrupts transferrin endocytosis. tTA HeLa cells were infected with adenovirus encoding tTA or HA-tagged K44A dyn1. Cells were incubated with fluorescein isothiocyanate (FITC)-labeled transferrin (5 µg/mL, green) for 8 min at 37◦C before fixation. K44A dyn1 expression was detected with the mAb hudy1 (red). Merged images include DAPI (blue) to mark cell nuclei, bar = 10 µm.

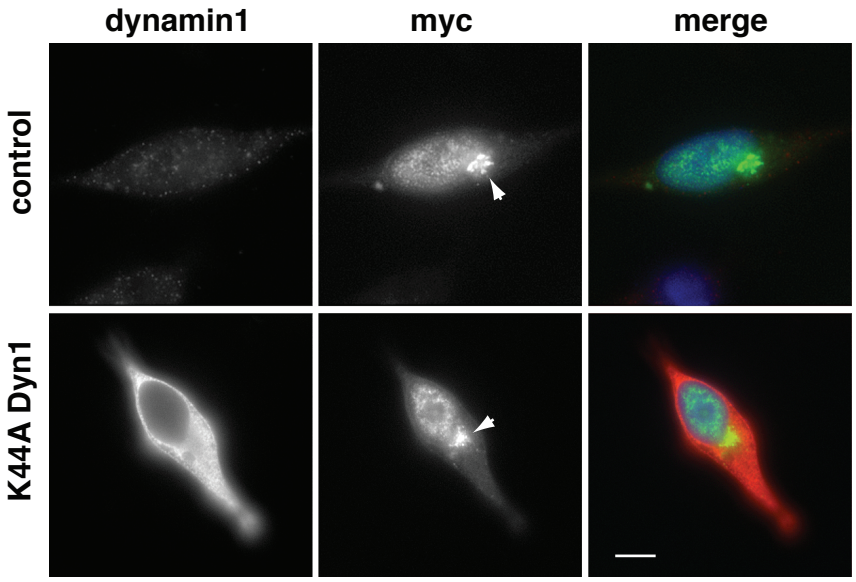

Figure S2: 4myc-NΔE-induced expression of endogenous c-myc is not disrupted by dominant-negative dynamin overexpression. 4myc-NΔE-expressing tTA HeLa cells were infected with adenovirus encoding tTA (control) or K44A dyn1. Endogenous c-myc expression (green, nuclear stain) was detected with FITC-tagged 9E10, which also recognizes endosome-localized 4myc-NΔE in the perinuclear compartment (arrowhead). Merged images include DAPI (blue) to mark cell nuclei, bar = 10 μm.

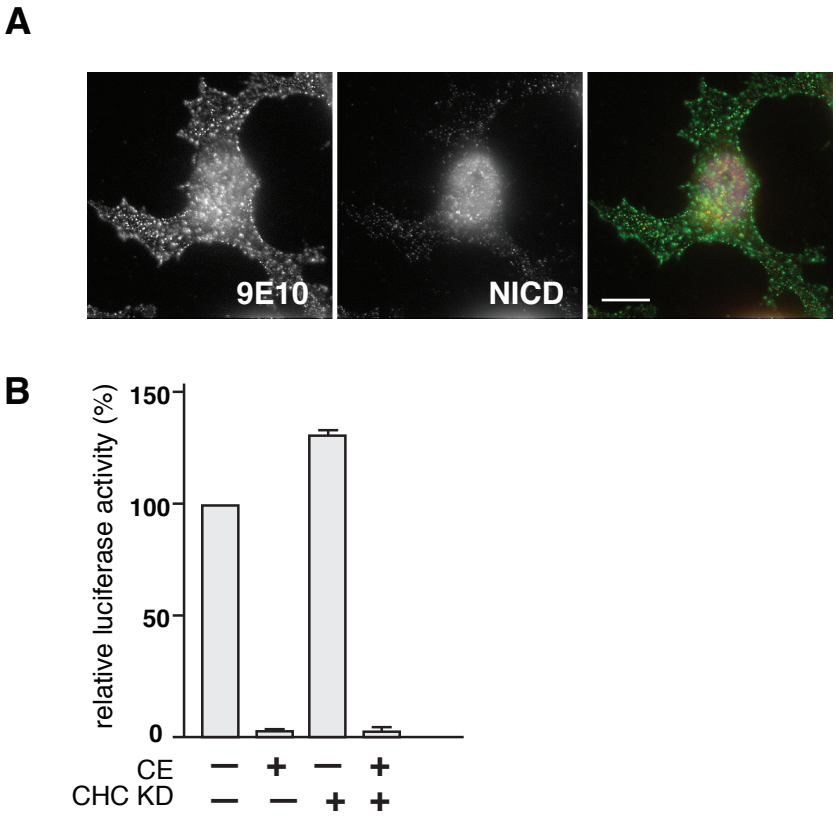

Figure S3: 4myc-NΔE is expressed at the cell surface and can be internalized. A) 4myc-NΔE-expressing tTA HeLa cells, grown on coverslips, were transferred to media containing 9E10 for 10 min at 37◦C. Cells were then fixed and probed for 9E10 using a fluorochrome-tagged secondary to mark 4myc-NΔE (green) and for the Notch cytoplasmic tail (red). Merged image includes DAPI (blue) to mark cell nuclei. Nuclear localized Notch reflects V1744NICD produced by γ-secretase-mediated cleavage of 4myc-NΔE. B) tTA HeLa cells were treated with control siRNA or CHC siRNA. After 50-h incubation, cells were then infected with adenovirus encoding 4myc-NΔE and incubated in the presence or absence of CE to inhibit γ-secretase activity. After an additional 16-h incubation, Notch signaling was evaluated using an RBP-Jk luciferase reporter assay as described in the Materials and Methods.

Chapter III: AAK1 Regulates Numb Function at an Early Step in Clathrin-Mediated Endocytosis

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Numb is an endocytic protein that is proposed to influence clathrin-coated pit assembly, although its mode of action and the mechanisms that regulate its activity are unknown. In this study, we show that Numb binds to and is phosphorylated by adaptor-associated kinase 1 (AAK1), a key endocytic kinase. We find that AAK1 redistributes Numb to perinuclear endosomes when overexpressed, while kinase depletion causes Numb to accumulate at the plasma membrane. Overexpression of a Numb point mutant (T102A) that lacks the AAK1 phosphorylation site potently disrupts transferrin and low-density lipoprotein internalization but does not impact EGF uptake. Consistent with Numb redistribution results, we find that T102A Numb no longer localizes to perinuclear endosomes. Instead, it is enriched at the plasma membrane where it shows elevated levels of colocalization with coated pit markers. Collectively, these observations demonstrate that Numb endocytic activity is regulated by AAK1 and that phosphorylation may be a critical step in promoting coated pit maturation.

Introduction:

The efficiency of receptor endocytosis through the clathrin-dependent pathway results from the tightly coordinated assembly of the internalization machinery. This is accomplished through the action of endocytic adaptors that nucleate and spatially organize clathrin and other endocytic components at the plasma membrane to promote receptor uptake (Maldonado-Baez & Wendland, 2006). Proteins encoding an amino-terminal phosphotyrosine-binding (PTB) domain constitute a major class of endocytic adaptors that includes Numb (Santolini et al., 2000), autosomal recessive hypercholesterolemia (ARH) (Garcia et al., 2001), disabled 2 (Dab2) (Mishra et al., 2002; Morris & Cooper, 2001) and GULP/CED-6 (Su et al., 2002). The independently folded PTB domain recruits these adaptors to the plasma membrane by simultaneously binding phospholipids and the cytoplasmic tails of receptors containing an NPXY internalization motif (Uhlik et al., 2005). Once there, PTB adaptors are thought to nucleate the assembly of additional endocytic factors through multiple peptide-binding motifs encoded within their highly divergent carboxy-terminal regions (Maldonado-Baez & Wendland, 2006).

Growing evidence demonstrates that both ARH and Dab2 perform cargo-selective roles in endocytosis where they promote low-density lipoprotein (LDL) receptor internalization by directly coupling the receptor to the endocytic machinery (Maurer & Cooper, 2006; Traub, 2003). Likewise, GULP/CED-6 is implicated in LDL-receptor-related protein uptake (Su et al., 2002). By contrast, Numb function as an endocytic adaptor does not appear to be cargo specific. Numb is best characterized as a Notch signaling antagonist in *Drosophila* (Wilkin and Baron, 2005) where it is postulated to promote Notch removal from the plasma membrane (Berdnik et al., 2002). However, Numb recruitment to clathrin-

coated pits and its presence in various endosomal compartments in mammalian cells suggest that Numb may function at multiple receptor transport steps (Santolini et al., 2000). Indeed, depletion studies show that Numb is required for integrin b1/b3 endocytosis (Nishimura & Kaibuchi, 2007) as well as interleukin 2a receptor recycling from the endosomal compartment (Smith et al., 2004).

Although the precise mode of action for Numb in receptor transport is currently unresolved, recent observations demonstrate that Numb activity is dynamic and regulated by phosphorylation. For example, phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) disrupts Numb interaction with the heterotetrameric adaptor complex AP2 *in vitro* (Tokumitsu et al., 2006). Likewise, phosphorylation by atypical protein kinase C (aPKC) releases Numb from clathrin-coated pits (Nishimura & Kaibuchi, 2007) and redistributes it to the cytosol (Smith et al., 2007). In addition to CaMKI and aPKC, yeast two-hybrid experiments reveal an interaction between Numb and Numb associated kinase (NAK) (Chien et al., 1998), which is the *Drosophila* homologue of the human endocytic kinase adaptor associated kinase 1 (AAK1) (Conner & Schmid, 2002). This observation led us to hypothesize that Numb activity as an endocytic adaptor might also be regulated by AAK1. We have tested this is a critical step that influences Numb activity during endocytosis.

Materials and Methods:

Reagents

The monoclonal antibody (mAb) 9E10 was used to recognize myc-tagged proteins. Where indicated, 9E10 was directly labeled with Alexa Fluor-488 using a monoclonal antibody labeling kit (Invitrogen). The mAb AP.6 (Affinity Bioreagents) was used to detect the a-adaptin subunit of AP-2, and TD.1 (Covance Research

Products) was used to identify clathrin heavy chain. The mAb E7 was used to identify β-tubulin. Rabbit antisera against Numb were obtained from Abcam (ab14140). Antibodies against Eps15 and epsin were a generous gift from Sandra Schmid (The Scripps Research Institute). AAK1 antisera were previously described (Conner & Schmid, 2003[2]). Human Numb cDNA was a generous gift from Dr Joseph Verdi (Maine Medical Center Research Institute, Scarborough, ME, USA). Numb 1 was used for experiments in this study as it was the only isoform to recapitulate the endogenous localization pattern in HeLa cells. AAK1 encoding amino acids 1–863 was used in this study.

Numb constructs

Human myc-tagged Numb in pcDNA3.1 was polymerase chain reaction (PCR) amplified and subcloned into the EcoRI sites of pADtet7 for adenovirus production (Damke et al., 1995) and pFastBac1 (Invitrogen) for baculovirus-mediated protein expression. T102A Numb was generated by site-directed mutagenesis using PCR, sequence verified and subcloned as the WT.

Protein production and isolation

The Bac-to-Bac expression system (Invitrogen) was used to produce recombinant Numb–myc–6HIS in SF9 cells according to manufacturer protocols. Recombinant protein was then isolated from insect cell lysates using a Ni^{2+}-Cam resin (Sigma). Recombinant AAK1–GST was generated and isolated as described (Conner & Schmid, 2002). Protein isolation for kinase assays was isolated from tTA HeLa cells infected with adenovirus-encoding WT and T102A Numb–myc for 18 h. Myc-tagged Numb was immunoprecipitated with 9E10 from cell lysates and then used for *in vitro* kinase assays.

Kinase assays

In vitro kinase assays employing recombinant proteins were performed

essentially as described (Henderson & Conner, 2007).

***In vitro* binding and coimmunoprecipitation**

For *in vitro* interaction experiments, baculovirus-expressed WT Numb was incubated with AAK1–GST immobilized on glutathione–Sepharose 4B beads (Amersham) for 1h at room temperature. Beads were pelleted and washed three times with phosphate buffered saline containing 0.1% Tween-20 (PBST) to remove unbound protein. Bead-bound proteins were then solubilized with protein sample buffer, resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted for Numb–myc–6HIS using 9E10. For coimmunoprecipitation experiments, 5 x 10^6 tTA HeLa cells were infected with Numb–myc–6HIS adenovirus alone or in combination with AAK1 adenovirus. Cells were then lysed with lysis buffer [100 mM (N-morpholino) ethanesulphonic acid (MES), pH 6.5; 0.2 mM EGTA; 0.5 mM MgCl2; 1% Nonidet P-40 and 0.2% NaN3] containing protease inhibitor cocktail (Sigma). Cell lysates were then incubated for 1.5 h at room temperature with preimmune or AAK1-specific antisera that had been prebound to protein A–sepharose (Zymed). Matrices were washed, and bead-bound proteins were then processed for immunoblot as described above.

Immunolocalization

For immunofluorescence experiments, tTA HeLa cells grown on coverslips and infected with recombinant adenovirus for 4–6 h. Cells were fixed with ice-cold acetone for 5 min and extracted with methanol or fixed with 3.7% formaldehyde for 20 min at room temperature. Cells were then washed with PBST and incubated with the indicated primary antibody for 1 h at room temperature. Cells were washed again and incubated for 1 h at room temperature with the appropriate secondary antibody conjugated to either Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen). Samples were visualized by epifluorescence using a Zeiss

Axioscop 2 microscope equipped with x 40 or x 100 objectives, an Axiocam MRm digital camera, and AXIOVISION IMAGE ACQUISION software 3.1. Images were then imported, cropped and assembled into panels using ADOBE PHOTOSHOP CS3. Panels were then illustrated using ADOBE ILLUSTRATOR CS3.

Endocytosis assays

Cell culture conditions and quantitative single-round transferrin internalization assays were preformed as previously described (Carter et al., 1993; Henderson & Conner, 2007). CD8-NΔE internalization experiments were preformed as described in Chapter 2. For internalization of fluorochrome-tagged ligands, cells grown on coverslips were infected with recombinant adenovirus for 18 h and then incubatedwith 10 mg/mL DiI-labeled LDL (Biomedical Technologies Inc.), 4 ng/mL rhodamine–EGF and 5 mg/mL Alexa Fluor 488–transferrin (Invitrogen) for 20 min at 378C. Coverslips were then transferred to ice, washed and fixed with ice-cold 3.7% formaldehyde. For colocalization with Numb, cells were then washed with PBST and incubated with the indicated primary antibody for 1 h at room temperature. Cells were washed again and incubated for 1 h at room temperature with the appropriate secondary antibody conjugated to Alexa Fluor 555 (Invitrogen). Samples were then visualized as described above.

Results:

Numb encodes a conserved AAK1 phosphorylation site

AAK1, along with other members of the Ark1/Prk1 family of serine/threonine kinases, is thought to selectively phosphorylate proteins containing an [LIVM] XX[QNTS]X*po*TG consensus site [X is any amino acid and 'poT' indicates the phosphorylated threonine (Huang et al., 2003; Zeng & Cai, 1999)]. In general agreement, AAK1 targets the μ1 (GHKLE*po*T^{154}G) and μ2

(ITSQV*po*T^{156}G) subunits of the heterotetrameric cargo adaptors AP1 and AP2, respectively (Conner & Schmid, 2002).

Biochemical studies revealed that μ2 phosphorylation by AAK1 increases AP2 affinity for receptors encoding a YXXϕ internalization motif, implicating AAK1 activity as a key step in regulating AP2 recruitment to endocytic cargo (Ricotta et al., 2002). However, we recently discovered that AAK1 functions at multiple steps in receptor transport (Henderson & Conner, 2007), suggesting that AAK1 has other targets, possibly other endocytic adaptors. Thus, we reasoned that if AAK1 functions to regulate the activities of additional components involved in receptor transport, we could use the phosphorylation consensus site sequence as a guide to identify additional AAK1 substrates. Using this approach, we discovered a slightly modified consensus site within the amino-terminal PTB domain of Numb (Santolini et al., 2000), which is also present in ARH (Garcia et al., 2001), Dab2 (Morris & Cooper, 2001) and GULP/CED-6 (Su et al., 2002). Aligning protein sequences from several species showed that the site is highly conserved and corresponds to the consensus ϕX[DE]XX[TS] (ϕ is hydrophobic [I/V/L] and X is any amino acid) (Figure 10A). Based on the resolved nuclear magnetic resonance (NMR) structure for the Numb PTB domain (Li et al., 1998), the predicted AAK1 phosphorylation site is accessible on the protein surface and situated within a linking region between two beta strands (β3 and β4). Thus, given that AAK1 and Numb localize to clathrin-coated pits and both have implicated roles in receptor endocytosis (Conner and Schmid, 2003[2]; Nishimura & Kaibuchi, 2007; Santolini et al., 2000), the presence of the highly conserved site suggests that Numb function might be regulated by AAK1.

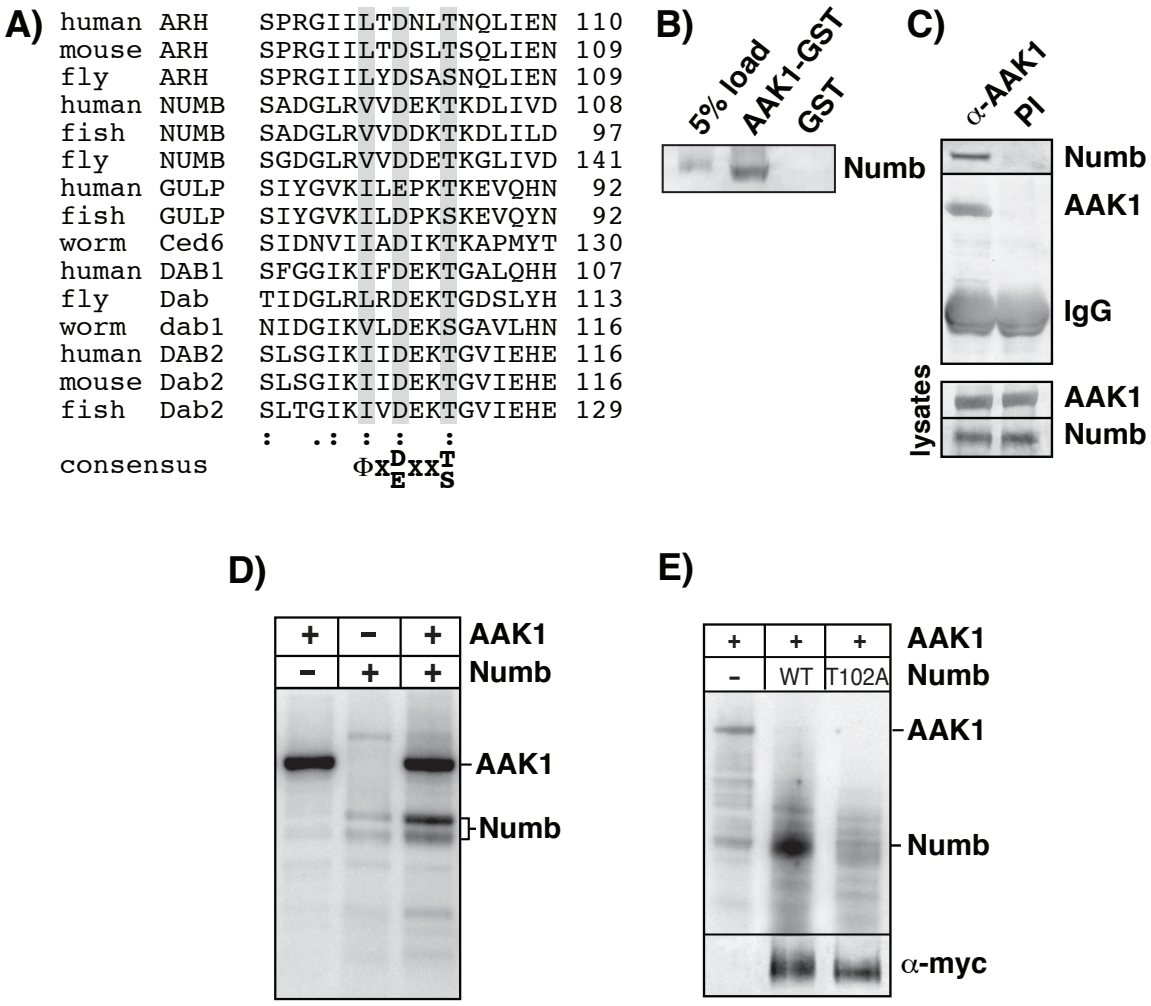

Figure 10: AAK1 interacts with and phosphorylates Numb. A) Abbreviated alignment of the AAK1 consensus site encoded within the PTB domain of the indicated endocytic adaptor proteins. The consensus site is shown below. B) Recombinant Numb–myc–6HIS was incubated with AAK1–GST immobilized on glutathione–Sepharose beads or GST beads. Numb binding was detected by immunoblot using 9E10. C) Numb coimmmunoprecipitates with AAK1. tTA HeLa cells were infected with adenovirus-encoding Numb alone or coinfected with AAK1 adenovirus. AAK1 was then immunoprecipitated with AAK1-specific antisera from cell lysates. Numb was detected by immunoblot. D) AAK1 was incubated in the presence or absence of Numb and [g32P]ATP. Numb phosphorylation and AAK1 autophosphorylation are indicated. E) tTA HeLa cells were infected with adenovirus-encoding WT and T102A Numb–myc for 18 h. Myc-tagged Numb was immunoprecipitated with 9E10 from cell lysates and then used for in vitro kinase assays (see Materials and Methods). Equal protein loading was determined by immunoblot as indicated. IgG, immunoglobulin G.

AAK1 binds and phosphorylates Numb

To explore the hypothesis that Numb activity is controlled by AAK1 phosphorylation, we initially performed a direct interaction test between Numb and AAK1. To do so, recombinant Numb–6HIS was incubated with AAK1–glutathione S-transferase (GST) and immobilized on a glutathione–agarose matrix or GST-bound beads. Following incubation, we found that Numb selectively interacts with AAK1 *in vitro*, while background binding to GST was not detectable (Figure 10B). To confirm the interaction *in vivo*, recombinant Numb and AAK1 were coexpressed in tetracycline transactivator (tTA) HeLa cells, and protein interaction was evaluated by coimmunoprecipitation from cell lysates. Following AAK1 isolation with AAK1-specific antisera, we analyzed the immunoprecipitant for Numb. Immunoblot analysis revealed that Numb specifically coimmunoprecipitated with AAK1 (Figure 10C), while Numb was not present in negative control immunoprecipitants using preimmune antisera.

To extend our analysis, we next tested Numb as an AAK1 substrate using baculovirus-generated Numb in kinase assays. Incubation of Numb–6HIS with [γ32P]ATP revealed a minor Numb phosphorylation product. The observed phosphorylation likely reflects the copurification of a contaminating kinase(s) from insect cells, possibly NAK that is the *Drosophila* homologue of AAK1 (Chien et al., 1998). Supplementing kinase reactions with recombinant AAK1 reveals a marked increase in Numb phosphorylation, demonstrating that AAK1 can indeed target Numb for phosphorylation (Figure 10D). To test if Numb is phosphorylated at the predicted AAK1 consensus site, we first mutated the conserved threonine at position 102 to a nonphosphorylatable alanine. Recombinant wild-type (WT) and T102A Numb were then expressed in mammalian cells, immunoprecipitated using antibodies against the engineered myc tag and immunoprecipitates were

used in kinase assays. Consistent with previous kinase assays (Figure 10D), immunoprecipitated WT Numb is phosphorylated by AAK1, while little AAK1-dependent phosphorylation is observed in T102A Numb immunoprecipitants (Figure 10E). Taken together, these observations demonstrate that AAK1 is capable of directly binding and phosphorylating Numb. Moreover, they support the idea that Numb function in endocytosis may be controlled by AAK1 activity.

AAK1 activity modulates Numb distribution within cells

In HeLa cells, Numb localizes to the plasma membrane and various endosomal compartments where it functions in receptor endocytosis and recycling, respectively (Santolini et al., 2000; Smith et al., 2004; Dho et al., 2006). Because our biochemical studies indicated that Numb interacts with and is phosphorylated by AAK1, we tested the prediction that AAK1 activity might influence Numb distribution within cells. Consistent with published reports, immunolocalization experiments reveal endogenous Numb at the plasma membrane and in association with perinuclear endosomes (Figure 11A). However, strong perinuclear Numb immunoreactivity was observed in ~15% of HeLa cells (Figure 11D). By comparison, WT AAK1 overexpression caused an almost 3.5-fold increase in robust Numb perinuclear staining (Figure 11B). The observed Numb redistribution was dependent on AAK1 kinase activity because overexpression of kinase-dead AAK1 mutants (K74A and D176A) (Conner & Schmid, 2003[2]) did not result in comparable changes in Numb localization (Figure 11C,D).

The endosomal recycling compartment (ERC) and trans Golgi network are found in the perinuclear region of HeLa cells. Therefore, to identify the compartment at which Numb becomes enriched following AAK1 expression, we

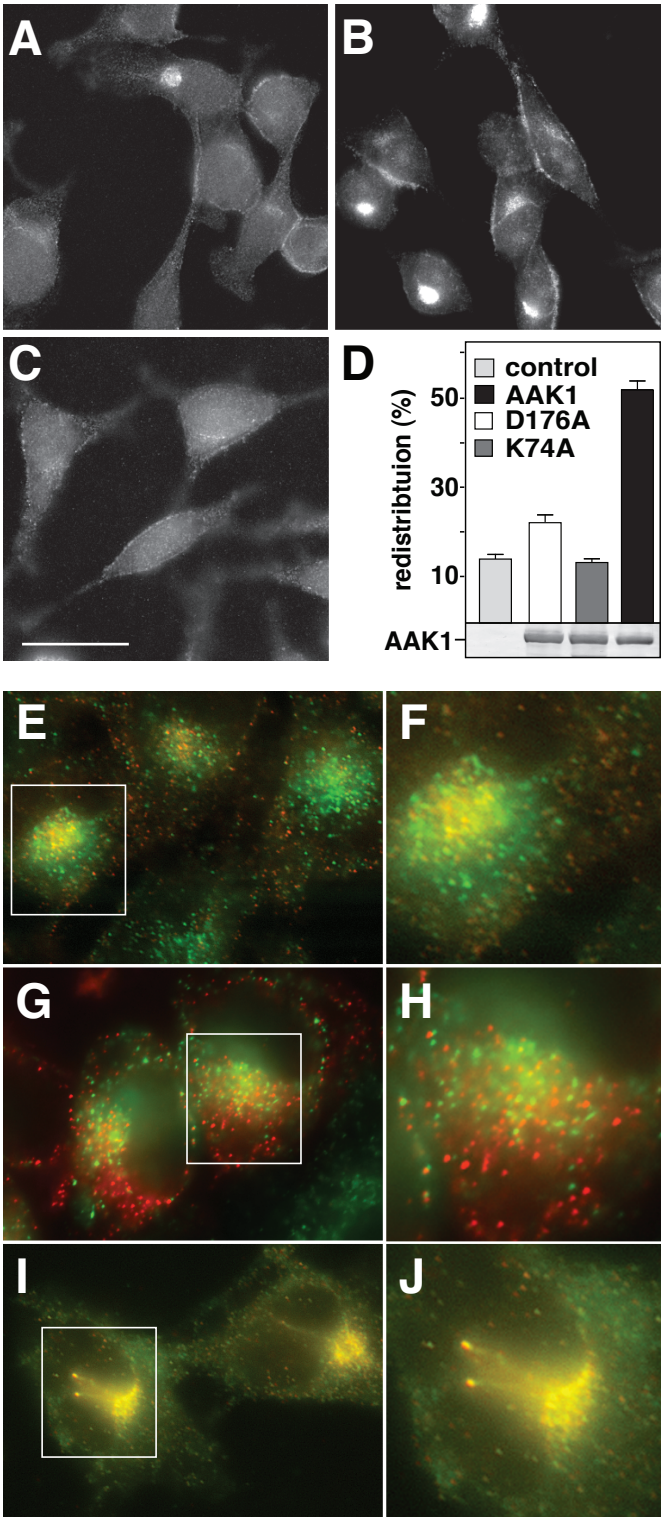

Figure 11: AAK1 overexpression redistributes Numb to the recycling endosome compartment.

Figure 11: AAK1 overexpression redistributes Numb to the recycling endosome compartment. tTA HeLa cells were infected with adenoviruses-encoding tTA (A, control), WT AAK1 (B) or a kinase-dead AAK1 mutant (C, K74A). After an 18-h incubation at 37˚C, cells were fixed and probed for endogenous Numb using polyclonal antisera. Strong perinuclear Numb enrichment was quantified by cell counting (D). Error bars indicate ±SD, and more than 800 cells were counted in each condition. Equivalent AAK1 expression levels validated by immunoblot (panel inset). Bar = 20 μm. tTA HeLa cells were infected with adenovirus-encoding tTA (E and F), myc-tagged Numb (G and H) or WT AAK1 (I and J). Following an 18-h infection, cells were incubated with FITC-labeled transferrin (10 mg/mL) for 45 min at 37˚C. Cells were fixed with 3.7% formaldehyde and probed for endogenous Numb using polyclonal antisera (E and F, I and J) or recombinant Numb using 9E10 (G and H) to visualize Numb. Boxed regions in (E, G and I) are shown in higher magnification in panels (F, H and J), respectively.

performed colocalization analysis. We found that Numb does not significantly overlap with Golgi-specific markers following AAK1 overexpression (data not shown). Therefore, we next tested Numb recruitment to the ERC, which can be marked by incubating cells with fluorescein isothiocyanate (FITC)-labeled transferrin. When focusing on control-infected cells with strong endogenous perinuclear Numb staining (Figure 11E) or cells expressing recombinant Numb (Figure 11G), we found little Numb colocalization with internalized FITC–transferrin. In contrast, in AAK1-expressing cells, we observed a significant endogenous Numb overlap with FITC–transferrin (Figure 11I), indicating that AAK1 promotes Numb localization to the ERC.

We next validated the impact of AAK1 activity on Numb distribution within cells by examining the consequence of AAK1 depletion on Numb localization. Given that AAK1 overexpression shifted endogenous Numb to the ERC, we predicted that if Numb was indeed a bona fide AAK1 target *in vivo*, RNA-mediated reduction in AAK1 expression would have the opposite effect. To test this, we infected tTA HeLa cells with adenovirus-encoding Numb and compared its localization in control and AAK1-depleted cells. Similar to that observed for endogenous Numb, recombinant Numb was found in punctate structures at the plasma membrane and in a perinuclear compartment (Figure 12A). However, in contrast to endogenous Numb, where ~15% of cells showed a strong perinuclear localization, nearly all cells expressing recombinant protein showed enriched perinuclear Numb. Following AAK1 depletion, we observed a marked reduction in Numb perinuclear staining and a significant increase at the plasma membrane (Figure 12A,B). Collectively, these observations support the idea that AAK1 activity influences Numb distribution within cells, possibly by influencing Numb interaction with other endocytic components, perhaps AP2 and/or Eps15

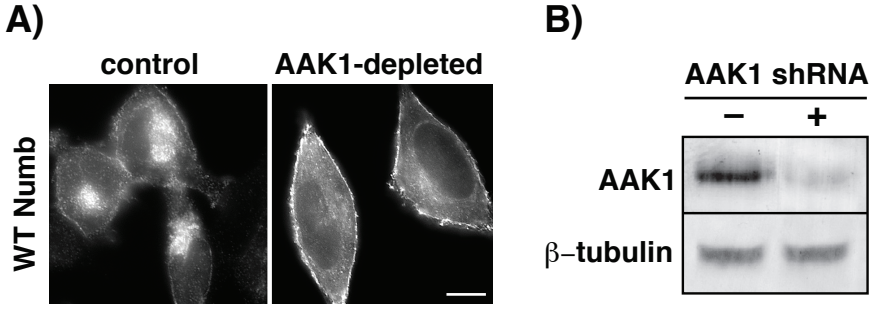

Figure 12: AAK1 depletion redistributes recombinant Numb to the plasma membrane. A) tTA HeLa cells were infected with adenovirus-encoding myc-tagged Numb for 6 h. The culture medium was then replaced with medium containing either tTA-encoded adenovirus (control) or adenovirus-encoding AAK1-specific small hairpin RNA (shRNA) to knockdown AAK1 expression. After an additional 30-h incubation, cells were fixed and recombinant Numb was visualized by staining for the myc tag (9E10). B) The extent of AAK1 depletion was determined by immunoblot with AAK1-specific antisera. Blots were also probed with antibodies against b-tubulin (E7) to ensure equal loading. Bar = 10 μm.

(Santolini et al., 2000; Tokumitsu et al., 2006). Alternatively, AAK1 may promote Numb recruitment to ERC by stabilizing Numb interaction with endosomal factors belonging to the Eps15 homology domain family (Smith et al., 2004).

T102A Numb overexpression impairs an early step in endocytosis

A role for Numb in receptor-mediated endocytosis is supported by Numb interaction and colocalization with AP2 in clathrin-coated pits as well as the endocytic defects in transferrin and EGF uptake that result from overexpressing truncated Numb fragments (Santolini et al., 2000). Because multiple lines of evidence indicate that AAK1 is most active in clathrin-coated pits (Jackson et al., 2003; Conner & Schmid, 2003[2]), we postulated that phosphorylation by AAK1 may be a key event in regulating Numb function in endocytosis. We tested this idea by employing an overexpression approach. We reasoned that if phosphorylation at the AAK1 consensus site was critical to Numb activity, overexpression of a nonphosphorylatable Numb mutant (T102A) might disrupt clathrin-dependent internalization of transferrin and EGF. Indeed, adenovirus-mediated overexpression of T102A Numb potently impaired FITC-labeled transferrin uptake, while no internalization defect was observed in WT-Numb-overexpressing cells (Figure 13A). Surprisingly, T102A Numb did not alter clathrin-dependent internalization of rhodamine-labeled EGF (Figure 13A), arguing that the observed transferrin internalization defect did not result from gross disruption of the endocytic machinery. This also suggested that Numb regulation by AAK1 might be limited to a subset of receptors, possibly those that are constitutively internalized (e.g. transferrin and LDL receptors). Consistent with this idea, we findthat 1,10-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate-labeled LDL (DiI-LDL) uptake is also disrupted in T102A Numb-

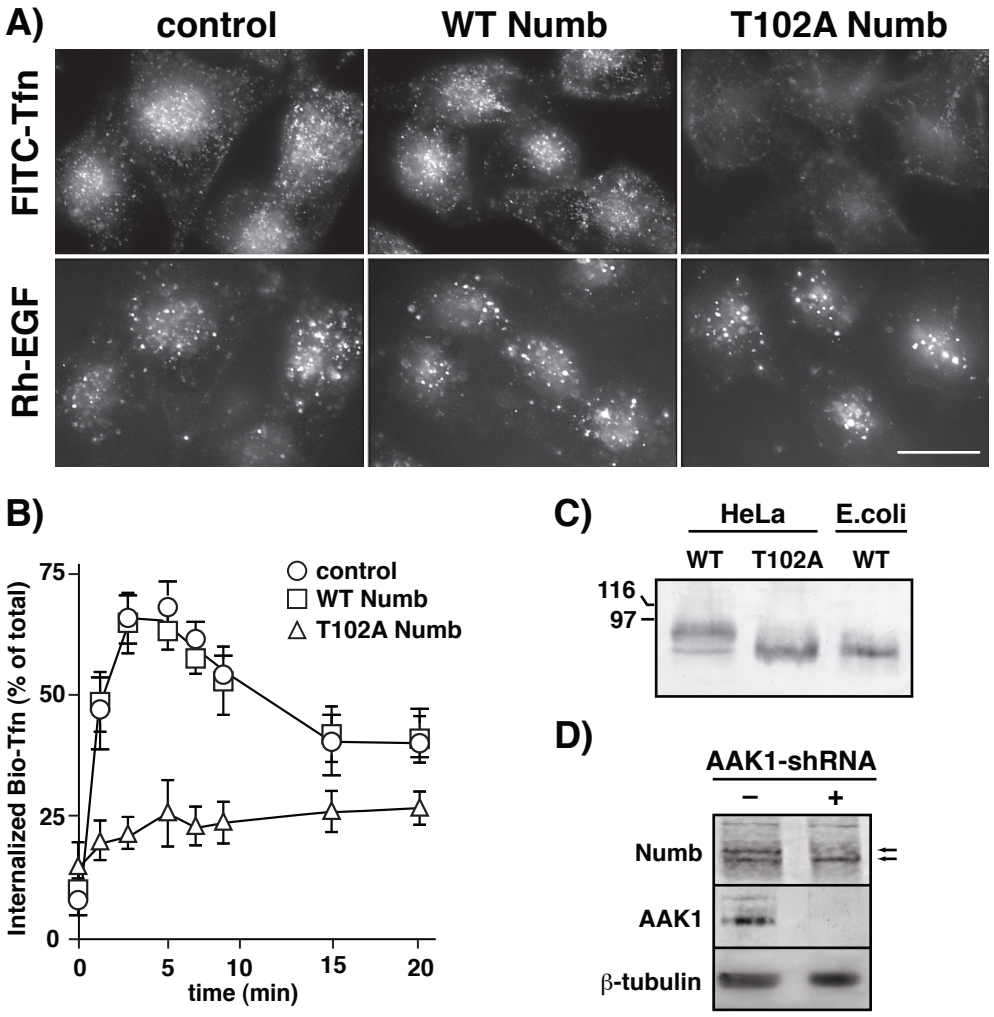

Figure 13: Phosphorylation is critical to Numb function at an early endocytic step. A) tTA HeLa cells were infected with the indicated adenoviruses for 18 h. Cells were then incubated with media containing FITC-transferrin (10 mg/mL) and Rh–EGF (4 ng/mL) for 20 min to assay endocytosis. Bar = 20 μm.B) The extent of transferrin internalization was determined using an enzyme-linked immunosorbent assay-based single round internalization assay following infection with the indicated adenoviruses. C) Protein expression levels in cells used for internalization assay levels were determined by immunoblot using 10% of total cells lysate. WT and T102A Numb mobility was compared with that of bacterially expressed WT Numb. D) tTA HeLa cells were infected with WT Numb adenovirus for 6 h. The media was replaced, and cells were infected with control or AAK1 small hairpin RNA (shRNA)-encoded adenovirus. After 36 h, cells lysates were analyzed by immunoblotfor recombinant myc-tagged Numb (arrows), AAK1 and b-tubulin as a loading control. E. coli, Escherichia coli; Rh, rhodamine.

expressing cells, while no internalization defect was observed following WT Numb overexpression (Figure S4). These observations are consistent with the proposed role for Numb as a general component of the endocytic machinery (Nishimura & Kaibuchi, 2007; Santolini et al., 2000). Moreover, the specific internalization defects that result from T102A Numb overexpression suggest that phosphorylation by AAK1 may be critical to controlling Numb activity.

To more precisely define the step at which transferrin uptake is impaired following T102A Numb overexpression, we took advantage of the fact that early steps in clathrin-mediated endocytosis can be distinguished from later steps by differential accessibility to large and small quenching agents (Schmid & Smythe, 1991). For example, at early steps, like clathrin-coated pit assembly, biotinylated transferrin linked by a disulfide bond (BSS-Tfn) is accessible to large exogenous quenching agents like avidin. By contrast, when the coated pit becomes constricted right before vesicle budding, the receptor-bound BSS-Tfn is no longer accessable to avidin but can still be quenched by small reducing agents like sodium 2-mercaptoethane sulfonate (MESNA). Using this approach, we find that BSS-Tfn remained assessable to quenching by avidin following T102A Numb overexpression in tTA HeLa cells. This was clearly indicated by the reduced rate of BSS-Tfn endocytosis (protection from sequestering agents) relative to virus control and WT-Numb-expressing cells (Figure 13B). This demonstrates that an early step in endocytosis, possibly clathrin-coated pit assembly or maturation, is disrupted by T102A Numb.

In the course of verifying equivalent Numb overexpression levels in tTA HeLa cells following transferrin internalization assays (Figure 13), we observed a significant shift in apparent molecular weight between the WT Numb and the T102A phosphomutant. Consistent with published reports, immunoblot

analysis revealed that recombinant WT Numb migrated as a doublet with the larger species migrating approximately 10 kDa slower than the smaller form (Figure 13C) (Nie et al., 2002; Santolini et al., 2000). By comparison, the T102A phosphomutant, like that of bacterially expressed unphosphorylated WT Numb, resolved as a single band that comigrated with the lower molecular weight band of WT Numb (Figure 13C).

In addition to AAK1, Numb is phosphorylated by CaMKI and aPKC at not less than nine unique sites (Smith et al., 2007, Tokumitsu et al., 2005, 2006). These multiple phosphorylation events are thought to retard Numb migration in SDS–PAGE gels. Consistent with this idea, phosphatase treatment causes isolated Numb to migrate as a single, lower molecular weight form (Santolini et al., 2000). Thus, based on the increased mobility of the T102A phosphomutant relative to WT Numb, we suspected that failure to phosphorylate at the AAK1 consensus site prevents subsequent modification by other kinases. If this is correct, WT Numb isolated from AAK1-depleted cells should also show increased mobility. To test this, tTA HeLa cells were infected with adenovirus-encoding WT Numb. They were then coinfected with either a control virus or a short hairpin RNA-encoding adenovirus to deplete endogenous AAK1. WT Numb from cell lysates was then analyzed by immunoblot. In agreement with our prediction, WT Numb migrated as a lower molecular weight species in AAK1-depleted cells (Figure 13D), while the expected doublet was observed in control cells (Figure 13D). Altogether, these observations support the conclusion that AAK1 phosphorylation is an important priming step that may be a necessary prerequisite for Numb phosphorylation by additional kinases (i.e. CaMKI and/or aPKC).

Numb phosphorylation by CaMKI *in vitro* destabilizes Numb interaction

with the endocytic adaptor protein complex AP2 (Tokumitsu et al., 2006). Similarly, phosphorylation by aPKC promotes Numb release from clathrin-coated pits (Nishimura & Kaibuchi, 2007; Smith et al., 2007). Thus, if AAK1 activity is an essential priming step for subsequent phosphorylation events, we predicted that a failure to phosphorylate at the AAK1 consensus site would lead to Numb accumulation at the plasma membrane. Indeed, cells depleted of AAK1 show increased localization of recombinant WT Numb at the plasma membrane (Figure 12A). However, to eliminate the impact of residual AAK1 activity that remains following incomplete AAK1 depletion (Figure 12B) and to more clearly resolve the consequence of AAK1 activity on Numb localization, we examined the distribution of the T102A Numb phosphomutant in cells relative to WT controls. In contrast to WT Numb, T102A Numb did not accumulate in a perinuclear compartment. Instead, the phosphomutant localized to punctate structures at the plasma membrane, reminiscent of clathrin-coated pits (Figure 14).

To establish if the T102A-Numb-positive structures were associated with clathrin-coated pits, we performed colocalization analysis with endocytic coat protein markers like AP2, Eps15 (Benmerah et al., 1998) and epsin (Chen et al., 1998). Coimmunolocalizations revealed a significant overlap between T102A Numb, AP2, Eps15 and epsin at the cell surface (Figure 15 and Figure S5). By comparison, the same degree of colocalization was not observed between these coated pit markers and WT Numb. This marked increase in colocalization with other endocytic components, combined with the restricted localization of the Numb phosphomutant, suggests that T102A Numb might stabilize clathrin-coated pits, slow their maturation and/or retard the formation of endocytic vesicles. This idea is consistent with the observed disruption in transferrin internalization at an early endocytic step following T102A Numb overexpression (Figure 13B).

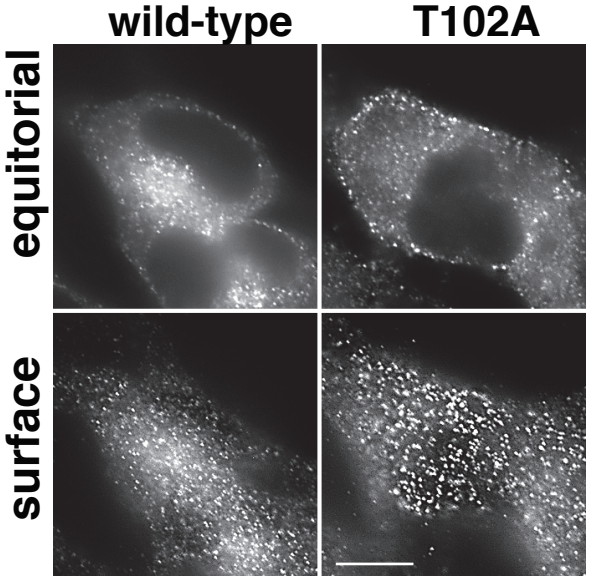

Figure 14: T102A Numb is localized to punctate structures. tTA HeLa cells were infected with adenoviruses-encoding WT Numb or T102A Numb for 6 h. Cells were fixed and probed for myc-tagged Numb with 9E10. Equatorial and cell surface images are shown to illustrate the absence of perinuclear T102A Numb relative to WT Numb. Bar = 5 μm.

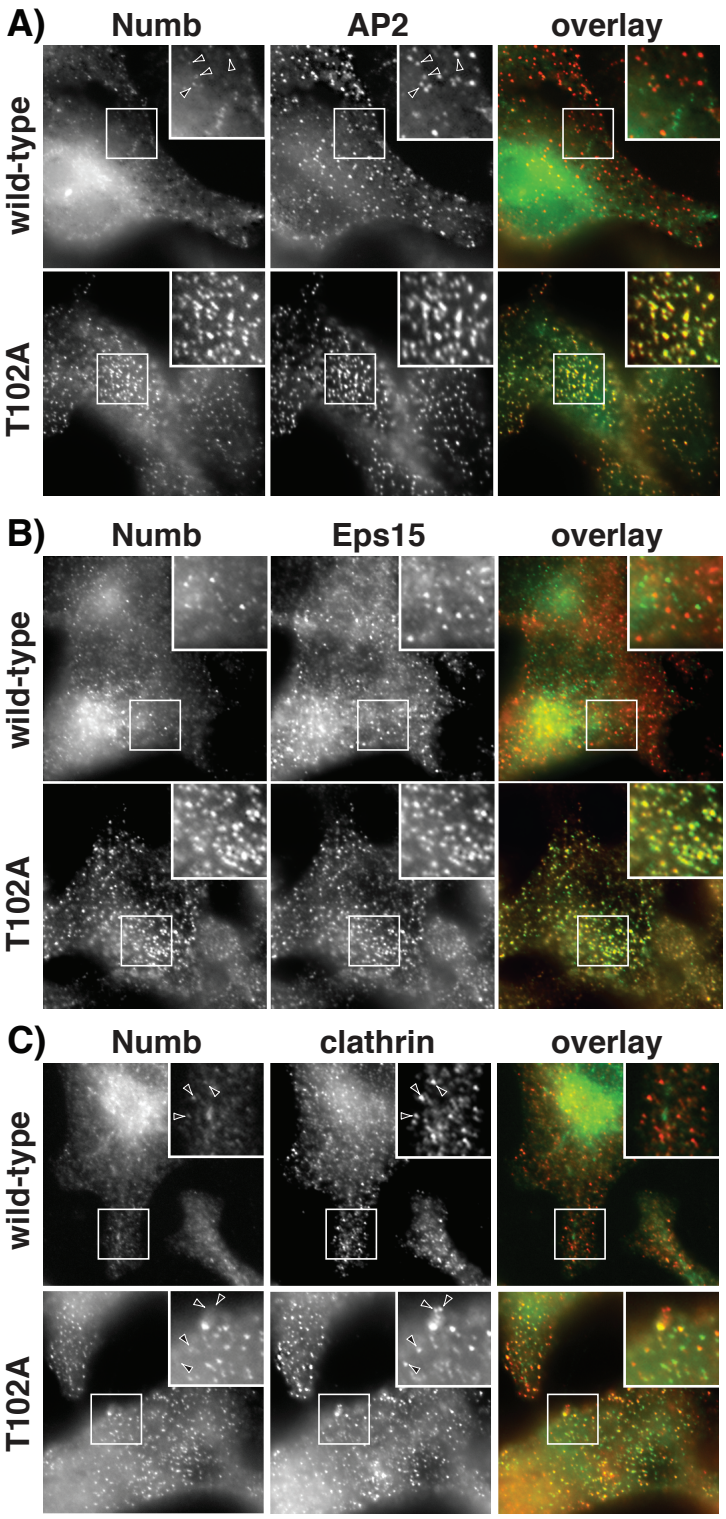

Figure 15: The Numb T102A phosphomutant accumulates in a subset of clathrin-coated pits.

Figure 15: The Numb T102A phosphomutant accumulates in a subset of clathrin-coated pits. Cells were infected with WT or T102ANumb-encoded adenovirus for 4.5 h and then processed for coimmunolocalization with A) AP2 (AP.6), B) Eps15 (pAb) or C) clathrin (TD.1). Myc-tagged Numb was visualized with Alexa Fluor 488-tagged 9E10. Boxed regions are shown in higher magnification (inset). Color over color (overlay) are shown where Numb is in green and AP2, Eps15 and clathrin are in red. Yellow indicates colocalization. Bar = 5 μm.

However, EGF uptake is not impaired following T102A Numb expression (Figure 13A), suggesting the possibility that the Numb phosphomutant selectively disrupts a subset of clathrin-coated pits. To test this, we evaluated the impact of T102A Numb on clathrin localization. Although we observe significant colocalization between T102A Numb and clathrin, a subset of clathrin-coated pits lacks the phosphomutant (Figure 15C). Thus, it is possible that the clathrin-mediated EGF uptake that occurs following Numb phosphomutant expression occurs through the subset of coated pits lacking T102A Numb. Altogether, these observations support the initially proposed role for Numb in facilitating clathrin-coated pit assembly (Santolini et al., 2000) and, importantly, also demonstrate that Numb phosphorylation by AAK1 is a critical regulatory step.

Notch internalization is independent of Numb and AAK1

Several studies propose that Numb and *Drosophila* Nak, the homologue of AAK1, regulate Notch receptor internalization (Berdnik et al., 2002; Chien et al., 1998; Guo et al., 1996; Santolini et al., 2000). Both Numb mutant and Nak overexpression flies exhibit a Notch gain-of-function phenotype during sensory organ precursor (SOP) development of the *Drosophila* peripheral nervous system. Given the genetic evidence linking AAK1 and Numb to Notch signaling and the general role of these proteins in clathrin-coated pit assembly, we tested if AAK1 and Numb are essential and/or Numb phosophorylation by AAK1 is critical for Notch receptor endocytosis. To test this, we employed the CD8-NΔE uptake (Chapter 2). Using this approach, we find that overexpression of T102A Numb1 phosphomutant (16A,B) or AAK1 (WT or kinase dead), disrupt CD8-NΔE uptake, as previously seen for transferrin internalization (13B; Conner and Schmid, 2003[2]). Since overexpression of these constructs disrupts clathrin-mediated

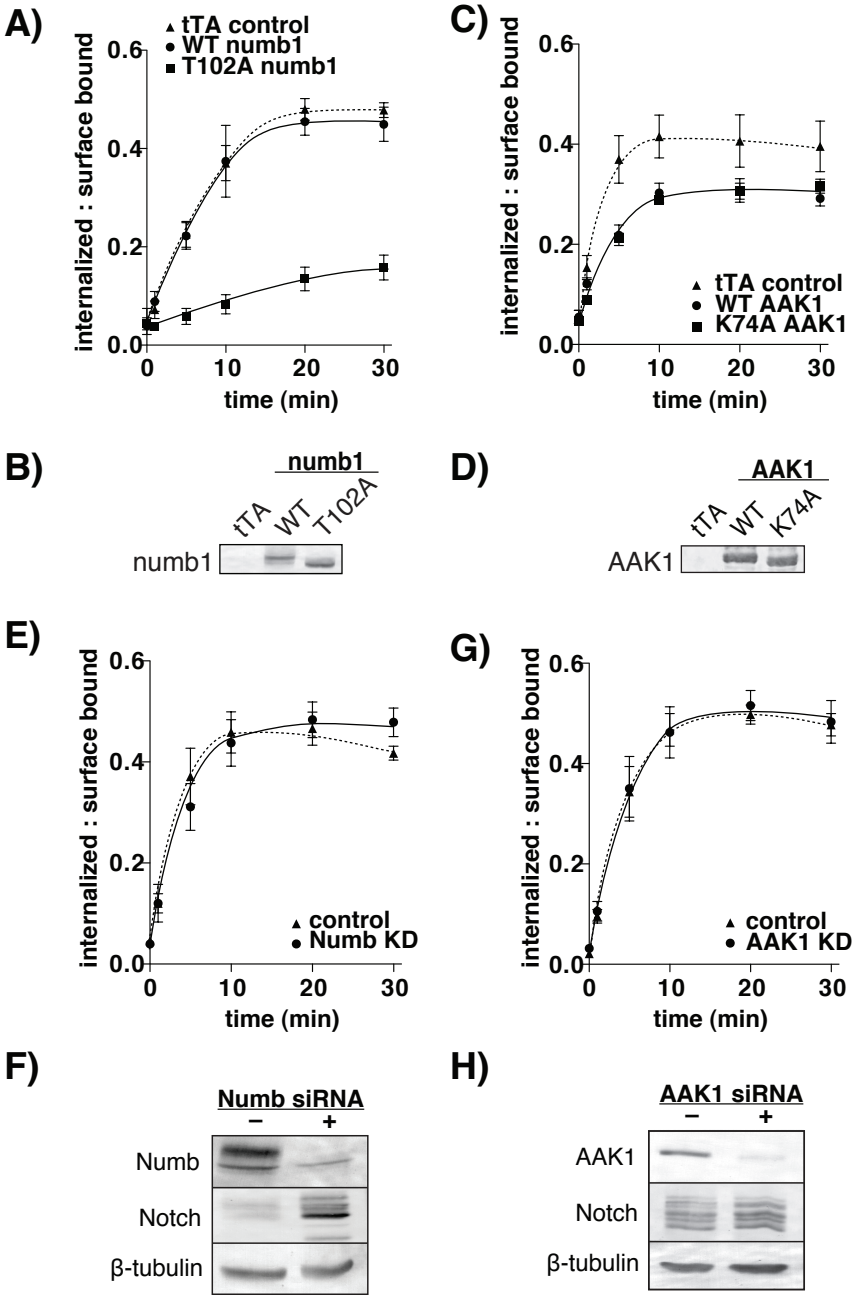

Figure 16: AAK1 and Numb are not required for Notch internalization but competition with core endocytic machinery impairs CD8-NΔE uptake. CD8-NΔE-expressing cells were infected with adenovirus encoding tTA (control), WT Numb1-myc or T102A Numb1-myc (A/B) or WT AAK1 or K74A AAK1 (C/D). tTa HeLa cells were transfected with control siRNA or siRNAs targeting Numb (E/F) or AAK1 (G/H). CD8-NΔE internalization was then scored and immunoblot analysis performed to validate protein expression and equal loading. Error bars represent ± SEM of four (A/C), six (E) or three (G) independent experiments.

endocytosis by competing with core endocytic machinery including AP2 and epsin, it is not clear if AAK1 and Numb are required for CD8-NΔE uptake. Using siRNA–mediated depletion, we find that Numb (16E,G) and AAK1 (16H,I) are not essential CD8-NΔE endocytosis.

Discussion:

What is the role of Numb phosphorylation by AAK1?

AAK1 localizes to coated pits where its extensive interactions with assembled clathrin stimulates its kinase activity (Conner et al., 2003; Jackson et al., 2003). Thus, AAK1 is ideally positioned to regulate endocytic coat protein function within coated pits. Our results indicating that phosphorylation by AAK1 influences Numb activity in endocytosis support this idea. However, the precise role of Numb in clathrin-mediated endocytosis is currently unresolved. Numb binds Eps15 and AP2, although not simultaneously (Santolini et al., 2000). Given Numb colocalization with AP2 and not with Eps15 in coated pits, Santolini et al. (2000) postulated that Numb might participate in the temporal hierarchy of clathrin-coated pit assembly. In general agreement with this hypothesis, we discovered that Numb functions at an early endocytic step, which is dependent on AAK1 phosphorylation. Accumulation of T102A Numb in coated pits and its increased colocalization with endocytic components suggest that phosphorylation by AAK1 may regulate Numb interaction with the coat protein machinery. Eps15 is progressively displaced from coated pits during coat assembly (Nishimura et al., 2003). Therefore, one possibility is that AAK1-dependent phosphorylation may increase Numb affinity for AP2. This might disengage Eps15 from AP2 to promote coated pit maturation. Alternatively, Numb phosphorylation may lead to the recruitment of accessory factors to coated pits that, in turn, serve to

enhance receptor internalization. SiRNA-mediated depletion studies indicate that AAK1 (Conner & Schmid, 2003[2]) and Numb (Nishimura et al., 2003), like Eps15 and epsin (Huang et al., 2004), are not essential for transferrin receptor internalization. Likewise, AAK1 and Numb are not essential for Notch receptor endocytosis. Instead, Numb and AAK1 may be redundant with other factors or they may perform higher order functions that influence the efficiency and/or fidelity of receptor endocytosis. Alternatively, they may be essential for endocytosis but only under certain conditions. This would be similar to what has been recently observed where dynamin 1 knockout mice manifest endocytic defects at the synapse only under strong exogenous stimulation (Ferguson et al., 2007). Thus, resolving the mechanism by which Numb contributes to clathrin-mediated internalization and the molecular consequence of AAK1 phosphorylation will require additional investigation.

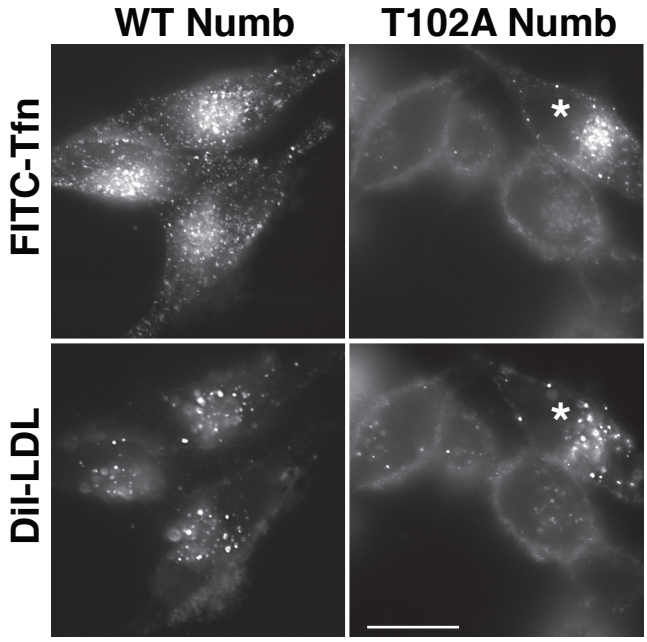

Figure S4: T102A Numb overexpression impairs nutrient receptor internalization. A) tTA HeLa cells were infected with WT or T102ANumb-encoded adenoviruses for 18 h. Cells were then incubated with media containing FITC–transferrin (10 mg/mL) and DiI-LDL (10 mg/mL) for 20 min to assay endocytosis. Asterisk indicates a cell that is likely not infected with adenovirus and thus not expressing T102A Numb. Bar = 20 μm.

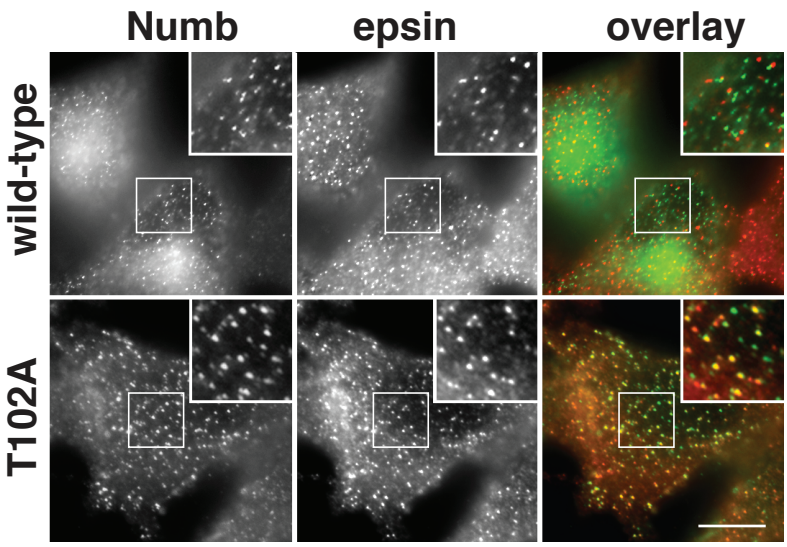

Figure S5: The Numb T102A phosphomutant accumulates in epsin positive structures. tTA HeLa cells were infected with WT or T102ANumb- encoded adenovirus for 4.5 h. Recombinant Numb–myc was visualized with 9E10 and co-stained for endogenous epsin. Boxed regions are shown in higher magnification to reveal colocalization between Numb–myc (green) and epsin (red). Yellow indicates colocalization. Bar = 5 μm.

Chapter IV: Conclusions and Significance

Core endocytic machinery coordinate CD8-NΔE Internalization

I find that robust CD8-NΔE internalization, in mammalian cells, requires clathrin, using an siRNA depletion strategy (Chapter 2). Consistent with my results, *Drosophila* imaginal discs null for clathrin have an accumulation of cell-surface labeled Notch at the cell cortex relative to wildtype controls (Windler & BIlder, 2010). Therefore, clathrin-dependent endocytosis is likely a conserved mechanism for Notch internalization. However, CD8-NΔE uptake following clathrin knockdown was not completely inhibited despite substantial clathrin heavy chain (CHC) depletion, verified by immunoblot analysis. Specifically, I observe an ~ 50% reduction in CD8-NΔE internalization following treatment with CHC siRNA relative to control siRNA. Although significant, if clathrin-mediated endocytosis is the only internalization pathway Notch utilizes, I would anticipate a greater uptake inhibition following clathrin-depletion than that observed. For example, other mammalian *ex vivo* internalization assays of receptors, (i.e. transferrin, LDL and EGF) known to be internalizied via clathrin-dependent pathways, reveal an ~80% reduction in endocytosis following CHC depletion relative to controls, using the same CHC siRNA sequence (Motley et al., 2003). Taken together, it is possible that in addition to clathrin-mediated endocytosis, CD8-NΔE may also utilize other clathrin-independent endocytic pathways.

Multiple endocytic pathways for a single receptor have been previously described. For example, EGF receptor (EGFR) undergoes both clathrin-dependent and clathrin-independent internalization depending on ligand concentration. In the presence of high concentrations of EGF, the clathrin-dependent pathway becomes readily saturated and therefore EGFR is internalized via a clathrin-independent mechanism (Jiang & Sorkin, 2003).

Therefore, CD8-NΔE may utilize caveolae-mediated or clathrin/caveolae-independent endocytosis in addition to the clathrin-dependent pathway.

It is possible that clathrin-dependent and clathrin-independent CD8-NΔE internalization send the receptor on different endocytic itineraries. Such that clathrin-mediated uptake may sort Notch to the lysosome for degradation; whereas, clathrin-independent uptake may sort Notch to a different endosomal compartment, possibly for storage. Different receptor destinations can have significant impacts on Notch signaling capacity. For example, receptor sorting to the lysosome for degradation will downregulate Notch signaling; whereas, receptor storage could permit future signaling because Notch can undergo ligand activation if returned to the plasma membrane. Exploration of caveolae-dependent, via siRNA-mediated depletion of caveolin1, or other endocytic pathways that may mediate CD8-NΔE uptake can be the focus of future studies.

I identified that robust CD8-NΔE endocytosis requires clathrin; however, clathrin must utilize adaptor molecules to engage receptor cytoplasmic tails and membrane lipids. One such adaptor molecule is heterotetrameric protein AP2. I observe a decrease in CD8-NΔE internalization following siRNA-depletion of AP2 (Chapter 2), consistent with the cell cortex accumulation of Notch in AP2 null imaginal discs relative to WT controls in *Drosophila* (Windler & Bilder, 2010). This is contrasted by mammalian internalization analysis of EGFR and CD8-LDLR, which reveal no defect in ligand uptake in cells following AP2 knockdown relative to control cells (Motley et al., 2003). However, the requirement for AP2 during Notch internalization is less than that observed for clathrin in my CD8-ΔE internalization assay. This demonstrates CD8-NΔE uptake requires multiple endocytic factors.

Phenotypic analysis in *Drosophila* and mice clearly implicate endoctyic

adaptor epsin1 in Notch signaling. Various genetic screens identified epsin mutants with similar phenotypes as those observed during loss of Notch signaling (Tian et al., 2004; Wang & Struhl, 2004). For example, Wang and Struhl (2004) identified *liquid facets* (*lqf*, the *Drosophila* epsin homolog) in a genetic screen for mutations affecting *Drosophila* wing development. Liquid facets mutants exhibit similar wing notching and vein thickening as loss-of-function Notch phenotypes (Wang and Struhl, 2004). Likewise, loss of epsin phenocopies Notch loss of function heart defects in both *Drosophila* (Tian et al., 2004) and mammals (Chen et al., 2009). But what are the molecular mechanisms by which epsin1 functions in Notch signaling?

Given that CD8-NΔE internalization requires additional endocytic factors and that loss of epsin1 phenocopies Notch signaling defects, I postulated that epsin1 may be critical for CD8-NΔE endocytosis. siRNA-mediated epsin1 depletion significantly impairs CD8-NΔE internalization in mammalian cells relative to control siRNA treatment (Chapter 2). This is the first robust requirement for epsin1 in receptor internalization as previous studies only observe a modest impairment of mammalian EGFR endocytosis following epsin1 depletion (Kazazic et al., 2008). CD8-NΔE endocytosis is epsin1-dependent. However, genetic data propose that epsin1 functions in Notch signaling to promote ligand endocytosis. Specifically, in addition to Notch signaling defects, *Drosophila* epsin mutant cells exhibit increased cell surface accumulation of Notch ligands by immunofluorescence (Overstreet et al., 2003, 2004; Wang & Struhl, 2005), suggesting that epsin-dependent ligand endocytosis is critical for Notch activation. Consistent with this hypothesis, mosaic analysis during *Drosophila* wing (Wang & Struhl, 2004) and eye development or (Overstreet et al., 2004) *C. elegans* germline development (Tian et al., 2004) reveal Notch signaling defects

when ligand-expressing cells are epsin-null but not when Notch-expressing cells are null for epsin. Consistent with these results, I observe no defect in Notch signaling when epsin1 is depleted in Notch-expressing cells (Chapter 2) (I will elaborate on this below: γ–secretase cleavage occurs at the cell surface). Therefore, epsin1 may play a dual role in Notch signaling by functioning to coordinate both receptor and ligand endocytosis.

Receptor modification is an important Notch endocytic/sorting signal

Epsins are endocytic adaptor proteins that contain central ubiquitin-interacting motifs (UIMs) to engage ubiquitinated transmembrane cargo (Chen et al., 1998, Wendland, 2002, Hawryluk et al., 2006). Given that CD8-NΔE internalization is epsin1-depedent, I propose that ubiquitin-modification may be a critical CD8-NΔE sorting signal with which epsin1 binds. To test this hypothesis, I performed siRNA-mediated depletion of E3 ubiquitin ligase Nedd4, in mammalian cells. I find that robust CD8-NΔE internalization requires Nedd4 (Chapter 2). Consistent with my result, Sakata et al. (2004) observed a decrease in the intracellular accumulation of Notch in *Drosophila* wing imaginal discs following overexpression of dominant-negative Nedd4, relative to control discs (Sakata et al., 2004). Moreover, Sakata et al. (2004) observed an interaction between Nedd4 and full-length Notch by coimmunoprecipiation from S2 cells and a decrease in the level of ubiquitinated full length Notch receptor immunuoprecipitated from Nedd4-depleted S2 cells (Sakata et al., 2004). Consistent with this result, I observe an increase in CD8-NΔE stability following siRNA-meidated Nedd4 knockdown in mammalian cells (Chapter 2). Taken together, these results propose that Notch receptor ubiquitination by Nedd4 marks the Notch receptor for internalization and then possibly degradation.

Therefore, Nedd4 functions to downregulate the Notch signaling pathway (Sakata et al., 2004).

Since both epsin1 and E3 ligase Nedd4 are required for robust CD8-NΔE internalization, I predicted that epsin1, an adaptor molecule capable of binding ubiquitinated-cargo, would engage the Notch receptor in a ubiquitin-dependent manner. If this model is correct, then mutations that disrupt epsin1 interactions with ubiquitinated cargo should prevent epsin1 and Notch binding. Indeed, expression of UIM mutant epsin1, previously shown to disrupt epsin1 interaction with ubiquitinated EGFR (Kazazic et al., 2009), did not appreciably coimmunoprecipitate with Notch, relative to wildtype epsin1 (Chapter 2).

Nedd4, epsin1 and AP2 promote CD8-NΔE internalization

Taken together, I propose the following model for CD8-NΔE internalization. CD8-NΔE at the plasma membrane is ubiquitinated by Nedd4, at one or multiple sites. Receptor ubiquitination promotes epsin1 interaction, with its ubiquitin-interacting motif (UIM). Epsin1 may subsequently recruit AP2 and clathrin to CD8-NΔE to create a clathrin-coated pit and promote receptor enodyctosis (Figure 17). I propose that this endocytic event fuctions to downregulate the Notch signaling pathway.

While my model predicts that CD8-NΔE ubiquitination, via Nedd4, is critical for receptor internalization, it is possible that Nedd4 instead ubiquinates an adaptor molecule involved in Notch internalization, possibly epsin1. For example, Nedd4 can ubiquitinate Eps15, another UIM containing endocytic adaptor. *In vitro* ubiquitination assays demonstrate that Nedd4 can monoubiquitinate Eps15 within its UIM, as a UIM deletion mutant is no longer undergoes monoubiquitination (Polo et al., 2002). Epsin1, which is critical for efficient CD8-NΔE endocytosis, is

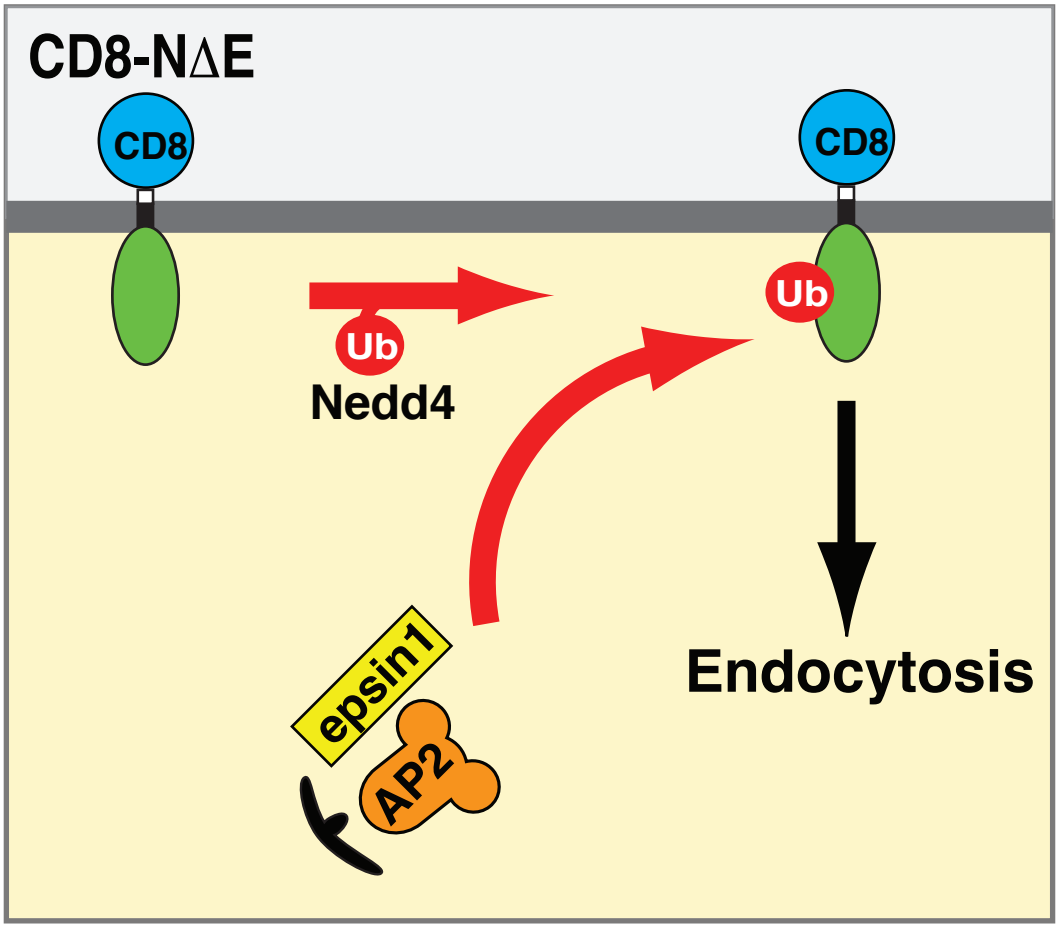

Figure 17 :Core endocytic machinery coordinate CD8-NΔE Internalization. Robust internalization is clathrin-dependent and requires E3 ligase Nedd4, AP2 and epsin1. I propose that Nedd4 ubiquitinates Notch, at one or multiple sites. This modification recruits epsin1, which can couple receptors to the internalization machinery, ie AP2 and clathrin, by engaging ubiquitinated cargo (Maldonado-Baez & Wendland, 2006). This endocytic event likely functions to downregulate the Notch signaling pathway.

itself monoubiquitinated within its UIM following treatment with EGF (Polo et al., 2002). Therefore, it is possible that Nedd4 ubiquitinates epsin1 within its UIM, the same motif responsible for ubiquitin-recognition, which may increase epsin1 affinity or specificity for Notch and promote receptor internalization. Future work to address the identity of Nedd4 substrate(s) as well as the identification of other post-translational modifications that regulate Notch receptor sorting decisions will be critical in determining Notch signaling activitiy.

Efficient CD8-NΔE endocytosis requires Nedd4, epsin1 and AP2, although CD8-NΔE uptake appears to be more heavily reliant on epsin1 than AP2. While my model predicts that AP2 and epsin1 coordinate CD8-NΔE internalization together, it is possible that AP2 and espin1 may function in separate endocytic pathways to internalize Notch. These different pathways may sort Notch towards distinct cellular fates. On one hand, ubiquitinated Notch uptake, via epsin1 and Nedd4, may internalize Notch into an endocytic pathway that sorts the receptor towards lysosomal degradation, which would downregulate Notch signaling. On the other hand, AP2-dependent Notch endocytosis may internalize Notch into a pathway that sorts the receptor to an endosomal storage compartment. Receptor storage allows Notch to “remain in play” such that Notch signaling events can occur if the receptor is recycled back to the plasma membrane and undergoes DSL-binding and γ-secretase cleavage.

γ–secretase-mediated Notch cleavage occurs at the cell surface

γ-secretase cleavage is the penultimate step in Notch signaling. This cleavage event releases the entire Notch intracellular domain (NICD) that will translocate to the nucleus and influence gene expression (Fortini, 2009; Struhl Adachi, 1998). While γ-secretase cleavage is clearly important for

Notch signaling, the cellular location where Notch undergoes γ-secretase cleavage is currently controversial. Limited studies suggest that γ-secretase cleavage occurs in the endosome following Notch receptor endocytosis. In mammalian cells expressing a constiutively active NEXT fragment (NΔE), overexpression of dominant-negative dynamin or Eps15, prevented generation of NICD by γ-secretase and NICD nuclear accumulation (Gupta-Rossi et al., 2004). Consistent with these observations, mosaic analysis of the developing *Drosophila* ovary reveals that follicle cells, which overexpress dominant-negative dynamin or those that lack clathrin or dynamin, do not express Hindsight, a Notch transcriptional target (Vacarri et al., 2008; Windler and Bilder, 2010). In contrast, others argue that γ-secretase cleavage occurs at the plasma membrane. Struhl and Adachi (2000) found that presenilin-dependent Notch processing, assayed by the expression of a reporter gene, is unaffected in *Drosophila* embryos expressing *shibire*^{ts} alleles, which cause an abrupt loss of dynamin activity when shifted to nonpermissive temperature. Moreover, studies of *C. elegans* LIN-12/Notch demonstrate that internalization-defective LIN12/Notch (ΔDTS) rescues the sterility and lethality of *Lin-12/Notch* null animals (Shaye and Greenwald, 2002, 2005). This demonstrates that receptor internalization is not required for Notch signaling in *C. elegans*.

To resolve the location where Notch undergoes γ-secretase cleavage, I developed a CD8-NΔE internalization assay, in mammalian cells. I find that CD8-NΔE endocytosis is impaired by expression of dominant-negative dynamin, consistent with results in flies (Vacarri et al., 2008) and mammalian cells (Gupta-Rossi et al., 2004). However, in each case where Notch internalization is disrupted (i.e. clathrin, AP2, epsin1 and Nedd4), I observe elevated production of ^{V1744}NICD, by immunoblot, and increased downstream signaling using a

Notch signaling reporter assay. How do I reconcile my observations with those showing a requirement for receptor internalization in Notch signaling activation? In mammalian cells, Gupta- Rossi et al. (2004) never specifically evaluated the rate of ^{V1744}NICD production in endocytosis-defective cells, relative to wildtype controls. Moreover, it is possible that endosomal transport of the truncated Notch form employed, which lacks nearly half the cytoplasmic tail, may not reflect the behavior of WT NEXT. By contrast, Vaccari et al. (2008) clearly show defective Notch signaling in Drosophila follicle cells lacking clathrin or dynamin (Windler & Bilder, 2010). Given the well-characterized requirement for clathrin and dynamin in endocytosis, the simple interpretation of these results is that Notch internalization is essential for signaling. However, it is possible that loss of dynamin and/or clathrin function, in addition to disrupting endocytosis, may also impact Notch transport to the plasma membrane. Therefore, the failure to observe Notch signaling in fly ovarian cells, which completely lack clathrin or dynamin, might result from limited endogenous Notch delivery to the plasma membrane (Windler & Bilder, 2010). Taken together, I favor the model that γ-secretase cleavage occurs at the plasma membrane.

Several additional studies support the model that γ-secretase cleavage occurs at the plasma membrane. Drug studies using a membrane-impermeable γ -secretase inhibitior, significantly reduces Notch signaling in mammalian cells expressing NEXT mimic (NΔE, Tarassishin et al., 2004). In addition, mass spectrometry analysis identified multiple γ-secretase cleavage sites within the Notch transmembrane domain (Tagami et al., 2008). Tagami et al. (2008) found that γ-secretase-dependent Notch cleavage within the endosome generates L-NICD or S-NICD, unstable cleavage products that are degraded by the proteosome. By contrast, γ-secretase-dependent cleavage at the plasma

membrane generates V-NICD, a stable cleavage product that is essential for robust signaling (Figure 18, Schroeter et al., 1998; Tagami et al., 2008). Given that γ-secretase cleavage produces a stable cleavage product at the plasma membrane, this positions endocytosis as crticial mechanism to downregulate Notch signaling.

Notch sorting within the endosome

Following receptor uptake, Notch endosomal transport decisions must be made to target the receptor for storage, recycling, or degradation within the lysosome. How these sorting decisions are made is unknown. One factor that may direct Notch sorting at the endosome is Numb. Numb is a well-described Notch signaling antagonist during *Drosophila* sensory organ precursor development. During *Drosophila* asymmetric cell division, Numb localizes asymmetrically into one of the two daughter cells, where it prevents Notch signaling (Berdnik et al., 2002). Given that Numb is required for the asymmetric localization of endocytic adaptor AP2 (Berdnik et al., 2002) and can directly interact with Notch (Guo et al., 1996), researchers propsed that Numb may antagonize Notch signaling by targeting Notch for endocytosis and subsequent degradation in the endosomal pathway (Berdnik et al., 2002, Guo et al., 1996, Santolini et al., 2000). Although studies show that Numb performs an endocytic role (Dho et al., 2006; McGill et al., 2010; Santolini et al., 2000; Smith et al., 2004), I did not observe a defect in CD8-NΔE internalization following Numb depletion, relative to controls (Chapter 3). Consistent with my observation, McGill et al. (2009) found that Numb depletion did not change full length Notch internalization compared with control. Therefore, Numb likely regulates the post-endocytic trafficking and degradation of Notch (McGill et al., 2009).

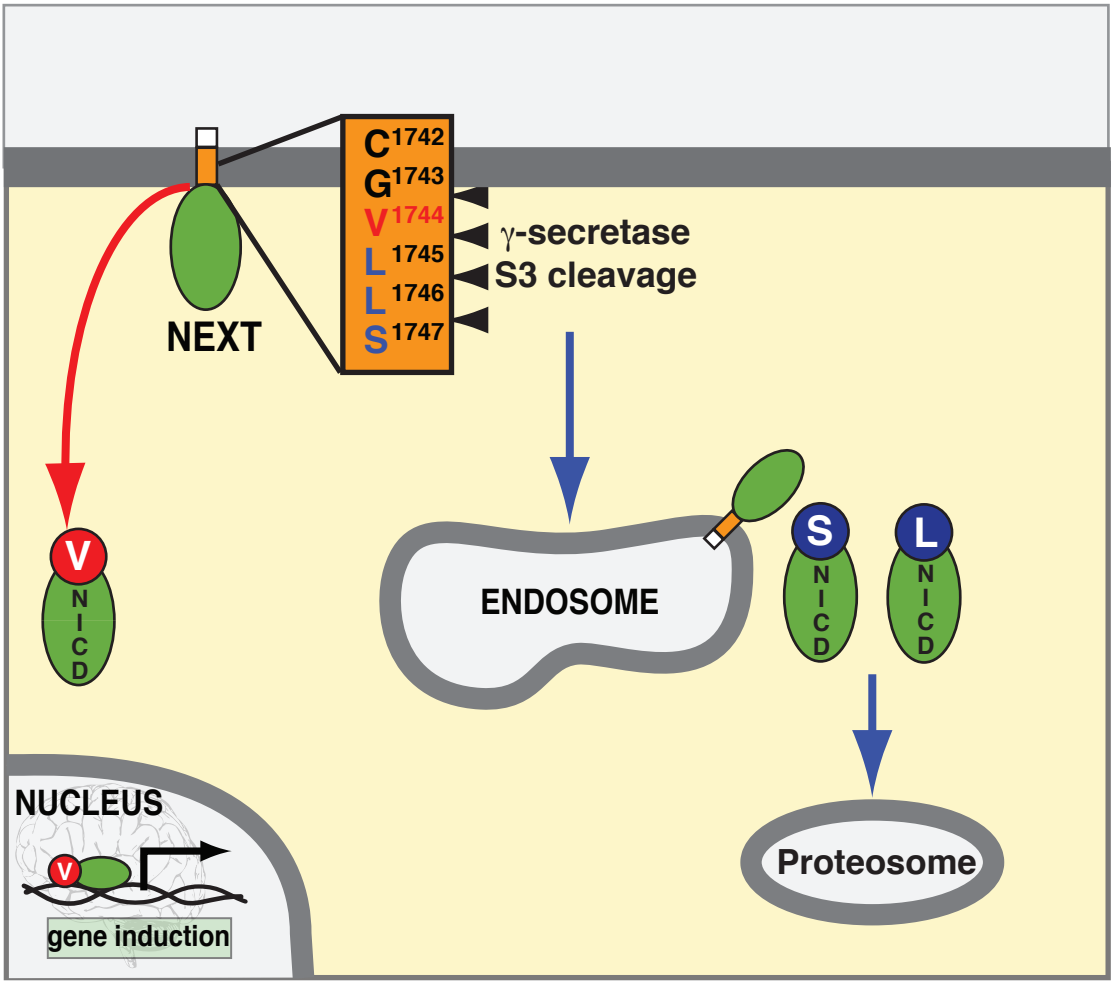

Figure 18: γ-secretase cleaves Notch at multiple sites. MALDI-TOF mass spepctrometry identified four γ-secretase cleavage sites within the Notch transmembrane domain (Tagami et al., 2008). V-NICD, originally described (Schroeter et al., 1998), is a stable cleavage product thought to mediate a majority of Notch signaling. Other cleavage sites generate L-NICD and S-NICD products. These products, are thought to be rapidly degraded in the proteosome as they only give rise to robust signaling in the presence of proteosome inhibitor. Cellular fraction experiments indicate that V-NICD is likely generated at the plasma membrane versus L-NICD and S-NICD which are produced at the level of the endsome. Therefore, the precision and location of γ-secretase cleavage can influence Notch signaling intensity (Tagami et al., 2008).

Another factor critical for directing receptor sorting decisions is AAK1 (adaptor-associated kinase 1). Mutations in *Sel5*, the *C. elegans* AAK1 homolgue, was identified in a genetic screen as a suppressor of constitutively active membrane-bound LIN-12/Notch but not an activated cytosolic LIN-12 (Fares & Greenwald, 1999). This positions Sel5 as a regulator of LIN-12/Notch prior to γ-secretase-mediated Notch cleavage, possibly by regulating of Notch receptor endocytosis or trafficking events (Fares & Greenwald, 1999). Therefore, I tested if AAK1 was critical for CD8-NΔE internalization, in mammalian cells. I did not observe defect in CD8-NΔE internalization following AAK1 depletion, relative to controls (Chapter 3). Therefore AAK1, like Numb, must function during Notch signaling at a step other than receptor endocytosis.

Although AAK1 and Numb are genetically linked to Notch signaling, how these proteins function mechanistically in Notch signaling was not known. A yeast two-hybrid screen identified NAK, the *Drosophila* AAK1 homologue, as a numb interacting partner (Chien et al., 1998) and studies in mammalian cells identified that AAK1, a serine/threonine kinase, phosphorylates adaptor protein AP2 (Conner & Schmid, 2002). These observations led to the hypothesis that AAK1-mediated phosphorylation may regulate Numb function during Notch signaling. However, I initially did not have the CD8-NΔE internalization assay in place and therefore tested if AAK1 could regulate Numb activity as a general endocytic adaptor in mammalian cells. I found, using an *in vitro* kinase assay with ^{32}P(ATP), that AAK1 can phosphorylate Numb at threonine 102 , as mutation of this threonine to a non-phosphorylatable alanine significantly reduces Numb phosphorylation, compared to wildtype (Chapter 3). Overexpression of AAK1 redistributes Numb to the perinuclear endosome; whereas AAK1 depletion restricts Numb to the plasma membrane. Likewise, expression of T102A Numb

enriches Numb at the cell cortex. These data suggest that AAK1-dependent Numb phosphorylation regulates Numb function by modulating its cellular distribution (Chapter 3).

What is the molecular mechanism for the AAK1/Numb/Notch relationship? Given that neither Numb nor AAK1 are essential for efficient CD8-NΔE internalization, I predict that AAK1 and Numb function within the endosomal pathway to regulate Notch receptor storage/recycling or sorting to the degradative lysosome. I propose the following two-step model. In step one, Numb interacts with full-length inactive Notch at the plasma membrane (Guo et al., 1996; Santolini et al., 2000). If this interaction persists within the endosome, then Notch will undergo endosomal sorting to the lysosome (Figure 19A). Several lines of evidence are consistent with this portion of the model. 1) Numb interacts with both Notch with E3 ligase ITCH, which cooperate to promote the ubiquitination of membrane-associated Notch (McGill and McGlade, 2003) and Notch targeting to the lysosome for degradation (Chastagner et al., 2008). 2) C. *elegans,* othologue NUM1-A overexpression phenocopies loss of *rme-1* (Nilsson et al.,2008), a protein that positively regulates recycling and from the endocytic recycling compartment (Smith et al., 2004). This suggests that NUM1-A inhibits endosomal recycling through negative regulation of RME-1 (Nilsson et al., 2008). 3) Numb siRNA depletion increases Notch recycling in mammalian cells suggesting that Numb promotes Notch sorting to the lysosome for degradation (McGill et al., 2009). In step two, if interacting Numb/Notch enter a clathrin-rich microdomain, where AAK1 kinase activity can be stimulated (Conner et al., 2003), AAK1 readily phosphorylates Numb. Phosphorylated Numb disengages the Notch receptor and redistributes to the endocytic recycling compartment. In the absence of Numb, Notch no longer undergoes lysosomal

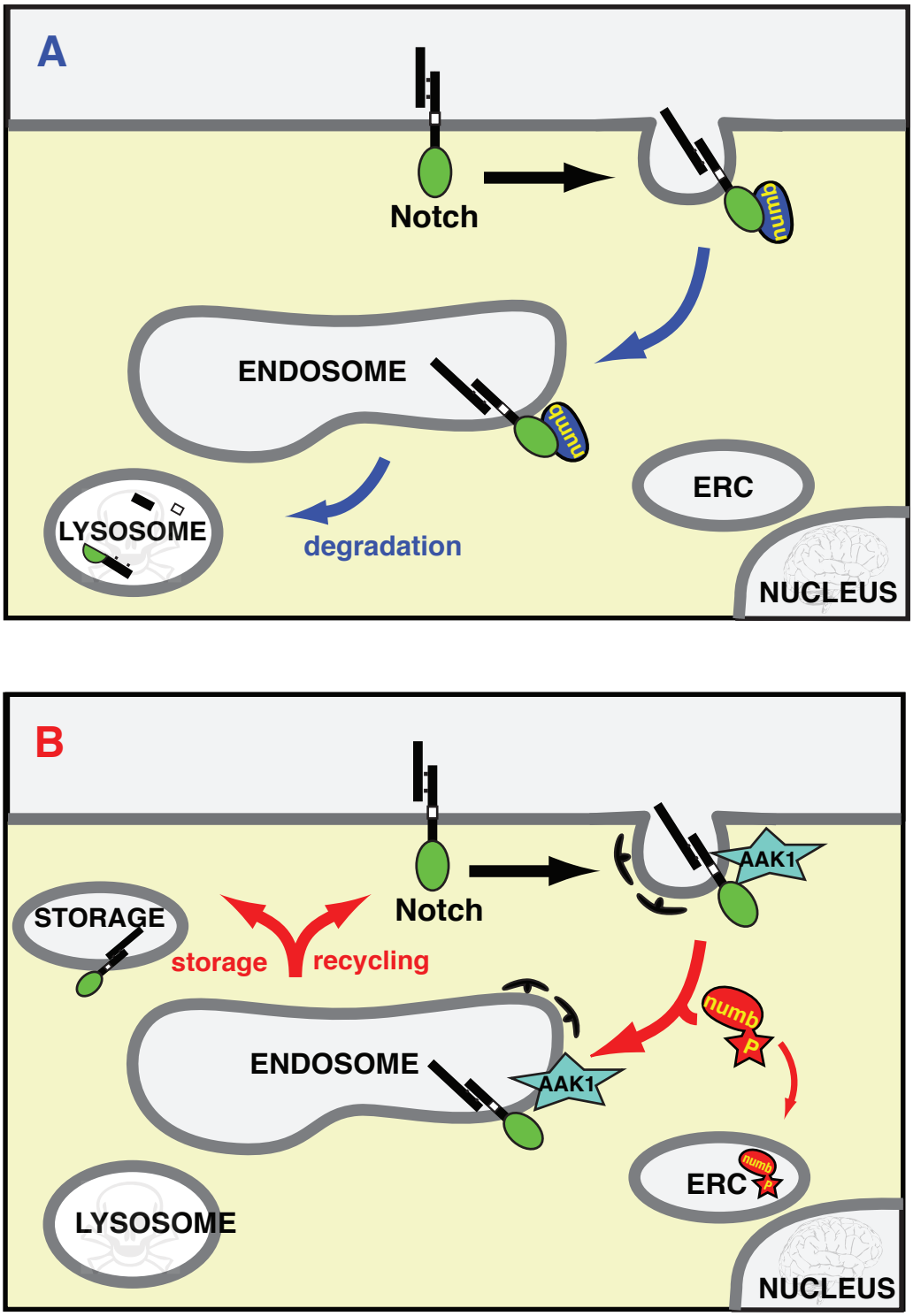

Figure 19 : AAK1-mediated Numb phosphorylation regulates Notch endosomal transport.

Figure 19 : AAK1-mediated Numb phosphorylation regulates Notch endosomal transport. A) During receptor downregulation, Numb interacts with full-length Notch, within a coated pit. If this interaction persists at the endosome, Notch sorts to the lysosome for degradation and signaling is attenuated. B) If the Numb/Notch interacting pair enter a clathrin-rich microdomain, where AAK1 kinase activity is stimulated (Conner et al., 2003), AAK1 phosphorylates Numb. Phosphorylated Numb redistributes to the endosomal recycling compartment (ERC). Notch, in the absence of Numb, instead sorts to a storage compartment or undergoes recycling to the plasma membrane. This gives Notch another opportunity to undergo signal activation by its ligand DSL and γ-secretase processing at the plasma membrane.

sorting. Instead, Notch can undergo sorting to a storage endosome or recycle to the plasma membrane. Therefore, Notch remains available for DSL activation and γ-secretase processing at the cell surface (Figure 19B). Consistent with this model, AAK1 plays a role in receptor recycling, as siRNA-mediated depletion of AAK1 in mammalian cells reduces transferrin receptor recycling from the early/sorting endosome (Henderson & Conner, 2007). Therefore, Numb and AAK1 may regulate Notch signaling by directing Notch endosomal transport. Endosomal sorting decisions are a critical mechanism for Notch signal regulation as these decisions will influence signal activation by DSL and γ-secretase, following receptor recycling or downregulation, by degradation within the lysosome.

Cytosolic factors that regulate Notch endosomal transport

This work identified 4 cytosolic components critical for CD8-NΔE internalization. However, additional factors are needed to regulate Notch sorting decisions that promote receptor degradation, recycling or sorting. The identification of the cytosolic components that regulate the Notch endosomal itinerary is critical to understanding signal activation as receptor recycling, endosomal storage or lysosomal degradation affects Notch signaling capacity (Fortini & BIlder, 2009; Furthauser & Gonzalez-Gaitan, 2009). To date, 5 *Drosophila* studies identify mutations in ESCRT (Endosomal Sorting Complex Required for Transport) protein complexes as critical Notch receptor sorting factors. ESCRT mutations exhibit ectopic Notch signaling as these complexes are critical for the recruitment and internalization of signaling receptor into multivesicular bodies for degradation (Childress et al., 2006; Gallagher & Knoblich, 2006; Herz et al., 2009; Moberg et al. 2005; Thompson et al., 2005).

Tools developed in Chapter 2 can identify additional factors critical in Notch receptor sorting and identify genes that are lethal upon complete knockout in genetic null mutants. For example, immunofluoroescence analysis of CD8-NΔE following clathrin-depletion reveals an accumulation of CD8-NΔE at the plasma membrane, as clathrin is needed for Notch internalization. Therefore, immuofluoroescence analysis of CD8-NΔE can identify the subcellular compartment where a protein functions in Notch sorting. Likewise, cytosolic components affecting the cell surface expression of Notch will change the signaling capacity of CD8-NΔE by luciferase reporter assay. For example, depletion conditions that impair Notch receptor endocytosis or increase receptor recycling will have elevated Notch signaling due to increased exposure to γ-secretase at the plasma membrane. In contrast, depletion conditions that prevent exposure of CD8-NΔE to the cell surface, i.e. accumulation in an intracellular compartment, will exhibit decreased Notch signaling by reporter assay. Therefore this work can be used as a launching point for identification of endocytic sorting components involved in Notch signaling regulation.

Identification of the intrinsic signals that regulate Notch internalization

Endocytosis and endosomal transport involves a two-part system. 1) cytosolic components critical for making these decisions and 2) the receptor sorting signals, either intrinsic or added by post-translational modification, that these cytosolic factors engage. The sorting signals within the mammalian Notch cytoplasmic tail, which direct receptor internalizaton and endosomal sorting decisions, are not currently defined. Shaye and Greenwald (2005) identified a di-leucine motif in C. *elegans* Lin-12 as a critical motif for endocytosis. Unfortunately, this motif is not highly conserved in *Drosophila* or mammalian

Notch1 and therefore it is unclear if this motif and/or other sorting signals are required for Notch internalization in other organisms. In addition, other motifs likely exist to regulate Notch post-internalization trafficking to endosomes/ lysosomes (Bonifacino & Traub, 2003). Using truncational analysis and site-directed mutagenesis, Cosmo Saunders (personal communication) identified a di-leucine motif critical for directing CD8-NΔE towards a storage compartment. This same approach can identify additional regions/motifs within the cytoplasmic tail that direct Notch endocytosis and endosomal transport.

In addition, results presented here and elsewhere implicate Notch post-translational modification (i.e. ubiquitination and phosphorylation) in signal regulation (Chastagner et al., 2008; Gupta-Rossi et al., 2001; McGill & McGlade, 2003; Sakata et al., 2004; Shaye & Greenwald, 2005; Wilkin et al., 2004). For example, robust CD8-NΔE internalization requires Nedd4, as siRNA depletion significantly reduces the Notch uptake and increases Notch signaling relative to control cells (Chapter 2). Although my results and others (Sakata et al., 2004) propose that Nedd4 directly ubiquitinates Notch, the modification site(s) are unknown. Therefore, future studies identifying modified residues in the cytoplasmic tail, by mass spectrometry, should be undertaken to better understand the signals that coordinate Notch receptor endocytosis and transport.

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