Endocytic Regulation of Notch Signaling in Drosophila Melanogaster Neural Progenitor Cells

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Abstract
Notch signaling is a ubiquitously used signaling pathway that is highly conserved and used throughout metazoan development. Understanding the regulation of Notch signaling is becoming increasingly important in determining the mechanism and treatment for the myriad of human Notch-related diseases. In Drosophila melanogaster, the development of external sensory organs provides a context in which Notch can be manipulated and phenotypes can be easily interpreted. Here, we expand upon the growing field of Notch regulation through endocytic trafficking by examining the role of Numb and Sara endosomes. Numb is a potent Notch inhibitor whose function is conserved in higher organisms, but whose mechanism of action has remained elusive. In this study, we dispel a previous hypothesis that Numb promotes Notch internalization and instead demonstrate that Numb is a suppressor of Notch endocytic recycling. In support of this, we show that Numb is necessary and sufficient for Notch trafficking to late endosomes/lysosomes to promote degradation. We do this by employing a novel technique that is able to distinguish recycled Notch from other populations within the cell. In addition, we show that the cell fate determinant Lethal (2) Giant Larvae, can also suppress Notch recycling, but at a step upstream of Numb. Results from this study help to answer a long-standing questions in the field of Notch signaling, by demonstrating the role of Numb in Drosophila. We also extended our investigation of endocytic Notch regulation by determining the role of a sub-population of early endosomes positive for Sara. We show that these Sara endosomes are trafficked preferentially to Notch activated cells, but do not contain appreciable levels of Notch. While we conclude that the Sara endosomes do not seem relevant to Notch signaling, we show that the mechanism of Sara endosome trafficking is likely tied to global anterior-posterior cues and not related to cell fate determinants. Results from our studies have important implications in the designing of treatments for Notch related dysfunctions that depend on an exquisite understanding of Notch regulation.

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ENDOCYTIC REGULATION OF NOTCH SIGNALING IN DROSOPHILA MELANOGASTER NEURAL PROGENITOR CELLS

Seth Andrew Johnson

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Notch signaling is a ubiquitously used signaling pathway that is highly conserved and used throughout metazoan development. Understanding the regulation of Notch signaling is becoming increasingly important in determining the mechanism and treatment for the myriad of human Notch-related diseases. In *Drosophila melanogaster*, the development of external sensory organs provides a context in which Notch can be manipulated and phenotypes can be easily interpreted. Here, we expand upon the growing field of Notch regulation through endocytic trafficking by examining the role of Numb and Sara endosomes. Numb is a potent Notch inhibitor whose function is conserved in higher organisms, but whose mechanism of action has remained elusive. In this study, we dispel a previous hypothesis that Numb promotes Notch internalization and instead demonstrate that Numb is a suppressor of Notch endocytic recycling. In support of this, we show that Numb is necessary and sufficient for Notch trafficking to late endosomes/lysosomes to promote degradation. We do this by employing a novel technique that is able to distinguish recycled Notch from other populations within the cell. In addition, we show that the cell fate determinant Lethal (2) Giant Larvae, can also
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS................................................................................................................... ii

ABSTRACT........................................................................................................................................ iv

LIST OF FIGURES AND TABLES...................................................................................................... vii

CHAPTER 1: INTRODUCTION........................................................................................................ 1
  1.1 Mechanics of Notch signaling ............................................................................................... 1
  1.2 Notch signaling during development ................................................................................. 9
  1.3 Establishment of polarity in external sensory organs ....................................................... 20
  1.4 Regulation of Notch signaling ........................................................................................... 30

CHAPTER 2: Numb regulates the balance between Notch recycling and late endosome targeting in Drosophila neural progenitor cells................................................................. 42
  2.1 Summary............................................................................................................................... 43
  2.2 Introduction.......................................................................................................................... 44
  2.3 Results.................................................................................................................................. 47
  2.4 Discussion............................................................................................................................ 67
  2.5 Supplementary Information............................................................................................... 71

CHAPTER 3: Determining the role of Sara endosomes in cell fate specification of external sensory organs.................................................................................................................. 76
  3.1 Summary............................................................................................................................... 77
  3.2 Introduction.......................................................................................................................... 78
  3.3 Results.................................................................................................................................. 81
  3.4 Discussion............................................................................................................................ 94

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS....................................................... 97
  4.1 Conclusions.......................................................................................................................... 97
  4.2 Remaining Questions and Future Directions.................................................................... 103
  4.3 Concluding remarks........................................................................................................... 108

CHAPTER 5: MATERIALS AND METHODS............................................................................. 109

REFERENCES................................................................................................................................. 117
LIST OF FIGURES AND TABLES

FIGURE 1.1 Summary of Notch signaling.................................................................4
FIGURE 1.2 Notch receptor schematic.................................................................8
FIGURE 1.3 SOP specification through lateral inhibition......................................13
FIGURE 1.4 Vulval precursor specification through lateral inhibition..................17
FIGURE 1.5 Alignment of the mitotic spindle pole............................................25
FIGURE 1.6 Sensory organ cell lineage...............................................................29
FIGURE 1.7 Endocytic trafficking of membrane receptors....................................34
FIGURE 2.1 Notch recruitment to early endosomes is Numb-independent..........49
FIGURE 2.2 Numb is required for asymmetric Notch trafficking to late endosomes..51
FIGURE 2.3 Notch recycling assay confirms Numb’s inhibition of Notch recycling...54
FIGURE 2.4 Rab5 dominant negative causes cell fate switch and accumulation of static Notch.................................................................58
FIGURE 2.5 Notch signaling requires Rab11 recycling endosome......................61
FIGURE 2.6 Notch recycling is suppressed by Lgl.................................................65
TABLE S2.1 Recycling assay p values.................................................................71
FIGURE S2.2 Wasp and Sec15 do not alter Notch trafficking.............................71
FIGURE S2.3 Rab7/Rab5 internalization assays..................................................73
FIGURE S2.4 Overactive or dominant negative Rab7 alters Notch localization.....74
FIGURE 3.1 Sara endosomes are asymmetrically trafficked.............................82
FIGURE 3.2 Rab5QL endosomes are symmetrically targeted............................85
FIGURE 3.3 Notch does not localize to Sara or large Rab5QL endosomes.........87
FIGURE 3.4 Insuteable reverses Sara asymmetry..........................................................89

FIGURE 3.5 Mitotic spindle projections are symmetric in pIIa/pIIb.................................93
CHAPTER 1: Introduction

1.1 Mechanics of Notch signaling

Just over 100 years ago, the notched wing phenotype was first observed in the lab of Thomas Hunt Morgan in 1913 while studying mutations in *Drosophila melanogaster*. He described the corresponding mutation as lethal when homozygous and at approximately position 2.6 on the X chromosome (Morgan and Bridges, 1916). From this unsuspectingly simple beginning, enormous advancements have been made in understanding the biological functions of Notch, its regulation, and the consequences that arise when Notch signaling is inappropriately regulated. Notch plays a central role in development where signaling is required for the preservation or specification of neural progenitor cells, a role that is conserved throughout metazoan evolution. Additional developmental roles for Notch signaling include cardiac, pancreatic and intestinal development, as well as angiogenesis and hematopoiesis. The strong evolutionary conservation of Notch structure and function makes model organisms a prime way of understanding Notch signaling in humans and identifying potential drug targets to treat the myriad of diseases associated with aberrant Notch signaling.

Notch as a signaling molecule

Notch is a large single-pass transmembrane protein that contains distinct extracellular (NECD) and intracellular (NICD) domains (Artavanis-Tsakonas *et al.*, 1983). Over its lifetime, Notch undergoes multiple modification and cleavage events that ensure appropriate transport and signaling specificity (Figure 1.1). After translation in the ER, Notch is transported to the Golgi where it undergoes O-fucosylation and O-
glycosylation modifications known to be essential for proper signaling (Okajima and Irvine, 2002; Sasamura et al., 2003); (Okajima et al., 2005). In Drosophila, the two possible ligands for the Notch receptor are Delta and Serrate. Elongation of O-fucose chains on Notch by Fringe promotes preferential binding of Delta to Notch and inhibits binding with Serrate, conferring specificity (Xu et al., 2007; Rana and Haltiwanger, 2011). Notch is also cleaved in the Golgi by Furin proteases, (S1 cleavage) causing the receptor to re-dimerimize on the plasma membrane (Logeat et al., 1998; Lake et al., 2009).

Binding of either Delta or Serrate with the NECD on the plasma membrane triggers a conformational change that allows a disintegrin and metalloproteinase (ADAM) to again cleave Notch at a revealed cleavage site (S2) (Figure 1.1)(Mumm et al., 2000; Parks et al., 2000a). This second cleavage occurs within the extracellular domain (ECD), leaving behind a portion of the Notch receptor still embedded in the plasma membrane termed the Notch extracellular truncation (NEXT). The NEXT fragment is the target for the gamma-secretase complex which performs the third Notch cleavage event (S3) (Figure 1.2) (De Strooper et al., 1999; Struhl and Greenwald, 1999). Regulation of gamma-secretase in the cleavage of NEXT is not entirely understood, but a recent study showed that a known regulator of Notch signaling, Sanpodo, forms a ternary complex with Notch and the gamma-secretase complex facilitating S3 cleavage (Upadhyay et al., 2013a). Upon cleavage by gamma secretase, the NICD is released, allowing it to translocate to the nucleus.
Once released, the NICD is guided to the nucleus by multiple nuclear localization sequences (NLS), and binds CSL (CBF1, Suppressor of Hairless, Lag-1) (Pursglove and Mackay, 2005; Kopan et al., 1994). Without NICD, CSL recruits Groucho, Hairless and CtBP corepressors, which help to block transcription of Notch target genes (Nagel et al., 2005; Morel et al., 2001). The RAM23 (RBP-jk associated molecule) and Ankyrin repeats of NICD bind CSL and recruit Mastermind to form a ternary complex activating histone acetyltransferases which allows transcription of Notch target genes (Kovall, 2007). In addition to the highly conserved RAM23 and Ankyrin repeat domains of the NICD, the C-terminal PEST (rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) domain also mediates proper Notch signaling, and is critical for proteasome-dependent degradation, which turns off the Notch signal. The E-3 ligase Sel-10 promotes degradation by binding within consensus sequences of the PEST domain and facilitates poly-ubiquitinylation, signaling proteasome degradation. (Öberg et al., 2001; Fryer et al., 2004; Gupta-Rossi et al., 2001). In mammalian cells, the PEST domain can also be marked for degradation by the E3 ligase Itch via an interaction facilitated by Numb (McGill and McGlade, 2003). Interestingly, this role for Numb appears to have evolved more recently, as Numb does not have this function in Drosophila.
Figure 1.1 Summary of Notch signaling
Figure 1.1 Summary of Notch signaling

1. Notch receptors (green/purple boxes) become glucosylated and fucosylated (yellow hexagons) in the Golgi.  2. S1 cleavage by Furin (dark blue oval) causes formation of the Notch heterodimer and is transported to the membrane. 3. Notch ligand (Delta/Serrate in *Drosophila*, red boxes) is activated through Rab11-dependent recycling and binds the Notch receptor extracellular domain (ECD, green). 4. Ligand is mono-ubiquitinylated (gray circle) by Neuralized (black oval) causing ligand internalization. Ligand endocytosis exerts a pulling force on Notch receptor allowing for S2 cleavage by ADAM (blue oval) leaving Notch extracellular truncation (NEXT). 5. NEXT fragments are cleaved by the gamma-secretase subunit of presenilin (light blue circle) allowing for release of Notch intracellular domain (NICD, purple boxes). The full-length receptor is internalized and degraded or recycled. 6. NICD travels to nucleus and recruits Mastermind (orange square) to convert CSL from a transcriptional repressor (red oval) to an activator (green oval).
Mechanics of Notch ligands Delta and Serrate

In addition to the Notch receptor, Notch ligands (Serrate, Delta in *Drosophila*) also undergo complex transport and activation mechanisms to properly regulate signaling. In *Drosophila*, Delta is mono-ubiquinylated by RING-finger E3 ligases Neuralized (Neur) and Mindbomb (Mib) (Lai et al., 2001; Yeh et al., 2000). Separate from the role of poly-ubiquitinylation of the PEST domain in the NICD for degradation, mono-ubiquitinylation of Delta is thought to be a signal for clathrin-mediated endocytosis (Hicke and Dunn, 2003). Endocytosis of Delta appears to have two distinct roles in its activation of Notch signaling. First, a Notch-independent endocytosis event causes Delta to be recycled back to the plasma membrane as a “priming” mechanism. The mechanism of this first endocytic priming event is not clear, as it does not appear to modify Delta. Instead, the recycling of Delta may be necessary for transporting and concentrating the ligand (transcytosis) in an apical microdomain. In support of this, trafficking regulators Rab11 and Sec15, are required for Notch signaling activation and Delta transcytosis to a subapical actin rich structure (ARS) (Benhra et al., 2010; Emery et al., 2005; Jafar-Nejad et al., 2005). In the signal sending cell, Notch activation also requires an AP-47 dependent transcytosis to a similar domain, suggesting that the ARS is the location of Notch activation and that concentration of Notch and ligand at that location is essential (Benhra et al., 2011). It is unclear, however, whether Rab11 is required only in signal sending cells for Delta transcytosis, or whether it serves a similar function in the signal receiving cell in transcytosis of Notch.
The second role of ligand endocytosis occurs after ligand binding with the NECD. Binding between Jagged and Notch in mammalian systems triggers ubiquitinylation by Mind Bomb and subsequent Jagged/NECD endocytosis (Hansson et al., 2010). In *Drosophila*, Delta ubiquitinylation by Neuralized triggers binding of epsin (liquid facets), dynamin and clathrin to mediate the endocytosis of Delta/NECD after Notch binding (Xie et al., 2012; Overstreet et al., 2004). After Delta/Notch binding, Delta/NECD become trans-endocytosed into the signal sending cell, a process that is required for Notch signal activation (Parks et al., 2000b). The current interpretation of this result is that epsin and clathrin elicit a change in membrane curvature that exerts a “pulling force” that is sufficient to change the conformation of Notch, allowing it to be cleaved by ADAM (Horvath et al., 2007; Windler and Bilder, 2010).
Figure 1.2 Notch receptor schematic

Representation of full-length *Drosophila* Notch receptor after S1 cleavage. The NECD is composed of 36 EGF-like repeats (green boxes), which facilitate ligand binding. LNR (Lin-12 Notch repeats, red boxes) and HD (heterodimerization domain, orange box) compose the NRR (negative regulatory region), which prevents S2 cleavage before ligand binding. S3 cleavage occurs just below the transmembrane domain after S2 cleavage. Three NLSs (nuclear localization sequences, red bars) facilitate transport of NICD to the nucleus. Once in the nucleus, Ram23 (blue box) and Ankyrin repeats (yellow boxes) recruit Mastermind and bind CSL activating Notch target genes. The PEST (Proline, Glutamic Acid, Serine, Threonine) domain recruits E3 ubiquitin ligases to facilitate proteasome-dependent degradation of NICD.
1.2 Notch signaling during development

Notch is a highly conserved signaling pathway that is used in many different contexts during development. In *Drosophila*, Notch plays a part in the development of almost all cell types, where its key role is to specify or maintain cell identity. Notch is perhaps most well studied in neurogenesis, where it adopts two distinct roles. The first role is in lateral inhibition, where Notch signaling amplifies genetic differences already present between adjacent cells over a large area (Figure 1.3). The second role is in binary cell fate choices, where asymmetric activation of Notch signaling generates daughter cells of differing cell fates (Figure 1.6). The development of the external sensory organs in the Drosophila PNS utilizes both of these Notch roles and will be the primary focus of this discussion. In addition, we will also discuss vulval development in *Caenorhabditis elegans* to highlight a role of Notch signaling in lateral inhibition outside of Drosophila. The critical role of Notch is also made evident by findings that errors in Notch signaling lead to a wide variety of diseases, most notably T-cell acute lymphoblastic leukemia (T-ALL), which will be discussed as an example of a disease caused by errors in Notch signaling during blood cell development.

Fate specification through lateral inhibition

In *Drosophila* development, lateral inhibition is used to pick out one cell from many to adopt a neural fate (Figure 1.3) and is used most prominently in specification of neuroblasts, photoreceptors of the eye, and external sensory organs. In the specification of external sensory organs, lateral inhibition involves antagonism between Notch signaling and a class of basic helix loop helix (bHLH) genes known as proneural genes.
The purpose of this process is to refine the pattern of cells that express proneural genes to ensure even and ordered specification of neural progenitors. The most widely known and understood proneural genes are those of the Achaete-Scute complex (AS-C), composed of Achaete (Ac), Scute (Sc), Lethal of scute (Lsc) and Asense (As) (Skeath and Carroll, 1994; Villares and Cabrera, 1987). Additional proneural genes, independent of the AS-C complex, include Atonal (Ato) (Jarman et al., 1993), Amos (Goulding et al., 2000b) and Cato (Goulding et al., 2000a). The presence of multiple proneural genes promotes some degree of tissue specificity, as some proneural genes are only used in certain cell types. For example, the AS-C genes are primarily used in SOPs, while Atonal is utilized in chordatonal and R8 photoreceptors of the eye (Treisman, 2013). Swapping the basic domains between Ato and As causes a cell fate switch from SOP to chordatonal organ, suggesting that the basic domain of bHLH neural proteins is responsible for dictating this specificity (Chien et al., 1996).

The interaction between Notch and proneural genes is facilitated by a negative feedback loop that gradually refines the number of cells expressing proneural genes from a proneural cluster (PNC) to a single SOP. Initially, all cells in the PNC express low levels of proneural genes. Despite the low concentration, proneural gene products bind to Delta enhances to promote its expression (Hinz et al., 1994). Increased expression of Delta causes transport to the cell membrane activating Notch receptors in adjacent cells. Activation of Notch triggers transcription of Enhancer of Split (E-Spl), which in turn causes a repression of proneural gene expression (Nakao and Campos-Ortega, 1996). Repression of the proneural genes essentially causes the particular cell to “lose” the
competition to become the last SOP. Ultimately, every cell but one in the PNC will express high levels of Notch, indicating it has lost proneural gene expression. The last cell to still express proneural genes maintains a low level of Notch receptor activity due to not being activated by the Delta of adjacent cells and thus becomes the SOP (Figure 1.3) (Campos-Ortega, 1995).

This relatively simple paradigm for the specification of the SOP has been well established, but more recent studies have uncovered the necessity of additional factors for this process. Using mathematical modeling, this negative feedback loop can indeed produce the grid-like pattern of SOPs seen in vivo, however, the models produce a pattern that is more densely packed than actually exists in vivo (Webb and Owen, 2004). A possible explanation for this discrepancy is the presence of dynamic, actin based filopodia that may transmit the Delta signal to activate Notch in non-adjacent cells (Cohen et al., 2010). These filopodia could inactivate cells farther away from their cell bodies to promote the sparser spacing observed than what is predicted using models. Another component in this process is the mechanism of Notch cis-inhibition, which occurs when Notch and Delta reside within the cell membrane of the same cell, and through binding of their respective extracellular domains, inactivate Notch signaling. The mechanism by which this cis binding leads to Notch inactivation is not entirely clear, but could result from either competition between the cis and trans Notch ligands, or possibly through inhibiting internalization signals (del Alamo et al., 2011). Cis-inhibition may also be provide the mechanism by which the error rate of the lateral
inhibition process is extremely low (less than 1%, with errors being defined as when adjacent cells each become SOPs) (Barad et al., 2010).
Figure 1.3 SOP specification through lateral inhibition
Figure 1.3 SOP specification through lateral inhibition

A. Cluster of equipotential cells (left panel, gray cells) express proneural genes of the Achaete-Scute complex (AS-C). AS-C (blue boxes) promotes Delta expression (red boxes) allowing transport of Delta to plasma membrane. B. Presentation of Delta on cell membranes activates Notch (green boxes) signaling in adjacent cells, promoting Enhancer of Split (E(spl), purple boxes) expression, which inhibits expression of AS-C. Dotted lines indicate reduced signal. Notch signaling causes refinement of the cell cluster into an arrangement of cells with high and low proneural gene expression (dark, light gray cells, respectively). C. Continued negative feedback inhibition causes one cell (the presumptive SOP cell) with AS-C expression to remain (black cell).
**Lateral inhibition in C.elegans**

In addition to *Drosophila*, Notch signaling is used in lateral inhibition in *C. elegans* to specify cell fates during vulval development. In *C. elegans*, the vulva is composed of six vulval precursor cells (VPCs) numbered P3.p through P8.p, which adopt three different cell fates (1°, 2°, and 3°) reviewed in Greenwald, (1997) (Figure 1.5). Specification of these cells types involves both signaling from a morphogen gradient and lateral inhibition between adjacent cells. The Anchor Cell (AC) of the somatic gonad is adjacent to the cell that eventually adopts the 1° cell fate and is necessary for specification of VPCs. The AC releases the morphogen LIN-3, which serves as a ligand for the receptor tyrosine kinase LET-23 (Hill and Sternberg, 1992). Binding of LET-23 triggers activation of a downstream signaling cascade first involving activation of *let-60* (Ras), which activates *lin-45* (Raf), *mek-2* (MEK), and *mpk-1/sur-1* (MAP kinase) (Han et al., 1990; Church et al., 1995). Activation of this signaling cascade in the P6.p cell promotes the 1° cell fate in the most proximal cell while eventually inhibiting the 1° fate in the adjacent VPCs. Laser ablation of P6.p (1° cell) causes adjacent cells, normally of the 2° fate, to instead adopt the 1° fate, implying an inhibitory mechanism originating from the 1° cell (Sternberg, 1988). This mechanism involves LIN-12, the worm homologue of the Notch receptor, and members of the DSL (delta, serrate, LAG-2) family of ligands. Loss of LIN-12 prevents the specification of the 2° cell fate of VPCs, while overactivation causes all VPCs to adopt 2° cell fates (Struhl et al., 1993), suggesting that LIN-12/Notch is required for this lateral inhibition signal. Activation of LET-23 triggers upregulation of three functionally redundant LIN-12/Notch ligands
including *apx-1, dsl-1*, and *lag-2*, all members of the DSL family of Notch ligands (Chen and Greenwald, 2004). Expression of these ligands in the 1° cell causes activation of LIN-12/Notch in the adjacent P5.p and P7.p cells, promoting the 2° cell fate in those cells. LIN-12/Notch is itself suppressed in the 1° cell in response to LET-23 activation to ensure that LIN-12/Notch signaling in the P6.p cell does not lead to 2° cell fate (Shaye and Greenwald, 2002). Suppression of LIN-12/Notch occurs through an endocytic mechanism that removes LIN-12/Notch from the membrane and targets it for degradation. Interestingly, a similar mechanism for Notch inactivation is observed in flies, where both involve ubiquitinylation by Su(dx)/Itch, the signal for proteasome degradation (Shaye and Greenwald, 2005). Thus, in both *Drosophila* and *C. elegans*, Notch signaling serves as a mediator of lateral inhibition and is both activated and inhibited by closely conserved mechanisms.
Six vulval precursor cells (VPCs) (P3.p- P8.p) are equipotent until expression of LIN-3 from the anchor cell (AC, blue circle). LIN-3 is received at the highest signal strength in P6.p and at lower strengths in P5.p and P7.p by LET-23 receptors (green circles).

Activation of LET-23 triggers activation of the 1° cell fate most strongly in P6.p causing DSL ligands (red bar) for LIN-12/Notch (purple bar). Activation of LIN-12/Notch in P5.p and P7.p triggers acquisition of the 2° cell fate (orange cell background). LIN-12/Notch is suppressed in P6.p by strong activation of LET-23 ensuring 1° cell fate (red cell background).
Fate specification through binary fate choices

In the Drosophila PNS, once the SOP is specified, Notch facilitates control of the binary cell-fate choices governing the development of the adult sensory organ cells (Figure 1.5). In Drosophila, there are approximately 220 thoracic bristles (microchaetes) that relay environmental information to the fly’s nervous system (Hartenstein and Posakony, 1989). Each microchaete begins as a SOP or pI cell, which divides along the anterior-posterior axis within the plane of the epithelium at approximately 14 hours after puparium formation (apf) at room temperature. The anterior and posterior daughter cells are termed pIIb and pIIa, respectively. The pIIa cell divides to create the shaft and socket cells, while the pIIb eventually leads to the sheath and neuron cells (Hartenstein and Posakony, 1989). The pIIb undergoes an intermediate step where it divides to create the pIIIb cell and the neuronal glial cell. The glial cell undergoes apoptosis, while the pIIIb cell divides again to create the sheath and neuron cells (Figure 1.6) (Gho et al., 1999; Fichelson and Gho, 2003).

Control of these binary cell fates is facilitated by the presence or absence of Notch signaling in the external sensory organ lineage. Using temperature sensitive Notch mutants, it was originally shown that Notch has two distinct functions depending on the time of heat shock. At 0-14 hour apf, inhibition of Notch signaling caused an over proliferation of sensory organs at the expense of the surrounding epithelial cells. If instead the heat shock was performed at 14-20 hours apf, extra neuronal cells (neuron and sheath) were found at the expense of external cell types (shaft and socket), demonstrating the necessity of Notch in both the lateral inhibition period (0-14 hours apf) and in the
correct specification of cell fates after SOP definition (14-20 hours apf) (Hartenstein and Posakony, 1990). Furthermore, when Notch is overexpressed at these early and late time points, it causes a loss of SOPs and a conversion from neural cell fates (neuron and sheath) to external cell fates (socket and shaft) (Guo et al., 1996). These early experiments led to the paradigm in which Notch signaling is necessary in the pIIa for specification of the external socket and shaft cells, while lack of Notch signaling in the pIIb cell is necessary for the specification of the internal neuron and sheath cells.

T-ALL: A consequence of aberrant Notch signaling during development

Perhaps the most widely studied developmental context for Notch in vertebrate systems is in hematopoiesis. Early on, Notch was proposed to be necessary for preserving the undifferentiated state of bone marrow progenitor cells (Milner et al., 1994). However, this notion was challenged when it was found that Notch signaling was dispensable for maintenance of hematopoietic stem cells (HSCs) (Maillard et al., 2008). Instead, Notch signaling appears to have a role in lymphoblastic rather than hematopoietic lineages (Besseyrias et al., 2007). Supporting evidence found that overactivation of Notch signaling promotes T-cell differentiation, while lack of Notch signaling promoted B-cell differentiation (Radtke et al., 1999).

The extensive research on Notch in blood cell development has produced a strong causative link between aberrant Notch signaling and T-ALL. T-ALL is characterized by an over production of T-cells at the expense of B-cells and is found most commonly in children and young adults. This condition is associated with a mutation in the human Notch1 gene that causes the receptor to be constitutively active (Ellisen et al.,
It was subsequently found that over 50% of all T-ALL cases are caused by mutations in the extracellular or PEST domains of Notch1 and Notch2 (Lee et al., 2009; Weng et al., 2004). Approximately 20-30% of all human T-ALL cases involve frameshift or nonsense mutations that introduce a stop codon, causing a Notch1 truncation that lacks the PEST domain leading to an over-stabilized receptor that resists degradation (Chiang et al., 2006). In support of this, mutations in the mammalian E3 ligase necessary for the degradation poly-ubiquitinylation signal, FBXW7, are also responsible for a large number of T-ALL cases (Thompson et al., 2007). The other most common region for mutation seen in T-ALL (40-45%) is within the homodimerization (HD) domain, which is normally responsible for preventing S2 cleavage without ligand interaction. Single base pair mutations in this region allows Notch to signal in the absence of ligand binding (Malecki et al., 2006).

1.3 Establishment of polarity for asymmetric cell division

One of the primary mechanisms by which a developing organism transitions from several equipotent cells to a fully differentiated adult is through asymmetric cell division. Intrinsic or extrinsic signals partition cell fate determinants in a way that is sufficient for daughter cells to adopt different cell fates. The master regulator of this process is the anterior-posterior axis which itself becomes positioned through planar cell polarity (PCP) specification. Cell fate determinants read cues from the anterior-posterior axis mediated through the Par complex and the mitotic spindle to become correctly partitioned into their appropriate daughter cells. Thus, cell fate specification requires interpretation from universal signals, communicated to specific contexts. Correct inheritance of these factors...
is critical for appropriate regulation of Notch signaling to control cell fate specification (Figure 1.6).

Establishment of the anterior-posterior axis

The establishment of polarity in *Drosophila* through PCP involves the asymmetric placement of a variety of factors that relay the global anterior-posterior polarity to local contexts. Establishment of the global signal is largely carried out by the core Frizzled/Van Gogh (Frz/Vang) pathway. Both Frz and Vang are transmembrane receptors that localize on opposite sides of the cells within epithelial sheets (Strutt, 2001; Bastock *et al.*, 2003). Asymmetric distribution of Frz and Vang depends on an intermediate linker protein, Flamingo, which facilitates binding between the extracellular domains of Frz and Vang on adjacent cells (Chen and Deng, 2009). Frz ECD binding with Flamingo causes crescents of Frz receptor to always be adjacent to Vang crescents on adjacent cells. This asymmetry is propagated through an antagonism between Diego/Dishevelled (Dgo/Dsh) and Prickled (Pk), cytosolic proteins that bind the intracellular domains of Frz and Vang, respectively (Axelrod, 2001; Jenny *et al.*, 2003). Experiments performed with clones for the core PCP pathway genes reveal that they are not cell autonomous and instead rely on cues from neighboring cells to achieve the correct orientation. Clones of Frz or Vang do not cause a randomization of orientation, instead causing trichomes to adopt orientations pointing either inward or outward in relation to the clone’s borders (Axelrod, 2001; Taylor *et al.*, 1998). What originally establishes the Frz/Vang asymmetry is unclear, although clues from early development implicate Wg/dWnt as a potential first cause. Before the establishment of orientations
based on Frz/Vang, trichomes orient towards the wing margin, the source of Wg/dWnt expression. In support of this, overexpression of Wg/dWnt can reorient the direction of the trichomes and the underlying Fz/Vang asymmetry (Wu et al., 2013).

**Positioning of the Mitotic Spindle**

The establishment of the apical-basal axis is critical for two main mechanisms that ensure proper development - the positioning of the mitotic spindle and the asymmetric distribution of factors before division. In the SOP, loss of Frz causes both errors in the positioning of the mitotic spindle and in the asymmetric distribution of cell fate determinants (Gho and Schweisguth, 1998)(Figure 1.5). In the neuroblast, positioning of the mitotic spindle is largely carried out through the anchor protein Partner of Inscuteable (Pins). Pins is normally asymmetrically localized to the anterior cortex, but upon mutation of the core PCP genes, becomes randomly distributed along the neuroblast/SOP cortex (Schaefer et al., 2000).

In order to correctly position the mitotic spindle, Pins receives many cues which help localize Pins to the anterior cortex. Pins normally exists in an inactive state that is only able to facilitate microtubule binding after activation through phosphorylation by anterior-localized Aurora-A kinase. Only anteriorly activated Pins recruits factors which binds microtubules to facilitate the pulling of the mitotic spindle, causing the spindle to align with the anterior-posterior axis (Johnston et al., 2009). Pins may also be restricted to the anterior cortex through phosphorylation by atypical protein kinase C (aPKC), which resides on the posterior cortex. Unlike phosphorylation by Aurora-A kinase, phosphorylation by aPKC causes Pins to dissociate from the plasma membrane, thereby
excluding Pins from posterior cortex (Hao et al., 2010). In support of this, aPKC was found to be necessary for both proper spindle orientation and apical/posterior exclusion of Pins (Guilgur et al., 2012).

Once positioned, Pins controls the spindle orientation by forming a complex with Mushroom Body Defect (Mud) and Discs Large (Dlg) (Siller, 2006, Izumi, 2006, Bowman, 2006). After activation by Aurora-A kinase, Pins and Mud recruit dynein, a microtubule motor protein that pulls the mitotic spindle towards the Pins crescent (Johnston, 2009). Dlg binds kinesin heavy chain 73 (Khc-73), another microtubule motor protein that is necessary for proper spindle alignment to the Pins crescent (Seigrist, 2005).

In addition to their roles in the recruitment of microtubule motors, Dlg and Mud also serve to reinforce the mitotic spindle along the anterior-posterior axis through communication with PCP factors. In the SOP, Pins/Mud/Dlg forms a crescent along the anterior basal cortex before division, which is dependent on the anterior positioning of Vang (Bellaïche et al., 2004). Indeed, loss of either Vang or Frz causes errors in spindle positioning along the anterior-posterior axis, suggesting a role for PCP in spindle alignment (Gomes et al., 2009). On the posterior cortex, Mud also accumulates at the posterior spindle pole, without Pins, where it is recruited by Dsh. Posterior positioning of Dsh depends on Frz, representing a link between PCP and mitotic spindle alignment (Segalen et al., 2010). This is consistent with another observation that loss of Frz, and by extension Dsh localization, causes a loss in the slight apical/basal tilt, indicating that apically localized Frz is playing a role in spindle orientation (David et al., 2005). In this
way, Mud controls spindle alignment which also helps to position the Pins crescent. In neuroblasts, when Pins is unanchored from the anterior/basal cortex through loss of Inscuteable (Insc), Pins crescents still form, but are correlated with the more erratic positioning of the mitotic spindle. Only when the spindles are destabilized with the addition of colecemid does Pins adopt a symmetrical distribution in Insc mutants (Siegrist and Doe, 2005).
Figure 1.5 Alignment of the mitotic spindle pole

Mud (brown oval) become positioned at the apical, posterior cortex through recruitment by Dsh (green oval), previously established by Frz. Pins (blue oval) becomes positioned at the anterior cortex by being phosphorylated by the Par complex on the anterior side, shifting its localization anteriorly. Pins binds Mud and Dlg (purple circle) to facilitate binding with mitotic spindle through Khc-73 (orange oval) and dynein (red oval). The pulling force generated by Khc-73 and dynein aligns the mitotic spindle along the anterior-posterior axis and at a slight apical-basal tilt in the SOP cell.
Partitioning of asymmetric cellular components

In addition to positioning the mitotic spindle, PCP is also critical for asymmetrically separating cell fate determinants needed in the daughter cells of the SOP (Figure 1.6). The SOP resides in the epithelial layer and divides along the anterior-posterior axis with a slight basal tilt on the anterior side. In order for the terminal sensory organs cells of the SOP to correctly differentiate, the SOP segregates different factors to either the anterior or posterior cortex for inheritance by their respective daughter cells. Three of these factors are Numb, Lethal (2) Giant Larvae (Lgl), and Neuralized, which are asymmetrically localized to the anterior cortex before division of the SOP (Betschinger et al., 2003; Rhyu et al., 1994). Numb is a cytosolic protein that localizes to endosomes and the cell cortex and is specifically inherited by the posterior pIIb cell. Presence of Numb in pIIb is required for the cell-autonomous inhibition of Notch signaling, though the mechanism is unclear (further discussed below) (Frise et al., 1996; Couturier et al., 2013b). Neuralized is required for Delta ubiquitinylation in pIIb, leading to activation of Notch signaling in pIIa (Lai et al., 2001). Lgl is similar to Numb in that they are both restricted to the anterior cortex during mitosis and are required for Notch inactivation in pIIb (Justice et al., 2003).

Asymmetric targeting of Numb, Neuralized and Lgl is thought to be facilitated by the Par complex, which itself is localized to the posterior cortex (Wirtz-Peitz et al., 2008). During interphase, the Par complex is composed of Par6, Lgl, and aPKC, which is inactive due to the presence of Lgl. Activation of aPKC occurs during mitosis, when Aurora A kinase phosphorylates Par6, a regulatory subunit of aPKC. Activated aPKC
then phosphorylates Lgl causing Bazooka (Baz) to swap for phospho-Lgl. Incorporation of Baz now allows aPKC to phosphorylate Numb and Neuralized, which releases them from the posterior cortex where the Par complex resides. In this way, Numb, Neuralized and Lgl are restricted to the anterior cortex and are positioned correctly in relation to the mitotic spindle to be inherited by the anterior daughter cell (Wirtz-Peitz et al., 2008).

While this mechanism for determining the asymmetry of Numb, Neuralized and Lgl is well understood, how PCP is connected is less clear. Key steps in regulating asymmetric distribution are the phosphorylation of Par6 by Aurora A kinase and phosphorylation of Lgl by aPKC at the posterior cortex. Aurora A kinase localizes to centrosomes and does not appear biased toward the posterior side (Berdnik and Knoblich, 2002). In contrast, aPKC/Par6/Lgl are asymmetrically localized towards the posterior centrosome, indicating that it is the Par complex that is responsible for breaking the symmetry at mitosis. PCP may be playing a role in the asymmetric distribution of the Par complex through the localization of Baz, which becomes more symmetrically distributed in Frz mutants (Bellaïche et al., 2001). It is unknown, however, whether loss of posterior Baz also causes the other Par complex members to lose their posterior localization. In addition, Strabismus (Van Gogh) physically interacts with Dlg to promote its anterior localization, while Dsh acts antagonistically to limit Pins from the posterior cortex (Bellaïche et al., 2004). Interestingly, Dlg/Pins may also be responsible for positioning the centrosome which recruits the Par complex, meaning that anterior recruitment of Dlg by Vang could serve as the link between PCP members Frz/Vang/Dsh and Par complex positioning. Thus, there may be redundant mechanisms of
communication between the PCP and cell fate determinants with Frz directing Baz and Dsh directing the Par complex.
Figure 1.6 Sensory organ cell lineage

A. The SOP (pI) cell is specified by lateral inhibition aligned along the anterior-posterior axis established by PCP (planar cell polarity) genes Frizzled (blue box) and Van Gogh (red box). Cues from PCP are received by the Par complex (yellow crescent) which segregates other cell fate determinants to the anterior side including Numb, Lgl, Neuralized, Dlg, Pins, and Mud (green crescent). B. SOP lineage with indicated anterior/posterior, apical/basal division patterns. Time indicates hours apf (after puparium formation). The SOP divides at 14 hrs apf generating pIIa/pIIb cells. The pIIb cell (internal cell lineage, blue cells) divides at 15 hrs apf generating pIIIb and glial cells, and the pIIa (external cell lineage, red cells) cell divides at 17 hrs apf generating socket and shaft cells. Glial cell undergoes apoptosis and pIIb cell divides at 18 hrs apf generating neuron and sheath cell.
1.4 Regulation of Notch Signaling

Notch signaling is critical for many cell processes but its regulation has been particularly well studied in sensory organ lineage. Through years of investigation including numerous genetic screens, key factors have been identified and characterized according to their effects in Notch signaling. In the context of sensory organ development, the most well-studied factors include Numb, Sanpodo, Lethal(2) Giant Larvae (Lgl), and Neuralized. The common characteristic of these factors appears to be regulation of some aspect of Notch endocytic trafficking, which is quickly arising as one of the foremost ways in which the cell in all contexts regulates Notch signaling.

Endocytic trafficking of membrane receptors

Movement of membrane-bound receptors and soluble proteins through the endocytic system is facilitated by a number of Rab GTPases (Figure 1.7). Rab GTPases are molecular switches that only become activated upon binding of GTP, which requires the release of the previously bound GDP. Hydrolysis of the GTP into GDP causes the Rab GTPases to become inactivated. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) regulate these steps by activating and inactivating Rab GTPases, respectively. In their active states, Rab proteins serve as mediators of membrane fusion events which are critical for trafficking receptors through the endocytic system. Each Rab protein recruits a different set of effectors that allows them to be associated with fusion of specific endosomal compartments (Mukherjee et al., 1997). The best characterized are Rab5 for early endosomes, Rab7 for late endosomes, and Rab11 for recycling endosomes (Novick and Zerial, 1997). By monitoring Notch in
these compartments and manipulating the Rab GTPases themselves, much can be learned about how the endocytic trafficking of Notch plays an important role in signaling.

Rab proteins serve as specific markers for their respective compartments and have critical roles in mediating proper trafficking of cargo. Rab5 is necessary for both clathrin-dependent endocytosis from the plasma membrane as well as homotypic fusion of early endosomes (Bucci et al., 1992). An important GEF of Rab5 is RME-6, which recruits the Alpha adaptin subunit of the AP-2 complex to facilitate internalization of membrane bound receptors (Sato et al., 2005). RME-6 as well as other GEFs such as EEA1 activate Rab5, allowing SNARE accessory factors to associate and promote vesicle tethering and fusion (Christoforidis et al., 1999). Transition between early and late endosomes is mediated by the differential associations of Rab5 and Rab7 with endocytic vesicles. Early endosomes with Rab5 recruit the HOPS complex, which is a GEF for Rab7. As endosomes mature, feedback from HOPS-activated Rab7 gradually causes a dissociation of Rab5 and further recruitment of Rab7 marking the transition to late endosomes (Rink et al., 2005). Rab7 itself is necessary for the maintenance of the late endosome/lysosome, as loss of Rab7 prevents the accumulation of acidified vesicles marked with cation-independent mannose 6-phosphate receptor, a known marker for lysosomes (Bucci et al., 2000).

Cargo that has been internalized through Rab5 may be degraded in the lysosome or recycled back to the plasma membrane. Cargo destined for recycling is trafficked to sorting endosomes, which represent the transitional period while Rab5 is being exchanged for Rab11. Sorting endosomes are characterized by long projections that have
a high proportion of surface area that was once plasma membrane. Membrane-bound receptors often localize to these projections and through pinching off, are transported to recycling endosomes through non-specific bulk flow (Mayor et al., 1993). Once in the recycling endosome, receptors are trafficked back to the plasma membrane through the activity of Rab11, which is required for fusion with plasma membrane (Wilcke et al., 2000; Ren et al., 1998). Transport from sorting endosomes to late endosomes/lysosomes occurs through selection by the ESCRT (endosomal sorting complex required for transport) complex. Ubiquitinylation of cargo is thought to be the primary signal that is recognized by HRS (hepatocyte-growth-factor-regulated tyrosine kinase substrate), considered part of the ESCRT complex (ESCRT-0), which routes cargo to internal vesicles within the maturing early endosomes (Raiborg and Stenmark, 2002; Raiborg et al., 2008). Multiple invagination events within the early endosomes cause transition to the multi-vesicular body (MVB), an increasingly acidic compartment where cargo is stored in intraluminal vesicles. Increasing acidity established by V-ATPase proton pumps recruited by Rab7, causes transition to late endosomes. Rab7 also facilitates heterotypic binding with pre-established, highly acidified lysosomes allowing for degradation of internalized cargo (Mullins and Bonifacino, 2001).

Although most of the experiments to understand endocytic trafficking were performed with the Transferrin receptor, Notch is also known to undergo internalization and recycling, with aberrations to this process leading to signaling defects. Some of the first signaling experiments with Notch showed that Dynamin (Shibire) was required in both the signal sending and receiving cells for ligand dependent Notch signaling (Seugnet
et al., 1997). Importantly, it was also established that overexpression of the NEXT fragment (post S2 cleaved Notch) caused overactivation of Notch signaling that was not dependent on Dynamin, suggesting that the requirement for endocytosis occurred before S2 cleavage (Mumm et al., 2000). In addition to S2 cleavage, endosomal localization may be necessary for gamma-secretase induced S3 cleavage. Loss of Avalanche (Avl), a factor necessary for early endosome formation, prevented the accumulation of Gamma secretase cleaved Notch, possibly suggesting that S3 cleavage occurs in endosomes (Vaccari et al., 2008). Due to disparate conclusions from several studies, the role of Notch trafficking after endocytosis is less clear. Loss of VPS25, a member of the ESCRT complex, prevented the formation of MVBs and subsequent late endosomes, leading to an accumulation of both Delta and Notch in early endosomes. Under these conditions, Notch signaling was increased, presumably due to Notch or Delta not being degraded in lysosomes (Vaccari and Bilder, 2005). In contrast, knockdown of Hrs (ESCRT-0) with RNAi caused an accumulation of Notch in Rab5 labeled early endosomes, but did not have an effect on Notch function, indicating separate functions for different members of the ESCRT complex (Jekely and Rorth, 2003; Thompson et al., 2005).

Mutant forms of Rab proteins have been employed as tools to understand how Notch trafficking is mediated. In Drosophila Rab proteins, mutations in the GDP binding domains prevent GTPase GEFs from exchanging GDP for GTP, rendering Rab5 and Rab11 constitutively inactive (Stenmark et al., 1994; Ullrich et al., 1996). Overexpression of these mutants causes a dominant negative effect due to competition
with the wild type forms and acts as a way to silence these particular proteins. Additionally, in *Drosophila*, Rab5 and Rab7, mutations were found in the GTP hydrolysis domain, preventing hydrolysis of GTP into GDP, causing these forms to be constitutively active.

Figure 1.7 Endocytic trafficking of membrane receptors
Figure 1.7 Endocytic trafficking of membrane receptors

Membrane-bound receptors (black bars) become internalized in Clathrin (yellow Ts) coated pits facilitated by AP-2 (green dots). Recruitment of Rab5 (red dots) and loss of Clathrin allows fusion of endocytic vesicles into early endosomes. Receptors in early endosome can be recruited or moved by bulk flow into sorting endosomes characterized by localization of Rab11 (purple dots). Budding and pinching off of sorting endosomes allows formation of recycling endosomes with Rab11. Recycling endosomes fuse with the plasma membrane allowing for release of cargo. Receptors that do not reach sorting endosomes move towards membrane surfaces with Rab7 (blue dots), which pinch off to form multivesicular bodies MVBs. Receptors are recruited to intraluminal vesicles by the ESCRT complex (orange circles). Maturation and acidification of MVBs leads to late endosome/lysosome formation where receptors are degraded.
The Roles of Numb and Lgl in Sensory Organ Development

As discussed previously, asymmetric distribution of Numb and Lgl into the pIIb cell is required for cell-autonomous Notch inhibition. However, the mechanism by which Numb or Lgl mediates this inhibition is poorly understood. One of the earliest studies looking into the mechanism of Numb concluded that Numb and Notch have a physical interaction between the Numb phosphotyrosine binding (PTB) domain and the Notch Ankyrin repeats (Guo et al., 1996). These conclusions have been called into question however, as this physical interaction has never been replicated. Later evidence would point to a role for Numb in Notch endocytic trafficking. Numb can bind the ear domain of Alpha-adaptin, a member of the AP-2 complex, as well as Epsin 15, factors which facilitate internalization of endocytic cargo (Santolini et al., 2000). Mutations in the ear domain of Alpha adaptin lead to Notch over-activation which cannot be suppressed by overexpression of Numb. Furthermore, Alpha-adaptin is also asymmetrically distributed with Numb to pIIb, and depends on Numb for this localization (Berdnik et al., 2002). Numb and Alpha-adaptin may achieve this localization due to the inability of Numb to bind AP-2 while phosphorylated. (Tokumitsu et al., 2006). Since Numb is specifically phosphorylated on the posterior cortex by aPKC, unphosphorylated Numb would only be able to bind AP-2 on the anterior cortex, leading to pIIb accumulation.

One of the potential mechanisms of Notch inhibition by Numb is through regulating the cellular localization of Sanpodo. From loss-of-function experiments, Sanpodo was originally characterized as a positive Notch regulator. Sanpodo mutants regularly exhibit multiple neurons, a phenotype consistent with loss of Notch function
(Dye et al., 1998). Numb was thought to be a potential regulator of Sanpodo from epistasis experiments that showed Sanpodo acting downstream of Numb (Skeath and Doe, 1998). Sanpodo appears to be equally segregated into the pIIa and pIIb daughter cells, however, Sanpodo’s localization within each cell is not identical. In the pIIa cell, Sanpodo is located near the plasma membrane, while in pIIb, Sanpodo is localized to intracellular endosomal compartments. Numb appears to be responsible for this difference as endosomal Sanpodo is dependent on Numb, and Numb overexpression causes endosomal Sanpodo localization in both cells (O’Connor-Giles and Skeath, 2003). This led to the hypothesis that Numb is inhibiting Notch by restricting the positive Notch regulator Sanpodo to endosomes in pIIb, where it would be presumably unable to activate Notch signaling. Indeed, Numb was shown to bind to Sanpodo through Numb’s PTB domain and to colocalize with Notch and Delta in early and late endosomes of the pIIb cell (Hutterer and Knoblich, 2005). Thus, it appeared that the function of Numb was to facilitate the endocytosis of Sanpodo, which would prevent Notch activation in pIIb.

However, there were several subsequent findings that did not support this model of Numb function. Opposition to this model first came from the observation that deletion of the motifs in dynamin that are required for endosomal Sanpodo had no effect on the ability of Numb to inhibit Notch signaling. To test whether additional endocytic factors could play a role, motifs in Numb known for binding endocytic cargo were mutated and were also not sufficient to prevent Numb inhibition (Tang et al., 2005). Additional scrutiny mounted following experiments to test the necessity of the motifs in Sanpodo for the binding of Numb and endocytic complexes. A binding site for Numb was identified.
near the amino terminus in Sanpodo through a conserved NPAF motif that is required for localization of endosomal Sanpodo. However, deletion of this motif had no functional effect on Notch signaling, suggesting that the Numb-dependent Sanpodo localization is irrelevant to Notch signaling (Tong et al., 2010). A Numb-independent role in Notch signaling for Sanpodo was proposed when Sanpodo was found to bind Notch directly and be responsible for removal of Notch from the membrane. Another Sanpodo motif, ELL, is known to bind endocytic sorting signals. Upon mutation of both the NPAF and ELL motifs, Sanpodo was blocked from both endocytosis and from inhibiting Notch signaling. These experiments suggest that Sanpodo may be binding other endocytic factors such as the AP-1 complex to facilitate Notch inhibition (Upadhyay et al., 2013b).

Similar to Numb, the role of Lgl in the regulation of Notch signaling is not entirely understood. As discussed previously, Lgl is an integral part of the complex that eventually segregates Numb to the anterior cortex. Unphosphorylated Lgl binds the Par complex, but becomes expelled after phosphorylation by Aurora-A kinase. Loss of Lgl allows recruitment of Baz into the Par complex, which is able to activate aPKC and phosphorylate Numb, triggering its release from the anterior cortex. In this way, Lgl appears to be necessary for partitioning Numb into the pIIb cell where it can inhibit Notch signaling (Wirtz-Peitz et al., 2008). However, this model conflicts with additional studies which find that the asymmetric crescent of Numb that forms at mitosis is not disrupted by loss of Lgl (Justice et al., 2003). A possible explanation may lie in a closer look at the temporal requirement of Lgl on the Numb crescent. Interestingly, loss of Lgl only delayed, rather than abolished formation of the Numb crescent until telophase.
Given these varying reports, it may be possible that Lgl has some yet undiscovered roles in Notch signaling that are not dependent on the positioning of the Numb crescent.

In support of a Numb-independent role for Lgl, additional mechanisms have been established that may be responsible for Notch regulation. Loss of Lgl was found to both cause accumulation of cleaved Notch in acidified compartments and to increase the size and number of those compartments. Decreasing vesicle acidification rescued the Notch overactivation phenotype, suggesting that the recruitment of Notch to these enlarged compartments by Lgl was responsible for Notch overactivation. Thus, it appears that Lgl may play a role in suppressing vesicle acidification or maturation, a role distinct from the regulation of Numb asymmetry (Parsons et al., 2014). A separate Numb-independent role for Lgl has also been proposed in relation to positioning of the mitotic spindle. During mitosis, Lgl becomes phosphorylated by Aurora-A kinase to facilitate release from the cell cortex. It now appears that removal of Lgl from the plasma membrane is necessary for the mitotic spindle to be positioned correctly via Dlg/Pins, although this effect appears to be context dependent (Bell et al., 2015).

**Current Model – Numb Regulates Notch Endocytic Trafficking**

*Drosophila* Numb shares a high degree of homology with Numb in higher organisms, and observations from these studies provide insight into the Numb mechanism in the *Drosophila* sensory organ context. In mice, NUMB (mNumb) is also asymmetrically targeted to the apical cortex of neural progenitors and physically interacts with Notch1. When expressed in *Drosophila*, NUMB is sufficient to rescue the Numb
loss-of-function phenotype (Zhong et al., 1996). In mice, NUMB is a Notch1 antagonist, and overexpression of NUMB inhibits Notch1-dependent neurite outgrowth (Berezovska et al., 1999). NUMB has a role in Notch1 trafficking through binding endocytic factors of the epsin homology domain (EHD) family to facilitate regulation of endosomal trafficking (Smith et al., 2004). When the role of NUMB trafficking was specifically examined in mice, NUMB overexpression caused Notch1 to be trafficked towards late endosomes to promote Notch1 degradation. Additionally, loss of NUMB caused Notch1 to become biased towards recycling endosomes, allowing Notch1 to escape degradation and return to the plasma membrane for signaling (McGill et al., 2009). NUMB also appears to have functions that are not conserved in Drosophila. NUMB binds the E3 ligase Itch, which ubiquitinylates the NICD to cause rapid degradation after S3 cleavage (McGill and McGlade, 2003). In Drosophila, Numb is not dependent on proteasome degradation, nor does Itch play a significant role in regulation of Notch signaling (Tang et al., 2005).

In Drosophila, further studies supported the role of Numb as a regulator of endocytic trafficking. Internalized Notch (iNotch) was detected more frequently in pIIb than pIIa, an asymmetry that is dependent on the presence of Numb in pIIb. In addition, loss of Numb function caused Notch and Sanpodo to accumulate on the apical interface of pIIa/pIIb, suggesting that Numb is required for accumulation of iNotch and Sanpodo in pIIb and preventing accumulation at the apical microdomain. Loss of Sanpodo was also sufficient for interface accumulation of iNotch, implying that both factors share a redundant, but independent role in Notch internalization (Couturier et al., 2012).
Analysis of GFP-Numb revealed that Numb localizes to endosomal compartments with Notch and Sanpodo and on the plasma membrane (Couturier et al., 2013a). The relationship between Numb and Sanpodo was further explored using an mCherry-tagged Sanpodo. mCherry Sanpodo accumulated in Rab7 late endosomes with a bias towards pIIb, an asymmetry that is dependent on Numb (Couturier et al., 2014). In addition, Sanpodo recycling to the plasma membrane was increased with loss of Numb, suggesting Numb has a role in blockage of endocytic recycling (Cotton et al., 2013). These experiments establish a clear role for Numb in endocytic regulation. Given these roles for Numb in the trafficking of Sanpodo, we wanted to explore the possibility that Numb was working through a similar mechanism with Notch signaling.

In summary, endocytic trafficking has been established as a key way to regulate Notch signaling. Current data suggests that Numb plays a role in the trafficking of Notch and Sanpodo, but Numb’s mechanism of Notch inhibition has remained elusive. The experiments in this work aim to explore the relationship between Numb and Notch endocytic trafficking and uncover how Numb contributes to cell fate specification. We examine the functional contributions of endocytic internalization and recycling to Notch signaling and seek to identify a role for Numb in these processes. In addition, we also aim to elucidate novel ways in which other factors regulate Notch signaling. Taken together, our results may have far-reaching implications beyond Drosophila with the development of new treatments for Notch related diseases.
CHAPTER 2
NUMB REGULATES THE BALANCE BETWEEN NOTCH RECYCLING AND LATE ENDOSOME TARGETING IN DROSOPHILA NEURAL PROGENITOR CELLS

This chapter is adapted from:


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2.1 Summary

The Notch signaling pathway plays essential roles in both animal development and human disease. Regulation of Notch receptor levels in membrane compartments has been shown to impact signaling in a variety of contexts. Here, we used steady state and pulse labeling techniques to follow Notch receptors in sensory organ precursor cells (SOP) in *Drosophila*. We find that the endosomal adaptor protein Numb regulates levels of Notch receptor trafficking to Rab7-labeled late endosomes, but not early endosomes. Using an assay we developed that labels different pools of Notch receptors as they move through the endocytic system, we show that Numb specifically suppresses a recycled Notch receptor subpopulation, and that excess Notch signaling in *numb* mutants requires the recycling endosome GTPase Rab11 activity. Our data therefore suggest that Numb controls the balance between Notch receptor recycling and receptor targeting to late endosomes to regulate signaling output following asymmetric cell division in *Drosophila* neural progenitors.
2.2 Introduction

The Notch signaling pathway is conserved throughout metazoan evolution and is used to control tissue patterning and cell fate determination in a diverse array of developmental contexts. Inappropriate activation of this pathway has been implicated in a variety of cancers as well as in human disease syndromes such as Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) (Louvi and Artavanis-Tsakonas, 2012). Notch signaling occurs when Notch, a transmembrane receptor protein in the signal-receiving cell, binds to ligands of the DSL (Delta/Serrate/Lag-2) family in the signal-sending cell, resulting in a conformational change in the receptor. The ligand-dependent conformational change permits proteolytic cleavage of the receptor by the gamma secretase complex, releasing the intracellular domain of Notch to travel to the nucleus and act as a transcriptional activator in the receiving cell (Kopan and Ilagan, 2009). Activation of the pathway therefore relies on mechanisms that control both the localization and abundance of the ligands and receptor in membrane compartments (Kandachar and Roegiers, 2012).

A longstanding model in the study of regulation of Notch signaling in development is the *Drosophila* sensory organ precursor (SOP) (Singhania and Grueber, 2014). The SOP cell divides four times to give rise to four terminally differentiated cells (hair, socket, neuron, sheath) that make up the external sensory organ (Fig. 2.1A). The SOP cell undergoes an asymmetric cell division along the anterior-posterior axis, characterized by targeting of a membrane-associated protein, Numb, to one side of the precursor cell during mitosis (Rhyu et al., 1994). Following division of the SOP, Numb is
exclusively inherited by one of the two daughter cells (the anterior pIIb cell), and is
excluded from the other cell (the posterior pIIa cell). Numb acts as a cell autonomous
inhibitor of Notch signaling in the pIIb cell, while in the pIIa cell, Notch signaling is
required for directing proper cell fate (Uemura et al., 1989; Rhyu et al., 1994; Frise et al.,
1996).

The Delta ligand is ubiquitinylated by the conserved ubiquitin ligase Neuralized,
then endocytosed and recycled through the Rab11 endosome and the Sec15-exocyst
complex back to the apical region of the pIIb cell to activate Notch signaling in the pIIa
cell (Lai and Rubin, 2001; Pavlopolous et al., 2001; Le Borgne and Schweisguth, 2003;
Emery et al., 2005; Benhra et al., 2010; Giagtzoglou et al., 2012). The apical cell
interface between the pIIa/pIIb is enriched with Arp2/3 complex and the Wiscott-Aldrich
syndrome protein (WASP)-dependent microvillar membrane projections required for
robust Notch activation in the pIIa cell (Ben-Yaacov et al., 2001; Rajan et al., 2009).

Control of membrane trafficking is not limited to the Delta ligand in the pIIb cell.
Sanpodo, a four-pass transmembrane protein that interacts with Notch, promotes Notch
receptor endocytosis (O’Connor-Giles and Skeath, 2003; Couturier et al., 2012;
Upadhyay et al., 2013), while Numb inhibits membrane targeting of Notch and Sanpodo
in the pIIb cell (Couturier et al., 2012; Cotton et al., 2013; Couturier et al., 2013). Notch-
Sanpodo oligomers appear to be recycled in SOP cells (Cotton et al., 2013; Couturier et
al., 2013, Upadhyay et al., 2013), but it remains unclear how Numb regulates membrane
levels of Notch to modulate signaling in this system. In mammalian cells, evidence points
to Numb acting on post-endocytic trafficking of Notch1 (McGill et al., 2009), and in
nematodes, Numb is linked genetically to a role in endocytic recycling (Nilsson et al., 2008). This raises the possibility that either one or both of these mechanisms contributes to SOP cell fate in *Drosophila*.

In this study, we sought to understand how vesicle trafficking controls targeting of Notch receptor pools in SOP cells during Notch-dependent cell fate decisions. We developed a technique to distinguish different populations of receptors as they trafficked from the plasma membrane to internal compartments or were recycled. Our observations confirm that Numb plays an important role in restricting recycling of a Notch receptor population, as opposed to promoting Notch endocytosis from the plasma membrane. Importantly, we find that Numb functions to re-route the receptor preferentially to Rab7-positive late endosomes in pIIb cells. Our analysis further shows that Notch recycling is unaffected in Sec15-exocyst and WASp mutants, but is regulated by conserved tumor suppressor and WD-repeat containing protein Lethal (2) giant larva (Lgl). Overall, our observations demonstrate that Numb plays an important role in restricting recycling of a Notch receptor population, as opposed to promoting Notch endocytosis from the plasma membrane.
2.3 Results

*Numb regulates Notch trafficking to late endosomes*

Notch receptors colocalize with markers of early and late endocytic compartments in pIIa/pIIb cells (Hutterer and Knoblich, 2005; Couturier et al., 2014). We hypothesized that Notch endosomal targeting may be regulated by Numb in SOP cells, as is observed in mammalian cells (McGill et al., 2009). We quantified the colocalization of Notch with the markers of early (Rab5) and late endosomes (Rab7) in wild type pIIa/pIIb cells in pulse chase experiments and at steady state. In pulse chase experiments in pIIb and pIIa cells, colocalization between Rab5 and Notch peaked at 20 minutes, while Notch colocalization with Rab7 peaked at 30 minutes (Fig. S2.3A, B). We saw no difference in pIIa and pIIb cell Notch colocalization with Rab5-GFP labeled early endosomes in steady state labeling experiments in wild type or *numb* mutants (Fig. 2.1B-D), confirming that Numb is unlikely to influence Notch trafficking through early endosomes (Couturier et al., 2013). In contrast, we observed a significantly higher level of Notch receptor colocalization with Rab7 punctae in Numb-positive pIIb cells when compared to the Numb-negative pIIa cell (Fig. 2.2A, C, E, F). Notch-Rab7 colocalization in pIIa/pIIb cells decreased in *numb* mutants (Fig. 2.2B, G), and increased in cells overexpressing Numb (Fig. 2.2D, H). Overexpression of Numb-myc results in loss of hair and socket cells in adult flies resulting in a virtually bald thorax, (data not shown). In both the *numb* mutant and overexpression samples, the asymmetry we observe in wild type pIIa and pIIb cells is abolished (Fig. 2.2G, H). However, in *numb* mutants, both the pIIa and pIIb cells had Notch-Rab7 colocalization levels comparable to the wild type pIIa cell (Fig. 2.2G).
contrast, the Notch-Rab7 colocalization in both cells was comparable to the wild type pIIb cell in Numb overexpression (Fig. 2.2H). Our findings demonstrate that Notch levels in late endosomes are Numb-dependent and higher in wild type pIIb than pIIa.
Figure 2.1: Notch recruitment to early endosomes is Numb-independent
Figure 2.1: Notch recruitment to early endosomes is Numb-independent.

(A) Sensory organ precursors (SOPs) divide to create the pIIa and pIIb cells, which divide again to produce the four terminal cell fates of which only the neuronal cell expresses ELAV. (B-C) Sections of wild type (B) (n= 23 cell pairs) and numb

² (C) (n=21 cell pairs) clonal tissue displaying pIIa/pIIb cells that express Rab5-GFP were stained with NECD. (D) Rab5-GFP endosomes that colocalized with NECD puncta (yellow arrows) were quantified and compared between pIIa and pIIb. Similar analyses were done for cells expressing Rab5-GFP in numb clones (C).
**Figure 2.2: Numb is required for asymmetric Notch trafficking to late endosomes**

(A) Wild type clones marked with Rab5-GFP were stained with antibody for NECD (red) and Rab7 (green) (n= 21). NECD and Rab7 puncta (white arrows) were most often colocalized (yellow arrow) in pIIb cells. This asymmetry was abolished in numb\(^2\) mutant clones (B) also stained for NECD (n= 20). (C) Wild type clones
Recycling assay distinguishes recycled from static Notch

In *Drosophila*, recent studies in SOP cells have shown that Numb inhibits Notch/Sanpodo oligomer membrane targeting (Benhra *et al*., 2011; Couturier *et al*., 2012; Cotton *et al*., 2013; Couturier *et al*., 2013; Upadhyay *et al*., 2013). From these studies, a model has emerged in which Numb has a conserved function in blocking endocytic recycling of Notch, thereby acting as a Notch signaling inhibitor (Couturier *et al*., 2013). However, testing this model has been challenging, as dynamic methods of following different populations of receptors have only recently been applied to understanding how Notch receptors are regulated (Coumailleau *et al*., 2009; Couturier *et al*., 2014). In this study, we developed an assay, adapted from a technique used previously for Sanpodo (Cotton *et al*., 2013), to specifically visualize the population of Notch receptors endocytosed and recycled back to the plasma membrane. We followed a multi-step pulse-chase procedure (described in detail in the materials and methods) in live tissue using an antibody that binds to the Notch extracellular domain (NECD), followed by a first and second secondary antibody, each coupled to a different fluorophore (FSA and SSA, respectively, Fig. 2.3A). This approach has the potential to identify three distinct populations of Notch receptors: 1) a static pool of receptors (labeled by both FSA and SSA) that remains at the cell surface throughout the double pulse-labeling assay, 2) the recycled population of the receptor (labeled by SSA alone) which is internalized in the first step of the assay, and subsequently returns to the plasma membrane, and 3) an internalized pool of receptors (labeled by FSA alone) that is endocytosed during the assay, but remains in intracellular compartments (see schematic, Fig. 2.3A). In contrast,
receptors sequestered in internal membrane compartments during the primary antibody incubation step (newly synthesized receptors that have not yet reached the membrane or receptors endocytosed prior to primary antibody addition) are excluded from the analysis due to the fact that only plasma membrane exposed receptors are labeled with primary antibody.

We conducted this assay on both wild type and numb mutant pIIa/pIIb cells. We analyzed Notch membrane levels at the interface between the pIIa and pIIb cell to exclude FSA and SSA signals from Notch receptors in neighboring epithelial cells. At the membrane interface of pIIa/pIIb cells, we found that FSA levels were low in both wild type and numb mutant cells (Fig. 2.3 B, C). In contrast, in a majority of cases in numb mutant cells, we detect a higher SSA signal at the subapical sections of the interface, as compared to controls that exhibit low levels SSA in pIIa and pIIb cells (Fig. 2.3B-E). These findings show, consistent with previous observations, that Notch membrane levels are higher in numb mutant pIIa/pIIb cells than in wild type (Couturier et al., 2012). Furthermore, the data from our assay suggests that the population of Notch receptors at the pIIa/pIIb cell interface in numb mutants represents a recycled pool of receptors, rather than a static pool of receptors that remains at the membrane surface throughout the assay.
Figure 2.3: Notch recycling assay confirms Numb’s inhibition of Notch recycling
Figure 2.3: Notch recycling assay confirms Numb’s inhibition of Notch recycling

(A) Schematic of the Notch recycling assay shows that living explants are incubated with NECD primary antibody (black circles) for 10 minutes allowing for internalization of bound receptors. After NECD antibody is removed, the first green-labeled secondary antibody (FSA) is added for 10 minutes at 4°C to allow internalization, but prevent recycling. FSA is removed and the sample is raised to room temperature to allow recycling of Notch receptors not labeled with the FSA. Samples are then fixed and stained with the second red-labeled secondary antibody (SSA). Samples which contain high levels of recycled Notch are those with prominent SSA signal that is not also represented by a similar FSA signal. Samples with high levels of static Notch are those with colocalized SSA and FSA signal. (B-D) Recycling assay image series through multiple z-planes. (B) Wild type clones marked with Actin-GFP showed low accumulation of FSA and SSA antibodies (cell pairs= 29). (C) Numb clones showed higher accumulation of SSA (white dotted circle) and low accumulation of FSA (cell pairs= 37). (D, E) Quantifications of SSA and FSA intensity for wild type and numb mutant clones are shown relative to background nuclear staining. Horizontal lines indicate averages. (F) Wild type and (G) numb mutant line graph quantification of FSA, SSA and Actin-GFP (blue, red, green lines) show intensity levels in a representative sample. Borders of pIIa and pIIb cells are shown with yellow and blue rectangles, respectively.
Rab5 activity is required to reduce membrane Notch levels to mediate Notch signaling

In order to further investigate the regulation of membrane Notch levels using our trafficking assay, we expressed a dominant-negative form of Rab5 (Rab5DN) in pIIa/pIIb cells. Rab5DN is a mutant form that locks the Rab5 GTPase in the inactive state, preventing the fusion of endocytic vesicles (Stenmark et al., 1994; Marois, 2005). Expression of Rab5DN blocks formation of early endosomes labeled with Rab5-GFP (Fig. 2.4A), and inhibits formation of large colocalized Notch-Sanpodo punctae seen in wild type cells (Fig. 2.4 B). Using our trafficking assay, we found that Rab5DN expression increases overlapping FSA and SSA signal levels at the pIIb/pIIa cell interface over wild type cell levels, indicating an increase in Notch receptors trapped at the membrane surface (Fig. 2.4 C and D). Furthermore, Rab5DN expression in numb mutant cells increased the FSA signal at the pIIa/pIIb cell interface (Fig. 2.4E) when compared to numb mutant cells (Fig. 2.3C). These findings suggest that the pool of endocytically recycled Notch receptors in numb mutant cells is dependent on Rab5 function.

We hypothesized that blocking early endosome formation would inhibit Notch signaling in pIIa cells. Surprisingly, we found that Rab5DN overexpression in SOP cells resulted in some bristle loss, but 18% of the remaining organs exhibited extra external cells (hair or socket, n=6 fly thoraces, Figure 2.4F, G). This result suggests that Rab5 activity is important for restricting Notch activation in the pIIb cell. We hypothesize that Rab5-dependent endocytosis of the Notch receptor is required to reduce overall plasma membrane levels of Notch, thereby reducing levels of Notch signaling in SOP cells. These findings would suggest that increasing the static pool of Notch at the plasma
membrane in Rab5DN expressing cells is likely sufficient to promote Notch signaling in the pIIb cell in some cases.
Figure 2.4: Rab5 dominant negative causes cell fate switch and accumulation of static Notch
Figure 2.4: Rab5 dominant negative causes cell fate switch and accumulation of static Notch

(A) Expression of Rab5-GFP in pIIa and pIIb causes formation of discrete puncta of Rab5-GFP labeled early endosomes (white arrowhead). Expression of Rab5 dominant negative (Rab5DN) prevents formation of Rab5 early endosomes. (B) NECD immunostainings in cells expressing Sanpodo-GFP (to mark early endosomes) in wild type cells show NECD in intracellular early endosomes. Rab5 dominant negative expressing cells (Rab5DN) show accumulation of NECD in subapical vesicles (white arrowhead). (C-D) Bristle phenotypes for wild type (C) and Rab5DN (D) showed multiple sockets and areas of balding with expression of Rab5DN. Adult cell phenotypes were confirmed with Su(H) staining in sensory organ clusters at 24 hours APF. Overexpression of RabDN resulted in clusters containing multiple (E) or zero (F) Su(H) stained cells. (G-H) Recycling assay of Rab5DN (G) and Rab5DN, numb2 (H) expressing cells displayed as z-plane stacks. Dotted circles indicate areas of overlap between FSA and SSA. (I-J) Quantifications of FSA and SSA from recycling assay in G, H. Intensity values represent the ratio of FSA or SSA relative to background nuclear staining. (I) n= 22, (J) n= 23. (K) Colocalization analysis of numb2 and Rab5DN,numb2. Colocalized pixels above separate channel intensity threshold are represented in yellow and show a higher likelihood in Rab5DN,numb2 double mutants.
Rab11 function is required for excess Notch signaling in numb mutant SOP cells

Our data above demonstrates that Numb plays a role in suppressing basolateral Notch receptor recycling in pIIa/pIIb cells. From this, we hypothesized that excess Notch recycling in *numb* mutants drives increased Notch signaling in *numb* mutant cells, resulting in loss of neuronal cell fates. Therefore, we tested whether disruption of Rab11-dependent Notch recycling by expression of a dominant negative Rab11 (Rab11DN) would restore neuronal cell fates to *numb* mutant clones. We found that overexpressing Rab11DN reduced SSA levels at the pIIa/pIIb interface in our recycling assay (data not shown). Next, we used the neuronal marker ELAV to label and quantify neuronal fates in *numb* mutant external sensory organs. External sensory organs in *numb* mosaic clones on the adult thorax showed the expected multiple socket phenotype we and others have reported previously (Frise *et al.*, 1996; Justice *et al.*, 2003). In wild type cells every organ contained a single ELAV–labeled neuron (Fig. 2.5 A and B). In contrast, 65% (n=76 cell clusters) of *numb* mutant organs had no detectable ELAV expression, indicating a pIIb to pIIa transformation (Fig. 2.5A and B). We found that Rab11DN expression in either wild type or *numb* mutant sensory organ cells significantly increased the number of sensory organs containing neurons (Fig. 2.5B). Surprisingly, approximately 10% of all *numb*/Rab11DN sensory organs exhibited multiple ELAV-expressing neuronal cells, a phenotype that was not observed in either wild type or *numb* mutant external sensory organs, but consistent with pIIa to pIIb cell fate transformations observed in Notch mutants (Guo *et al.*, 1996). From these observations, we conclude that Rab11 activity contributes to excess Notch signaling activity in *numb* mutant pIIb cells.
Figure 2.5: Notch signaling requires Rab11 recycling endosome
Figure 2.5: Notch signaling requires Rab11 recycling endosome

Notal tissue was staged to 22 hours after puparium formation (apf) to capture the 4-cell stage and immunostained with neuronal marker ELAV. (A) Wild type GFP-expressing differentiated sensory organs showed a single ELAV positive (blue) cell, (red asterisks) with 3 non-neuronal cells (white asterisks) (n=55). Magnified cells are shown in right panels. numb MARCM clones showed clusters with zero and one ELAV positive cell clusters (n=76). Rab11SN, numb double clones showed zero, one and two ELAV positive cell clusters (n=89). Dominant-negative Rab11 (Rab11DN), showed one and two ELAV positive cell clusters (2 ELAV cells: yellow arrows, n=31). (B) Quantification of the number of ELAV-positive cell clusters in each background.
Lgl, but not WASp or exocyst component Sec15, regulates Notch recycling in SOP cells

In the pIIb cell, Delta trafficking through the Rab11-dependent recycling endosome promotes Notch activation in the neighboring pIIa cell (Emery et al., 2005). Delta furthermore requires exocyst complex and WASp activity for recycling and membrane targeting in pIIa/pIIb cells (Rajan et al., 2009). Since we found that Notch recycling in numb mutant cells also requires Rab11 in SOP cells, we speculated whether Notch, like Delta, requires exocyst complex and WASp activity for recycling in pIIa/pIIb cells. Using our recycling assay, we determined that FSA and SSA signal levels in sec15 and wasp mutants (Fig S2) were indistinguishable from wild type cells (Fig 1B).

We next expanded our analysis to explore the role of Lethal (2) giant larvae (Lgl) in regulating Notch trafficking in SOP lineage cells. Lgl is an evolutionarily-conserved tumor suppressor that plays important roles in apical-basal cell polarity, asymmetric targeting of cell fate determinants, and membrane trafficking (Vasioukhin, 2006; Wirtz-Peitz and Knoblich, 2006; Portela et al., 2015). In SOP cells, Lgl regulates cell fate: in lgl mutants, sensory organ differentiation is disrupted, resulting increased hair and socket cells at the expense of neurons, which is a phenotype reminiscent of numb mutants (Ohshiro et al., 2000); Justice et al., 2003; Langevin et al., 2005). We hypothesized that Lgl may play a role in regulating Notch trafficking in SOP cells. We therefore performed the recycling assay in cell tissue containing lgl mutant MARCM clones. Compared to wild type cells, lgl mutant pIIa/pIIb cells had increased SSA signal (but no change in FSA levels) at the membrane interface (Fig. 2.6A- E), similar to that observed in numb mutant cells (Fig.2.3C). However, in the lgl mutant cells, in contrast to numb cells, the
recycled Notch signal was shifted basolaterally. In lgl and numb double mutant cells, we observed a higher signal intensity in the SSA signal than in either wild type of lgl alone. However, lgl, numb double mutant cells displayed lower SSA intensity than in numb alone, suggesting a suppressive role for Lgl. (Fig. 2.6B and E). These finding suggests that Lgl and Numb may have independent roles in suppressing the pool of recycled Notch receptors at the pIIa/pIIb cell interface, and that Lgl may regulate the apical-basal polarity of the recycled pool receptors.
Figure 2.6: Notch recycling is suppressed by Lgl
Figure 2.6: Notch recycling is suppressed by Lgl

Sensory organ precursor cells were staged to 16 hours apf (2-cell stage) analyzed using Notch recycling assay of *lgl* (A, n= 36) and *lgl, numb* (B, n= 27) clones showed accumulation of SSA but not FSA, indicating recycled Notch, at the pIIa/pIIb interface (circled region). (C-E) Z-stack representation of the recycling assay for wild type, *lgl* (A), and *lgl, numb* (B) clones. White dotted area indicates region of uncolocalized SSA. (F,G) Quantifications of *lgl* (F) and *lgl, numb* (G). Intensity values represent ratio of FSA or SSA relative to background nuclear staining. Averages for FSA and SSA are represented by gold and blue bars, respectively.
2.3 Discussion

In order to elucidate how Notch signaling is controlled during development, and how Notch signaling can be dysregulated in disease, an understanding of the mechanisms underlying control of membrane levels of Notch pathway components is essential (Vaccari et al., 2008; Fortini and Bilder, 2009). From recent studies, it is clear that cellular context plays an important role in regulation of Notch receptor levels and in signaling output. In SOP cells, Numb, a known endocytic regulator, is asymmetrically localized during progenitor mitosis and acts to block Notch pathway activation cell-autonomously in the pIIb daughter cell that inherits it. Recent evidence in Drosophila and C. elegans has implicated Numb in inhibiting Notch receptor recycling, thereby decreasing Notch plasma membrane levels (Nilsson et al., 2008; Cotton et al., 2013; Couturier et al., 2013). In mammalian cells, evidence points to Numb promoting Notch targeting to late endosome compartments through the ubiquitin ligase Itch (McGill and McGlade, 2003; McGill et al., 2009). These two functions may not be mutually exclusive. In this study, we analyzed the Notch levels in endocytic compartments and developed an assay that allows us to identify pools of Notch (recycled, static, internalized) in SOP cells in vivo. Our findings reveal that Numb is responsible for regulating Notch accumulation in Rab7-positive late endosomes, and that Numb restricts a population of recycled Notch receptors in SOP daughter cells.

In mammalian cells, Numb promotes Notch targeting to late endosome compartments through the ubiquitin ligase Itch (McGill and McGlade, 2003; McGill et al., 2009). Our study confirms previous observations that Numb does not influence Notch
colocalization with the early endosomes in pIIa/pIIb cells (Couturier et al., 2013).

However, our observation of a Numb-dependent Notch asymmetry in late endosomes, while consistent with findings in mammalian cells, is at odds with results obtained using Notch-GFP and Notch-Cherry fusion proteins in pIIa/pIIb cells, where no asymmetry was detected (Couturier et al., 2014). This may be due to our use of different approaches: our study followed Notch by antibody labeling of the extracellular domain of the receptor, while Couturier et al. used receptors fluorescently tagged within the intracellular domain. Furthermore, our marker for late endosomes, Rab7 may have defined a slightly different population of endosomes from those defined by Couturier et al. based on differences in Notch-GFP and Notch-Cherry fusion protein signals (Couturier et al., 2014).

Nonetheless, our data indicates that, under our assay conditions, Numb has a conserved role in influencing Notch trafficking to late endosomes. Numb localizes to late endosomes in pIIb cells (Couturier et al., 2013), however, whether Numb regulates Notch trafficking through a ubiquitin-dependent mechanism or by direct interaction with the Notch receptor remains unclear.

We also assessed the role of Lgl in regulating Notch trafficking, as Lgl plays an important role in restricting Notch activation, therefore promoting pIIb cell fate in the sensory lineage (Justice et al., 2003). In its role as polarity regulator, Lgl functions to regulate asymmetric targeting of Numb in both neuroblasts and SOP cells during metaphase of mitosis (Ohshiro et al., 2000; Peng et al., 2000; Langevin et al., 2005). However, Lgl is not required for Numb asymmetry to the pIIb cell at telophase, resulting in a delay, but not failure, to segregate Numb to pIIb (Justice et al., 2003; Langevin et al., 2005).
2005). Studies from yeast, flies, and vertebrate neurons have implicated Lgl in membrane fusion events and vesicle trafficking, including trafficking of Sanpodo (in SOP cells) and regulating Notch signaling by controlling endosome acidification in the Drosophila eye (Lehman et al., 1999; Langevin et al., 2005; Roegiers et al., 2005; Zhang, 2005; Grosshans, 2006; Wang et al., 2011; Parsons et al., 2014). We show here that loss of Lgl increases Notch membrane recycling suggesting that Lgl may play a role in suppressing Notch recycling, and therefore may be a mechanism by which Lgl regulates pIIb cell fate. It is interesting to note that we see an increased level of recycled Notch in lgl, numb mutant cells compared to wild type, but is reduced when compared to numb alone. These findings indicate that Lgl is having a suppressive effect on Numb, suggesting that these two factors may be performing different roles in the same pathway.

Taken together, we propose that control of Notch signaling in pIIb/pIIa cells is dependent on the balance between the membrane and endosomal pools of Notch receptors. Rab5 and Sanpodo function to shunt Notch to the endosomal pool in the SOP. After the asymmetric cell division, the presence of Numb in the pIIb cell promotes trafficking of internalized Notch receptors to late endosomes, either directly or by decreased trafficking through the Rab11-dependent recycling endosome. Delta, on the other hand, is recycled in a Neuralized/Rab11/Sec15-dependent manner. The case in pIIa is different, where Sanpodo promotes Notch internalization to early endosomes at the same rate as that observed in pIIb. However, the absence of Numb in pIIa cells prevents sequestration of Notch in late endosomes, resulting instead in Rab11-dependent basolateral membrane Notch recycling and activation of Notch signaling. Interestingly,
Notch recycling is not strictly required for Notch signaling activity in the pIIa cell, as disrupting early endosomes blocks Notch recycling but does not affect pIIa cell fate determination. However, blocking early endosome function does result in both accumulation of static Notch at the plasma membrane and cell fate changes in the pIIb. In conclusion, this study provides direct evidence that Numb is responsible for regulating the endosomal sorting of Notch, putting forth an answer to the long-standing question of the function of Numb.
2.5 Supplementary Information

### Table S2.1: Recycling assay

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Wasp and Sec15 do not alter Notch trafficking

### Figures S2.2

- SSA
- sec15
- Wasp
- Merge

71
Table S2.1: Recycling assay p values
P values in all cases were obtained using a Wilcoxon rank sum test for use with paired samples of unequal variance. p values are given for corresponding FSA or SSA data sets of compared genotypes.

Figures S2.2 Wasp and Sec15 do not alter Notch trafficking
SOPs were marked at the two cell stage with Rab5-GFP (green). Expression of Rab5-GFP did not alter the loss of bristle phenotype observed in sec15 and wasp mutants. The Notch recycling assay was performed on wasp (A) and sec15 (B) mutant clones. (C,D) Vertical montage representation of the recycling assay from A,B. SSA showed no appreciable difference in either mutant compared to wild type.
Figure S2.3: Rab7/Rab5 internalization assays

(A) Notch flux assay with expression of Rab5-GFP (n= 27). Notch flux through early endosomes was fixed and measured at the indicated times. NECD colocalization with early endosomes peaked at 15 mins and did not show significant differences between pIIa/pIIb at any time point. (B) Rab7GFP internalization assay (n=25). Tissue was dissected, then allowed to internalize NECD antibody for the indicated times and fixed. Colocalization between Rab7-GFP and NECD was quantified, showing the greatest amount of colocalization after 30 minutes of internalization.
Figure S2.4 Overactive or dominant negative Rab7 alters Notch localization
Figure S2.4 Overactive or dominant negative Rab7 alters Notch localization

(A) Rab7QL-GFP was overexpressed using Apterous-Gal4 to create an expression boundary in wing disc epithelial cells. Dotted yellow line delineates Rab7QL-GFP expression (lower half) from wild type tissue (upper half). Notch (red) was stained with NECD antibody. (B) Quantification of average NECD (red) and Rab7QL-GFP intensities from area indicated in dotted blue box from (A), x-axis represents distance from the dotted yellow line. (C) Rab7 dominant negative (Rab7TN-GFP) was overexpressed in wing disc epithelial cells using apterous. Yellow dotted line delineates tissue overexpressing Rab7DN-GFP (right side) from wild type tissue (left half). NECD staining (red) reveals Notch in larger and more numerous endosomes.
CHAPTER 3

DETERMINING THE ROLE OF SARA ENDOSONES IN CELL FATE

SPECIFICATION OF EXTERNAL SENSORY ORGANS
3.1 Summary

Notch is an extremely well conserved signaling pathway that has a role throughout all metazoan development. Understanding how Notch signaling is controlled is essential for elucidating the developmental mechanisms that control cell fate specification. In *Drosophila*, the external sensory organ lineage provides an exquisite context for studying Notch signaling in both lateral inhibition and in asymmetric cell division. Sensory organ precursor cells (SOPs) generate two daughter cells whose specification depends on activation or inhibition of Notch signaling. Here we explore a new mechanism of Notch regulation, described previously in Coumailleau et al., 2009, where activation of the Notch-dependent cell fate is promoted by directional trafficking of Notch to one daughter cell before division. We find that a sub-population of early endosomes containing Smad anchor for receptor activation (Sara) are asymmetrically targeted to the Notch-activated cell before division. However, we were unable to show that this sub-population of endosomes contained Notch suggesting this mechanism may not be required for Notch-dependent cell fate specification. Nevertheless, we find that the asymmetry in Sara-positive endosomes may be dependent on cues from the global anterior-posterior axis rather than SOP intrinsic factors. While we were not able to show the relevance of Sara endosomes in cell fate decisions of the external sensory organ, understanding the basis of Sara endosome asymmetry may lead to new findings in other mechanisms controlling asymmetric cell division.
3.2 Introduction

Development within sensory organ clusters depends on the precise activation of Notch signaling in pIIa and suppression of its activation in pIIb. A recently proposed model to explain this differential activation is through directional trafficking of Delta and Notch to the pIIa within Smad anchor for receptor activation (Sara) positive endosomes. Sara is an endosomal protein that localizes to phosphatidylinositol 3-phosphate (PI(3)P) positive early endosomes via binding of its FYVE domain. Sara was found to colocalize with a short 10-minute dextran pulse chase, but not with a longer 40-minute pulse chase, indicating localization in early, but not late endosomes. In wing disc epithelia, Sara endosomes associate with acetylated tubulin within the mitotic spindle. During cytokinesis, extension of the mitotic spindle ensures equal segregation of Sara endosomes in both daughter cells (Bokel et al., 2006).

In the SOP context, Sara has been reported to localize to the mitotic spindle. In addition, Sara endosomes have been reported to be preferentially localized to pIIa, rather than pIIb (Coumailleau et al., 2009). Furthermore, these Sara endosomes contain internalized Notch and Delta, and are directionally trafficked to pIIa. From these observations, Coumailleau et al concluded that the asymmetry in Sara endosomes may represent a contributing factor to activation of Notch in pIIa. To test the hypothesis that Sara/Notch/Delta positive endosomes promote Notch activation and therefore influence cell fate, the investigators overexpressed a dominant active form of Rab5, Rab5QL, which caused enlargement of all early endosomes. In the majority of cell pairs (60%), this enlarged Sara endosome was targeted to the pIIa, which gave rise to a normal external
sensory organ. In contrast, when this large Sara endosome was targeted to pIIb (40% of cell pairs), multiple socket cells were observed indicating a duplication of the pIIa cell fate. This result suggested that this enlarged endosome was capable of eliciting a positive Notch signal; a finding which could also apply to wild type Sara endosomes as well (Coumailleau et al., 2009).

In addition to the external sensory organ context, Notch directionally traffics through Sara endosomes in other cell types. Intestinal stem cells (ISCs) of the *Drosophila* midgut divide asymmetrically to create an enteroblast cell (EB) and to maintain the ISC. Similar to the external sensory organ, Notch and Delta are required for proper cell fate specification and are trafficked in Sara endosomes. Sara mutants cannot properly differentiate ISCs, suggesting a requirement of Sara-based directional trafficking on Notch signaling (Montagne and Gonzalez-Gaitan, 2014). Recent studies have also revealed a potential role for Sara in asymmetric division in vertebrate systems. In asymmetrically dividing cells of zebrafish, accumulation of Sara/Notch/Delta endosomes correlated with the p cell fate (double neuron) as opposed to the n cell fate (single neuron). These cell fates require both Notch and Sara, suggesting a similar mechanism involving Sara-dependent directional targeting of Notch (Kressmann et al., 2015).

While there is data to support the role of Notch/Sara/Delta endosomes in Notch-activated pIIa specification, some recent studies have challenged the notion of asymmetric Notch localization within endosomes as a mechanism for pIIa cell fate specification. When internalized Notch itself is quantified between pIIa/pIIb, no additional accumulation in pIIa was detected (Couturier et al., 2012). Moreover,
knockdown or mutants for Sara show no cell fate phenotypes that would be expected if Sara-mediated directional Notch trafficking was required for activation (Mummery-Widmer et al., 2009a; Coumailleau et al., 2009). In addition, there is a lack of understanding on how the SOP lineage would achieve the reported asymmetry in Sara endosome localization and whether Notch itself is required. Mutants for uninflatable, a protein necessary for tracheal inflation, do not asymmetrically target Sara endosomes to pIIa. Uninflatable binds Notch and both are individually required for Sara endosome asymmetry (Loubery et al., 2014). However, this finding conflicts with previous reports that did not find that Notch was required for asymmetric Sara endosome targeting (Coumailleau et al., 2009). Given the contradictory data regarding Sara endosomes, we sought to determine the role of Sara endosomes in Notch activation as well as the mechanism that is responsible for this asymmetry.
3.3 Results

*Ubi*-Sara GFP shows a bias for pIIa accumulation

We were unable to acquire the reagents used in the original (Coumailleau et al.) study, but were able to acquire a Ubi-Sara-RFP fly line, a generous gift from Ben Ohlstein at Columbia University. To determine if this reagent displayed a similar asymmetry in segregation, we examined localization of Sara-endosomes in pIIa/pIIb at time points around cytokinesis. To distinguish pIIa/pIIb, we overexpressed GFP-tagged partner of numb (Pon) using the SOP specific Neuralized driver. Because Sara asymmetry was previously reported to occur concurrently with establishment of the Pon crescent (Coumailleau et al., 2009), we confined our analysis to approximately 5 minutes around the time of cytokinesis (abscission). When quantified at time points relative to cytokinesis, we observed a significant accumulation of Sara endosomes in pIIa, relative to pIIb (Figure 3.1A-C). However, this accumulation was diminished at later time points, consistent with previous reports of Sara endosomes arising *de novo* in pIIb after division (Figure 3.1C). At early time points (0-60s), approximately 60% of Sara endosomes in the cell were localized to pIIa, and diminished to non-significant differences after two minutes. To illustrate the full breadth of our data set, we separated our data into two classes: those in which Sara was clearly biased to pIIa or pIIb (Figure 3.1 A,B).
Figure 3.1 Sara endosomes are asymmetrically targeted to the pIIa cell
Figure 3.1 Sara endosomes are asymmetrically targeted to the pIIa cell

(A) Live cell imaging of Ubi-Sara-RFP with Neuralized-Gal4, UAS-Pon-GFP given as time points after abscission. Posterior pIIb cells were marked with Pon-GFP, while pIIa cells are indicated with white dotted lines. (B) Quantification of Sara endosome localization at the corresponding time points given as average Sara endosomes per cell. (**p=.032 *p=.045, n=24)
**Enlarged Sara endosomes are symmetrically localized**

Given that our Ubi-Sara-RFP showed a localization bias to pIIa, similar to what had been previously reported, we next wanted to determine if enlarged early endosomes would also be biased toward pIIa. To do this, we overexpressed Rab5QL-RFP using Neuralized-Gal4 to generate enlarged early endosomes in sensory organ cluster cells. We observed that in pIIa/pIIb daughter cells, contrary to previous findings, overexpression of Rab5QL produced multiple enlarged early endosomes (Figure 3.2A). Unexpectedly, we did not observe a significant bias in the trafficking of this endosome towards a single cell. Instead, there appeared to be a roughly equal chance of either pIIa/pIIb receiving enlarged early endosomes (Figure 3.2B). In addition, we did not observe any bristle defects in cell pairs in which enlarged endosomes were trafficked to pIIb cells (data not shown). Therefore, we conclude that overexpression of Rab5QL did not elicit a discernable phenotype in the context of adult sensory organ cell fates.

**NECD colocalizes with Rab5QL, but not with Sara**

Given that we observed Sara endosomes being directionally trafficked to pIIa, we wanted to determine whether full-length Notch was being localized to these compartments. Using our Ubi-Sara-RFP, we overexpressed Pon-GFP using Neuralized-Gal4 to mark sensory organ cells and stained for Notch using NECD antibody. Upon visualization, we did not detect any clear localization of Notch in Sara-positive endosomes within either pIIa or pIIb (Figure 3.3A). As a control, we expressed Rab5-GFP to mark all early endosomes of which Sara represents a subpopulation. Upon staining with NECD, we detected colocalization with some endosomes, indicating that
Notch could localize to Sara-negative early endosomes. To determine if Notch resides within the enlarged early endosomes, we overexpressed Rab5QL-GFP using Neuralized-Gal4.

Figure 3.2 Rab5QL endosomes are symmetrically targeted
Figure 3.2 Rab5QL endosomes are symmetrically targeted. (A) Rab5QL-RFP and Pon-GFP were overexpressed with Neuralized-Gal4 to mark sensory organ cells. Times represents seconds after abscission. Top panels show a representative division in which Rab5QL endosomes travel to pIIb, bottom panels show localization in pIIa. (B) Quantification of average Rab5QL endosomes per cell in pIIa/pIIb at the indicated time points in seconds after abscission and stained for Notch using NECD. Surprisingly, Notch was found to colocalize with small Rab5QL early endosomes primarily in pIIb cells (Figure 3.3B). Furthermore, NECD did not colocalize with large Rab5QL endosomes in either cell. These findings indicate that full-length Notch is unlikely to be primarily within endosomes which asymmetrically localize between pIIa and pIIb cells.
Figure 3.3 Notch does not localize to Sara or large Rab5QL endosomes. (A) Sensory organ cells marked with Pon-GFP and expressing Ubi-Sara-RFP were stained with NECD antibody (red). Lower panels show colocalization between Rab5-GFP and NECD antibody staining. (B) Rab5QL expressing sensory organ cells marked with Neuralized-GFP were stained with NECD (red). Top panels show cells in which the large Rab5QL localized to pIIa, while lower panels show cells depicting equal segregation of small Rab5QL endosomes.
Inscuteable reverses Sara asymmetry

Inscuteable (Insc) is normally expressed in neuroblasts and helps to anchor the mitotic spindle with Pins/Dlg. Insc is not normally expressed in SOPs, but can be ectopically expressed to reverse the polarity of the Par complex. Ectopic Insc expression causes Baz, Numb, and Lgl to accumulate on the posterior cortex causing the pIIa/pIIb to switch positions and be localized anteriorly and posteriorly, respectively (Bellaïche et al., 2001). In order to test whether the Sara endosome asymmetry depends on the polarity of cell fate determinants, we overexpressed Insc in sensory organ clusters and tracked Sara endosomes over time. Live cell imaging of dividing SOPs revealed that overexpression of Insc caused a bias in Sara endosomes toward the pIIb cell, but became equally distributed in both cells after two minutes. This result suggests that Sara endosomes asymmetrically migrate posteriorly regardless of pIIa/pIIb cell identity. Cell polarity reversal upon Insc overexpression was confirmed by noting that the normally larger posterior pIIa cell, was now located anteriorly.
Figure 3.4 Insuteable reverses Sara asymmetry
**Figure 3.4 Inscuteable reverses Sara asymmetry.** (A) Live cell imaging of sensory organ precursor cells overexpressing Insc and Pon-GFP with Neuralized-Gal4. Time points indicate seconds after abscission and show higher Sara endosome localizations in posterior pIIb cells. (B) Quantification of Sara endosomes in pIIa/pIIb with overexpression of Insc.
Mitotic spindle plus-ends project symmetrically in pIIa/pIIb

Sara endosomes have recently been shown to bind the mitotic spindle, which itself has been proposed to be asymmetrically localized to the pIIa cell. However, how the spindle achieves this asymmetry is not fully understood. EB1 is microtubule plus-end binding protein that serves as a reliable marker for identifying extending microtubules (Berrueta et al., 1998). Given that Sara binds microtubules, we used EB1 as a marker to determine whether an asymmetry existed in the extension of microtubules into pIIa. To assess the localization of EB1, we ubiquitously overexpressed EB1-RFP and evaluated mitotic spindle lengths in epithelial and SOP cells. In dividing epithelial cells, we detected EB1-RFP at the mitotic spindle and as expected, no significant difference in mitotic spindle projection was detected between daughter cells (Figure 3.4A). Next, we examined EB1-RFP in dividing SOP cells and observed no significant difference in mitotic spindle projections between pIIa/pIIb, similar to epithelial cells. Thus, our results suggest that there is no bias in extending microtubules which could account for the accumulation of Sara endosomes in pIIa.
Figure 3.5 Mitotic spindle projections are symmetric in pIIa/pIIb.
Figure 3.5 Mitotic spindle projections are symmetric in pIIa/pIIb. (A) Live cell imaging of dividing SOP cells expressing EB1-RFP revealed equal EB1 marked microtubule projections in pIIa (top cell) and pIIb (bottom cell). Indicated time points are seconds relative to abscission. (B) Dividing epithelial cells with EB1-RFP also show symmetric EB1-RFP projections.
3.4 Discussion

Many binary cell fate decision contexts in both *Drosophila* and vertebrates require asymmetric activation of Notch signaling. Therefore, determining how Notch is activated or repressed is important for understanding regulation of Notch in many contexts. A potential novel mechanism for regulating Notch signaling is the directional trafficking of Notch and Delta towards the cell in which Notch activation occurs. In the SOP context, a long-standing assumption was that the amount of Notch receptors in pIIa/pIIb were equal in both cells. However recent studies have questioned this paradigm by suggesting that Notch and Delta can be trafficked to pIIa in a subpopulation of early endosomes marked with Sara (Coumailleau *et al.*, 2009). In this study, we have examined these claims using independent regents and sought to assign a mechanism for why this asymmetry may exist. In agreement with previous studies, we found that Sara endosomes do indeed traffic primarily to pIIa. However, we did not find full-length Notch localization to Sara endosomes to be reproducible with our reagents, indicating that additional confirmation is needed. Independent of Notch, we find that Sara asymmetry may be related to global anterior-posterior polarity and not a result of intrinsic cell fate determinants.

Given the potential importance of the reported finding in Notch asymmetry, it is imperative that results are confirmed with multiple reagents. We used a ubiquitously expressed Sara-RFP in conjunction with Pon-GFP to mark pIIa/pIIb cells in sensory organ clusters. Live cell imaging analysis using these reagents revealed asymmetrical localization of Sara endosomes does occur, albeit at a less pronounced bias than
previously reported. Specifically, the original study found that a maximum of 90% of
Sara endosomes migrated posteriorly into pIIa, while we find a more modest maximum
of 60% of Sara endosomes in pIIa. It is likely that this difference arises from a difference
in reagents, but which (if either) represent physiological conditions is unclear.

Our study also showed that Notch did not colocalize with the Sara subpopulation
of early endosomes. However, our experiments were done using an antibody to NECD,
while the original study used a NICD antibody. These differing results suggest that the
Notch in Sara endosomes is most likely the NEXT fragment, though why the Notch in
these compartments did not undergo S3 cleavage is unclear. Experiments using GFP-
tagged Notch would help confirm Notch in Sara endosomes, as GFP-Notch is more
observable than antibody staining. In addition, the original study monitored
colocalization of Sara with internalized Notch and Delta, while our studied assessed
colocalization at steady state. Internalized Notch and Delta are rapidly trafficked out of
early endosomes (Bokel et al., 2006) potentially causing over-representation of
colocalization when not given time to equilibrate. Overall, the difference in results
brings into question the degree of Sara asymmetry in pIIa/pIIb and its relevance to Notch-
dependent cell fate decisions.

To assess the functional relevance of asymmetric Sara endosomes, we
overexpressed Rab5QL to create enlarged endosomes which become symmetrically
localized between pIIa/pIIb. The previous study reported a bias in Rab5QL localization
to pIIa and a pIIa cell fate duplication when this endosome was mislocalized to pIIb.
Through cell cluster tracking, we did not observe any correlation between errors and cell
fate and localization of enlarged early endosomes to pIIb. We then tested for Notch localization within the enlarged endosomes, but did not detect NECD staining in the largest endosomes. Interestingly, we primarily detected localization of Notch in smaller Rab5QL-RFP endosomes of pIIb. The significance of this asymmetry in localization is unclear, though could represent increased levels of Notch internalization in pIIb due to Alpha-adaptin or Sanpodo (Berdnik and Knoblich, 2002; Upadhyay et al., 2013a).

We next tried to ascertain the mechanism behind this asymmetry in Sara endosome localization. Overexpression of Insc in the SOP caused a flip in the anterior/posterior localization of pIIa/pIIb. In wild type and in Insc overexpressing cells, Sara endosomes showed a posterior bias. This demonstrates that the Sara asymmetry is not dependent on pIIa/pIIb cell placement along the anterior-posterior axis. Instead, it is likely that a more global signal, such as PCP relaying information directly to the mitotic spindle, is responsible (Gho and Schweisguth, 1998). This is supported by the recent finding that Sara endosome asymmetry may be linked to positioning of the mitotic spindle and not to cell fate determinants like Numb and Lgl (Kressmann et al., 2015). Although the link between Notch and Sara endosomes may be more tenuous than previously thought, determining how Sara asymmetry is established in the SOP may still provide clues to Sara endosome localization in other, Notch relevant contexts.
CHAPTER 4: Conclusions and Future Directions

4.1 Conclusions

Notch signaling is used in variety of contexts throughout all metazoans. The mechanisms of its regulation are often consistent among different contexts and conserved between species. Understanding the regulation of this pathway is critical for the design of treatments where appropriate Notch signaling has been compromised. To examine Notch signaling regulation, we utilized the external sensory organ development in *Drosophila*. This context uses binary cell fate decisions which are dependent on the cell-intrinsic activation state of Notch. Aberrations in these fate decisions result in phenotypes that are easily detectable and can be further characterized. Due to the high evolutionary conservation of Notch, conclusions made in this context have far-reaching implications in higher organisms. In the present work, we examined multiple ways in which Notch signaling is regulated and significantly added to the understanding of how these contribute to Notch function.

A long standing question in the field of Notch regulation is how the negative regulator Numb inhibits Notch signaling. It had previously been proposed that Numb and Notch physically interacted through the phosphotyrosine (PTB) domain of Numb and the Ram23 or Ankyrin repeats in Notch (Guo et al., 1996). However, due to the lack of supporting evidence from subsequent screens for Notch interactors, this finding remains controversial (Go and Artavanis-Tsakonas, 1998; Mummery-Widmer et al., 2009a). The observation that Notch accumulated on plasma membranes in Numb mutants supports the
current prevailing hypothesis that Numb prevents Notch membrane localization (Couturier et al., 2012). Prior to this work, two central hypotheses competed to explain how Numb could limit the amount of membrane Notch: 1) Numb promotes Notch internalization from the plasma membrane, or 2) Numb prevents recycling of internalized Notch back to the plasma membrane. The first hypothesis was supported by evidence that Numb bound Alpha-adaptin and Sanpodo, factors that are known to facilitate internalization of membrane-bound receptors (Berdnik et al., 2002; O’Connor-Giles and Skeath, 2003). The second hypothesis was supported by mammalian studies in which Numb regulated Notch and Sanpodo trafficking after internalization (McGill et al., 2009; McGill and McGlade, 2003; Cotton et al., 2013; Santolini et al., 2000).

To address the validity of these hypotheses, we first examined the flux of Notch through the endocytic system by monitoring the colocalization of internalized Notch with early endosomes (Rab5) and late endosomes (Rab7). We determined that the flux of Notch into early and late endosomes is similar in pIIa/pIIb, suggesting that Numb may not be responsible for movement into these compartments. Our results also agree with previous work showing that endocytic cargo traffics to late endosomes about 15 minutes after it would traffic to early endosomes (Thilo et al., 1995). We next looked at Notch at steady state in early and late endosomes and showed that Numb has no effect on the localization of Notch with Rab5 early endosomes. Furthermore, loss of Numb has no effect on the number of Notch positive early endosomes. This demonstrates that Numb is not required for Notch internalization and provides strong evidence contrary to the first hypothesis.
To determine if the second hypothesis was correct, we needed to assess the functional role of recycling on Notch signaling. We blocked Notch recycling by overexpressing a Rab11 dominant negative that caused a cell fate change, indicating loss of Notch activity. This demonstrated that Notch required Rab11-dependent recycling to become activated. Previously, Rab11-dependent recycling of Delta was shown to be necessary for ligand activation (Emery et al., 2005). It is possible that our observation was due to the inability of Delta to activate, causing loss of Notch signaling. However, our observation that the recycling assay detected recycled Notch levels below that of wild type levels supports a direct blockage of Notch recycling. Given that recycling is necessary for Notch activation, we assessed the role of Numb, Lgl, and Rab5DN on levels of recycled Notch. We discovered that the amount of recycled Notch was increased in Numb mutants and that this could be suppressed by blocking Notch internalization using Rab5DN. Previously, Lgl had been implicated in recruiting Notch to acidified compartments, specifically lysosomes, in Drosophila eye tissue (Parsons et al., 2014). Our novel finding that Lgl can also limit Notch recycling offers an explanation for this previous observation and demonstrates a novel role for Lgl in Notch regulation. These results strongly support the second hypothesis that Numb and Lgl are responsible for suppressing Notch recycling to the plasma membrane.

We have thus shown that Numb can block Notch Rab11-dependent recycling. An important implication of this finding is whether blocking of Notch recycling also causes an accumulation of Notch in late endosomes. Results from our steady state analysis reveal an asymmetry in the amount of Notch-positive late endosomes marked with Rab7.
By marking late endosomes by expressing Rab7-GFP or antibody staining for Rab7, we found that Notch accumulated more abundantly in late endosomes of the Numb-positive pIIb cell. Notch accumulation in late endosomes of pIIb was diminished to wild type pIIa levels in numb mutant clones, suggesting that this asymmetry is Numb dependent. In addition, overexpression of numb caused the reverse effect of increasing Notch accumulation in late endosomes of pIIa to wild type pIIb levels. Overall, these data demonstrate that Numb is necessary and sufficient for asymmetric recruitment of Notch to late endosomes.

Our conclusion that Numb inhibits Notch recycling is consistent with other studies. It was recently shown that Numb is required for Sanpodo recycling to the plasma membrane. Using an assay similar to our recycling assay, the authors showed that Numb was required specifically for Sanpodo recycling (Cotton et al., 2013). Moreover, Numb was also shown to traffic Sanpodo to late endosomes (Couturier et al., 2014). These results support our dual role model where Numb may be responsible for suppressing recycling and/or promoting late endosome trafficking.

A common assumption in studying Notch in the SOP context of Drosophila is that the amount of Notch is equal in both daughter cells. This is not an unreasonable assumption as staining for Notch or use of GFP-tagged Notch has not detected an asymmetry in Notch levels between daughter cells (Couturier et al., 2012). However, another study has shown that directional Notch trafficking may be occurring in a subpopulation of endosomes marked with Sara. In the proposed model, Notch and Delta localize in Sara endosomes, which become asymmetrically targeted toward the pIIa cell.
where Notch can undergo ligand-dependent activation (Coumailleau et al., 2009). In this way, increased level of Notch in pIIa are sufficient for specification of the Notch-activated cell fate, while in pIIb, insufficient levels of Notch signaling trigger specification of the Notch-inhibited cell fate. As these findings could have far-reaching implications, we sought to confirm these results and to determine the underlying mechanism for the perceived asymmetry.

We began by using live cell imaging to analyze the dynamics of Sara endosomes within pIIa/pIIb and found that the majority of Sara endosomes did traffic to pIIa as previously reported. However, when we sought to determine if these endosomes contained Notch, we were unable to detect any significant colocalization between Sara and full-length Notch. An important difference between our approaches was in the method of Notch detection. The original study allowed Notch antibody to internalize for a predetermined 10-minute period, since this was the internalization time that yielded the greatest colocalization. In contrast, we probed for Notch at steady state, which reports the amount of Notch present under normal conditions. Our analysis has the advantage of accounting for mechanisms that would traffic Notch away from Sara endosomes. Another important consideration that could account for the difference is that we utilized alternative reagents for the assay, as we were unable to attain those used in the original study. If directional trafficking of Notch is indeed a relevant mechanism of regulation, affirmation of these results utilizing different reagents would be beneficial. Nevertheless, Sara endosome asymmetry has been linked to Notch regulation in other contexts,
indicating that determining the mechanism of Sara asymmetry in the SOP context is still a relevant inquiry (Kressmann et al., 2015; Montagne and Gonzalez-Gaitan, 2014).

In order to elucidate the mechanism of Sara asymmetry, we first overexpressed Insc, a polarity determinant in neuroblasts that is not normally found in SOP cells. However, when Insc is overexpressed in the SOP context, the polarity of cell fate determinants is reversed due to a relocalization of the Par complex (Roegiers et al., 2001; Bellaïche et al., 2001). We found that overexpression of Insc still causes Sara endosomes to migrate posteriorly, though now into the pIIb cell. This suggests that the Sara asymmetry is not reliant on the polarity of pIIa/pIIb cell fate determinants that are dependent on the Par complex. Instead, we propose that Sara asymmetry is likely linked to some aspect of global polarity establishment. To explore this mechanism further, we tested the idea that extension of the mitotic spindle may be biased toward pIIa using a marker for plus-end microtubules, EB1. We found that EB1 was symmetrically partitioned into pIIa/pIIb, suggesting that extension of microtubules is not the cause of Sara asymmetry. A recent study also examined the mitotic spindle in pIIa/pIIb cells and reported an asymmetry in the microtubule marker, Jupiter (Derivery et al., 2015). In addition, they reported that that Sara is recruited to microtubules and then transported preferentially to the pIIa cell. The reason for this discrepancy is likely that EB1 and Jupiter mark different regions of the mitotic spindle and this Jupiter asymmetry was not apparent to us using EB1. However, this study does support the hypothesis that the Sara asymmetry is unrelated to the Par complex, as the mitotic spindle receives cues directly from the anterior-posterior axis established by PCP (Gho and Schweisguth, 1998).
4.2 Remaining Questions and Future Directions

*What is the role of increased late endosomal accumulation of Notch in pIIb?*

We have shown that at steady state, there is an asymmetry in Notch/Rab7 late endosome colocalization, but not in Notch/Rab5 early endosome colocalization. From this study, we also observed that there was significantly more Notch in pIIb endosomes than in pIIa endosomes, which was also reported using GFP-tagged Notch (Couturier *et al.*, 2012). The reason and significance for this asymmetry remains an important question. Our data addresses this in part by mutation or overexpression of Numb, which equalized endosomal Notch between pIIa/pIIb. However, the mechanism by which Numb facilitates this is not clear. Since Numb was not found to impact Notch internalization, the most likely explanation is a Numb-dependent accumulation of Notch in late endosomes in pIIb. This is supported by our finding that the majority of Notch within either cell is contained within late endosomes. However, the functional relevance of Notch in late endosome recruitment is also not clear. Knockdown of HOPS complex members required for transport of cargo to late endosomes appears to have no effect on cell fate decisions (Mummery-Widmer *et al.*, 2009a; Akbar *et al.*, 2009). However, due to the use of RNAi, the efficiency of the knockdown was not reported, nor is it clear whether Notch specifically was blocked from late endosome trafficking. It is possible that increased pIIb Notch accumulation has no function and is simply a byproduct of reduced recycling. Whether lysosome degradation is required for Notch inhibition could be tested by addition of chloroquine to living tissue, then observing any changes in cell fate.
What is the relationship between recycling and late endosome trafficking?

We have shown that the function of Numb in sensory organ cells is to limit Notch on the plasma membrane by blocking recycling. We also show that Numb is responsible for promoting Notch recruitment to late endosomes. It is not known whether Numb is actively performing both of these functions, or if one is indirectly causing the other. It is possible that blocking the recycling of endocytic cargo causes an indirect accumulation of late endosomes, or that promotion of late endosome targeting indirectly depletes recycling. We began to answer these questions by overexpressing a dominant active form of Rab7 (Rab7QL), which has been reported to increase the rate of transport through late endosomes (Mukhopadhyay et al., 1997). Rab7QL appeared to reduce the amount of membrane Notch when overexpressed in wing disc epithelial cells, suggesting that membrane levels of Notch could be altered by late endosome trafficking. It would be important to next establish whether the pool of recycled Notch is specifically being diminished with Rab7QL overexpression. If so, that would demonstrate that recycled Notch could be directly affected by promotion of late endosome trafficking. However, overexpression of Rab7QL had only a mild effect on cell fate, suggesting that late endosome trafficking has a minor role in Notch signaling. We also made use of a dominant negative Rab7 (Rab7TN) which although never confirmed, is assumed to block late endosome transport (Zhang et al., 2007a). Overexpression of Rab7TN caused Notch to accumulate in large endosomes, but had no effect on membrane Notch levels or cell fate. If Rab7TN were confirmed to function as intended, it would demonstrate that blocking late endosome trafficking alone is not sufficient to increase Notch recycling.
However, all of these experiments would need to be repeated in SOP sensory organ cells where Notch levels and trafficking are quite different.

In order to address the question of whether altering recycling is sufficient for altering late endosome dynamics, similar experiments could be performed using overactive and dominant negative Rab11. Dominant negative or overactive Rab11 should block or increase the recycling of Notch to the plasma membrane, which may have an effect on late endosome recruitment (Zhang et al., 2007b). This experiment would determine whether Numb could be acting directly on recycling and if the Notch/Rab7 asymmetry would then follow as a passive byproduct. This question could also be answered biochemically by performing a screen for Numb interactors. If Numb were found to interact with factors known to facilitate recycling or late endosome recruitment, it could lend support to Numb performing either or both of those roles.

*What is the function of Lgl?*  
Utilizing our recycling assay, we showed that in *lgl* and *lgl, numb* mutants the amount of recycled Notch increased, demonstrating that Lgl suppressed Notch recycling. However, it is unclear whether this a direct effect of Lgl or an indirect effect caused by Numb mislocalization. Lgl is thought to be the determinant that relays polarity information from the Par complex to Numb. In this way, Lgl is considered a Notch antagonist by positioning Numb in pIIb where Numb can block Notch signaling (Wirtz-Peitz et al., 2008). However, it was also reported that proper positioning of the Numb crescent in dividing SOP cells is only delayed, achieving proper distribution in pIIb after mitosis (Langevin et al., 2005b). Thus, two competing hypotheses for the role of Lgl are
1) exclusively positioning Numb into pIIb to inhibit Notch or 2) inhibiting Notch through a Numb independent mechanism. Evaluating \( lgl \) mutants alone helps answer this question. If the sole role of Lgl is to direct asymmetric Numb localization, then loss of Lgl should elicit no effect on the amount of total Numb present in pIIa/pIIb combined. For this reason, and from our finding that Numb inhibits Notch recycling, total Notch recycling in pIIa/pIIb should be unchanged in \( lgl \) mutants. However, we find that \( lgl \) mutants have significantly increased levels of Notch recycling, suggesting Lgl may be normally suppressing recycling, independent of Numb. This data supports the second hypothesis that Lgl may have Numb-independent roles in regulation of Notch. To further confirm this, Lgl could be overexpressed in numb mutant clones. If Lgl overexpression results in a change in Notch trafficking and/or cell fate in contrast to numb mutant clones alone, this would also suggest Numb-independent roles for Lgl.

In addition to our findings, other studies have begun to explore Numb-independent roles for Lgl. In the developing eye, Lgl was reported to promote trafficking of Notch to acidified compartments identified with lysotracker (Parsons et al., 2014). Similar experiments could be done in the SOP context to determine if the number of acidified compartments is dependent on Lgl expression. If overexpression of Lgl increased the quantity of compartments identified by lysotracker, it would suggest a similar mechanism may be working in the SOP context. Additionally, it would be useful to assess the role of Lgl in the trafficking of Notch to late endosomes. Similar experiments could be done with Lgl as with Numb, to determine how Notch/Rab7 colocalization changes with Lgl overexpression or mutations. To determine where Lgl
functions in relation to Rab7, an epistasis experiment could be performed to measure how Rab7TN and/or Lgl mutants alter the trafficking of Notch to late endosomes. It was also proposed that Lgl may directly promote the acidification of vesicles by the recruitment of vacuolar ATPases (Parsons et al., 2014). To test if a similar mechanism is at work in the SOP contexts, mutations in V-ATPase pumps could be introduced to determine if overexpression of Lgl could be suppressed. Given these results, it is likely that novel roles exist for Lgl that could provide valuable insight into Notch regulation.

What other factors regulate Notch recycling?

In order to distinguish recycled Notch from other cellular populations, we needed to develop an assay that could provide a quantifiable readout for recycled Notch. Thus, we applied the assay from (Cotton et al., 2013) to determine the role of Numb, Lgl, Rab5DN, Rab11, Sec15, and WASP on the Notch recycling levels. Given the success of this assay in detecting differences in Notch recycling between some of these genotypes, it is likely that many other factors exist for which their effect on Notch recycling could be tested. Interesting candidate genes include Sec6, Chmp1, Ap-1, Neuralized and Sanpodo, all factors that have been reported to alter some aspect of Notch membrane trafficking (Mummery-Widmer et al., 2009b; Benhra et al., 2011; Upadhyay et al., 2013b). Sec6 is of particular interest as it is necessary for DE-Cadherin recycling via the exocyst complex (Langevin et al., 2005a). Loss of Sec6 also causes a cell fate switch to multiple sockets, suggesting overactivation of Notch signaling. Confirmation of Sec6 as a Notch signaling suppressor would be interesting given its known role as a recycling effector.
4.3 Concluding Remarks

In this investigation, we address a long-standing question in the field of Notch research by demonstrating the role of Numb in Notch signaling. In addition, we show novel ways in which signaling can be regulated and provide a valuable assay that can be used to identify additional factors. However, the significance of our findings extend beyond an understanding of Notch signaling in *Drosophila*. Notch is a ubiquitously expressed and well conserved signaling protein that has been implicated in a wide variety of dysfunctions in humans. Currently, the most common treatments for Notch-related cancers are use of gamma-secretase inhibitors (GSI), siRNA, and monoclonal antibodies against Notch receptors (Yuan *et al.*, 2015). GSIs, while shown to be somewhat effective at suppressing Notch related cancer, are often plagued with off-target gastrointestinal effects that hinder efficacy (Staal and Langerak, 2008). Inhibiting Notch signaling through knowledge of Notch endocytic trafficking regulation has recently emerged as an additional therapeutic strategy (Kobia *et al.*, 2014). Our research has shown that Numb, a highly conserved Notch-inhibitor, inhibits Notch by blocking membrane recycling. Novel treatments could utilize this mechanism and potentially replicate Numb’s mechanism of action by routing Notch towards the late endosome causing Notch inhibition. In conclusion, our work not only expands the current understanding of Notch signaling regulation in *Drosophila*, but also has important implications for treatments of human disease.
CHAPTER 5: Materials and Methods

Pupal selection and dissection

White pre-pupae were selected from cross vials based on the presence of selection markers relevant to the particular cross. Pupae were then placed into humidified collection chambers and aged for an amount of time appropriate for the desired developmental stage at 25°C. The following incubation times were used for proper staging; SOP: 14 hours, 2-cell (pIIa/pIIb):16 hours, 4-cell: 18 hours. Staged pupae were then mounted on glass slides using double-stick tape and had their pupal cases removed to display the head and thorax. Pupae were selected for further dissection by visualization under fluorescent microscopy at 10x to determine the presence of clonal tissue marked with GFP. Pupae positive for GFP-labeled clones were fully removed from their pupal cases and transferred to silica gel dissection dishes and immobilized with steel pins. Pupae were then further dissected according to the appropriate protocol (see below).

Clone generation (MARCM)

Creating homozygotes of many of the mutants we required resulted in lethality that prevented analysis of pupal or adult phenotypes. Therefore, we utilized the Mosaic Analysis of a Repressible Cell Marker (MARCM) system which enabled us to produce individually labeled homozygous cells in an otherwise heterozygous fly. In this way, we generated homozygous mutant clones that allowed the organism to survive but still elicit a mutant phenotype. To do this, we crossed flies with the mutant allele recombined with
a flippase recognition target (FRT) to flies that contained Gal80 also recombined with FRT. In these flies, there was also a ubiquitously expressed flippase gene (Ubx-FLP) and a tissue-specific Gal4 element with UAS-GFP. Crossing these flies together caused their progeny to inherit the FRT Gal80 with the FRT mutant allele. Expression of FLP caused recombination at the FRT sites during mitosis allowing some cells to become homozygous for the mutant gene while simultaneously losing the Gal80. Removal of Gal80, which normally inhibits Gal4, allowed for expression of the tissue-specific Gal4 to drive the UAS-GFP marker for visualization of homozygous mutant clones. Utilizing this system, we were able to generate and mark mutant cells that could be characterized for their mutant phenotype.

Recycling assay

The recycling assay we used to distinguish recycled, internalized and static Notch was adapted from a protocol used similarly for investigating the recycling of Sanpodo (Cotton et al., 2013). After appropriate staging, pupae were immobilized in dissection dishes and immersed in S2 insect cell media (Sigma-Aldrich) Pupae heads were removed using microdissection scissors and cuts were made on the flanks to remove ventral thoracic tissue. Remaining tissue was then flushed to remove fat bodies and trachea and was then transferred to glass dishes containing NECD primary antibody (Developmental Studies Hybridoma Bank (DSHB)) at 1:50 in S2 media. Samples were placed in humidified chambers and allowed to incubate for 10 minutes at room temperature with agitation. After the appropriate time, samples were then washed with additional S2 media 3 times for 1 minute per wash. After washing, samples were then transferred to a
glass dish at 4°C containing S2 media with the first secondary antibody (FSA) (Alexa fluor 488, Thermo-Fisher) at 1:100 for 10 minutes with agitation and maintained at 4°C. Samples were washed with 4°C S2 media 3 times for 1 minute per wash and then transferred to S2 media at room temperature for 15 minutes. After 15 minutes, S2 media was removed and samples were fixed using 4% paraformaldehyde for 20 minutes at room temperature. Samples were then washed with phosphate buffered saline (PBS) 3 times and then immersed in PBS with second secondary antibody (SSA) (Alexa fluor 568, Thermo-Fisher) at 1:50 for 1 hour at room temperature. After 1 hour, samples were washed 5 times with PBS and transferred back to the silicon dissection dish for the removal of the notum from other tissues. Nota were then transferred to glass slides with PBS and Vectashield.

*Flux internalization assay*

To monitor the rate at which Notch moves through the endocytic pathway, we made use of an internalization assay. Pupae were staged to an appropriate time and then transferred to silicone dissection dishes with S2 media at room temperature. Heads of pupae were removed with microdissection scissors and cuts were made along the flanks to allow removal of the ventral thoracic tissue. Dissected samples were then transferred to glass dishes with S2 media containing NECD primary antibody at 1:100. Samples were incubated with NECD for 10 minutes at room temperature with agitation. After incubation, samples were washed with S2 media and chased with S2 for 0, 5, 10, 15, or 30 minutes. After the established time, samples were fixed with 4% paraformaldehyde for 20 minutes. Samples were then transferred to PBS with 0.1% Triton X-100 (PBT)
and washed 3 times with PBT. Secondary antibody was then added in PBT with 5% normal goat serum (NGS)(Sigma-Aldrich) at 1:1000 and incubated with the samples overnight at 4°C. Samples were then washed 5 times with PBT and transferred back to dissection dishes to remove the notum from the remaining tissue. Nota were mounted with Vectashield and PBS.

_Drosophila stocks_

**Fig 2.1:** (B-D) _Neuralized-Gal4 UAS-Rab5-GFP_ (Zhang et al., 2007b) _numb^2frt40A_ (Frise et al., 1996). (C) _Ubx-flp; frt40Gal80, Neuralized-Gal4, UAS-Rab5-GFP_

**Fig 2.2:** (A) _Ubx-flp; frt40Gal80, Neuralized-Gal4, Rab5-GFP ckfrt40/Cyo_, (B) _ckfrt40numb^2/Cyo_, (C) _NeuralizedGal4 UAS-Rab7GFP_, (D) _UAS-numb-myc_ (Wang et al., 1997)

**Fig 2.3:** (A-C) _UAS-ActinGFP_ (Ritzenthaler et al., 2000),(B) _Neuralized-Gal4 Ubx-flp; ckfrt40A; NeuralizedGal4_, (C) _Ubx-flp; numb^2frt40A; Neuralized-Gal4_ (C)

**Fig 2.4:** (A,C) _UAS-Sanpodo-GFP_ (Tong et al., 2010) _Neuralized-Gal4_ , (C-E) _UAS-Rab5SN_ (Stenmark et al., 1994), _Gal80frt40; Neuralized-Gal4_, (D-E) _UAS-Actin-GFP_

**Fig 2.5:** (A) _UAS-Rab5-GFP, Neuralized-Gal4 UAS-Rab11SN-YFP_ (Zhang et al., 2007b) _Ubx-flp; numb^2frt40, Gal80frt40, Neuralized-Gal4, Rab5-GFP/TM6_

**Fig 2.6:** (A-B) _Ubx-flp; NeuralizedActin-Gal4, Gal80FRT82b_, (C) _Ubx-flp; Gal80frt40; Neur-Gal4, Rab5-GFP_, (D) _lg1^334frt40a_ (Mechler et al., 1985), (D) _lg1^334, numbfrt40A_

**Fig S2.1:** (A) _wsp^3frt82b_ (Ben-Yaacov et al., 2001), (B) _sec15^2frt82b_ (Mehta et al., 2005)

**Fig S2.3:** (A) _Neuralized-Gal4, UAS-Rab5-GFP_, (B) _Neuralized-Gal4, UAS Rab7GFP_
**Fig S2.4:** (A) *apterous-Gal4, UAS-Rab7QL* (Zhang et al., 2007a), (B) *apterous-Gal4, UAS Rab7TN-GFP* (Zhang et al., 2007a)

**Fig 3.1:** (A) *Ubi-Sara-RFP* (Ben Ohlstein, Columbia University), *NeuralizedGal4 UAS-Pon-GFP*

**Fig 3.2:** (A) *Neuralized-Gal4 UAS-Rab5QL-RFP* (Zhang et al., 2007a), *UAS-Pon-RFP*

**Fig 3.3:** (A) *Neuralized-Gal4, UAS Pon-GFP, Ubi-Sara-RFP*, (B) *Neuralized-Gal4, UAS-Pon-GFP, UAS Rab5QL-RFP*

**Fig 3.4:** (A) *Ubi-Sara-RFP, Neuralized-Gal4, UAS-Insc* (Schober et al., 1999)

**Fig3.5:** (A,B) *Scabrous-Gal4, UAS-EB1-RFP* (Alana O’Reilly, Fox Chase Cancer Center)

All *Drosophila* stocks without labeling or labeled previously were obtained from the Bloomington Stock Center, Indiana University.

**Data quantification**

The recycling assay quantifications were performed using ImageJ software. To quantify levels of recycled Notch, antibody staining in the SSA channel was evaluated for its intensity at the interface between pIla/pIIb cells. The SSA interface signal level was normalized to the level of background SSA found in the nucleus. To quantify internalized Notch, FSA signal was also measured at the interface and normalized to the background nuclear levels.

To represent static Notch, colocalization of FSA and SSA was assessed. To determine colocalization, signal intensity from FSA and SSA was set to a threshold equal
to approximately 30% of maximum intensity. Masks of thresholds were then taken and colocalized pixels were counted and represented in yellow.

In order to quantify the number of endosomes that contained Notch, we defined an endosome as being a roughly spherical object between 0.5-1 microns in diameter. Image analysis was done using Metamorph software (Molecular Devices). NECD punctal intensity was measured and was considered a Notch endosome if its intensity was at least 33% that of the maximum intensity and its diameter was greater than 0.5 µm. If Notch endosomes overlapped with at least half of the Rab5-GFP or Rab7-GFP labeled endosome, they were considered colocalized. NECD alone and NECD colocalized with Rab5 or Rab7 was quantified for pIIa and pIIb cells separately.

**Live cell imaging**

In order to observe developing cells in real time, we made use of a live cell imaging technique from (Zitserman and Roegiers, 2011). Pupae were staged to the SOP time point (16 hours at 25°C). Pupal cases were then partially dissected to reveal heads and thoraces and then selected using fluorescent microscopy to determine pupae with GFP labeled clones. Those with GFP clones were then fully dissected and placed on glass slides and sealed with a small layer of vacuum grease which supported a glass cover slip. Sealed slides were then visualized under 63x magnification using confocal microscopy. SOP cells were then tracked until division at which point movies were taken of endosome dynamics during mitosis.

Live cell imaging of Sara endosomes was performed in the same way, except that pupae were staged to the SOP time point and then enclosed in a humidified chamber
between a slide and coverslip. SOP cells marked with Neuralized-RFP were observed until the beginning of mitosis, then imaged for 30-second intervals. Imaging was stopped after approximately 5 minutes from the end of cytokinesis. Live imaging of EB1 labeled microtubule dynamics were performed in a similar way. SOP cells were labeled with scabrous driven EB1-RFP, then imaged until approximately five minutes after cytokinesis. Epithelial cells were staged to a similar time point, then examined for presently dividing cells. Dividing cells were then imaged until the completion of cytokinesis.

**Wing disc dissection**

In order to visualize expression borders in epithelial cells, wing discs were dissected from third-instar larvae. Larvae were attached to silicone dishes in PBS and cut in a way that reveals imaginal discs. Wing discs were carefully dissected and immediately fixed in 4% PFA for 20 minutes. After fixation, wing discs were washed with PBT and incubated with NECD antibody (DSHB) overnight at 4°C. Wing discs were then washed in PBT and incubated with Alexa fluor 568 (Thermo Fisher) for two hours at room temperature. Tissue was then washed with PBT and mounted for visualization. Images were taken at the expression borders, marked with GFP that had been generated by Apterous-Gal4 expression.

**Data representation and statistics**

Data from our recycling assay was presented as scatter plots as advised in (Weissgerber et al., 2015). This method of representation allows for a more unbiased summary of all data points when the data set is not expected to be in a normal
distribution. In these graphs, the average is indicated by a colored solid bar corresponding to the respective dots it represents.

Statistical analyses of the Notch recycling assay, punctal colocalization assay, and internalization assays were performed using a Wilcoxon Rank-Sum test as advised by the Statistics Facility at Fox Chase Cancer Center. The Wilcoxon test does not assume a normal distribution and is the most appropriate analysis given our data set.
REFERENCES


118


