Copyright

by

Susan Marie-Louise Banks

2012

The Dissertation Committee for Susan Marie-Louise Banks Certifies that this is the approved version of the following dissertation:

The Role of Auxilin and Endocytosis in Delta Signaling

Committee:

Janice Fischer, Supervisor

Jon Huibregtse

Paul Macdonald

Jennifer Morgan

Theresa O'Halloran

The Role of Auxilin and Endocytosis in Delta Signaling

by

Susan Marie-Louise Banks, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2012

Dedication

To my family and friends.

Acknowledgements

Thank you to Janice Fischer for her guidance through this project. Thank you to my committee members for their thoughtful comments and discussions. Thank you to the University of Texas at Austin fly community for sharing reagents, equipment, and helpful comments on my project. Thank you to the many Fischer laboratory members both past and present for sharing reagents and thoughtful discussion in the laboratory. Thank you to Will Stoutt, an undergraduate who was integral to completion of the screen. This project would not have been possible without the work of many other undergraduates and technicians in the laboratory: Sakina Karimjee, Khasim Syed, Emily Beck, Markay Isaac, Brian Nyquist, Alan Te, Samantha Marion, and Stephen Fleenor. Thank you to my family for their love and encouragement over the years. Thank you also to my friends, who were always there to support me.

The Role of Auxilin and Endocytosis in Delta Signaling

Susan Marie-Louise Banks, Ph.D.

The University of Texas at Austin, 2012

Supervisor: Janice Fischer

Notch signaling is important for cell-cell signaling during development. Notch signaling is highly conserved across all metazoans and failure in Notch signaling is causative in many human diseases. In the *Drosophila* eye, activation of the Notch pathway requires Lqf (*Drosophila* Epsin)-dependent and Clathrin-dependent internalization of the Notch receptor ligands, Delta or Serrate, by the signal-sending cells. However, it is unclear why ligand must be internalized into the signal-sending cells to activate Notch signaling in the signal-receiving cells. Evidence suggests that in addition to Clathrin and Epsin, Auxilin is essential for signaling and is indirectly required for internalization of the Notch receptor ligand Delta. Auxilin functions in uncoating Clathrin-coated vesicles to maintain a pool of free Clathrin and Epsin in the cell. *auxilin* mutants were used as an entryway to identify previously unknown components of the Notch signaling pathway. An F1, *FLP/FRT,* EMS screen was performed and enhancers of an *auxilin* mutant rough eye defect were isolated. The enhancers ultimately formed one complementation group on the 2nd chromosome and fourteen complementation groups on the 3rd chromosome. Three of the 3rd chromosome complementation groups were each identified as *Delta, lqf,* or *hsc70*. A single allele was identified as *faf*. Delta and Epsin have known roles in signaling cells to activate Notch as described above.

Hsc70 is an ATPase that functions with Auxilin to uncoat Clathrin-coated vesicles and Faf is a deubiquitinating enzyme that maintains levels of active Epsin in the cell. These results suggest I have isolated mutations in genes closely tied to Notch signaling or functioning directly with Auxilin. Mutations in two genes previously undescribed in Notch signaling in the developing *Drosophila* eye were also isolated from the screen and identified. The second chromosome complementation group was identified as *α-adaptin*. α-Adaptin is a subunit of the heterotetrameric Clathrin adaptor protein AP-2. One of the third chromosome complementation groups was identified as *crumbs*. Crumbs is an integral membrane protein that functions at adherens junctions and in establishing apical/basal polarity in cells. Characterizing roles for α-Adaptin and Crumbs during Notch signaling may elucidate the purpose for Delta internalization to activate Notch signaling.

Table of Contents

List of Tables xii

List of Figures xiii

Chapter 1: Introduction 1

1.1. Notch signaling 1

1.1.1. The role of Notch signaling in *Drosophila* egg formation, wing disc, SOP, and eye development 2

1.1.2. Notch signaling and cell-cell communication 3

1.2. Notch receptor and Delta/Serrate ligands 5

1.2.1. Receptor structure, processing, and modification 5

1.2.2. Receptor activation and downstream targets 8

1.2.3. *Drosophila* ligand structure and modification 10

1.2.3.1. The role of Neur and Ubiquitination 12

1.2.4. Endocytosis of ligand is required to activate Notch signaling 13

1.3. Endocytosis 15

1.3.1. Clathrin-independent endocytosis 16

1.3.2. Clathrin-dependent endocytosis 19

1.3.3. Endosomal pathway 21

1.3.4.1. Rab GTPases 23

1.4. Adaptor proteins 23

1.4.1. Epsin structure, function, and regulation 24

1.4.1.1. Epsin regulation via the deubiquitinating enzyme fat facets 27

1.4.2. AP-2 structure and function 27

1.5. Auxilin and uncoating 30

1.5.1. Auxilin structure and function 31

1.5.2. *auxilin* loss of function mutations dominantly enhance the mutant eye defect caused by Epsin overexpression. 32

Chapter 2: Auxilin functions during the endocytosis of some Delta 34
2.1 Introduction 34
2.1.1. Drosophila 3rd instar eye disc development 34
2.1.2. Generating clones in developing *Drosophila* eye discs 37
2.1.3. Auxilin functions during eye development in signal-sending cells to activate Notch signaling 39
2.1.3.1. auxilin loss of function mutations result in aberrant photoreceptor numbering and patterning 39
2.1.3.2. Delta transcription is increased at the furrow in auxilin mutants 41
2.1.3.3. Auxilin is required in signal-sending cells during Notch signaling in the developing Drosophila eye 41
2.1.3.4. The Clathrin-binding domain and DNA-J domain together alone are sufficient for Auxilin function 42
2.1.3.5. Chc overexpression partially rescues auxilin hypomorphic mutants 43
2.2 Results 44
2.2.1. *auxilin* mutants show a Delta endocytosis defect 44
2.3. Discussion 47

Chapter 3: The Role of Auxilin in Delta signaling is not for the purpose of recycling 50
3.1. Introduction 50
3.1.1. Evidence supporting the pulling model and the recycling model50
3.1.2. Rab11 is not required for Notch signaling in the developing *Drosophila* eye 53
3.1.3. Auxilin is not required for signal-sending in the developing oocyte 53
3.2. Results 54
3.2.1. $glqf^{+}$ partially rescues aux^{hypo} 55
3.2.2. $glqf^{+}$ and $gChc^{+}$ almost completely rescue aux^{hypo} 56
3.3. Discussion 59
3.3.1. The canonical recycling pathway is not required for Notch signaling 59

Chapter 4: Identification of enhancers of an *auxilin* loss of function eye defect .61
4.1 Introduction 61
4.1.1 Screen rationale 61
4.1.2. EMS mutagenesis 62
4.1.3. F1 GMR-hid screen 64
4.2. Results 66
4.2.1. Screen numbers 68
4.2.2. Segregation testing 70
4.2.3. Complementation testing 70
4.2.3.1. 2nd chromosome 70
4.2.3.2. 3rd chromosome 71
4.2.4. Positive controls identified 80
4.2.5. Mapping 81
4.2.5.1. Meiotic mapping 2nd and 3rd chromosomes 81
4.2.5.2. Deficiency mapping 2nd and 3rd chromosomes 82
4.2.6. Identified enhancers 83
4.2.6.1. Validation of isolated α-adaptin alleles 83
4.2.6.2. α-adaptin mutations enhance the auxilin mutant photoreceptor defect 85
4.2.6.3. Validation of isolated crumbs alleles 87
4.2.6.4. crumbs mutations enhance the auxilin mutant photoreceptor defect 88
4.3 Discussion 90
4.3.1. Extent of screen coverage of the genome 91
4.3.2. Possible types of enhancers to be isolated from the screen 91
4.3.3. Role of α-Adaptin in photoreceptor differentiation 94
Chapter 5: Discussion and future directions 95
5.1. Discussion 95
5.1.1. Possible role for α-Adaptin in Notch signaling 95
5.1.2. Possible role for Crumbs in Notch signaling 98

5.2. Future Directions 103
5.2.1. Identification of other enhancers isolated from the screen and testing genetic interactions 103
5.2.2. Delta localization in *α-adaptin* mutants 105
5.2.3. Function of α-Adaptin during Notch signaling in signal-sending cells, signal-receiving cells or both 106
5.2.4. Further experiments to characterize α-Adaptin function in Notch signaling 109
5.2.5. Determining a role for Crumbs in Notch signaling during photoreceptor differentiation 111
5.2.6. Further experiments to characterize Crumbs function in Notch signaling 112
5.3. New model for Delta endocytosis to activate Notch signaling 113

Appendices 116
Appendix 1: Materials 116
A.1.1. *Drosophila* stocks 116
A.1.2. Antibodies 127
A.1.4. PCR Primers 127
Appendix 2: Methods 129
A.2.1. Eye disc dissection 129
A.2.2. EMS mutagenesis 129
A.2.3. RNA isolation and RT PCR 130

References 131

Vita 155

List of Tables

Table 3. Rescue of lethality of *aux* mutants by overexpression of Epsin and/or Clathrin heavy chain. 58

Table 4. Summary of complementation groups 73

List of Figures

Figure 1.0. Notch signaling events 4

Figure 1.1. Structure of the *Drosophila* Notch receptor 6

Figure 1.2. Activation of the Notch receptor via ligand binding 9

Figure 1.3. Structure of Notch ligands Delta and Serrate 11

Figure 1.4. Models for why Delta is endocytosed 14

Figure 1.5. Clathrin-dependent, Clathrin-independent endocytosis and the endosomal pathway 17

Figure 1.6. Clathrin Triskelion and Lattice 20

Figure 1.7. Structure of Epsin 25

Figure 1.8. Epsin regulation by ubiquitination and Faf 28

Figure 1.9. AP-2 structure 29

Figure 1.10. *Drosophila* Auxilin structure 33

Figure 2.0. Notch signaling events during photoreceptor differentiation 35

Figure 2.1. FLP/FRT mitotic recombination 38

Figure 2.2. *auxilin* hypomorphic mutants have an eye defect 40

Figure 2.3. Delta trafficking in 3rd instar eye discs 45

Figure 2.4. Auxilin functions in signal-sending cells to activate Notch signaling. 49

Figure 3.0. Overexpression of Clathrin heavy chain and/or Epsin suppresses the adult eye defects in *aux* loss-of-function mutants. 57

Figure 4.0. EMS ethylation affects G-C base pairing 63

Figure 4.1. Cross scheme for an F1 EMS *FLP/FRT* mutagenesis screen for enhancers of an *auxilin* mutant eye defect. 67

Figure 4.2. Examples of enhancers isolated......69

Figure 4.3. 2nd Chromosome complementation matrix.....72

Figure 4.4. 3rd chromosome complementation matrix, Part I of Parts I-VI ..74

Figure 4.4. 3rd chromosome complementation matrix, Part II 75

Figure 4.4. 3rd chromosome complementation matrix, Part III.....76

Figure 4.4. 3rd chromosome complementation matrix, Part IV 77

Figure 4.4. 3rd chromosome complementation matrix, Part V.....78

Figure 4.4. 3rd chromosome complementation matrix, Part VI 79

Figure 4.5. *α-adaptin* mutations enhance the *auxilin* hypomorphic photoreceptor defect 86

Figure 4.6. *crumbs* mutations enhance the *auxilin* hypomorphic photoreceptor defect 89

Figure 5.0. New model for Delta endocytosis and Notch signaling 97

Figure 5.1. Crumbs structure 100

Chapter 1: Introduction

1.1. NOTCH SIGNALING

Notch signaling is a cell-cell communication pathway that functions during development of all metazoans to determine cell fate. A failure of Notch signaling was first observed in *Drosophila* female flies with notches on the margins of their wings (Dexter, 1914). *Notch* was determined to be sex-linked, haploinsufficient, and homozygous lethal, because only females with the wing defect were observed and *Notch* mutant males were absent from the viable adult population (Dexter, 1914). Further studies showed that Notch signaling was important not only in wing development, but also during *Drosophila* neurogenesis (Poulson, 1937). Since then, the importance of Notch signaling during development of many different tissues and organisms has been determined. *Drosophila* only have one *Notch* gene, but several *Notch* homologs have been identified in other organisms, such as *LIN-12* and *GLP-1* in *C. elegans* (Greenwald et al., 1983) or mammalian *Notch1*, *Notch2, Notch3* and *Notch4* (Ellisen et al., 1991; Robbins et al., 1992; Weinmaster et al., 1992). Failure of Notch signaling during human development is catastrophic as it is required for proper differentiation of cells in many different tissues, such as the heart and blood vessels (Lee et al., 2009; Phng and Gerhardt, 2009). The Notch pathway and its components have also been linked to Alzheimer's (Levitan and Greenwald, 1995; Nakayama et al., 2011), leukemia (Roy et al., 2007; Shelly et al., 1999), and influenza viral infection of cells (Ito et al., 2011).

Characterization of the *Notch* gene locus revealed that it encodes a single-pass transmembrane protein most likely functioning as a receptor, based on its similarity to the Epidermal Growth Factor Receptor (EGFR), insulin receptor, and Low-Density Lipoprotein Receptor (LDLR) (Kidd et al., 1986; Wharton et al., 1985). Notch signaling

cannot occur unless the Notch receptor binds its ligand, which in *Drosophila* is either Delta or Serrate (Figure 1.4) (Fehon et al., 1990; Fleming et al., 1990; Thomas et al., 1991). The Notch ligand in *C. elegans* is LAG-2 (Tax et al., 1994). In mammals, the Notch ligands homologous to Delta are Delta-like1 (Bettenhausen et al., 1995), Delta-like3 (Dunwoodie et al., 1997), and Delta-like4 (Shutter et al., 2000). The mammalian Serrate homologues are Jagged1 (Lindsell et al., 1995) and Jagged2 (Shawber et al., 1996). Notch signaling is not only important for cell fate determination during development, but is important for maintenance of cell identity (Chao et al., 2005; Nelson et al., 2011), can result in cell movement (Fanto and Mlodzik, 1999; Hermann et al., 2000; Zheng et al., 1995), and may regulate mitotic events (Ohata et al., 2011). The experiments described in this dissertation were performed in *Drosophila* and specifically address Delta activation of Notch during photoreceptor differentiation.

1.1.1. The role of Notch signaling in *Drosophila* egg formation, wing disc, SOP, and eye development

Notch signaling is required during *Drosophila* development in different contexts and tissues, such as the eye, the oocyte, sensory organ precursor (SOP) or bristle cells, the wing, and other tissues. In the oocyte, Notch signaling is essential in somatic follicle cell specification and to inhibit follicle cell over-proliferation (Deng et al., 2001). During SOP development Notch signaling is important for differentiation of the neural bristle cells from the cells that become the non-neural epithelial cells. When Notch signaling fails during bristle development, the result is too many bristles spaced closely together (Le Borgne, 2005). In the developing wing, Notch signaling is important for establishment of the dorsal-ventral boundary (Irvine and Vogt, 1997). During eye development Notch signaling is essential for differentiation of several cells that make up

the eye, including photoreceptors (Baker and Yu, 1997), cone cells (Flores et al., 2000), and pigment cells (Parks et al., 2000).

1.1.2. Notch signaling and cell-cell communication

Cell-cell communication is essential to relay information required to function as a multi-cellular organism. During development Notch signaling is important in communicating cell fate, growth, proliferation, survival, and morphogenesis (Artavanis-Tsakonas, 1999). While there is some evidence that Notch signaling can occur at a distance through secretion of ligand (Chen and Greenwald, 2004), it is mostly thought to occur between two adjacent cells. Notch signaling events have been classified into three different types, depending on the context of the signaling event (Figure 1.0).

For simplicity, these signaling events are described in a two-cell model to differentiate a neural cell and a non-neural cell. Lateral inhibition is a Notch signaling event that results in equivalent progenitor cells adopting distinct cell fates. Lateral inhibition involves mutual inhibition, biased inhibition, and unidirectional inhibition. Mutual inhibition occurs when both equivalent cells send inhibitory Notch signals. Following a complex series of feedback loops, biased inhibition occurs in which one cell becomes biased to send a stronger inhibitory signal than the other cell. Finally, unidirectional inhibition occurs when the biased cell becomes the signal-sending cell and sends an inhibitory Notch signal to the signal-receiving cell.

A second Notch signaling event, known as induction, results in a fate change when the two cells are not equivalent. Induction usually occurs to establish an organizer or boundary. One cell sends a signal to activate Notch in the other cell. Upon activation of Notch in the second cell, a change in gene expression then directs the cell to adopt a specific fate (Schweisguth, 2004). A third Notch signaling event results in establishment

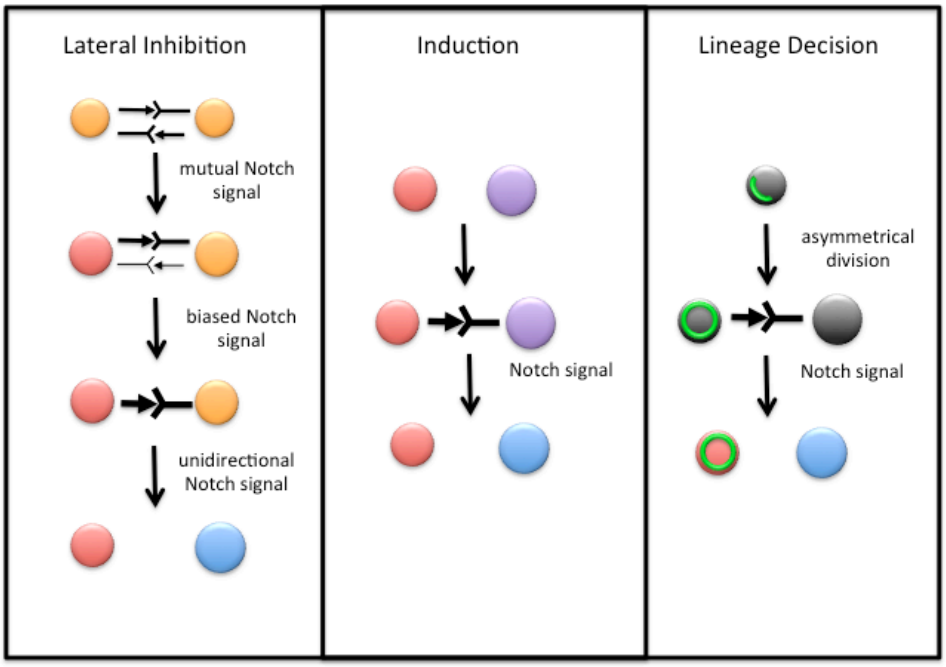

Figure 1.0. Notch signaling events

Notch signaling events vary depending on the context. (a) Lateral inhibition occurs in a pool of equivalent progenitor cells. Notch signaling first occurs to mutually inhibit both cells. Next, a biased inhibitory Notch signal occurs due to feedback loop biasing one of the cells. Finally, a unidirectional Notch signal results in differentiation of a neural cell and a non-neural cell. (b) Induction occurs when two different cell types undergo a Notch signaling event to establish a boundary. (c) Lineage decisions occur when a single cell undergoes an asymmetrical division in which Notch components are asymmetrically localized to one daughter cell. This daughter cell is biased to send an inhibitory Notch signal to the other daughter cell [Modified from (Schweisguth, 2004) and (Bray, 2006)].

of a new lineage of cells. Notch signaling in lineage decisions occurs when a single cell undergoes an asymmetrical division. During the division, specific Notch components are localized into one daughter cell over the other. This asymmetrical localization of Notch components biases one daughter cell to become the signal-sending cell and one daughter cell to become the signal-receiving cell (Bray, 2006).

1.2. NOTCH RECEPTOR AND DELTA/SERRATE LIGANDS

In canonical Notch signaling, a signal cannot be sent without binding of the receptor to one of its ligands. Modifications to the Notch receptor are required for interaction with the ligands Delta and Serrate. Ligand/receptor interaction initiates a series of cleavage events resulting in the intracellular domain of the receptor being released. The intracellular domain is then trafficked to the nucleus where it functions to affect gene expression, which leads to changes in cell fate. It is not just binding of the receptor to the ligand that is necessary to initiate activation. Though it is unclear why this occurs, the ligand must be endocytosed to activate Notch signaling.

1.2.1. Receptor structure, processing, and modification

The Notch receptor is a single-pass transmembrane protein consisting of the Notch ExtraCellular Domain (NECD) and the Notch IntraCellular Domain (NICD) (Figure 1.1). The Notch receptor also contains a transmembrane domain. The S1 cleavage event described below cleaves the receptor and the portion of the receptor that contains the transmembrane domain and the NICD is referred to as the Notch Transmembrane (NTM) subunit.

The NECD has a series of cysteine-rich epidermal growth factor (EGF)-like repeats that function in binding EGF-like repeats found on Notch ligands (Fleming, 1998). Lin-12 Notch Repeat (LNR) motifs and heterodimerization domains function in

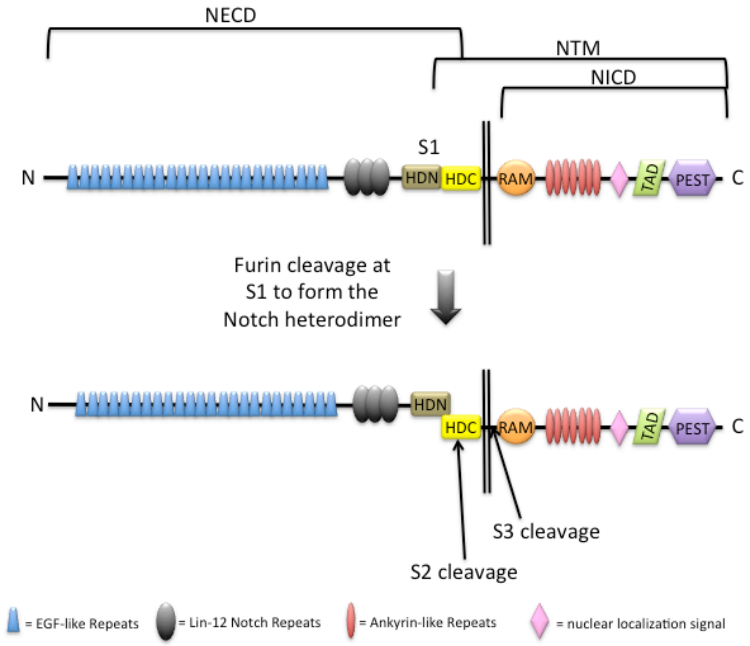

Figure 1.1. Structure of the *Drosophila* Notch receptor

The Notch receptor is shown with the NECD, NTM, and NICD regions identified. There are several EGF-like repeats, LIN12-Notch repeats (LNR), and heterodimerization domains (HDN/HDC) on the extracellular portion of the receptor. On the intracellular portion of the receptor are a RAM domain, ankyrin-like repeats, a transactivation domain (TAD) and a PEST domain. Cleavage at the S1 site by a furin-like enzyme allows for formation of the Notch receptor heterodimer [modified from (Roy et al., 2007), (Kurooka et al., 1998) , and (Kageyama and Ohtsuka, 1999)].

maintaining the Notch receptor heterodimer and inhibiting ectopic Notch activation in a calcium-dependent fashion (Rand et al., 2000). The NICD contains an RBP-Jκ-associated-molecule (RAM) docking site, which has a high affinity for binding RBP-Jκ protein, also known as CSL (CBF1 in mammals, Suppressor of Hairless (Su(H)) in *Drosophila*, and Lag-1 in *C. elegans*). CSL binding the Notch receptor is required for transmitting the signal (Johnson et al., 2010). An ankyrin-repeat domain is also required to bind CSL to transmit the signal (Rebay et al., 1993; Roehl and Kimble, 1993). The TAD region contains a poly-glutamine region and functions with the RAM docking site and the ankyrin-repeats to transmit the signal (Kurooka et al., 1998). At the C-terminus is a proline (P), glutamate (E), serine (S), Threonine (T) or PEST domain that serves as a site for phosphorylation and possibly ubiquitination to regulate the NICD (Hubbard et al., 1997) (Figure 1.1).

Post-translational modifications to the NECD are required for ligand binding. The NECD is modified through the addition of sugars to the cysteine rich EGF-repeats. Two enzymes important for the initial sugar modifications are an O-glucosyltransferase and GDP-fucose Protein *O*-fucosyltransferase 1, which add O-glucose and O-fucose respectively (Acar et al., 2008; Moloney et al., 2000; Wang et al., 2001). After this modification, the Notch receptor can be trafficked to the plasma membrane, where it has the potential to bind Serrate. However, the previously described modifications are not sufficient for binding Delta. The enzyme Fringe is a glycosyltransferase that adds an N-acetylglucosamine followed by additions of galactose and sialic acid, enabling the receptor to bind Delta (Moloney et al., 2000). Fringe is not present in all tissues, but is limited to dorsal or ventral regions for the purpose of establishing a boundary (Brückner et al., 2000; Panin et al., 1997). Fringe modification inhibits binding of Serrate to the Notch receptor (Fleming et al., 1997). This difference in sugar additions to the Notch

receptor, as well as variations in modifier expression, can provide a level of regulation through ligand binding specificity. For example, Fringe expression on the ventral side of the developing *Drosophila* eye disc, allows for Serrate activation of Notch from the ventral side and Delta activation of Notch from the dorsal side. This differential signaling establishes the dorsal/ventral boundary in the developing eye disc (Cho and Choi, 1998; Domínguez et al., 1998). Other types of protein-protein interaction could serve to regulate receptor activity as a homodimer could be formed by the NECD (Kelly et al., 2010), which facilitates receptor-ligand interaction and may inhibit ligand-independent Notch activation (Sakamoto et al., 2005).

1.2.2. Receptor activation and downstream targets

In mammalian Golgi, a furin-like convertase enzyme cleaves off a portion of the extracellular domain of the Notch receptor precursor in the S1 cleavage (Logeat et al., 1998). Binding of the Notch receptor to ligand initiates subsequent cleavage events (Figure 1.2)(Lieber et al., 1993).

The S2 cleavage occurs when the A Disintegrin And Metalloprotease (ADAM)-metalloprotease/ TNF-alpha Converting Enzyme (TACE) enzyme separates the NECD from the rest of the receptor. Next, the γ-secretase complex, composed of Presenilin, Nicastrin, Aph1, and Pen2, initiates the S3 cleavage that separates the NICD from the rest of the receptor. The NICD is then trafficked to the nucleus (Figure 1.2) (Bray, 2006; Fortini, 2002; Schweisguth, 2004; Struhl, 1998; Struhl and Greenwald, 1999). Once in the nucleus, the NICD interacts with a transcription factor CSL and its co-activator Mastermind (Mam). This complex then binds CSL specific DNA binding sites in the regulatory region of target genes (Fryer et al., 2002). The best understood example in *Drosophila*, occurs when the NICD/Su(H)/Mam complex binds the regulatory region of

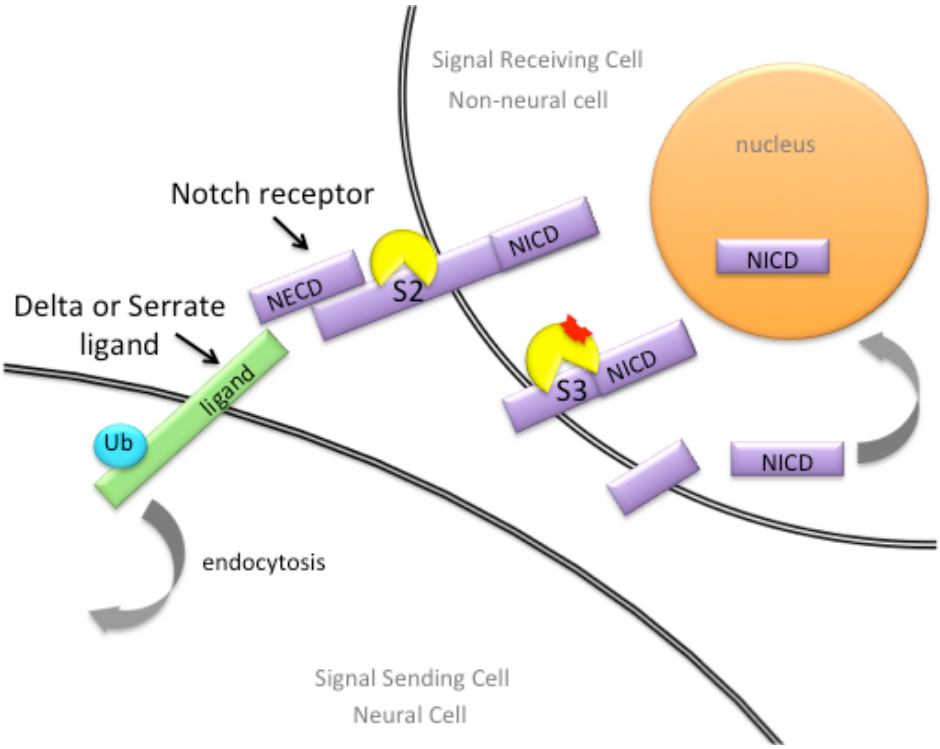

Figure 1.2. Activation of the Notch receptor via ligand binding

Upon ligand binding the Notch receptor undergoes a series of cleavage events that results in the release of the NICD. Activation of Notch target genes occurs when the NICD is trafficked to the nucleus where it functions as a transcription factor. Ubiquitinated (Ub) ligand has to be endocytosed into the signal-sending cell to activate Notch signaling [modified from (Fischer et al., 2006)].

Enhancer of split (E(spl)) and up-regulates transcription of the seven basic helix-loop-helix (bHLH) proteins encoded by that gene region (Jennings et al., 1994; Ligoxygakis et al., 1998). These bHLH proteins then regulate other target genes, either through repression of proneural genes (e.g. *achaete*, *scute*) or through activation of epidermal fate genes (e.g. *wingless*, *cut*, *vestigial*). The transmitted Notch signal results in adoption of a specific cell fate due to changes in gene transcription (Bray and Furriols, 2001; Heitzler et al., 1996).

Notch signaling is different from most other signaling pathways not only because the NICD acts in complex as a transcription factor, but also due to the fact that there is some evidence that it must be endocytosed to activate signaling (Seugnet et al., 1997). Rab5, a Rab-GTPase involved in endosomal trafficking was shown to be required in the signal-receiving cells in the developing oocyte (Windler and Bilder, 2010). Endocytosis of receptors in other signaling pathways often occurs to down-regulate the signaling pathway. While Notch signaling is unique due to processing and function of the receptor, the ligands are also unique in that they must be endocytosed to activate Notch signaling (Bray, 2006).

1.2.3. *Drosophila* ligand structure and modification

Delta and Serrate are single-pass transmembrane proteins that consist of differing numbers of extracellular EGF repeats. Delta has fewer EGF repeats than Serrate and Delta lacks the cysteine rich (CR) repeats found in Serrate (Figure 1.3) (Artavanis-Tsakonas et al., 1995; Fleming, 1998).

Though it has been suggested that cleavage of the ligand can occur (Klueg et al., 1998; LaVoie and Selkoe, 2003), no clear functional role for a cleavage product has been identified. Cleavage may be linked to down regulation of the signaling pathway through

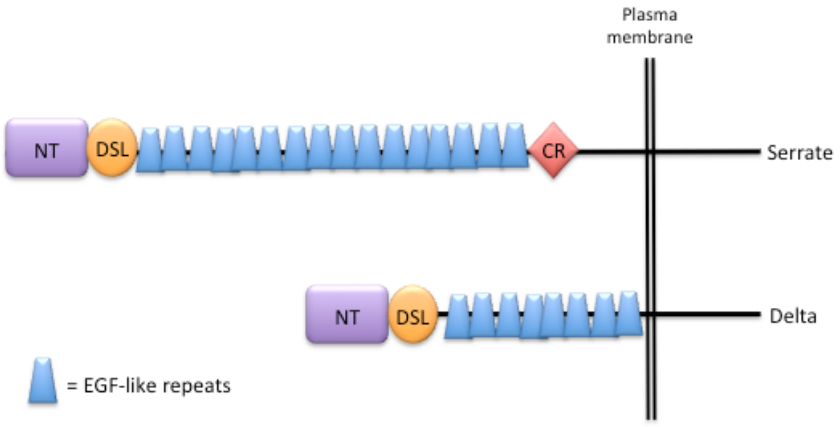

Figure 1.3. Structure of Notch ligands Delta and Serrate

Serrate and Delta are both Notch ligands. They contain an N-terminal (NT) domain and a Delta-Serrate-Lag2 (DSL) domain. While they both have EGF-like repeats, Serrate has more repeats than Delta. Serrate has a cysteine-rich (CR) region that is not present in Delta [modified from (Fleming, 1998)].

degradation of ligand (Mishra-Gorur, 2002). Its also possible that cleaved and secreted ligand serves to block Notch activation (Sun and Artavanis-Tsakonas, 1997). Another idea is that long range signaling occurs due to production of cleaved ligand. Once cleaved, the extracellular portion of the ligand is free to interact with receptor nearby or at a distance. *In vitro* experiments have suggested the cleaved extracellular product is capable of inducing signaling (Qi, 1999). Other experiments suggest cleaved ligand signaling in a bi-directional way through trafficking of cleaved ligand into the nucleus where it could function in a feedback mechanism (Bland et al., 2003). Though this aspect of ligand processing is not well understood, it is known that ubiquitination of the ligand is required to activate signaling (Itoh et al., 2003; Lai et al., 2001).

1.2.3.1. The role of Neur and Ubiquitination

Ubiquitination occurs when ubiquitins are added to substrates. Ubiquitins are a post-translational modification that are highly conserved and function to direct protein trafficking and regulation. Consisting of 76 amino acids, ubiquitins can be added as either monomers or in chains with specific linkages. The type of linkage determines if the protein is trafficked to a specific location in the cell or if it is targeted to the proteasome for degradation. The process of ubiquitination begins with a ubiquitin-activation enzyme or an E1, which binds a ubiquitin. Then, the ubiquitin is transferred to a ubiquitin-conjugating enzyme or an E2. Next a ubiquitin-ligase or E3 facilitates transfer of the ubiquitin to the substrate. The E3 enzymes typically contain either a HECT domain or a RING domain. HECT E3 enzymes involve transfer of the ubiquitin to the E3 before it is transferred to the substrate. RING E3 enzymes serve as scaffolds to facilitate transfer of the ubiquitin directly from the E2 to the substrate (Hicke and Dunn, 2003; Weissman, 2001).

In Notch signaling, ligand ubiquitination occurs via the redundant RING domain containing E3 ubiquitin ligases Neuralized (Neur) or Mindbomb (Mib) (Itoh et al., 2003; Lai et al., 2001). Neur and Mib have different patterns of expression, however specific preference for one ligand or another has not been documented (Lai, 2005). In the developing *Drosophila* eye, Neur is important in ubiquitinating Delta to target it for internalization (Figure 1.4) (Lai et al., 2001).

1.2.4. Endocytosis of ligand is required to activate Notch signaling

After modification of both receptor and interaction with the ligand, it is still not possible to activate Notch signaling without the cellular process, endocytosis (Wang, 2005). Endocytosis is the method by which cells internalize plasma membrane, which results in the uptake of extracellular and membrane bound proteins (Doherty and McMahon, 2009). More specifically, endocytosis of Delta to activate Notch signaling requires ubiquitination of the intracellular portion of Delta (Wang and Struhl, 2004). There are a couple of popular models about how and why ligand must be endocytosed to activate signaling (Figure 1.4).

One model is the pulling model. X-ray crystallography was performed to solve the structure of the area around the S2 cleavage portion of the Notch receptor. It was determined that the LNR modules each form a loop structure that shields the cleavage site from protease access. A pulling force may be generated to induce a conformational change in the Notch receptor adjusting the position of the loops to provide the ADAM-metalloprotease/TACE protease access to the S2 cleavage site (Gordon et al., 2007; Musse et al., 2012). It is also possible a pulling force is generated to physically separate the NECD from the rest of the Notch receptor heterodimer (Nichols et al., 2007), thereby inducing further cleavage events. Other evidence supporting the pulling model is that the

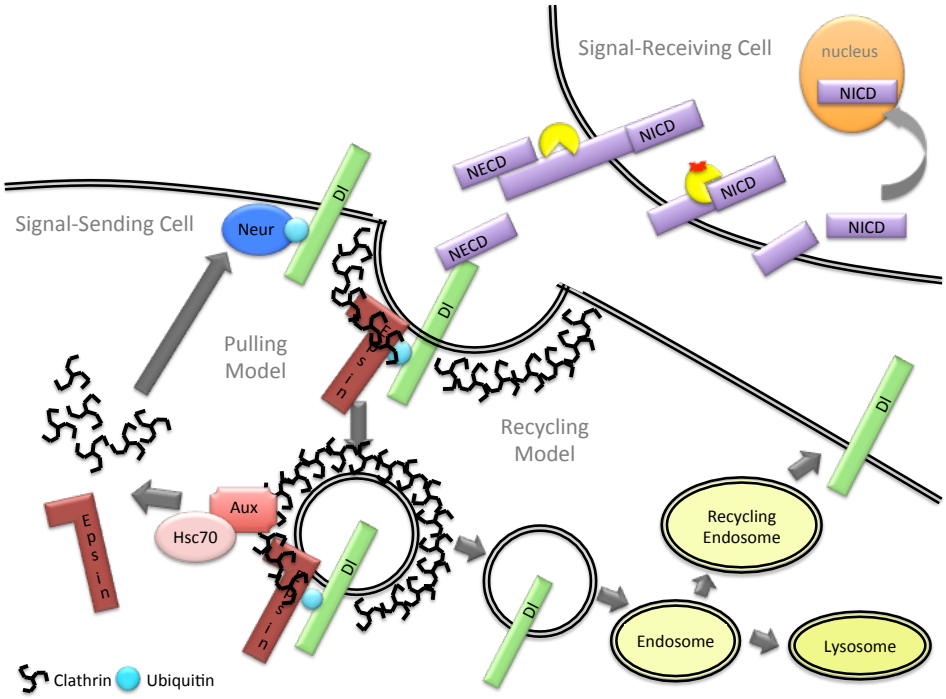

Figure 1.4. Models for why Delta is endocytosed

The E3 ubiquitin ligase Neur ubiquitinates Delta, which is recognized by Epsin and targeted for internalization. Auxilin and Hsc70 uncoat Clathrin-coated Dl-containing vesicles to release Clathrin and Epsin, which can be trafficked to the plasma membrane to be used in future endocytic events. Auxilin and Hsc70 uncoating also generates uncoated Dl-containing vesicles that, which fuse with the endosomal pathway where it can be recycled to the plasma membrane. In the pulling model, it is suggested that Delta is endocytosed to generate a pulling force on the Notch receptor to facilitate activation. The recycling model suggests that Delta has to be internalized and processed through the endosomal pathway where it is recycled to the plasma membrane.

NECD has been observed in endosomes with Delta (Nichols et al., 2007; Parks et al., 2000; Parks et al., 1995). This result would be expected if the ligand is binding the receptor and pulling on it to separate the NECD, then being endocytosed together.

Another model is the recycling model, which suggests that ligand must first be internalized, processed through the endosomal pathway and then recycled to the plasma membrane before it can bind Notch and activate signaling. Rab11, a Rab GTPase that functions in trafficking of cargo through recycling endosomes, is required in some signaling cells to endocytose Delta and activate Notch signaling (Emery et al., 2005; Jafar-Nejad et al., 2005). Also, Delta may be present on the plasma membrane in a position where it is difficult to interact with the Notch receptor, it may need to be transcytosed to a different position where it an interact with Notch (Benhra et al., 2010; Rajan et al., 2009).

It could also be a combination of these two models, in that ligand present on the plasma membrane must first be internalized and recycled to the plasma membrane. Then, ligand can bind the Notch receptor and generate a pulling force to initiate cleavage and activate signaling. While the requirement of endocytosis in Notch signaling is not well understood, this dissertation will attempt to further clarify the role of endocytosis in Notch signaling.

1.3. ENDOCYTOSIS

Endocytosis, as mentioned above, is a process that results in the internalization of proteins and is required for many cellular functions, including Notch signaling (González-Gaitán, 2003). Endocytosis can occur in several ways, but this dissertation will focus on Clathrin-independent or Clathrin-dependent. Clathrin-independent endocytosis can encompass bulk endocytosis, macropinocytosis, caveolae-dependent

endocytosis, and Flotillin-dependent endocytosis (Doherty and McMahon, 2009; Kiss and Botos, 2009). Along with Clathrin, endocytosis may require other proteins to function in regulation. Also, adaptor proteins may be required to recognize cargo in both Clathrin-dependent or Clathrin-independent endocytosis. Adaptor proteins functioning during Clathrin-dependent endocytosis may be required to initiate membrane curvature, as well as facilitate interaction between Clathrin and cargo for formation and internalization of Clathrin-coated vesicles (Figure 1.5) (Doherty and McMahon, 2009). There is some evidence that adaptor proteins may be important for recognition of cargoes and targeting them for Clathrin-independent endocytosis (Sigismund et al., 2005).

Once a vesicle has been internalized, it is processed through the endosomal pathway, which can lead to recycling or degradation (Doherty and McMahon, 2009).

1.3.1. Clathrin-independent endocytosis

Clathrin-independent endocytosis occurs through caveolae, which in mammals are enriched in Caveolin1. Proteins and other structural components such as cholesterol are clustered at the caveolae. At the caveolae, the plasma membrane undergoes an initiation of curvature (Rothberg et al., 1992). This invagination continues to enlarge, but remains attached to the plasma membrane. The GTPase dynamin (*Drosophila* Shibire) is recruited to pinch off the vesicle (Henley et al., 1998). Once the vesicle is released from the plasma membrane, it is trafficked through the endosomal pathway (Figure 1.5) described in section 1.3.3. It is not clear whether *Drosophila* have caveolae, because a gene encoding Caveolin has yet to be identified. However, other known proteins integral to caveolae, Flotillin1 and Flotillin2, have been identified in flies (Bickel et al., 1997; Galbiati et al., 1998).

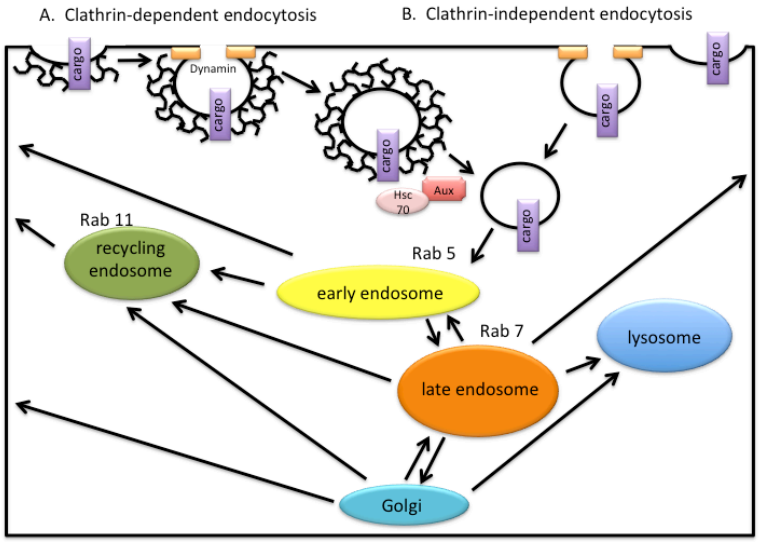

Figure 1.5. Clathrin-dependent, Clathrin-independent endocytosis and the endosomal pathway

(A) Clathrin-dependent endocytosis can occur to internalize a cargo. Initially a Clathrin-coated pit forms. Then, it continues to invaginate until it is pinched off by the GTPase Dynamin. Next, Auxilin and Hsc70 function to uncoat Clathrin-coated vesicles. Then the vesicle can fuse with the early endosome for sorting. (B) Clathrin-independent endocytosis can occur to internalize cargo. Caveolae form a curvature in the plasma membrane and it continues to invaginate until it is pinched off by dynamin. When cargo is internalized, it is trafficked to the early endosome where it is sorted and either processed through the recycling endosome or the late endosome. If cargo is sent to the late endosome, it can be sent on to the Golgi or to the lysosome for degradation. The vesicle can fuse with the early endosome for sorting. Rab GTPases function at different steps throughout the endosomal pathway. Rab5 functions at the early endosomal, while Rab7 functions in vesicle trafficking between early and late endosomes, and Rab11 functions at recycling endosomes [Modified from (Sorkin, 2000)].

This dissertation will focus on Clathrin-dependent Delta endocytosis, which is implicated in Notch activation in development of bristles (Hutterer and Knoblich, 2005), the wing (Wang and Struhl, 2004), and the eye (Banks et al., 2011). However, there is some evidence that Delta can be endocytosed in a Clathrin-independent manner in the developing oocyte. During oocyte development, the oocyte sends a Delta signal to activate Notch signaling in the surrounding follicle cells. The Notch target Hindsight (Hnt) is used as a read out of Notch signal activation. In a wild type oocyte, Hnt expression can be observed in the follicle cells with an anti-Hnt antibody. When *Clathrin* germline mutant clones were made, Hnt expression was still observed in the neighboring follicle cells. This result suggests that the oocyte is still able to send a Delta signal and activate Notch signaling. However, it was observed that when *Clathrin* mutant clones were generated in the follicle cells, Hnt expression was not observed in the neighboring follicle cells. This result suggests that Clathrin is required to receive the signal from the oocyte to activate Notch signaling (Windler and Bilder, 2010).

Bulk endocytosis involves a larger invagination of the plasma membrane than other vesicles and it occurs in a Clathrin-independent manner. Bulk endocytosis has been studied mostly at the synapse, but it is thought to serve several functions (Cousin, 2009). One possibility is to clear large amounts of membrane quickly, either as a way to quickly down regulate a signaling pathway or to reorganize the membrane components through internalization and recycling. Another possibility is to ensure endocytosis occurs even in the absence of the components necessary for Clathrin-dependent endocytosis (Grant and Donaldson, 2009). There is evidence that Delta is cleared from the membrane in the developing eye disc via bulk endocytosis. However, Delta internalized via bulk endocytosis does not appear to have a role in Notch signaling (Wang and Struhl, 2004).

1.3.2. Clathrin-dependent endocytosis

Clathrin-dependent endocytosis involves Clathrin and other adaptor proteins. Clathrin is composed of light and heavy chains that form a triskelion. Triskelia assemble into a lattice structure surrounding invaginations of the plasma membrane called Clathrin-coated pits (Figure 1.6) (Kirchhausen, 2000).

Clathrin cannot bind the plasma membrane directly and is not capable of binding cargoes directly (Kirchhausen 2000); so membrane-Clathrin and cargo-Clathrin interactions are facilitated cargo through accessory and adaptor proteins (Kirchhausen et al., 1997; Robinson, 1992). Examples of accessory proteins and Clathrin adaptors are Eps-15 (Benmerah et al., 1996), Adaptor Protein-180 (AP-180) or Clathrin Assembly Lymphoid Myeloid Leukemia protein (CALM) (Morris et al., 1990; Tebar et al., 1999), Epsin (Chen et al., 1998a) and Adaptor Protein-2 (AP-2) (Keen and Beck, 1989). The adaptor proteins, specifically Epsin and AP-2, will be discussed in greater detail in 1.4. Different cargoes may require specific combinations of the above listed accessory or adaptor proteins, as well as others, for Clathrin-mediated endocytosis.

Clathrin-mediated endocytosis is initiated when Clathrin is recruited to a pre-determined site on the plasma membrane. Clathrin is recruited by AP-2 and AP-180. AP-2 and AP-180 interact with the plasma membrane through binding phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) (Ford et al., 2001; Gaidarov et al., 1996; Rapoport et al., 1997; Rohde et al., 2002). $PI(4,5)\ P_2$ is an inositol lipid found at the plasma membrane that is required for Clathrin-coated endocytosis (Jost et al., 1998). Epsin will be described in more detail in 1.4.1., but it has been shown to be targeted to the plasma membrane via interactions with $PI(4,5)P_2$ (Itoh et al., 2001). Epsin also interacts with AP-2 and Clathrin (Chen et al., 1998a). One function of Epsin is to initiate membrane curvature (Ford et al., 2002). Epsin and AP-2 also interact with the EH-

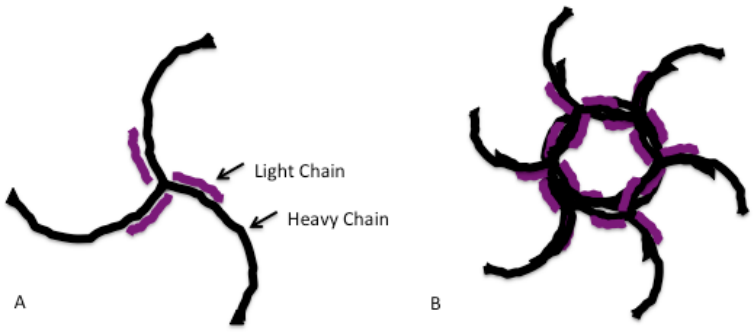

Figure 1.6. Clathrin Triskelion and Lattice

(A) Each Clathrin triskelion is composed of three Clathrin heavy chains and three Clathrin light chains. (B) These triskelia assemble into a lattice structure [modified from (Kirchhausen, 2000)].

domain containing protein Eps-15. Eps-15 may facilitate Clathrin rearrangement during Clathrin-coated pit formation, invagination, and ultimately scission of the Clathrin-coated vesicle (Chen et al., 1998a; Chen et al., 1999).

At full maturity of the invaginated vesicle, AP-2 recruits the GTPase Dynamin (*Drosophila* Shibire) (Wang et al., 1995), which binds the plasma membrane (Lin and Gilman, 1996; Zheng et al., 1996) and encircles the neck of the budding vesicle (Hinshaw and Schmid, 1995; Roux et al., 2006; Takei et al., 1995). Dynamin then facilitates scission of the Clathrin-coated vesicle from the plasma membrane (Figure 1.5) (Chen et al., 1991; De Camilli et al., 1995; van der Bliek and Meyerowitz, 1991). Actin polymerization may also be required to generate forces to dynamically form a vesicle (Giardini et al., 2003; Upadhyaya et al., 2003; Yarar et al., 2005). Endocytosis defects are observed in *actin* mutants (Kübler and Riezman, 1993), in mutants of Actin regulators such as *Arp2/3* (Moreau et al., 1997) and *BAR* (Itoh et al., 2005), and in mutants of the Actin based motor *myosin VI* (Buss et al., 2001).

Upon internalization the Clathrin-coated pit must be uncoated in order to fuse with endosomes (Helenius et al., 1983). Auxilin and the ATPase Heat Shock Cognate 70 (Hsc70) function in uncoating Clathrin-coated vesicles, generating uncoated vesicles and free Clathrin and other components used in generating the Clathrin coat (Figure 1.4, Figure 1.5) (Holstein et al., 1996; Jiang et al., 1997). Auxilin will be discussed in further detail in section 1.5.

1.3.3. Endosomal pathway

After internalization, uncoated vesicles first fuse with the early endosome and are sorted depending on the cargoes present in the vesicle. The cargo can be directed next to other intracellular compartments such as the recycling endosome, the trans Golgi

network, or to the late endosome also known as the multivesicular body (MVB). Cargoes are trafficked through the recycling endosome to be put back on the plasma membrane (Doherty and McMahon, 2009). There is some evidence that Delta present on the membrane is internalized and trafficked through the recycling endosome to be placed back on the plasma membrane (Emery et al., 2005; Jafar-Nejad et al., 2005).

If cargoes are sent to the trans Golgi network, further processing could occur. Then the cargo can be sent to several other locations in the cell, such as the recycling endosome, late endosome, early endosome, or directly back to the plasma membrane (Doherty and McMahon, 2009). Though Fringe has only been shown to function on modification of the Notch receptor, there is some *in vitro* evidence that Delta could be modified in a similar manner (Panin et al., 2002). As Fringe and similar proteins function in the Golgi, perhaps it is necessary for Delta to be internalized and processed through the Golgi to activate Delta, which can then be trafficked to the plasma membrane to interact with Notch.

If cargoes are sent to the late endosome, they can be processed and sent back to the plasma membrane. As a cargo progresses from endosome to endosome, it encounters increasingly acidic environments. For example, the late endosome is more acidic than the early endosome. This acidification can also play a role in modification of particular cargoes (Mellman et al., 1986). There is some evidence that as Delta is endocytosed it is internalized with the NECD of the Notch receptor still attached to the extracellular domain of Delta (Nichols et al., 2007; Parks et al., 2000; Parks et al., 1995). Once Delta is trafficked to the late endosome, perhaps the acidic environment facilitates removal of the NECD. Delta could then trafficked back to the plasma membrane where it can interact with a different Notch receptor. A cargo may be targeted to the late endosome to be trafficked to the lysosome where it is degraded (Figure 1.4) (Doherty and McMahon,

2009). Specific proteins present, such as Rabs, can identify each of the endosomal compartments to which cargoes can be trafficked (Zhang et al., 2007).

1.3.4.1. Rab GTPases

The endosomal pathway is marked with several different families of proteins that are important for establishment and maintenance of the endosomes. Rabs are members of the Ras-like GTPase family of proteins that function in vesicle trafficking with in the cell. While vertebrates have more than 75 identified Rabs, *Drosophila* have 33 identified Rabs (Zhang et al., 2007). Each Rab tends to be localized to specific endosomes, which allows them to be used as markers for each of the endosomes.

However, some Rabs have been shown to mark more than one endosome type. Early endosomes are typically identified by the presence of proteins such as Rab5, which functions in the fusion of recently endocytosed vesicles with the early endosome (Figure 1.5). Rab5 has also been thought to localize to the lysosome (Hirota et al., 2007). Late endosomes are typically identified by the presence of Rab7, which functions in vesicular trafficking from early to late endosomes (Feng et al., 1995). Recycling endosomes are typically identified by the presence of Rab 11, which facilitates trafficking of vesicles from the early endosome through a recycling endosome and to the plasma membrane (Stenmark, 2009). Rab11 has also been thought to function in trafficking from the Golgi (Chen et al., 1998b). While there is some indication that Rabs may play a role in Notch signaling (Emery et al., 2005; Jafar-Nejad et al., 2005; Windler and Bilder, 2010), their role during *Drosophila* eye development is as yet not understood.

1.4. ADAPTOR PROTEINS

As mentioned previously, it is sometimes necessary to facilitate interaction between Clathrin and cargoes to be endocytosed. This interaction occurs through adaptor

proteins that have domains or motifs to bind the cargo and other domains or motifs to recruit and bind Clathrin (Kirchhausen, 2000). The adaptor protein Epsin has been shown to function in Clathrin-dependent endocytosis (Chen et al., 1998a). Epsin is also required for Delta endocytosis to activate Notch signaling (Figure 1.4) (Overstreet et al., 2004; Wang and Struhl, 2004). Therefore, it is possible that Epsin could be functioning to endocytose Delta in a Clathrin-dependent manner to activate Notch signaling. However, there is some evidence that Epsin-dependent Clathrin-independent endocytosis of Delta can activate Notch signaling in the developing *Drosophila melanogaster* oocyte (Windler and Bilder, 2010). If Delta is endocytosed in a Clathrin-dependent manner, it is also possible that other adaptor proteins known to be required for Clathrin-coat formation, such as AP-2 may be involved as well. However, the role of AP-2 during Notch signaling in the developing *Drosophila* eye is not currently understood.

1.4.1. Epsin structure, function, and regulation

The *liquid facets* (*lqf*) gene in *Drosophila* encodes the protein Epsin. It is homologous to vertebrate Epsin (Cadavid et al., 2000). Epsin was identified based on its interaction with the EH-domain containing protein Eps15 (Chen et al., 1998a). Epsin is composed of an Epsin N-Terminal Homology (ENTH_ domain and several identified motifs (Figure 1.7). The ENTH domain is at the N-terminus of the protein and is the only portion of the protein with a higher order structure. The ENTH domain facilitates plasma membrane localization through interaction with $PI(4,5)P_2$ (Aguilar et al., 2003; De Camilli et al., 2002). It is thought that the ENTH domain not only interacts with the plasma membrane, but can also insert itself into the plasma membrane and initiate curvature. The initiation of curvature is a crucial step in order for endocytosis to occur (Ford et al., 2002).

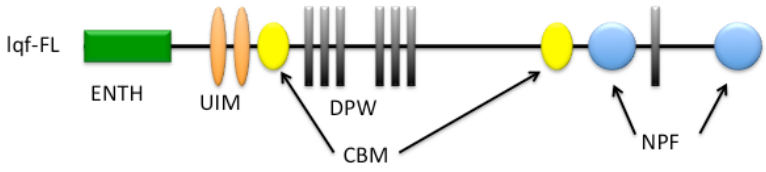

Figure 1.7. Structure of Epsin

Epsin contains an ENTH domain, two UIM motifs, two CBM motifs, seven DPW motifs, and two NPF motifs [Modified from (Overstreet et al., 2003)].

Epsin recognizes ubiquitinated cargoes, such as Delta, and functions to internalize the cargoes (Overstreet et al., 2003; Wang and Struhl, 2004). The motif on Epsin that recognizes ubiquitin is the Ubiquitin Interacting Motif (UIM) (Polo et al., 2002). Epsin has two UIMs, and experiments in our laboratory have shown that the UIMs are essential for Epsin function during Notch signaling (Figure 1.7). However, having just one UIM is sufficient for Epsin function during Notch signaling (Xie et al., 2012). Epsin could be functioning to recognize ubiquitinated cargoes via the UIMs to facilitate internalization and activation. To function as a Clathrin adaptor to endocytose Delta, Epsin not only needs to bind the ubiquitinated Delta cargo, it also needs to bind and Clathrin. Epsin has three possible motifs that function in Clathrin recruitment. One is a direct interaction with Clathrin via the two Clathrin Binding Motifs (CBMs) (Figure 1.7). However, the CBMs have been shown to be non-essential for Epsin function during Notch signaling (Xie et al., 2012). This result could suggest that either Epsin is not functioning as a Clathrin-adaptor, or that it is recruiting Clathrin via indirect interactions at other motifs.

A second way Epsin could recruit Clathrin is indirectly via another adaptor protein, AP-2. AP-2 will be discussed further in the next section, but it is an adaptor protein that binds Clathrin and recruits it to sites of Clathrin-coated vesicle formation (Keen and Beck, 1989). AP-2 is thought to interact with Epsin via DPW motifs of which it has seven (Figure 1.7) (De Camilli et al., 2002). It is also possible that Epsin is recruiting Clathrin indirectly via the two NPF motifs. The NPF motifs interact with the EH-domain containing protein Eps-15 (Morgan, 2003; Salcini et al., 1997). Eps-15 can bind AP-2, which then recruits Clathrin for Clathrin-dependent endocytosis (Benmerah et al., 1996).

Overexpression of Epsin results in a rough eye due to aberrant photoreceptor number and pattern (Eun et al., 2007). *lqf* (*Drosophila melanogaster epsin)* loss of

function mutations result a rough eye due to aberrant photoreceptor number and pattern (Cadavid et al., 2000). In *lqf* mutants this defect is tied specifically to a failure of Notch signaling (Overstreet et al., 2003; Wang and Struhl, 2004).

1.4.1.1. Epsin regulation via the deubiquitinating enzyme fat facets

Epsin is regulated via ubiquitination. Ubiquitination of Epsin either renders it non-functional or targets it for degradation (Figure 1.8). The ubiquitin ligase responsible for ubiquitination of Epsin is currently unknown. However, the deubiquitinating enzyme Fat Facets (Faf) functions to remove the ubiquitins on Epsin (Figure 1.8). Loss of Faf leads to an increase in ubiquitinated Epsin (Chen, 2002) resulting in a rough eye. The *faf* loss of function mutation results in an eye defect that is similar to that of a *lqf* mutant eye defect (Cadavid et al., 2000).

1.4.2. AP-2 structure and function

Though Epsin is important for recognizing ubiquitinated Delta and targeting it for internalization, there are other adaptor proteins (APs) that function during Clathrin-dependent endocytosis. There are five different hetero-tetramer AP complexes that have been identified. Each one functions in a different capacity and is targeted to different membranes within the cell (Hirst et al., 2011). AP-1 is found at the trans-Golgi network (TGN) (Doray et al., 2002; Robinson et al., 2010). AP-3 and AP-4 are found at the TGN and may have roles in trafficking to endosomes (Burgos et al., 2010; Dell'Angelica et al., 1997; Hirst and Robinson, 1998; Simpson et al., 1997). AP-5 plays a role in endosomal trafficking (Hirst et al., 2011). AP-2 functions as a Clathrin adaptor during Clathrin-dependent endocytosis at the plasma membrane (Beck and Keen, 1991; Keen, 1987; Keen and Beck, 1989). AP-2 consists of α, $\beta 2$, $\sigma 2$, and $\mu 2$ subunits (Figure 1.9) (Hirst and Robinson, 1998; Kirchhausen, 1999).

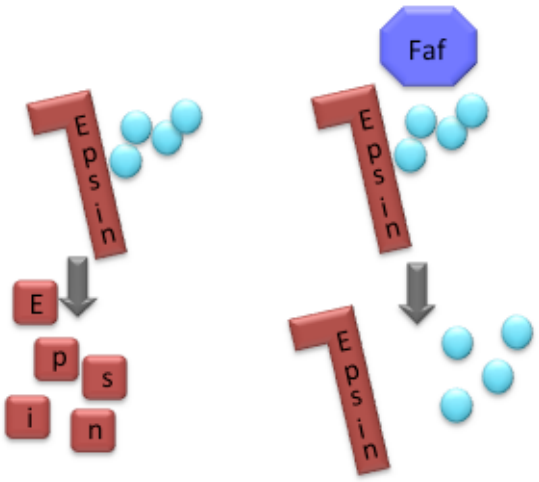

Figure 1.8. Epsin regulation by ubiquitination and Faf

Epsin and be rendered non-functional or degraded when it is ubiquitinated. Faf, a deubiquitinating enzyme removes the ubiquitins and frees Epsin to function in the cell.

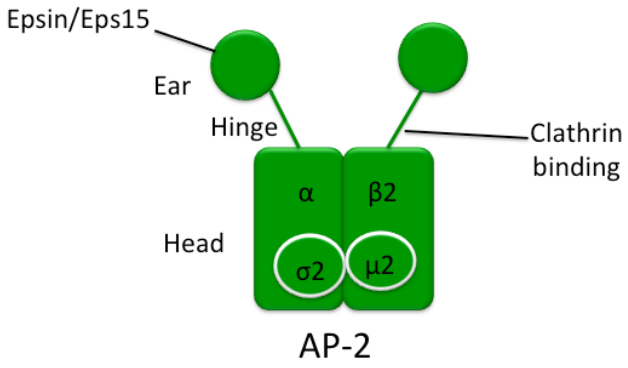

Figure 1.9. AP-2 structure

AP-2 is a heterotetramer composed of an α subunit, a β2 subunit, a σ2 subunit, and a μ2 subunit. The α subunit is known as α-adaptin. α-adaptin consists of a head region, a hinge region and an ear region. The ear region of α-adaptin binds other adaptor proteins such as Epsin and Eps15. The β2 subunit, also known as β adaptin, has been shown to bind Clathrin [Modified from (Hirst et al., 2011)].

The α subunit is known as α-adaptin and the protein is composed of an N-terminal head region connected by a hinge or linker to a C-terminal ear region (Heuser and Keen, 1988; Owen et al., 1999) (Figure 1.9). The ear region is important for interaction with Epsin, Eps-15, and other proteins important for Clathrin-coated vesicle formation and scission (Benmerah et al., 1996; Benmerah et al., 1998; Chen et al., 1998a; Owen et al., 1999; Slepnev and De Camilli, 2000). The head region is important for binding $PI(4,5)P_2$ at the plasma membrane (Beck and Keen, 1991). The β subunit, known as β-adaptin, is important for AP-2 interaction with Clathrin (Gallusser and Kirchhausen, 1993; Shih et al., 1995). The σ and μ subunits are important for cargo recognition and targeting to the plasma membrane via interaction with $PI(4,5)P_2$ (Owen et al., 2004). The role that AP-2 plays during Notch signaling in the developing *Drosophila* eye is as yet unknown. As Clathrin-dependent endocytosis is required for Notch signaling, it would be plausible for AP-2 to be important. However, previous experiments have failed to identify a role for AP-2 during Notch signaling or have been inconclusive.

1.5. AUXILIN AND UNCOATING

That Clathrin-dependent endocytosis of Delta is required for Notch signaling is clear, but it not fully understood why. One possible reason for Delta to be endocytosed is to process it through the endosomal pathway and recycle it back to the plasma membrane. In order to be processed through the endosomal pathway, a Clathrin-coated vesicle must be uncoated. Auxilin functions with Hsc70 to uncoat Clathrin-coated vesicles (Holstein et al., 1996; Jiang et al., 1997; Lemmon, 2001), may function in chaperoning Clathrin to inhibit aggregation and in rearrangement of Clathrin on forming vesicles at the plasma membrane (Eisenberg and Greene, 2007).

1.5.1. Auxilin structure and function

Auxilin was first isolated from bovine brain tissue. Auxilin was associated with Clathrin-coated vesicles. Mammals and zebrafish (Bai et al., 2010) have two *auxilin* genes, neuron specific *auxilin1* (Ahle and Ungewickell, 1990) and ubiquitously expressed *auxilin 2* or *cyclin-G Associated Kinase* (*GAK*) (Kanaoka et al., 1997; Kimura et al., 1997). Cyclin-G is a cell cycle regulator that is a target of the known tumor suppressor p53 (Okamoto and Beach, 1994), but the role of GAK with Cyclin-G is as yet undetermined. Only one *auxilin* has been identified in *S. cerevisiae* (Gall et al., 2000), *C. elegans* (Greener et al., 2001), and *Drosophila,* which is homologous to mammalian *GAK* (Chang, 2004; Eun et al., 2007; Hagedorn, 2006). Like GAK, *Drosophila* Auxilin has a protein kinase domain, a Phosphatase and tensin homolog (PTEN)-like phosphatase domain, a Clathrin binding domain and a DNA-J domain. Auxilins present in other species contain different combinations of these domains, however, they all contain at least the Clathrin binding domain and the DNA-J domain (Lemmon, 2001). The function of the protein kinase and the PTEN-like domain is unclear, but the kinase domain is important for Notch signaling during neurogenesis in zebrafish (Bai et al., 2010). The PTEN-like domain has yet to be studied, but it lacks key conserved residues required to function as a phosphatase (Greener et al., 2000). When cDNA transgenes containing only specific Auxilin domains were expressed in an *auxilin* mutant background, the Clathrin-binding and DNA-J domain together were sufficient for Auxilin function (Eun et al., 2008). Auxilin binds Hsc70 via the DNA-J domain (Figure 1.10) (Greener et al., 2000; Holstein et al., 1996; Umeda, 2000).

Hsc70 is an ATPase that facilitates uncoating (Chappell et al., 1986; Ungewickell, 1985). However, Hsc70 cannot bind directly, so it binds to Auxilin via the DNA-J domain and Auxilin binds Clathrin through its Clathrin-binding domain (Greener et al.,

2000; Holstein et al., 1996; Umeda, 2000). Clathrin-heavy chain, but not Clathrin-light chain, is essential for uncoating Clathrin-coated vesicles (Ungewickell et al., 1995). Auxilin binds Clathrin-heavy chain at the intersection between two Clathrin triskelia and recruits Hsc70. This binding induces a conformational change initiating destabilization of the lattice, leading to uncoating (Fotin et al., 2004).

1.5.2. *auxilin* loss of function mutations dominantly enhance the mutant eye defect caused by Epsin overexpression.

A screen was performed to identify modifiers of an Epsin overexpression eye defect. Enhancers isolated from the screen include loss of function mutations in *auxilin*, *Clathrin-heavy chain*, *Ral*, and *Spen*. Auxilin and Clathrin heavy chain appear to positively regulate Notch signaling, while Ral and Spen appear to negatively regulate Notch signaling (Eun et al., 2007). Auxilin could provide more information about regulation of Delta endocytosis. This dissertation will discuss the role of Auxilin during Delta endocytosis to activate Notch signaling.

Figure 1.10. *Drosophila* Auxilin structure

Drosophila Auxilin, like mammalian GAK, consists of a protein kinase domain, a PTEN-like domain, a Clathrin binding domain and a DNA-J domain [Modified from (Eun et al., 2008)].

Chapter 2: Auxilin functions during the endocytosis of some Delta

2.1 INTRODUCTION

auxilin loss of function mutations were identified as enhancers of Epsin overexpression rough eyes (Eun et al., 2007). Auxilin is known to function with Hsc70 in uncoating Clathrin-coated vesicles (Fotin et al., 2004; Greener et al., 2000; Ungewickell, 1985). Experiments were performed to characterize the role Auxilin may be playing during Notch signaling. These experiments, performed by myself and others, were published (Eun et al., 2008) and most were included in S.H. Eun's dissertation. A summary of the experiments performed by others will be presented in the introduction to this chapter. However, the experiments performed by myself will be described in the results section of this chapter.

2.1.1. Drosophila 3rd instar eye disc development

The developing *Drosophila* eye was used to determine if Auxilin has a role in Notch signaling. *Drosophila* larvae have imaginal discs for legs, wings, antennae, eyes, etc. These imaginal discs undergo a morphological change during pupation from a monolayer of epithelial cells to a fully formed appendage or organ (Wolff and Ready, 1993). Imaginal disc development involves signaling through many pathways such as decapentaplegic (Dpp) (Heberlein et al., 1993; Spencer et al., 1982), hedgehog (Hh) (Heberlein et al., 1993), epidermal growth factor (EGFR) (Domínguez and de Celis, 1998) and Notch signaling (Fehon et al., 1991). The third instar eye disc was used to study early Notch signaling events. Initially, cells in the eye disc go through many rounds of division. Next, a wave of morphogenetic signaling called the morphogenetic furrow travels from the posterior to the anterior of the eye disc initiating differentiation (Figure 2.0.a).

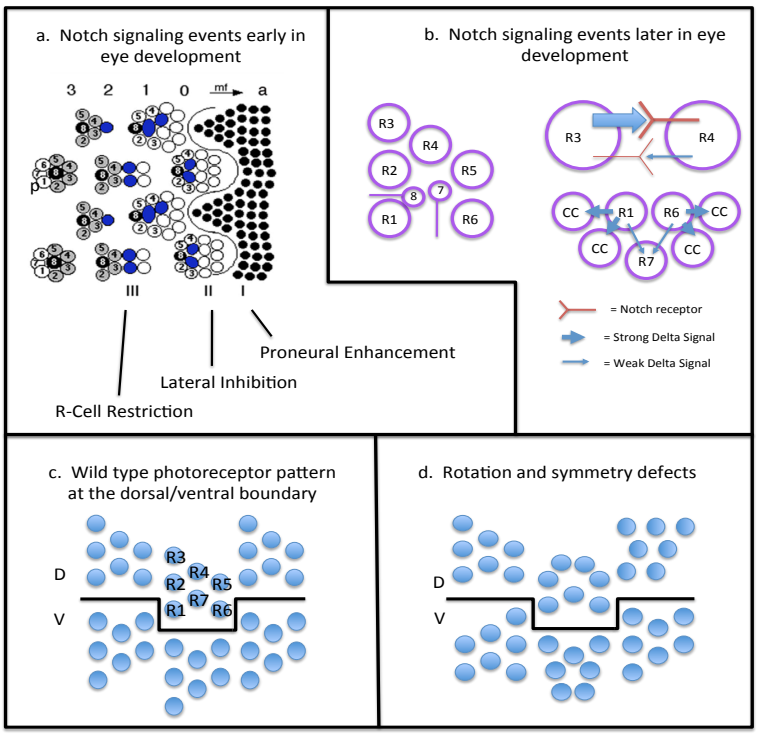

Figure 2.0. Notch signaling events during photoreceptor differentiation

(a) During *Drosophila* eye development, the morphogenetic furrow moves through the eye disc from the posterior to the anterior. A Notch signaling event at the furrow called proneural enhancement increases the number of proneural cells. A couple of rows posterior to the furrow, a Notch signaling event called lateral inhibition establishes the patterning and inhibits R-cell recruitment into the ommatidium. Next, a Notch signaling event called R-cell restriction further limits the number of photoreceptors recruited to the ommatidium to 8. (b) During photoreceptor differentiation the R8 cell is first recruited into the ommatidium, followed by recruitment of R2/R5, R3/R4, and finally R1/R6/R7. Following recruitment to the ommatidium Notch signaling events further differentiate the photoreceptors into specific R-cells as with R3/R4 and R1/R6/R7 cells. R1/R6/R7 also participate in differentiation of the cone cells (cc). (c) Wild type orientation of photoreceptors within an ommatidium. Along the dorsal (D) /ventral (V) border, the photoreceptors are organized into mirror image orientation. (d) When Notch signaling defects can result in rotation defects in the photoreceptors, depicted in the D/V pairs of ommatidia on the left and right. Symmetry defects occur when R3 and R4 do not differentiate from one another. These defects are observed in the middle ommatidia. [modified from (Eun et al., 2008), (Cho and Fischer, 2011)].

Posterior to the morphogenetic furrow, a second wave of mitosis occurs, but any further divisions are perturbed. Eight neural photoreceptors are recruited into each ommatidium beginning with photoreceptor R8, followed by photoreceptors R2/R5, then photoreceptors R3/R4 and finally photoreceptors R1/R6/R7. The photoreceptors are positioned in specific locations that allows for them to be identified based on their location within the ommatidium. However, in adult eye sections, it is not always possible to observe R8 as it lies beneath R7 (Figure 2.0.b) (Wolff and Ready, 1993).

Notch signaling events occur several times during development and each signaling event has a distinct defect observed when Notch signaling fails. These distinct Notch signaling events can provide a read-out to determine when Notch signaling fails. Proneural enhancement is a Notch signaling event that occurs at the morphogenetic furrow. Notch signaling for proneural enhancement increases the pool of neural progenitor cells present. If Notch signaling fails at this step, it results in a complete lack of photoreceptors present in the eye. After the morphogenetic furrow has progressed anteriorly, another Notch signaling event begins to inhibit the equivalent proneural cells from being recruited to the ommatidia. This event is called lateral inhibition and if Notch signaling fails at this step, it results in a general disorganization of the photoreceptors and extra photoreceptors being present within each ommatidium (Fehon et al., 1991; Li and Baker, 2001; Ligoxygakis et al., 1998). A few rows posterior to the furrow, a third Notch signaling event called R-cell restriction occurs. R-cell restriction further restricts the number of photoreceptors recruited into an ommatidium. If R-cell restriction fails, photoreceptors are organized into distinct ommatidial clusters, but extra photoreceptors are present (Figure 2.0.a) (Overstreet et al., 2004).

The eye disc is divided into a dorsal half and a ventral half. Within each half, the photoreceptors in each ommatidium are organized in a specific orientation. On the dorsal

half, the R3 cell in each ommatidium positioned towards the dorsal part of the eye and in the ventral half a mirror image of the photoreceptors has the R3 positioned towards the ventral side. At the border this is a natural line with one orientation of photoreceptors on one side and a mirror image orientation of photoreceptors on the other side. When Notch signaling fails late in photoreceptor differentiation, it can result in orientation changes (Figure 2.0.c). Though the final patterning of the photoreceptors in the adult eye is asymmetrical, sometimes Notch signaling can fail at R3/R4 differentiation, resulting in photoreceptors within ommatidia to appear symmetrical (Figure 2.0.d) (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Wolff and Ready, 1993).

2.1.2. Generating clones in developing *Drosophila* eye discs

Many proteins important during eye development are pleiotropic. When those genes are mutated, it can result in embryonic lethality, making it impossible to look at later stages of development. Using the mitotic recombination *Flipase (FLP)/ Flipase Recombination Target (FRT)* system, it is possible to generate flies that are viable as a heterozygote for a mutant allele, but have clones that are homozygous for the mutation. (Figure 2.1).

The mutation of interest is recombined onto a chromosome arm with a specific FRT. Another chromosome is generated that contains the same specific FRT and a cell marker of some sort, often lacZ or *GFP*. These two chromosomes are crossed into a fly that is expressing a spatially or temporally regulated FLP. The FLP induces recombination at the FRT, which results in homozygosing the chromosome arm with the FRT. Following mitosis, three possible cells can result. If recombination did not occur, then a cell will be heterozygous for the mutation and express GFP. If recombination did occur and the inherited recombinant chromosome contains the *ubi-GFP* transgene, then

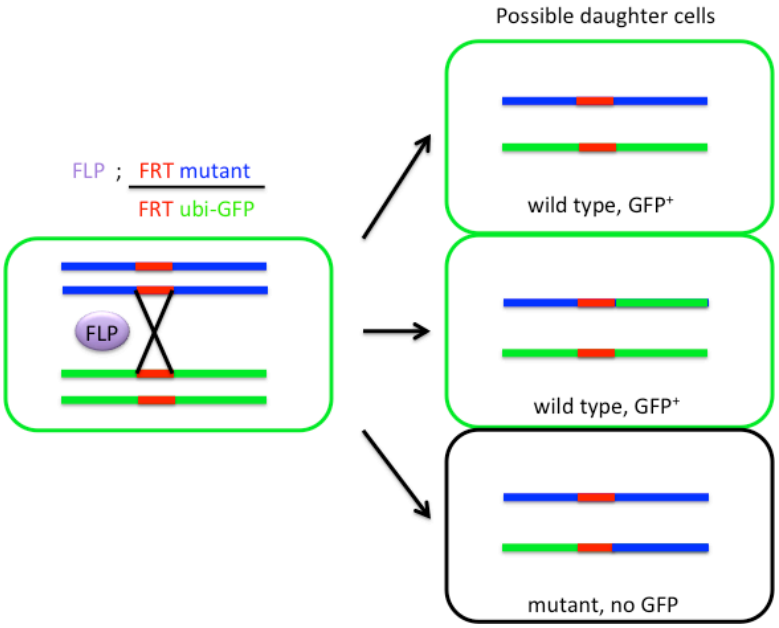

Figure 2.1. FLP/FRT mitotic recombination

A source of FLP is expressed in a cell that contains two chromosomes. Each contains the same FRT (indicated by red line), but one has a mutation on the same arm with the FRT and the other has a *ubi-GFP* transgene on the same arm with the FRT. FLP induces recombination at the FRT and mitosis results in the generation of three possible daughter cells. One possible type of daughter cell has chromosomes that have undergone no recombination. A second possible type of daughter cell has one non-recombinant GFP-containing chromosome and one recombinant GFP-containing chromosome. A third possible type of daughter cell has one non-recombinant mutation-containing chromosome and one recombinant mutation-containing chromosome.

that chromosome arm is homozygosed. The cells will express GFP, but will be wild type for the mutation. Sometimes these cells that are expressing two copies of GFP can be distinguished from the heterozygote cells that only have one copy of *ubi-GFP*. If recombination did occur and the inherited recombinant chromosome contains the mutation, then that chromosome arm is homozygosed. The cells will be identifiable due to the lack of GFP and will be homozygous for the mutation. It is also possible to use the FLP/FRT system with the cell marker present on the same arm as the mutation, such that any cells that contain the mutation are distinguishable due to marker expression and any cells that do not express the marker are wild type for the gene (Dang and Perrimon, 1992; Xu and Rubin, 1993).

2.1.3. Auxilin functions during eye development in signal-sending cells to activate Notch signaling

Experiments were performed to determine how Auxilin functions during Notch signaling during photoreceptor differentiation.

2.1.3.1. *auxilin* loss of function mutations result in aberrant photoreceptor numbering and patterning

Several *auxilin* mutations were isolated in the screen; two mutations were missense mutations and nine were nonsense mutations (Figure 2.2).

auxilin null and hypomorphic mutant flies generated by combinations of these alleles were completely rescued by either a *gaux*$^{+}$ or an *aux* cDNA transgenes. The eyes of adult flies overexpressing *lqf* along with one copy of a loss of function *auxilin* mutation were dissected, fixed, embedded and sectioned to observe photoreceptors. The enhancement observed was due to an increase in aberrant photoreceptor number and patterning (Eun et al., 2007). Therefore, Auxilin may be functioning in a pathway in the

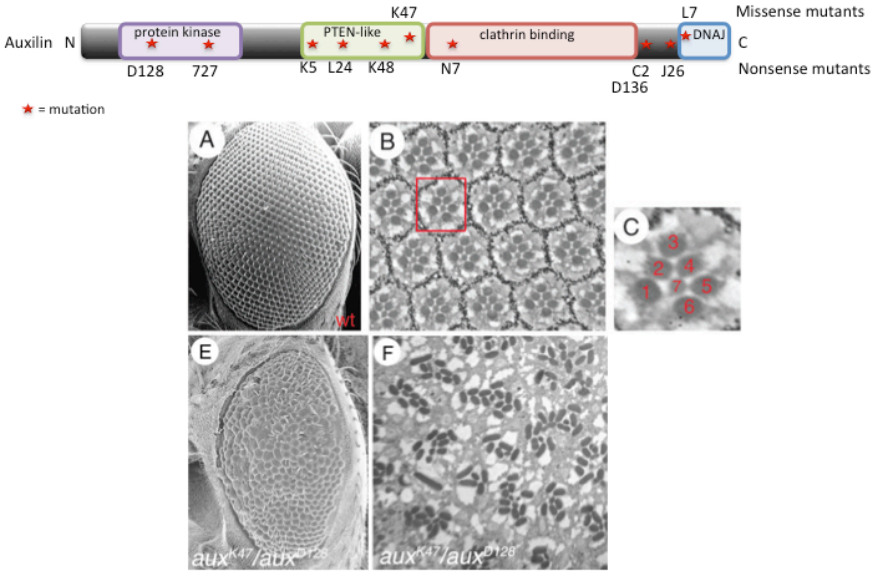

Figure 2.2. *auxilin* hypomorphic mutants have an eye defect

Locations of the *auxilin* missense and nonsense mutations isolated from the screen. (A) Scanning Electron Micrograph (SEM) of a wild type external eye. (B) Tangential section of a wild type eye shows wild type photoreceptor patterning. Red box is shown in close up (C) indicates the photoreceptors visible and their position within the facet. (E) SEM of $auxilin^{K47}/auxilin^{D128}$ mutant rough adult eye. (F) Tangential section of a $auxilin^{K47}/auxilin^{D128}$ eye shows aberrant photoreceptor number and pattern [Modified from (Eun et al., 2007)].

same direction as Epsin during photoreceptor development during Notch signaling. Experiments were performed to test this hypothesis.

auxilin loss of function mutations enhance the Epsin overexpression photoreceptor defects. However, it must be determined if *auxilin* mutations result in a rough eye defect when not combined with Epsin overexpression. Flies were generated using combinations of the isolated mutations that most likely generate a strong hypomorphic *auxilin* mutant, $auxilin^{K47}/auxilin^{D128}$. The $auxilin^{K47}/auxilin^{D128}$ mutants have a rough eye compared to wild type (Figure 2.2. A, E). It was observed that $auxilin^{K47}/auxilin^{D128}$ mutants have aberrant photoreceptor number and patterning (Figure 2.2. B, F). These defects are observed in both the adult eye and larval eye disc stages, suggesting Auxilin is important throughout photoreceptor differentiation.

2.1.3.2. Delta transcription is increased at the furrow in *auxilin* mutants

Auxilin is important in photoreceptor differentiation, but its role in Notch signaling is not known. When Notch signaling fails in the developing eye disc, there is an increase of Delta transcription observed at the morphogenetic furrow (Wang and Struhl, 2004). To better understand how Auxilin might be functioning in the Notch pathway in developing eye discs, Delta transcription expression was observed using a *Delta-lacZ* reporter transgene. An increase in Delta transcript expression was also observed in *auxilin* mutants, suggesting Auxilin is playing a role in Notch signaling.

2.1.3.3. Auxilin is required in signal-sending cells during Notch signaling in the developing Drosophila eye

Auxilin could be functioning in Notch signal-receiving cells, signal-receiving cells, or both. To determine the cell type in which Auxilin is functioning, strong alleles

of *auxilin* (*auxilin*^{727}/*auxilin*^{D128}) were used to generate mutant clones. Expression of E(spl), a downstream target of Notch, was observed by antibody staining and served as a readout of Notch activation. If Auxilin is functioning solely in signal-receiving cells, then it will not be possible for *auxilin*^{727}/*auxilin*^{D128} mutant cells to receive a signal from their wild type neighbors and activate Notch signaling. Therefore, inside the mutant clone, there will not be any Notch activation readout observed. If Auxilin is functioning solely in signal-sending cells, then it will be possible for *auxilin*^{727}/*auxilin*^{D128} mutant cells along the border of the mutant clone to receiving a Notch signal from their wild type neighbors, but they will be unable to send the signal to their neighboring mutant cells in the middle of the clone. Notch activation was observed in *auxilin*^{727}/*auxilin*^{D128} mutant cells along the border of the clone, but a lack of E(spl) staining in the middle of the clone suggested Notch was not activated in those cells. This result suggests that *auxilin*^{727}/*auxilin*^{D128} mutant cells are capable of receiving a signal from their wild type neighbors, but cannot send a signal to their neighboring *auxilin*^{727}/*auxilin*^{D128} mutant cells. Therefore, Auxilin is functioning in signal-sending cells to activate Notch signaling.

2.1.3.4. The Clathrin-binding domain and DNA-J domain together alone are sufficient for Auxilin function

Auxilin contains four known domains. If certain domains are required, but others are dispensable, it may provide some information regarding the function of Auxilin in signal-sending cells. Structure/function analysis was performed to determine which domains of Auxilin are required for its function. *auxilin* mutant flies, *auxilin*^{727}/*auxilin*^{D128}, that are strong loss of function possibly null alleles, were used for these experiments. Genomic and cDNA constructs were generated of full-length *auxilin*, deletion of the Clathrin binding and DNA-J domains, deletion of the kinase and PTEN-like domains, and just the DNA-J domain deleted. Transgenic flies were generated and

each transgene was expressed in *auxilin*^{727}/*auxilin*^{D128} mutant flies. The *auxilin* mutant flies are lethal and failed to be rescued by expression of the kinase and PTEN-like domains deletion transgene, or the DNA-J domain only deletion transgene. However, expression of full-length Auxilin and the Auxilin transgene that consists of only the portion of the protein containing the Clathrin-binding domain and the DNA-J domain resulted in complete rescue of lethality and the eye defect. This result suggests that the Clathrin-binding domain and the DNA-J domain together are sufficient for Auxilin function in the fly. As these domains are important for binding Clathrin and Hsc70 during uncoating, it further suggests Auxilin may be functioning in uncoating Clathrin-coated vesicles.

2.1.3.5. Chc overexpression partially rescues *auxilin* hypomorphic mutants

The same screen that identified *auxilin* loss of function mutations as enhancers of an Epsin overexpression eye defect also identified loss of function mutations in *Clathrin heavy chain* (*Chc*) as enhancers. It suggests Clathrin-dependent endocytosis is important during Notch signaling and that Auxilin functions to uncoat Clathrin-coated vesicles. If Auxilin is failing to efficiently uncoat Clathrin-coated vesicles, it is possible that Clathrin is remaining on the vesicles. If Clathrin is not removed via uncoating, then the level of Clathrin in the cell to be used in Clathrin-dependent endocytosis could be reduced. Therefore, it was hypothesized that overexpressing Chc in an *auxilin* mutant could bypass the need for Auxilin to uncoat vesicles and generate free Clathrin. If Clathrin is necessary for Notch signaling and was limiting in *auxilin* mutants, then it is possible that adding extra Clathrin could rescue the Notch-like eye defects observed in *auxilin* mutants.

Chc was used specifically for this experiment as opposed to Clc or Chc and Clc expressed together, because Auxilin targets Chc for uncoating (Fotin et al., 2004; Heymann, 2005; Lindner and Ungewickell, 1991). The full-length genomic *Chc (gChc$^+$)* transgene completely rescues *Chc* null flies (Bazinet et al., 1993). Overexpression of g*Chc*$^+$ did rescue the Notch-like eye defects in *auxilin*^{k47}/*auxilin*^{D128} hypomorphic mutants, but not completely. It is possible that one copy of the *gChc*$^+$ transgene was not overexpressing Chc at a level high enough to overcome a certain threshold level of free Chc needed in the cell. Flies were generated containing multiple copies of the g*Chc*$^+$ transgene in an *auxilin* mutant background. While rescue was observed, it still did not completely rescue the *auxilin* hypomorphic mutant, suggesting either the level of Chc present was still not enough to completely overcome the *auxilin* mutant eye defect or there could be other components functioning during Clathrin-dependent endocytosis that are also limiting when Auxilin is not efficiently uncoating Clathrin-coated vesicles.

2.2 RESULTS

Auxilin has a function in signal-sending cells to activate Notch signaling. However, what role Auxilin plays within the signal-sending cell is not understood. Delta must be endocytosed into the signal-sending cell to activate Notch. Auxilin may be functioning in signal-sending cells to uncoat Clathrin-coated Delta-containing vesicles. If Auxilin is functioning in this capacity, then Delta protein expression and localization may be abnormal in *auxilin* mutants.

2.2.1. *auxilin* mutants show a Delta endocytosis defect

Eye discs were dissected and stained with an anti-Delta antibody to observe Delta localization (Overstreet et al., 2003; Qi, 1999). In wild type discs, Delta is not observed at the furrow, but is observed in intracellular puncta posterior to the furrow (Figure 2.3).

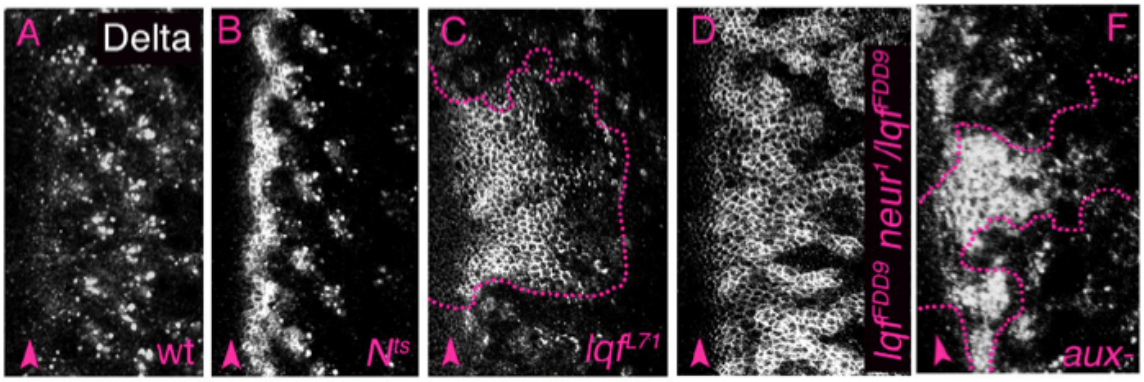

Figure 2.3. Delta trafficking in 3rd instar eye discs

Delta protein is observed in 3rd instar eye discs using an anti-Delta antibody. (A) In wild type eye discs, Delta cannot be observed at the morphogenetic furrow (pink arrowhead), but it is observed in intracellular puncta posterior to the furrow. (B) In *Notch*^{ts} mutants, Delta expression is increased and Delta is observed at the furrow on the plasma membrane. However, it is cleared into intracellular puncta posterior to the furrow. Clones for (C) *lqf* strong loss of function mutants show not only an increase of Delta at the furrow, but Delta remains on the plasma membrane for several rows posterior to the furrow. (D) Weak *lqf* mutants in combination with one copy of a strong loss of function *neur* mutant results in an increase of Delta at the furrow and perdurance on the plasma membrane longer than just *lqf* mutants alone. (F) *aux* strong loss of function mutants show not only an increase of Delta at the furrow, but Delta remains on the plasma membrane for several rows posterior to the furrow.

These intracellular puncta suggest that Delta is internalized. In the eye disc, when Notch signaling is perturbed, it leads to an increase of Delta expression and protein at the furrow. Delta is present not only in intracellular puncta, but is observed on the plasma membrane at the furrow. This increase is observed in *Notch*^{ts} mutant eye discs (Figure 2.3. B). Delta expression is higher, but is internalized posterior to the furrow. A slight perdurance may occur, but that could be due to the larger volume of Delta present on the membrane needing longer to be removed via endocytosis. Epsin functions in Delta internalization to activate Notch signaling. Strong alleles of *lqf* in are lethal in combination with other *lqf* alleles, so it is necessary to generate clones to make observations of Delta internalization. Clones were generated in flies using eye specific expression of Flipase (*eyFLP)* to induce mitotic recombination between an FRT chromosome containing a *lqf* allele and an FRT chromosome containing ubi-GFP. The clones are identified by presence of GFP (wild type cells) and absence of GFP (mutant cells). When clones of strong *lqf* mutants are generated in the developing eye disc, there is excess Delta observed at the furrow due to perturbed Notch signaling (Figure 2.3. C). While a few Delta puncta are observed, most of the Delta appears to be on the plasma membrane and instead of being cleared from the membrane, it perdures for several rows posterior to the morphogenetic furrow. However, it is eventually cleared from the membrane into intracellular puncta (Figure 2.3) (Overstreet et al., 2003).

Flies were generated in which two components required for Delta endocytosis were mutant. One strong *neur* mutant was crossed into a *lqf*^{FDD9} homozygous mutant background. It is possible that in these flies a reduction in Neur ubiquitination of Delta in combination with reduced Epsin targeting ubiquitinated Delta for internalization will result in a reduction of Delta endocytosis. In eye discs from these flies, Delta was observed increased both at the furrow and on the plasma membrane of cells posterior to

the furrow. However, compared to the *lqf* strong loss of function mutant clones, the Delta is not being cleared from the plasma membrane. This result suggests that Delta endocytosis is either not occurring, or at a much slower rate than just *lqf* mutants alone.

I generated *auxilin* mutant clones in a manner similar to that described for generating *lqf* clones. However, at the time, *auxilin* was not present on an FRT chromosome due to its proximity to the 3rd chromosome centromere. Therefore, *auxilin* alleles were combined to produce a strong loss of function background, then a $gaux^{+}$ rescue fragment was recombined onto the chromosome containing the FRT and ubi-GFP. Using *eyFLP* to induce mitotic recombination, the $gaux^{+}$ is only present in GFP positive wild type cells and mutant cells are identified due to the lack of GFP. Delta internalization in *auxilin* mutants looks similar to that of the *lqf* mutant clones. While there are some puncta present, most of the Delta appears to be on the plasma membrane and it perdures for several rows posterior to the furrow before being internalized.

2.3. DISCUSSION

auxilin loss of function mutations were identified as enhancers of an Epsin overexpression eye defect (Eun et al., 2007). Auxilin, like Epsin, functions during photoreceptor differentiation for proper number and patterning. As an enhancer of Epsin overexpression with a similar photoreceptor defect, Auxilin could be functioning in the same pathway with Epsin during *Drosophila* eye development. *auxilin* mutants show an increase of Delta transcript at the furrow, like other mutants that affect Notch signaling. *auxilin* clones show Notch activation along the border, similar to other genes involved in sending signals. Auxilin is functioning in signal-sending cells to activate Notch signaling, but is not essential for Notch activation in signal-receiving cells. The Clathrin-binding domain and the DNA-J domain of Auxilin together alone are sufficient for

Auxilin function. Auxilin binds Chc through its Clathrin-binding domain and functions with Hsc70 interaction at the DNA-J domain to uncoat Clathrin-coated vesicles (Greener et al., 2000; Holstein et al., 1996; Umeda, 2000; Ungewickell et al., 1995). Auxilin could be functioning during photoreceptor differentiation in Clathrin-mediated endocytosis to uncoat Clathrin-coated vesicles. When Chc is overexpressed in an *auxilin* mutant background, it partially rescues the eye defect. This result suggests that Auxilin may be function in uncoating Clathrin-coated vesicles to free Clathrin for use in other endocytic events (Figure 2.4).

Experiments I performed showed that Auxilin is important for endocytosing Delta. The published experiments suggest that Auxilin is important for Clathrin-dependent Delta endocytosis in signal-sending cells to activate Notch signaling during photoreceptor differentiation (Eun et al., 2008). Auxilin may be a useful tool for supporting or arguing against the recycling model for Delta endocytosis.

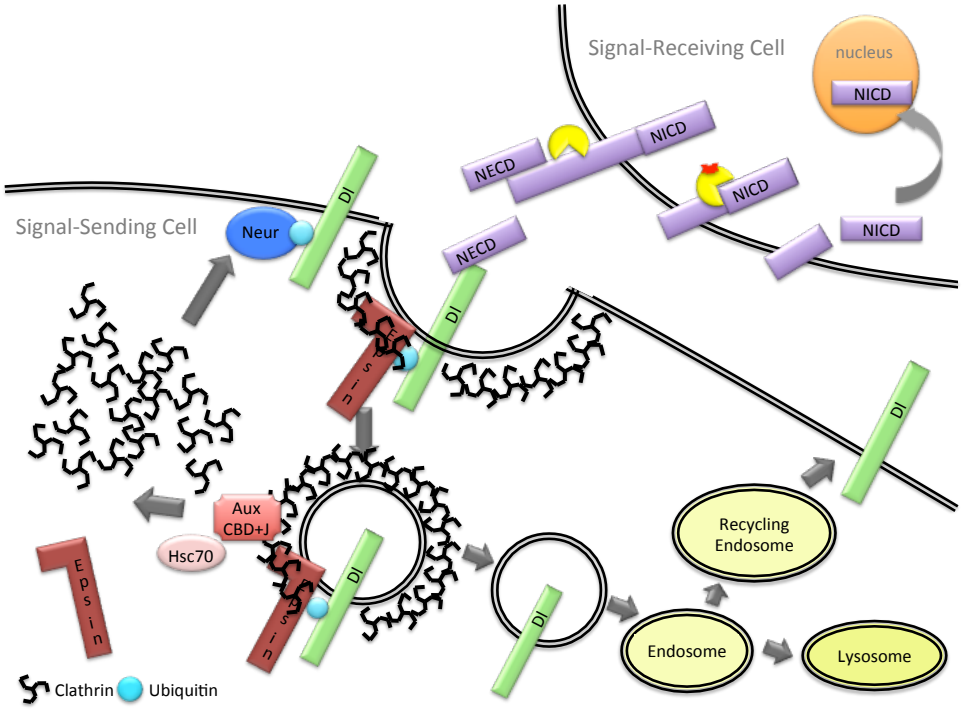

Figure 2.4. Auxilin functions in signal-sending cells to activate Notch signaling.

A model for Auxilin function in signal-sending cells to activate Notch signaling. Expressing only the Clathrin-binding domain (CBD) and DNA-J domain (J) of Auxilin together is sufficient for its function. Overexpressing Clathrin in *auxilin* mutants partially rescues the Notch signaling defects. Auxilin may function in signal-sending cells for uncoating Clathrin-coated vesicles at least partially to maintain free Clathrin in the cell.

Chapter 3: The Role of Auxilin in Delta signaling is not for the purpose of recycling

3.1. INTRODUCTION

Experiments in the previous chapter suggest that Auxilin is functioning in photoreceptor differentiation and Delta endocytosis is inhibited in *auxilin* mutant clones. However, it is still not clear why Delta must be endocytosed to activate Notch signaling. Auxilin may be functioning in uncoating Delta-containing Clathrin-coated vesicles to generate uncoated Delta-containing vesicles and pools of free Clathrin and Epsin. *auxilin* mutants my be inefficiently uncoating Clathrin-coated vesicles. Generation of uncoated Delta-containing vesicles is required if Delta is being recycled as suggested by the recycling model. Therefore, Auxilin can serve as a tool to test the recycling model. The experiments described in this chapter were performed by myself and others and were published (Banks et al., 2011). The work of the other authors on this paper will be summarized in the introduction to this chapter. The experiments I performed will be presented in the results section of this chapter.

3.1.1. Evidence supporting the pulling model and the recycling model

Experimental evidence has been combined to generate two popular models for why Delta must be endocytosed to activate Notch signaling. The pulling model suggests that a pulling force must be generated to promote S2 cleavage. If the force generated is great enough it may be responsible for physically separating the NECD from the rest of the receptor (Nichols et al., 2007). However, experiments suggest a requirement for an ADAM-metalloprotease/TACE enzyme having access to and acting on the cleavage site (Gordon et al., 2007; Pan and Rubin, 1997). Though its unclear exactly how it is

happening, separation of the NECD domain from the rest of the receptor at the S2 cleavage site is required to initiate other cleavage events to activate the Notch receptor.

Other evidence to support the pulling model is the observation that the NECD is in endosomes with Delta, indicating the NECD remains bound to Delta after it is separated from the rest of the Notch receptor (Nichols et al., 2007; Parks et al., 2000; Parks et al., 1995). This observation suggests the Delta-NECD interaction must be strong enough that the NECD remains bound to Delta and is internalized into the signal-sending cell. The strength of the Delta-NECD interaction may be due to the requirement of Delta generating a pulling force on the Notch receptor. However, papers showing a measurable force being generated by a Delta-Notch interaction have yet to be published.

The recycling model suggests that ligand is endocytosed, processed through the endosomal pathway, and recycled to the plasma membrane where it can bind Notch and activate signaling. A domain or motif specifically linked to recycling has yet to be identified on Delta. However, specific lysines and NNL/NPL motifs have been identified that affect Delta and Serrate internalization and function in Notch signaling. The lysines implicated in Delta/Serrate function during Notch signaling are putative sites of ubiquitination by Neur and Mib (Glittenberg et al., 2006; Parks et al., 2006). Internalization and recycling signals have been identified on the LDL receptor. Replacing the intracellular portion of Delta with the internalization and recycling sequence, GSRLRNINSIN**FDNPVY**QRTT, still results in activation of Notch. The bold portion of the sequence was identified as being specifically required for endocytosis of the fusion protein and subsequent activation of the Notch receptor. Other experiments indicate Epsin is no longer required for the endocytosis of the Delta-LDL fusion protein to activate Notch signaling (Wang and Struhl, 2004). While this data further suggests that Delta endocytosis is essential to activate Notch and recycling may play a role, the

experiments were performed with a non-endogenous fusion protein that had reduced ability to signal. Also, though these results further support the role of Delta endocytosis in Notch signaling, it is not clear if Delta was recycled for Notch activation in this instance.

Another way to test the role recycling of Delta may play is to look at the requirement of Rab11 during Notch activation. Rab11 was shown to be is required in signal-sending cells during SOP differentiation to endocytose Delta and activate Notch signaling (Emery et al., 2005; Jafar-Nejad et al., 2005). However, experiments suggest Rab11 is not required in the signal-sending cells to activate Notch in the developing oocyte (Windler and Bilder, 2010). Recycling may only be required in certain cell types and contexts, so Rab11 may have tissue or context specific roles during Delta endocytosis for Notch signaling. Finally, Delta may not be present on the plasma membrane in a position where it can interact with the Notch receptor. Recycling of Delta could result in it being transcytosed to a different position on the plasma membrane opposite Notch on the adjacent cell (Benhra et al., 2010; Rajan et al., 2009).

Experiments presented in the previous chapter suggest that, in the developing *Drosophila* eye, Auxilin is functioning to endocytose Delta most likely in a Clathrin-dependent manner to activate signaling. In *auxilin* loss of function mutants, vesicle uncoating is probably happening inefficiently. It is possible that Delta containing vesicles are getting held up at the uncoating phase. If uncoating is necessary for the vesicle to fuse with the early endosome and enter the pathway to be recycled to the plasma membrane, then recycling of Delta may be inhibited in *auxilin* mutants. Therefore, *auxilin* mutants may provide a unique way to test the validity of the recycling model.

3.1.2. Rab11 is not required for Notch signaling in the developing *Drosophila* eye

Rab11 is linked to recycling endosomes as described in 1.3.4.1. However, a Rab11 requirement for Notch signaling varies from tissue to tissue. The requirement for Rab11 during Notch signaling in the developing *Drosophila* eye was tested. *Notch, Delta, lqf, aux,* and *Rab11* mutant clones were generated in the eye. The mutant cells were marked by the absence of GFP. Elav expression was used as a neural photoreceptor marker (Robinow and White, 1991) and *mδ0.5-lacZ* was used as a read out of Notch activation (Cooper and Bray, 1999; Fanto and Mlodzik, 1999).

The *Notch-* clone shows an expected complete lack of *mδ0.5-lacZ* expression within the mutant clone cells, suggesting the *Notch* mutant cells are unable to receive a signal from the wild type neighboring cells. In the *Delta-*, *lqf-,* and *aux-* clones, the expected pattern of *mδ0.5-lacZ* expression along the borders of the clone, but not in the middle, suggests the cells near the border can receive signals from the neighboring wild-type cells. However, they are unable to signal to the surrounding mutant cells. In the *Rab11-* clone, β-Gal staining is observed throughout the mutant clone, suggesting no defects in either signal-sending or signal-receiving. Analysis of Elav staining also shows almost normal photoreceptor differentiation and patterning. This result suggests that Rab11 is not required to for Notch signaling in the developing *Drosophila* eye.

3.1.3. Auxilin is not required for signal-sending in the developing oocyte

In the developing oocyte, Epsin is required to send a signal from the oocyte to the surrounding follicle cells, but neither Chc nor AP-2 is required (Windler and Bilder, 2010). These results suggest that endocytosis of ligand to activate signaling is occurring in a Clathrin-independent manner in the oocyte. If Auxilin is functioning during Delta endocytosis to uncoat Clathrin-coated vesicles, it may not be required for sending a signal in the developing oocyte. However, the requirement for Auxilin must be tested. The

results may further confirm the idea that Clathrin-dependent endocytosis is not required for sending a signal and to ensure there is no unknown secondary function of Auxilin required for oocyte development.

Germline clones mutant for *aux* were generated and an antibody to Hindsight (Hnt), an indicator of Notch activation, was used to label Hindsight expression in the follicle cells. In a wild type oocyte, hindsight staining is observed in the follicle cells. Hindsight expression is also observed in the follicle cells surrounding $aux^{D136/727}$ and aux^{F956*} germline clone mutants. If Hindsight staining is observed in these experiments, it suggests the oocyte is still able to send a signal to activate Notch in the surrounding follicle cells. This result suggests Auxilin does not function to activate Notch signaling during oocyte development.

3.2. RESULTS

Auxilin and Hsc70 uncoating of Clathrin-coated vesicles results in uncoated Delta-containing vesicles and pools of Clathrin. In *auxilin* mutants, Notch signaling is perturbed. If the recycling model is correct, Auxilin could be functioning in Notch signaling to generate uncoated Delta containing vesicles. Uncoating produces not only uncoated Delta containing vesicles, but pools of Clathrin and other endocytic components as well. The Clathrin and other endocytic components could be trafficked to the membrane and used in future endocytic events. In *auxilin* mutants, uncoating is not occurring efficiently and Delta-containing vesicles could remain in a coated state, resulting in a failure to produce uncoated Delta-containing vesicles as well as limit the free Clathrin and other endocytic components in the cell.

The failure to produce uncoated Delta-containing vesicles makes *auxilin* mutants an ideal tool to study the recycling model. It is not possible to overexpress uncoated

Delta-containing vesicles in an attempt to rescue *auxilin* mutants. However, it is possible to overexpress other by products of uncoating such as Clathrin. If overexpression of Clathrin cannot rescue the Notch signaling defects observed in *auxilin* mutants, then uncoated Delta-containing vesicles may be required and it could support the recycling model. If overexpression of Clathrin can rescue the Notch signaling defects observed in *auxilin* mutants, then it would suggest that production of uncoated Delta-containing vesicles is not required and it would argue against the recycling model.

Expression of a $gChc^{+}$ transgene partially rescues *auxilin* hypomorphic mutants, as described in 2.1.3.5. (Eun et al., 2007). This result supports the idea that uncoated Delta-containing vesicles are not the only required product of Auxilin uncoating. However, complete rescue was not observed. One possible reason for a lack of complete rescue could be due to a requirement not just of excess Chc, but also another endocytic component that becomes limiting in an *auxilin* mutant. Epsin is also required to endocytose Delta. Experiments were performed to determine if it was possible to completely bypass the requirement of Auxilin to uncoat Clathrin-coated vesicles through overexpression of $glqf^{+}$ alone and $gChc^{+}$ together with $glqf^{+}$.

3.2.1. $glqf^{+}$ partially rescues aux^{hypo}

A $glqf^{+}$ construct was generated and injected into flies. The transgene is located on the 2nd chromosome and is called $glqf^{+}$*-1C*. The expression level was tested via western blotting and determined to be at almost endogenous levels. One copy of $glqf^{+}$*-1C* was put into both lqf^{hypo} and lqf^{null} backgrounds and completely rescued the mutants to wild type (Xie et al., 2012). $glqf^{+}$*-1C* was put into an aux^{hypo} background. The alleles used to generate an aux^{hypo} background were aux^{K47}/aux^{D128}, which are the same alleles used for the $gChc^{+}$ rescue experiments (Eun et al., 2008). At 25ºC, aux^{K47}/aux^{D128} are

pupal lethal with few escapers. The escapers have rough eyes with patches of cell death (Figure 3.2.1.) One copy of *glqf*$^{+}$*-1C* in an *aux*^{hypo} mutant partially rescues the *aux* mutant eye defect. Lethality was rescued (Table 3.2.1) and while the eye is still rough, the eye defect is less severe.

When *glqf*$^{+}$ is expressed in this *auxilin* mutant background, it results in a dramatic increase of viable flies to a point near expected Mendelian levels. As controls, viability of each of the transgenes individually and combined in *aux*$^{-}$ /+ flies was tested. There is a slight reduction in the number of flies that do not contain either of the transgenes, but are *aux*$^{-}$/+. However, *glqf*$^{+}$ expressed in *aux*$^{-}$ /+ flies results in near expected Mendelian levels of viability (Table 3). Next, ability to rescue the eye defect was tested and quantified.

Adult eyes were dissected, fixed, embedded and sectioned (Tomlinson and Ready, 1987). Sectioning of the adult eyes shows a change in percentage of wild type facets from 8 ± 5% in *aux*^{hypo} to 62 ± 3% in *glqf*$^{+}$*-1C ; aux*^{hypo} (Figure 3.0). Overexpressing *glqf*$^{+}$ in an *aux*^{hypo} mutant dramatically rescues viability and increases the number of wild type ommatidia observed in the adult eye. However, complete rescue of the eye defects was not observed.

3.2.2. *glqf*$^{+}$ and *gChc*$^{+}$ almost completely rescue *aux*^{hypo}

Complete rescue of the *aux*^{hypo} eye defect did not occur when either Chc or Epsin were overexpressed individually (Figure 3.0). However, as both Chc and Epsin are required for Clathrin-dependent Delta endocytosis, overexpression of the transgenes together in an *aux*^{hypo} could result in a greater, possibly complete, rescue of the mutant eye defect than either transgene separately.

A line with a second chromosome *gChc*$^{+}$ transgene was crossed into a fly with

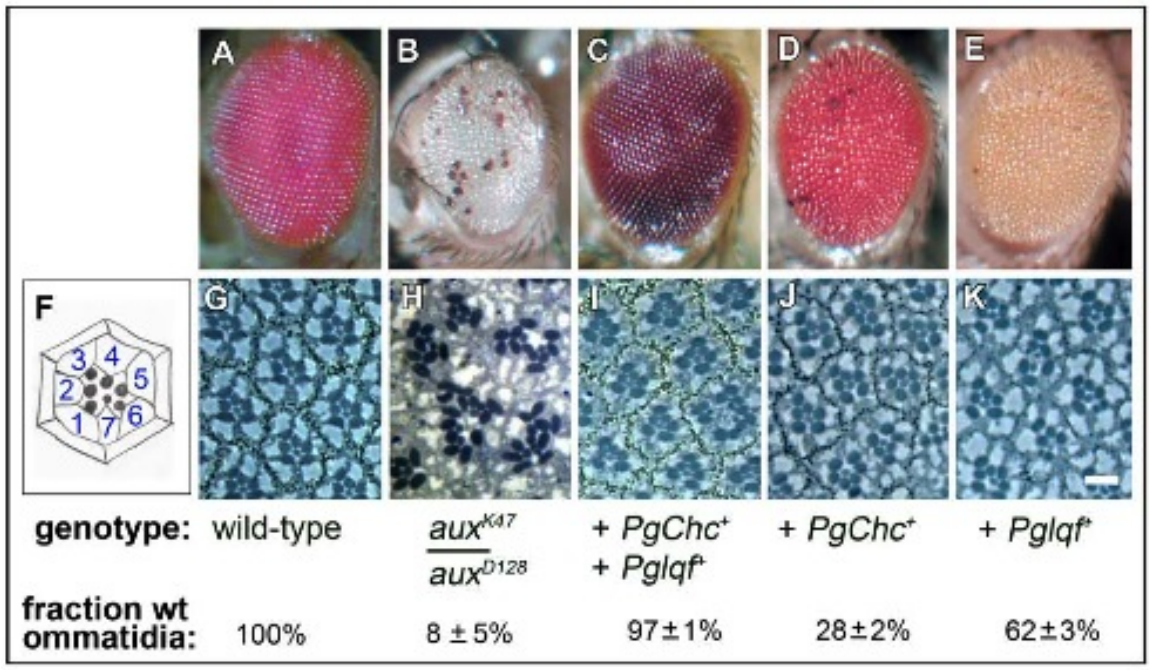

Figure 3.0. Overexpression of Clathrin heavy chain and/or Epsin suppresses the adult eye defects in *aux* loss-of-function mutants.

(A-E) Light micrographs of adult external eyes of the genotypes indicated beneath are shown. (F) A diagram of an apical tangential section of a single ommatidium is shown. The numbers are photoreceptor cells R1-R7. The black circular projections from each cell are the light-gathering organelles called rhabdomeres. The hexagonal shape is formed by pigment cells. (G-K) Small fields of apical tangential sections of adult eyes are shown. (H) Ommatidia of *aux* hypomorphs are usually disorganized, and often have extra photoreceptors. (I-K) Addition of genomic DNA transgenes that express $gChc^{+}$ or $glqf^{+}$ suppresses the eye morphology defects of *aux* hypomorphs. The fraction of phenotypically wild-type (wt) ommatidia was determined by observing 300-500 ommatidia in 4-5 eyes of each genotype. The error is one standard deviation. Scale bar 10 μm (G-K) and 60 μm (A-E).

genotype[a]	# flies[c]	# expected[d]
w; +/CyO ; aux^{K47}/aux^{D128}	2	0
w; Pglqf+/+ ; aux^{K47}/aux^{D128}	84	61
w; PgChc+/CyO ; aux^{K47}/aux^{D128}	44	61
w; PgChc+/Pglqf+ ; aux^{K47}/aux^{D128}	52	61
w; +/CyO ; aux[b]/TM6B	69	122
w; P glqf+/+ ; aux/TM6B	114	122
w; PgChc+/CyO ; aux/TM6B	193	122
w; PgChc+/Pglqf+ ; aux/TM6B	114	122
Total	672	671

Table 3. Rescue of lethality of *aux* mutants by overexpression of Epsin and/or Clathrin heavy chain.

[a]The flies of the genotypic classes listed were obtained from crosses of three *w ; gChc^{+}/+ ; aux^{K47}/TM6B* males with eight *w ; glqf^{+}/CyO ; aux^{D128}/TM6B* virgin females, kept at 25ºC, and transferred to new food vials every 2-3 days for 5 days. Flies with *glqf^{+}* only were differentiated from *gChc^{+}/glqf^{+}* flies by the latter having darker eye color.

[b]*aux* means either *aux^{K47}/aux^{D128}*

[c]The important comparison is between the first row and the three rows beneath. Addition of either or both *glqf^{+}* or *gChc^{+}* transgenes increases drastically the viability of *aux^{K47}/aux^{D128}* adults. It is not clear why the effect of both transgenes is not greater than the effect of a single transgene. One possibility, suggested by the expected frequency of adults (see d below) is that each transgene rescues viability completely. In this case, the differences from expectation would be due to the effects of other aspects of the genotype, such as the presence or absence of *CyO*, and transgene insertion sites.

[d]The expected numbers were calculated making three simplifying assumptions: (1) *aux^{K47}/aux^{D128}* is completely lethal; (2) one copy of either transgene rescues viability fully; (3) no aspect of the genotype other than *aux^{K47}/aux^{D128}* affects viability.

glqf^{+}*-1C* in the *aux*^{K47}/ *aux*^{D128} hypomorphic background. The experiment was constructed such that it was possible to obtain flies that were mutant for *auxilin*, but did not have either of the transgenes, had one or the other transgene, or both transgenes. Obtaining all of these flies from the same cross served as a control allowing for comparison of level of viability rescue. Having both transgenes present together in the *aux*^{hypo} background resulted in rescue of lethality, but did not rescue the lethality at a greater level than each transgene individually (Table 3). Though complete rescue of the eye defect was not observed, greater rescue of the eye defect was observed; as determined through sectioning of adult eyes and comparison of number of wild type facets. Rescue with each transgene individually was not more than 65% wild type facets. However, in the flies with both genomic transgenes present, the eyes had 97 ± 1% wild type facets (Figure 3.0).

3.3. DISCUSSION

3.3.1. The canonical recycling pathway is not required for Notch signaling

Rab11 is not required for Notch signaling in the developing *Drosophila* eye. Though Rab11 is not required, recycling of Delta could be occurring in a non-canonical Rab11-independent manner. While Auxilin has a function in signal-sending cells in the eye, it does not have a function in signal-sending during oocyte development. Both of these results support the idea that Notch signaling occurs differently depending on the tissue and/or context.

The recycling model suggests that Delta-containing vesicles must be uncoated, fuse with and processed through the endosomal pathway, and then recycled to the plasma membrane. Using *auxilin* mutants, the endocytic process and ultimately recycling was most likely slowed or inhibited due to inefficient uncoating. If the Delta containing

uncoated vesicles were essential for signaling, overexpression of Chc or Epsin should not rescue the mutant phenotype. However, we were able to show that by overexpressing Chc and Epsin together resulted in almost complete rescue of the *auxilin*^{hypo} mutant phenotype. This result suggests that Auxilin functions in uncoating Clathrin-coated Delta-containing vesicles not for the purpose of generating uncoated-Delta containing vesicles *per se*, but instead to release Clathrin and Epsin from the coat. If production of uncoated Delta-containing vesicles to be recycled through the endosomal pathway is not required, it argues against the recycling model.

The lack of complete rescue of the hypomorph is most likely due to another endocytic factor required in addition to Chc and Epsin. It is possible that a small amount of Delta-containing uncoated vesicles are required, therefore, we cannot completely rule out the recycling model. However, the extent of the rescue observed is significant and argues strongly against recycling being a large part of Delta signaling. It is also possible that another endocytic component, such as AP-2, Eps-15 or some other adaptor/accessory protein, is limiting in Notch signaling when Auxilin is not efficiently uncoating Clathrin-coated vesicles. Future experiments could be performed overexpressing other components involved in Delta endocytosis, in an attempt to observe complete rescue. However, AP-2 overexpression will be almost impossible due to it being a heterotetrameric complex.

Auxilin is most likely functioning in the developing *Drosophila* eye to uncoat Clathrin-coated vesicles, not to produce Delta-containing uncoated vesicles, but rather to generate pools of free Clathrin and Epsin.

Chapter 4: Identification of enhancers of an *auxilin* loss of function eye defect

4.1 INTRODUCTION

In characterizing the role of Auxilin in the signal-sending cells to endocytose Delta and activate Notch signaling, it was possible to provide evidence against the recycling model. However, it is still not entirely clear how and why Delta has to be endocytosed to activate Notch signaling. Auxilin can be used as a new entry point into the pathway to identify other components not previously known to function during Notch signaling. Our laboratory has had success in the past using forward genetic screens to identify new components functioning in the pathway, i.e. Epsin and Auxilin (Eun et al., 2007; Fischer et al., 1997). Identification of novel components may provide more information with regard to pathway specificity, function and regulation.

4.1.1 Screen rationale

The premise of a forward genetic screen for dominant modifiers relies on having a background that is sensitized for the pathway of interest. Typically, that sensitized background results in a mutant phenotype on its own. Then upon random mutagenesis of the genome, modifiers are isolated that either enhance or suppress the background phenotype. These modifiers are dominant modifiers in that with only one copy of the gene being mutant in a sensitized screen background, an effect is still observed. However, these dominant modifier mutations, which could be as much as halving the level of gene activity, show no mutant phenotype in a wild type background. A background sensitized for a specific pathway may lead to the identification of components closely tied to the pathway. Based on pilot screen results, enhancers were

isolated at a much higher rate than suppressors. Ultimately, only enhancers were isolated from this screen.

4.1.2. EMS mutagenesis

Random mutations can be generated in the genome by either a physical mutagen such as X-ray mutagenesis (Muller, 1928) or a chemical mutagen. The mutagen chosen for this screen was the chemical mutagen ethyl methanesulfonate (EMS). EMS functions to generate point mutations or small deletions in the genome. EMS causes point mutations via ethylation of the DNA base Guanine (G) (Figure 4.0).

G forms three hydrogen bonds to base pair with Cytosine (C). Upon ethylation, guanine is only capable of forming two hydrogen bonds to base pair, resulting in an inability to base pair with a Cytosine (C). During DNA synthesis, the ethylated G will be paired with a T. However, the ethylated G-T pairing will be identified as a mismatch and when the repair machinery attempts to fix the mismatch, it cannot determine which was the original base. Therefore, the ethylated G could be removed and replaced with an adenine (A) to pair with the T. The original G-C base pairing is now converted to an A-T base pairing, resulting in a point mutation. EMS causes deletions when a glycoside linkage at an ethylated guanine is weakened and results in hydrolysis, which removes that base. Upon replication, a base can be randomly inserted in the gap either resulting in no change, or a point mutation, or the gap can be skipped resulting in a deletion (Jenkins, 1967; Krieg, 1962).

EMS dosage affects the number of mutations generated in the genome of the organism. *Drosophila melanogaster* have approximately 13,000 genes and approximately one third of those genes are essential. For this screen EMS was used at a concentration of 25mM, which results in roughly one lethal mutation per chromosome

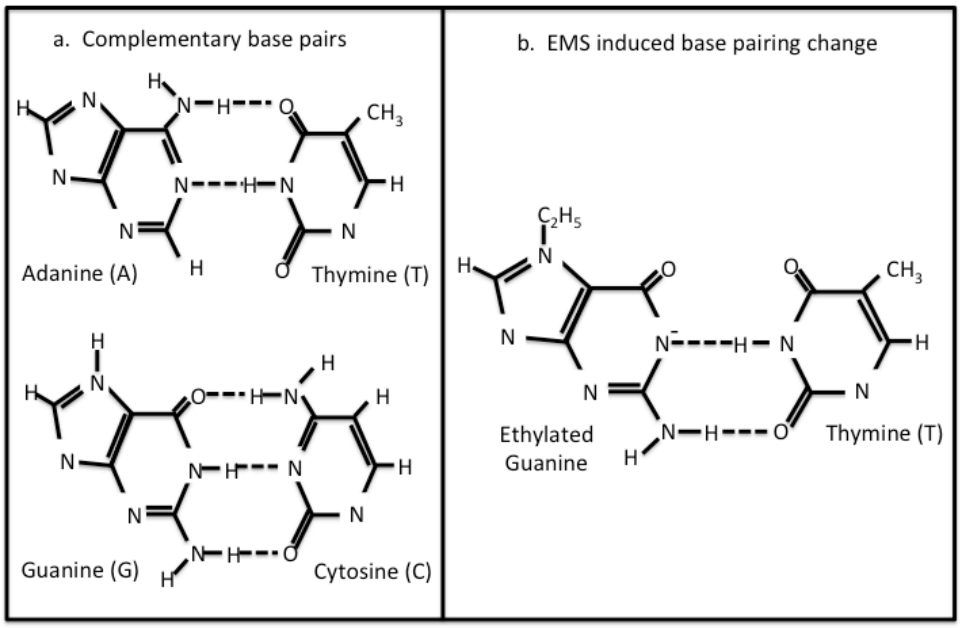

Figure 4.0. EMS ethylation affects G-C base pairing

Adenine base pairs with Thymine via two hydrogen bonds. Guanine base pairs with Cytosine via three base pairs. When Guanine is ethylated by EMS, instead of pairing with Cytosine, it now pairs with Thymine. This base pair mismatch can either undergo repair to the original G-C pairing or it can undergo repair to an A-T pairing, introducing a point mutation [Modified from (Krieg, 1962)].

arm. That mutation rate would result in 3 total mutations per chromosome arm (Lewis 1968 (Koundakjian et al., 2004). Mutations will be generated on all of the chromosomes of the fly, but I only isolated those generated on the 2nd and 3rd chromosomes. There are approximately 10,000 genes present on the 2nd and 3rd chromosomes, and each chromosome has 2 chromosome arms. If three mutations are possible per chromosome arm and there are four possible arms, it suggests a total of 12 mutations possible per fly isolated. I screened 20,000 flies, which resulted in covering the genome approximately 24 times (20,000 flies * 12 mutations / 10,000 genes = 24). Covering the genome that many times should result in mutating each gene at least once, perhaps multiple times. Isolating multiple alleles of a gene allows for ease in mapping and identifying the gene that has been mutated.

4.1.3. F1 GMR-hid screen

It was determined that identification of the enhancers at the F1 generation would ultimately reduce the workload due to early detection and not carrying through non-enhancers longer than necessary. To perform the screen, a combination of *auxilin* alleles had to be identified for use as the sensitized background. Strong *auxilin* mutants are lethal. If I am trying to isolate enhancers of a mutant phenotype that make the phenotype more severe, the background must be viable to allow for identification of an enhanced phenotype. A background that is lethal in the absence of an enhancer makes it impossible to observe a more severe phenotype in the presence of an enhancer, because the flies would be dead whether an enhancer was present or not. Therefore, a weaker combination of *auxilin* mutant alleles had to be used. Weaker hypomorphic *auxilin* alleles chosen for this screen are $auxilin^{C2}$ and $auxilin^{K47}$, which are viable with a slightly rough eye. However, there are still problems in isolating enhancers in this background. In one

instance the mutant enhances the *auxilin* mutant to lethality. If the enhanced line is dead, it is not possible to isolate the enhancer in a stock for identification. Also, these weak *auxilin* mutants are sterile. If an enhancer is identified, it wouldn't be heritable and a stable stock could not be established.

A genomic *auxilin* (*gaux*$^+$) transgene was generated in the laboratory that completely rescues an *auxilin* null mutant. This transgene consists of a genomic region that contains the *auxilin* gene and it completely rescues the *auxilin* hypomorphic screen background. However, *gaux*$^+$ expression removes the sensitized background (Eun et al., 2007). Therefore, it is necessary to generate a fly that has the *gaux*$^+$ transgene in the whole body to ensure fertility, but does not have the *gaux*$^+$ transgene in the eye. Removing the to *gaux*$^+$ transgene in the eye reveals the sensitized background eye defect. The FLP/FRT system described in 2.1.2. was used to remove *gaux*$^+$ from the eye. Flipase (FLP) was expressed under control of the eye specific *eyeless* (*ey*) enhancer. The *gaux*$^+$ transgene was recombined onto the right arm of the second chromosome, which also contains the cell lethal gene *hid* under control by the eye specific promoter/enhancer *GMR*. An FRT site was also recombined onto the chromosome with both *GMR-hid* and *gaux*$^+$. This chromosome is crossed into a fly that has a chromosome with just the FRT present on a chromosome. When FLP is induced in the eye of a fly with these two chromosomes in trans, it results in site-specific mitotic recombination at the FRT. The right arm of the second chromosome is homozygosed. One daughter cell is homozygous for the chromosome containing *GMR-hid gaux*$^+$ and the *hid* expression induces cell death. The other daughter cell is homozygous for the chromosome lacking both *GMR-hid* and *gaux*$^+$ and the cells survive. This specific cell death results in an entire eye that no longer has a copy of the rescue fragment and it is now possible to see the screen background mutant eye defect in a viable fly.

EMS was fed to the parental males and they were mated to the parental females . The F1 male progeny were screened for an enhanced rough eye. At this point, the flies are mosaic for the mutation, so they must be crossed again to ensure the enhancement mutation is present in the cells in the germline and is therefore heritable. The enhanced males were crossed to females of the same genotype as the parental females. F2 males were screened for enhancement. The enhanced F2 males are crossed to flies that are double balanced on the second chromosome and have an *auxilin* mutant marked with *Sb* balanced on the third chromosome. At this point in the screen, enhancement has only been observed in the eye. By only observing enhancement in the eye, it could also be leading to false enhancers due to homozygosing the right arm of the second chromosome. The F3 progeny have the enhancement mutation in a whole fly mutant for *auxilin* and the *gaux*$^{+}$ transgene is not present. The males were once again been screened for enhancement. Stable stocks were be established using balanced siblings of the enhanced males. If the flies are enhanced to lethality, the mutation can still be recovered due to balanced siblings present in the same vial.

4.2. RESULTS

EMS mutagenesis was performed and flies were screened (Figure 4.1). Enhancers were isolated and stable stocks were established. Identification of the enhancers isolated began.

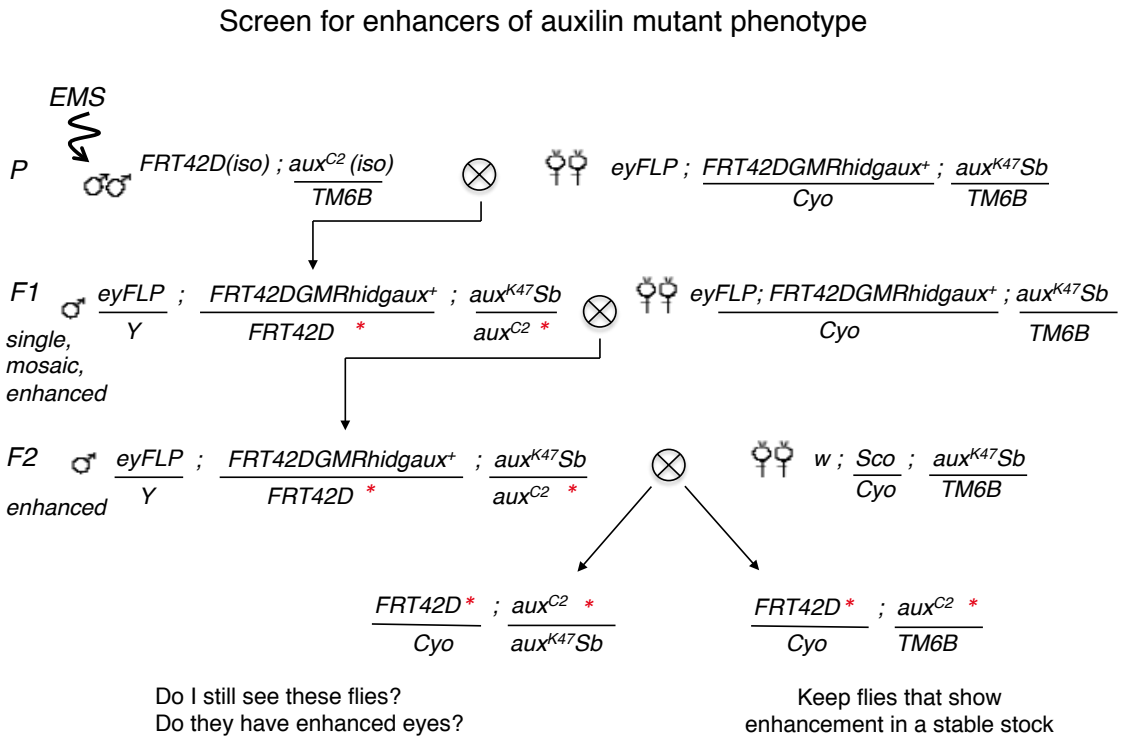

Figure 4.1. Cross scheme for an F1 EMS *FLP/FRT* mutagenesis screen for enhancers of an *auxilin* mutant eye defect.

EMS mutagenized adult males with *FRT42D* and a 3rd chromosome balanced *auxilin*^{C2} allele were crossed to females that provided an eye specific source of FLP, an *FRT* chromosome that contained a *gaux*$^{+}$ rescue transgene and *GMR-hid*, and a 3rd chromosome balanced *auxilin*^{K47}marked with *Sb*. Males were screened in the F1 and single, enhanced, mosaic males were crossed to females of the same genotype as the females used in the parental cross. Males still enhanced at the F2 show the mutation is heritable and they were crossed to a fly balanced on the second chromosome with a 3rd chromosome balanced *auxilin*^{K47}marked with *Sb*. Stable stocks were established of balanced siblings of enhanced flies.

4.2.1. Screen numbers

The goal was to screen 20,000 flies to cover the genome approximately 24 times. I screened 19,984 F1 males of which 7,256 were enhanced. The enhanced F1 males were then crossed (Figure 4.1), and 1,596 F2 males showed heritability of the enhancement. The enhanced F2 males were crossed (Figure 4.1), and 136 stable stocks balanced by *CyO* on the second chromosome and *TM6B* on the third chromosome were established. Based on the mutagenesis information presented in 4.1.2., I would expect at least one third of the F1 progeny screened to be enhanced. The number drops dramatically again between the F1 and F2, because many of the enhanced males were rendered sterile due to the EMS mutagenesis. Also, many of the mutations affect somatic cells, but are not carried in the germline. Finally, the smaller number of stable stocks results from the loss of false enhancers that result from homozygosing the right arm of the second chromosome.

The enhancers isolated were grouped into three categories (Figure 4.2). The first category includes the 15 mutations that enhanced the *auxilin* screen mutant background to lethality. The second category includes the 54 mutations that were strong enhancers. Strong enhancers were identified as lines that were viable, but had eyes that were rougher and contained more patches of cell death as compared to the screen background. The third category includes the 65 mutations that were weak enhancers. Weak enhancers were identified as lines that were viable, but had eyes that were only slightly rougher than the screen background. Next segregation testing was performed to determine if the isolated mutation was present on the 2nd or 3rd chromosome.

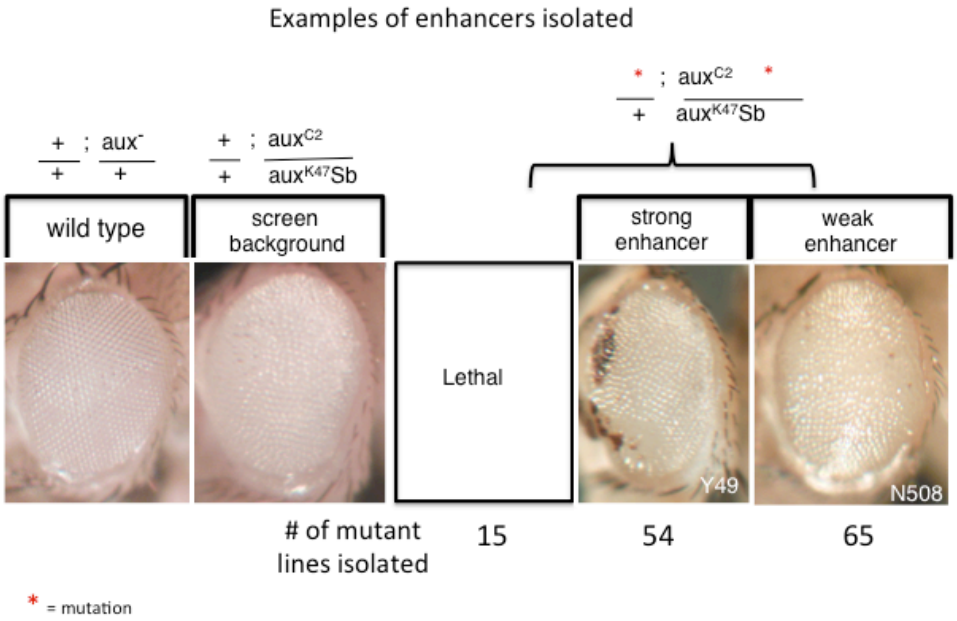

Figure 4.2. Examples of enhancers isolated.

Flies with only one mutant copy of *auxilin* have wild type eyes. In the screen background, the *auxilin* hypomorphic mutants have a slightly rough eye. 15 enhancers were isolated that enhance the background to lethality. 54 enhancers were isolated that strongly enhance the background. 65 enhancers were isolated that weakly enhance the background. The red asterisks indicate the potential chromosome location of the mutation.

4.2.2. Segregation testing

The stable stocks were crossed to a fly line that had a wild type second chromosome and a 3rd chromosome balanced *auxilin*^{K47}*Sb* allele. Then all *CyO*, non-*TM6B* progeny were screened for enhancement. If the flies are *CyO*, then the mutagenized second chromosome is no longer present. However, the mutagenized third chromosome, which also contains an *auxilin* allele, is present. If the *CyO*, non-*TM6B* progeny are enhanced, it suggests the mutation is on the third chromosome. If the *CyO*, non-*TM6B* are not enhanced, it suggests the mutation is on the second chromosome. Segregation testing resulted in 15 enhancer stocks having mutations on the second chromosome and 119 enhancer stocks having mutations on the third chromosome. Next, it is necessary to determine if multiple mutations in a single gene were isolated.

4.2.3. Complementation testing

Complementation testing involves crossing each mutation to other mutations segregated to the same chromosome. If the progeny containing both mutations are viable, they are said to complement and it suggests they are mutations in different genes. If the progeny containing both mutations are lethal or have a morphological defect, they are said to fail to complement and it suggests they are mutations in the same genes. To set up these complementation matrices of crosses, only homozygous lethal lines were used. Including only homozygous lethal lines is intended to ease interpretation of the results. If a line is not homozygous lethal, it is most likely not going to be lethal in combination with another allele.

4.2.3.1. 2nd chromosome

Of the 15 enhancers isolated on the 2nd chromosome, 13 enhancers were homozygous lethal. These mutations were crossed to one another and ability to

complement was determined (Figure 4.3). One cross failed to complement, thereby forming a complementation group. The alleles in the complementation group are Y49 and G512.

4.2.3.2. 3rd chromosome

The 3rd chromosome complementation testing was complicated by the presence of an *auxilin* mutation on the chromosome with the mutation. To perform complementation testing, it would result in homozygosing the *auxilin* mutation, which is lethal. To bypass the lethality caused by the *auxilin* mutation, a *gaux*$^{+}$ rescue transgene was crossed onto the X chromosome. It completely rescues the *auxilin* mutant and ensures that any lethality observed is due to the combination of enhancers and not *auxilin*. Of the 119 mutations isolated on the 3rd chromosome, 93 were homozygous lethal and underwent complementation testing (Figure 4.4). Several lines failed to complement one another, resulting in the formation of 11 complementation groups (Table 4). Three mutations isolated enhanced the *auxilin* mutant background to lethality and were identified as separate complementation groups with only one member. Mutations that enhance the *auxilin* mutant background to lethality suggest a strong interaction and were tested further, even if not grouped with other enhancer mutations. Only complementation groups underwent further testing. However, there were also several mutations that enhanced the *auxilin* screen background to lethality, but failed to be identified as a member of a complementation group. These enhancers could be interesting if they have a strong enough interaction with the pathway to cause lethality. Therefore, they also underwent further testing.

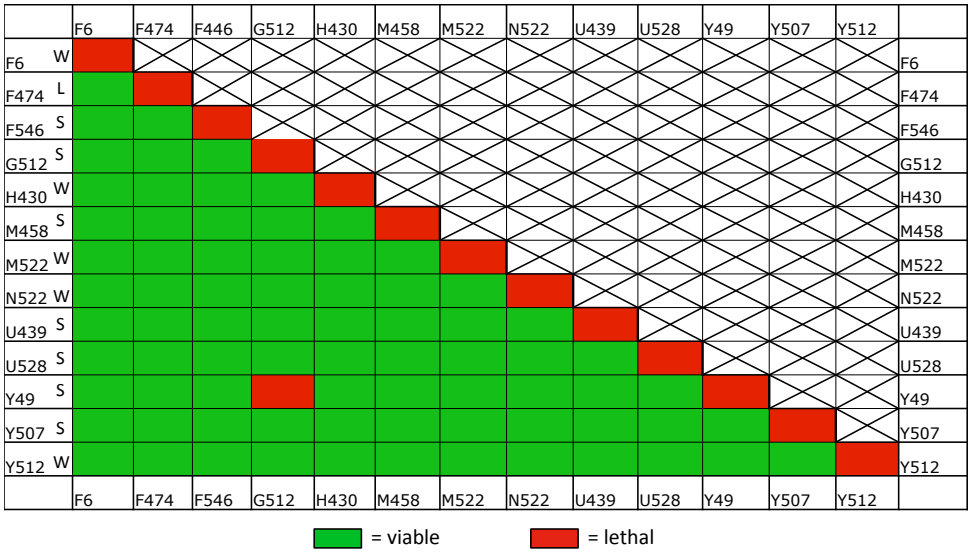

Figure 4.3. 2nd Chromosome complementation matrix

Homozygous lethal 2nd chromosome enhancers were crossed to one another. The strength of the enhancer is indicated as lethal (L), strong (S) or weak (W) in the column on the left. The outcome of the cross is indicated by the color of the box. Green indicates the progeny from the cross were viable. Red indicates the progeny from the cross were lethal.

	Groups	Enhancers	Identified genes
2nd Chromosome	1	G512 (S), Y49 (W)	*α-adaptin*
3rd Chromosome	1	X225 (W), I225 (W), M10 (W)	
	2	R426 (W), D292 (L), E514 (S)	
	3	K504 (S), X138 (S)	
	4	S432 (S), O114 (W)	
	5	N514 (S), Y301 (S), X119 (S) T283(W) A541 (W) J340 (W)	
	6	M411 (S), S2123 (S), V16 (S) D339 (W), Q417 (S) Z74 (S) Q403 (S) F8 (W) F508 (S) R531 (W)	
	7	Q325 (W), R34 (S)	*Delta*
	8	N564 (W) T596 (W) Y214 (S)	
	9	W256 (W) E414 (S) X516 (W) D270 (W) K413 (L) N435 (L)	
	10	R32 (S) R308 (S) F311 (L) E511 (S) C529 (S) S427 (W)	
	11	T297 (L), N508 (W), E227 (L) D467 (L)	*Crumbs*
	12	I230 (L)	
	13	W465 (L)	*lqf/Epsin*
	14	A287(L)	*Hsc70*

Table 4. Summary of complementation groups

Enhancers are grouped with other enhancers that failed to complement one another. The strength of the enhancement is indicated as lethal (L), strong (S), and weak (W) in parentheses following each enhancer. Group 12 is not a traditional complementation group, as it consists of single lethal enhancers that do not fall into any other complementation group.

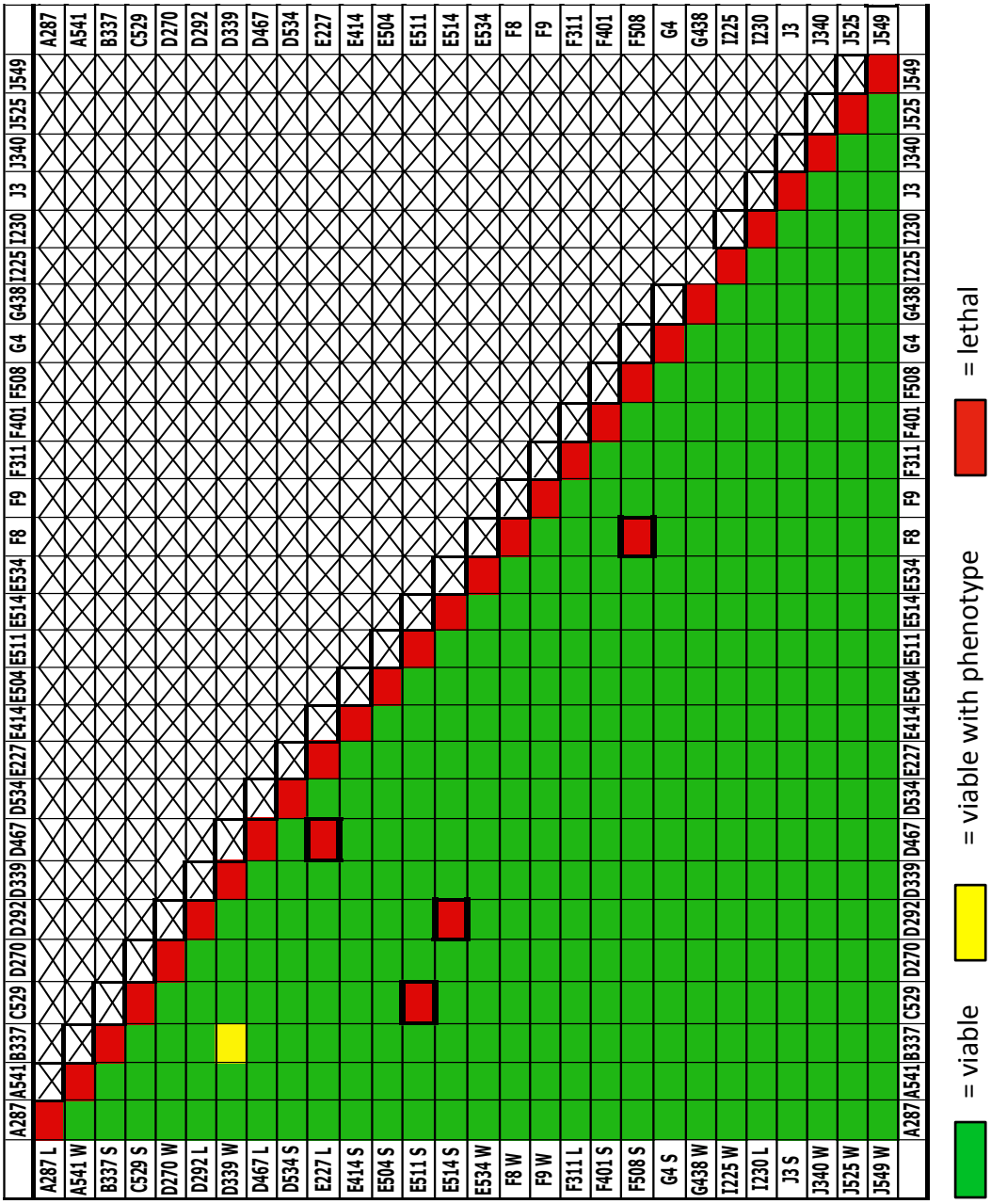

Figure 4.4. 3rd chromosome complementation matrix, Part I of Parts I-VI

Homozygous lethal 3rd chromosome enhancers were crossed to one another. The strength of the enhancer is indicated as lethal (L), strong (S) or weak (W) in the column on the left of Parts I, II, and IV. The outcome of the cross is indicated by the color of the box. Green indicates the progeny from the cross were viable. Yellow indicates the progeny from the cross were viable, but had an eye defect. Red indicates the progeny from the cross were lethal.

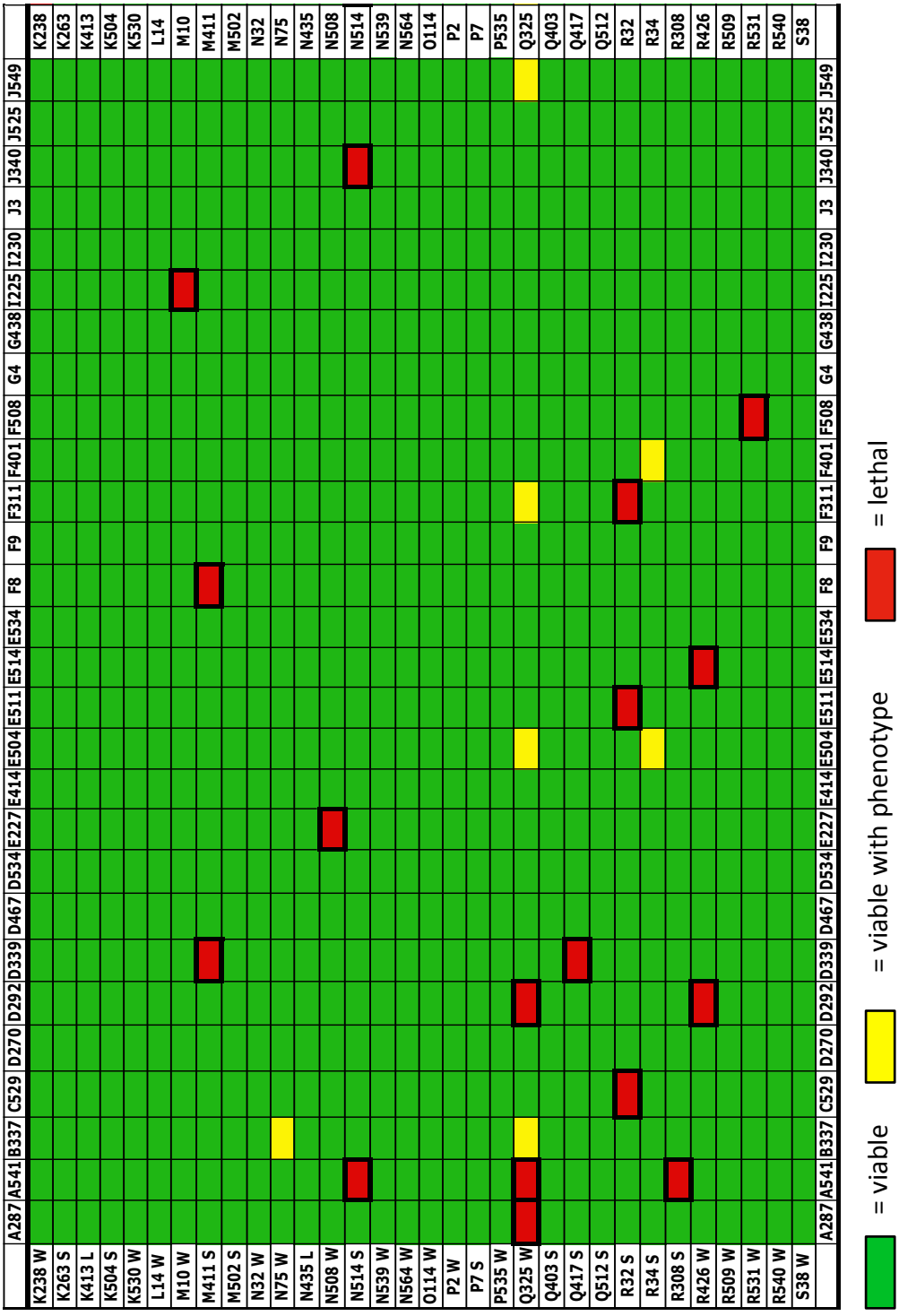

Figure 4.4. 3rd chromosome complementation matrix, Part II

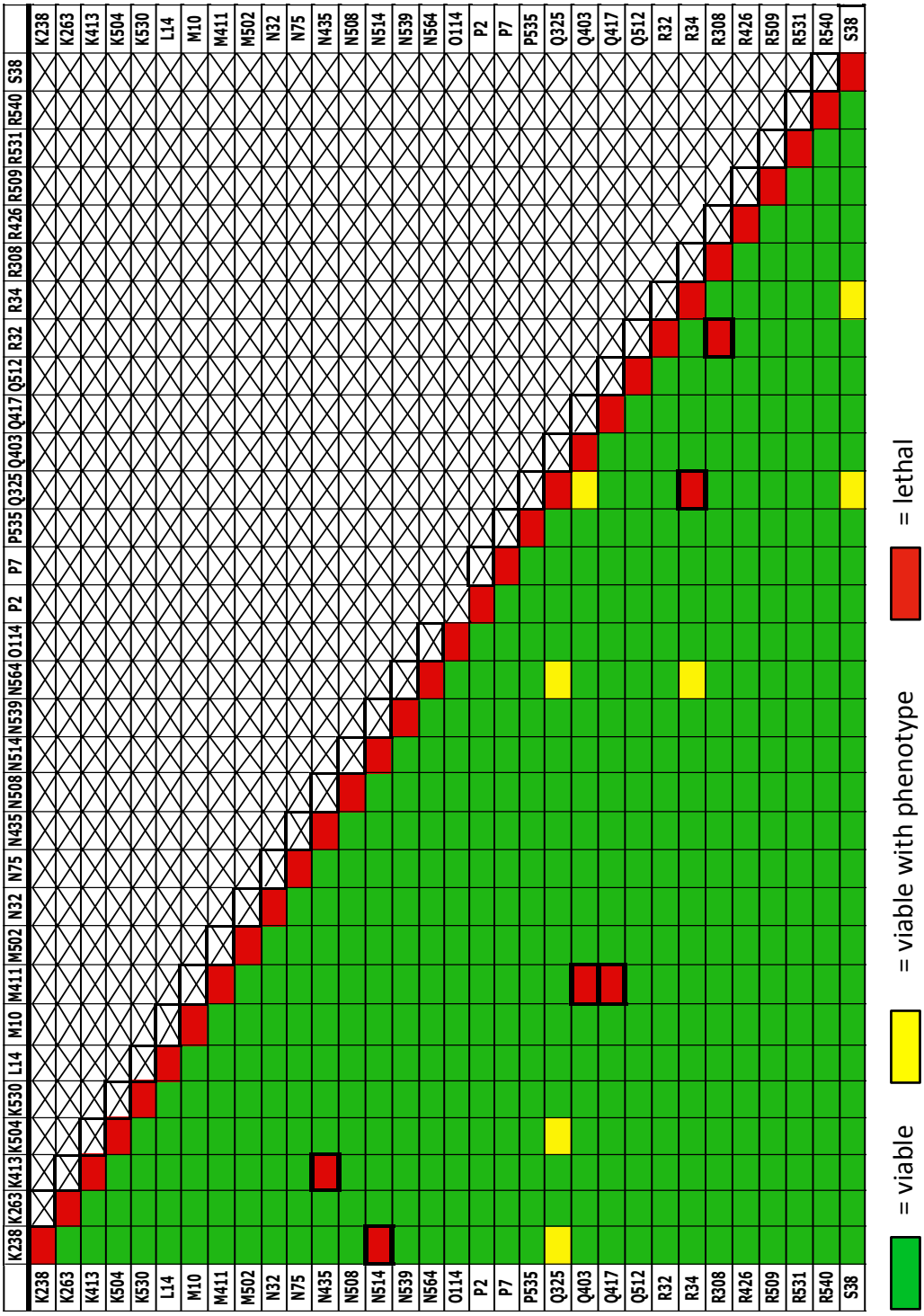

Figure 4.4. 3rd chromosome complementation matrix, Part III

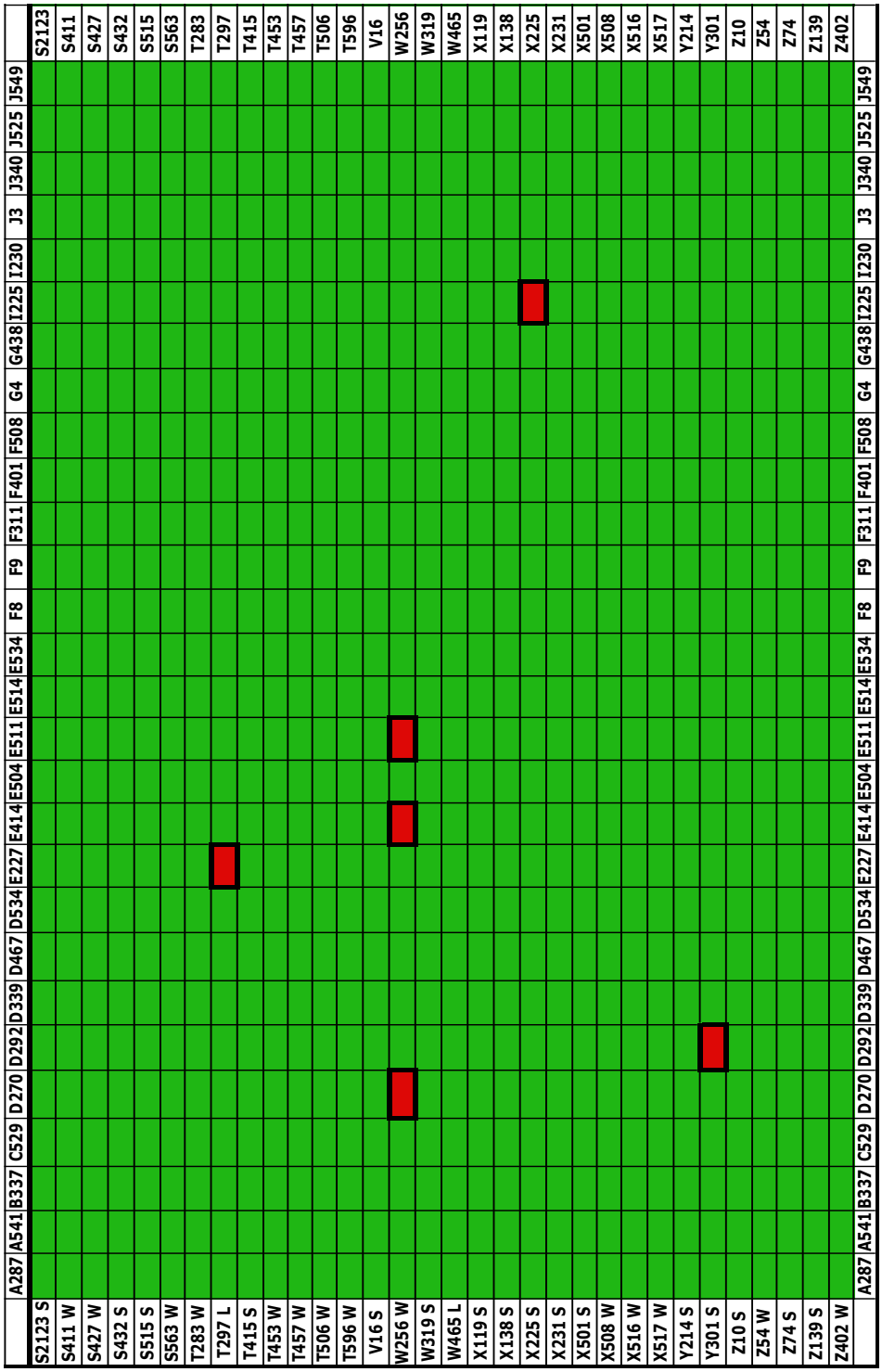

Figure 4.4. 3rd chromosome complementation matrix, Part IV

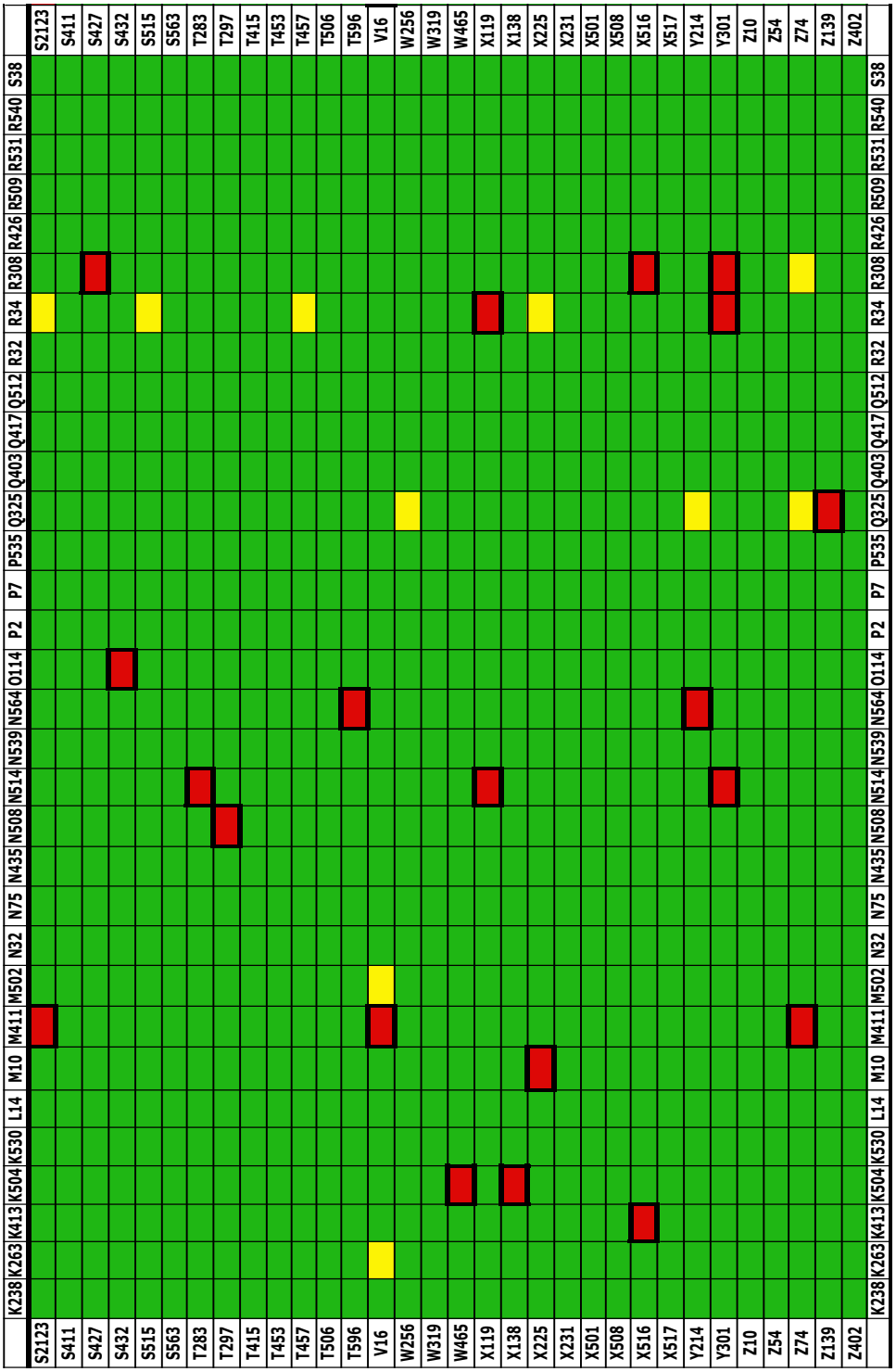

Figure 4.4. 3rd chromosome complementation matrix, Part V

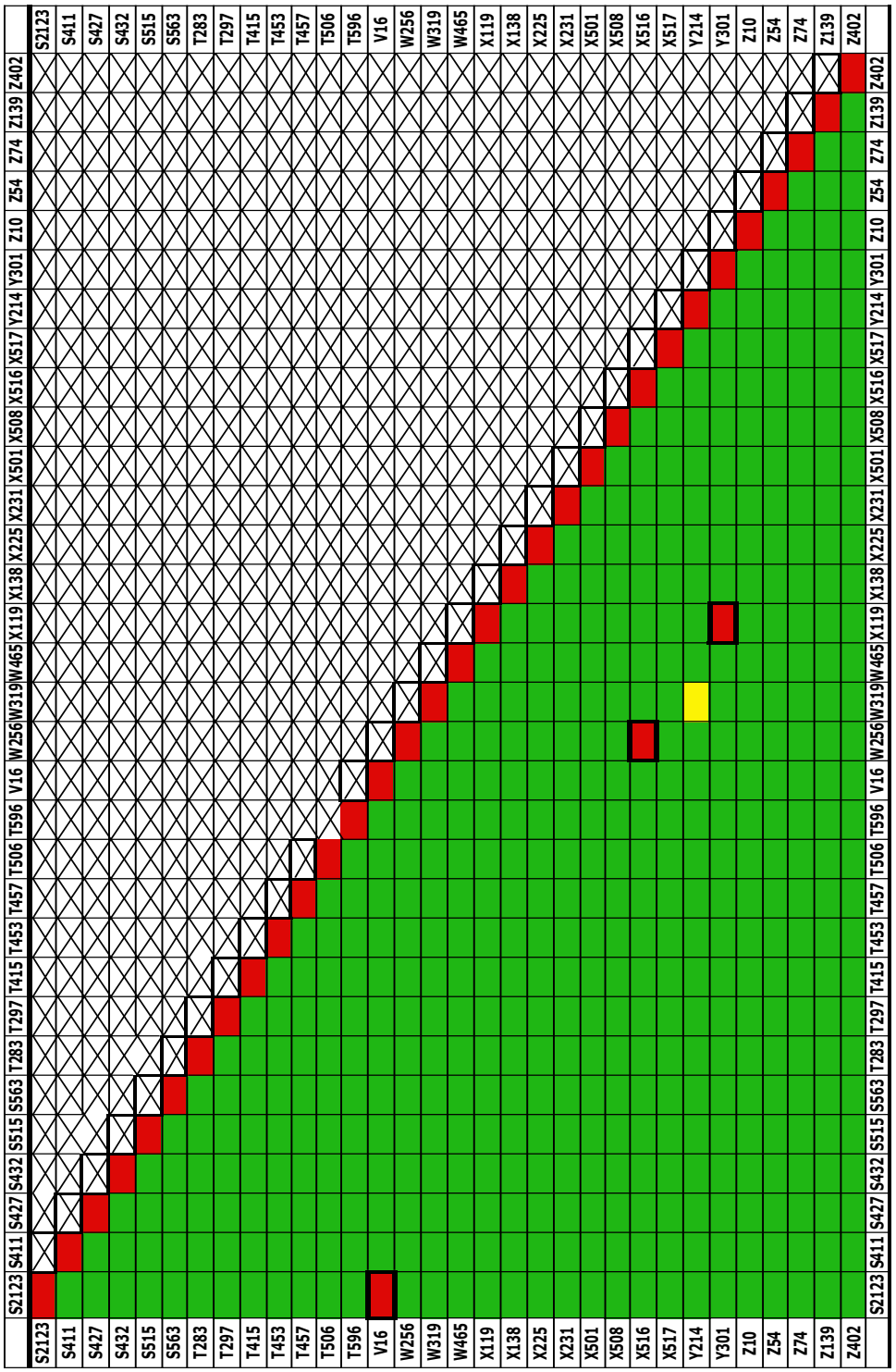

Figure 4.4. 3rd chromosome complementation matrix, Part VI

4.2.4. Positive controls identified

Prior to performing the screen, certain members of the Notch pathway were tested for their ability to enhance the screen background. This test showed the *auxilin* background could be enhanced and provided a list of possible positive controls of enhancers to be isolated in the screen. The known enhancers of *auxilin* are *lqf, neur,* and *hsc70*. Alleles of these known enhancers and other components of the Notch pathway found on the 2nd and 3rd chromosomes were tested. Alleles of the Notch components on the 2nd chromosome that were tested were, *mastermind* (a Notch coactivator) (Xu et al., 1990), *kuzbanian* (an ADAM protease required to cleave the Notch receptor) (Pan and Rubin, 1997), *spen* (a negative regulator of Notch signaling) (Kuang et al., 2000). Alleles of the Notch components on the 3rd chromosome that were tested were *lqf, neur*, *hsc70*, *faf*, and *Dl*.

The crosses with the 2nd chromosome stocks did not result in identification of any of the mutations as being alleles in the genes that were tested. The crosses with the 3rd chromosome stocks identified complementation group 7 (*Q325*, *R34*) as being alleles in *Dl* (Table 4). None of the other complementation groups were identified as being alleles of any of the other possible positive controls. However, single alleles of some of the known enhancers were isolated. Mutation *W465* was identified as an allele of *lqf* (Table 4), because it failed to complement both weak and strong alleles of *lqf*. Also, sequencing of the *lqf* locus revealed a point mutation converting a C to a T, which changes a CAG glutamine codon to a UAG stop codon. The stop codon may generate a protein truncated after the first DPW motif. Mutation *A287* was identified as an allele of *hsc70* (Table 4), because it failed to complement the strong $Hsc70^{4\text{-}R447H}$ allele. Mutation *K263* was identified as an allele of *faf* (Table 4), because it failed to complement the strong faf^{F08} allele.

4.2.5. Mapping

All complementation groups that were not identified through testing with previously known components of the Notch signaling pathway were mapped as an initial step to identification of the mutated gene. Two complementation groups were selected for mapping. The only second chromosome group was mapped. Complementation group 11 on the 3rd chromosome was mapped, because 3 of the 4 mutations enhanced the *auxilin* mutant background to lethality. First meiotic mapping was performed, followed by deficiency mapping to narrow down the chromosomal region in which the mutation is located.

4.2.5.1. Meiotic mapping 2nd and 3rd chromosomes

Meiotic mapping takes advantage of known markers present on the chromosome that can be recombined during meiosis with the mutant chromosome (Fogel and Hurst, 1967). The second chromosome mutations were mapped using a chromosome marked with *aristaless* (*al*), *dumpy* (*dp*), *black* (*b*), *curved* (*c*), and *plexis* (*pl*). The third chromosome mutations were mapped using a chromosome marked with *roughoid* (*ru*), *hairy* (*h*), *thread* (*th*), *curled* (*cu*), *striped* (*sr*), *ebony* (*e*), and *Prickle* (*Pr*). Meiotic recombination produces multiply marked chromosomes with different markers present depending on the location of the recombination. These recombined chromosomes may or may not contain the enhancer mutation. They are crossed to another member of the complementation group. If they complement the lethality associated with the enhancer mutation, it suggests the mutation is not present with that combination of markers. If they fail to complement, it suggests the mutation is present on that marked chromosome and the mutation is mapped between two markers. However, the region between two markers could still represent a large number of genes.

One line was selected from each complementation group to be mapped. Typically, the strongest enhancer of the group was chosen. *G512* from the second chromosome complementation group mapped to the region near *al*. It was not possible to map all of the complementation groups on the third chromosome, so initially only 3 groups were mapped. *X225* from complementation group 1 and *D292* from complementation group 2 did not map cleanly, but appeared to be located between *h* and *sr*. *E227* from complementation group 11 mapped to the right of *e* near *Pr*.

4.2.5.2. Deficiency mapping 2nd and 3rd chromosomes

Deficiency mapping serves to further refine the area of the chromosome in which the mutation is located. Many deficiencies are available that in combination uncover almost the entire genome. If the deficiency is crossed to an enhancer mutation and it complements, it suggests the mutation is not located in the region deleted in the deficiency. If it fails to complement, it suggests the mutation is located in the region deleted in the deficiency.

Deficiencies to the left of and just to the right of *al* were tested against *G512*. The deficiency *Df(2L)BSC106* failed to complement both *G512* and *Y49*. The deficiency uncovers a region containing approximately 20 genes. Many are conceptual genes with unknown function. However, it also uncovers *Hop* (Hsp70/Hsp90 organizing homolog protein), *Pi3K21B* (a kinase binding protein), *Plc21C* (phosphatidylinositol phospholipase C activity), *ebi* (RNA polymerase II transcription co-repressor activity), and α-adaptin (a subunit of AP-2).

Deficiencies between *h* and *sr* were crossed to *X225* and *D292*, but none of them failed to complement. Identification of these complementation groups was not pursued further. Deficiencies to the right of *e* were crossed to *E227*. The deficiencies

Df(3R)Exel6199 and *Df(3R)Exel8178* failed to complement *E227*, as well as the other 3 members of the complementation group. This region contains approximately 33 genes. Most of the genes are conceptual genes with no known function. However, it also uncovers *crumbs* (involved in adherens junction formation and apical/basal polarity), *BRWD3* (mitotic cell cycle G2/M transition) and *Dis3* (involved in RNA surveillance and neurogenesis).

4.2.6. Identified enhancers

When possible, alleles of genes in a region uncovered by the deficiencies that failed to complement were obtained and crossed to the members of the complementation group.

4.2.6.1. Validation of isolated *α-adaptin* alleles

One of the genes in the region on the 2nd chromosome uncovered by the deficiency is *α-adaptin*. α-Adaptin is a subunit of the AP-2 heterotetrameric protein (Kirchhausen et al., 1989). One way to determine the identity of the gene affected by the mutations isolated it to cross the members of the complementation group to other characterized alleles of that gene obtained from other sources. If the members of the complementation group are not alleles of that gene, then the enhancer mutation should complement the characterized allele. If the members of the complementation group are alleles of that gene, then the enhancer mutation should fail to complement the characterized allele. Strong (ada^{3}, $ada^{40\text{-}31}$) and weak (ada^{06694}) alleles of *α-adaptin* failed to complement both *G512* and *Y49*, suggesting *G512* and *Y49* are alleles of *α-adaptin* (Table 4). To further confirm they are alleles of *α-adaptin*, both *G512* and *Y49* were sequenced to identify any lesions present. *G512* and *Y49* are homozygous lethal at an early developmental stage, making it impossible to obtain homozygous mutant larvae for

sequencing. Both alleles were crossed the weak *α-adaptin* allele ada^{06694}. *G512* or *Y49* in trans to ada^{06694} are viable at least through the larval stage. Larvae were obtained, total RNA was isolated, and RT-PCR was performed to generate cDNA. cDNA was also generated from the screen background and wild type (w^{1118}) flies to serve as controls. Then, the cDNA was used as the template for PCR with primers specific to *α-adaptin*. The ada^{06694} allele results from the insertion of an approximately 14.5 kb P-element in the first exon of *α-adaptin*. ada^{06694} in trans to other *α-adaptin* alleles exhibits an *α-adaptin* mutant phenotype (González-Gaitán and Jäckle, 1997). The ada^{06694} P-element insertion greatly increases the length of the transcript as compared to the approximately 2.3 kb RA transcript. *G512* and *Y49* do not have the P-element insertion and should only have the transcript. The parameters for PCR were set to only amplify the approximately 2.3 kb transcript, ensuring only the *G512* or *Y49* transcript was isolated and sequenced. Following the PCR with *α-adaptin* specific primers, the PCR product was run on a gel, the appropriate size band was excised, purified and sequenced.

Sequencing of *G512* identified a point mutation from a G to an A. A UGG tryptophan codon was mutated to a UAG stop codon. Sequencing of *Y49* identified a point mutation from a G to an A. An in frame codon of UGG encoding the amino acid tryptophan was mutated to the UGA stop codon. These mutations occur within the same codon, but affect a different G nucleotide in each instance. Isolating two mutations in neighboring nucleotides is rare, but it does occur. α-Adaptin has an N-terminal head region, a hinge, and a C-terminal ear region (Robinson, 1993). The stop codons in these alleles are located in the head region and would result in a truncated protein without part of the head region, a hinge, or ear region. It is not known if this truncated α-Adaptin protein is stable, whether or not it can form a heterotetramer, or if it does form a heterotetramer whether or not it retains any function.

4.2.6.2. *α-adaptin* mutations enhance the *auxilin* mutant photoreceptor defect

α-adaptin mutations isolated from the screen enhanced the screen background rough eye defect. A rough eye defect can be manifested by defects in several types of cells. The slightly rough eye of the screen background is due to aberrant photoreceptor number and patterning (Figure 4.5).

Adult eyes were dissected, fixed, stained, embedded and sectioned (Tomlinson and Ready, 1987) to observe the photoreceptors. Facets were counted in a single section from 2-3 different eyes of each genotype. The facets counted were characterized as wild type or mutant depending on photoreceptor number and patterning. From that data a percentage of wild type facets was determined for each section. The result for each section was averaged with other sections of the same genotype. These averages were charted with standard error. For the wild type genotype, facets were counted in a single section from two different flies. Of the facets counted, all were wild type. Sections of the screen background slightly rough adult eyes consist mostly of wild type facets with an average of 82±7% wild type facets observed. Non-wild type facets were observed with too many photoreceptors, too few photoreceptors, and defects in facet organization. Sections of adult eyes from flies that have one copy of *G512* in the screen background show a decrease in the number of wild type facets with an average of 57±4% wild type facets observed. Sections of adult eyes from flies that have one copy of *Y49* in the screen background show a decrease in the number of wild type facets with an average of 34±13% wild type facets observed. In both the *G512* and *Y49* experiments, non-wild type facets were observed with too many photoreceptors, too few photoreceptors, symmetry defects, and orientation defects. The percent of wild type facets was determined for each section of a genotype. Within each genotype, the mean number of wild type facets was determined and charted with standard error.

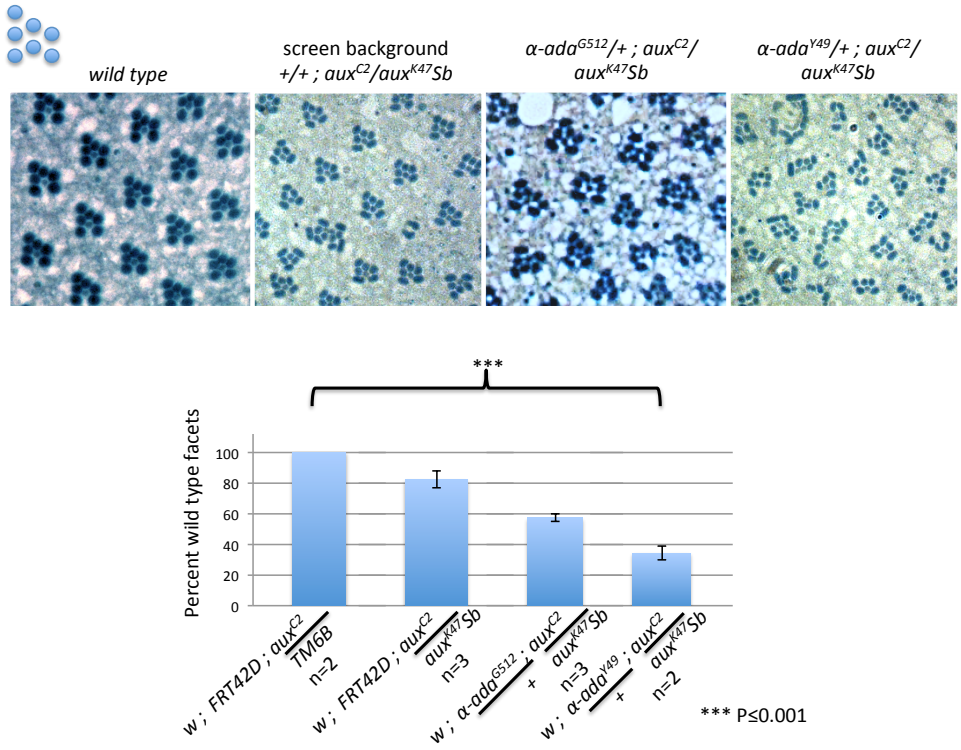

Figure 4.5. *α-adaptin* mutations enhance the *auxilin* hypomorphic photoreceptor defect

A cartoon depicts the pattern of wild type receptors. Adult eye sections show the wild type photoreceptor pattern, the photoreceptor defects observed in the screen background, and the photoreceptor defects observed in one copy of each of the *α-adaptin* mutations in the screen background. Wild type facets were counted in sections of 2 or 3 different eyes of each genotype. The sections appear to show a change in the number of wild type facets. An ANOVA test was performed to determine the significance of the difference among the samples. The difference observed is significant at a P≤0.001.

Results were analyzed by an ANOVA test to determine the significance of differences observed among the means of the genotypes (Davenport, 1940). ANOVA analysis revealed the difference observed among the means of the genotypes is highly significant ($p \leq 0.001$). This analysis does not indicate whether or not the difference in means between any two groups is significant. It is interesting that the averages for the two *α-adaptin* mutations vary, even though they affect the same codon. This difference is most likely due to other mutations generated during EMS mutagenesis. The presence of these mutations within the background of each of the enhancer lines isolated could alter the phenotype slightly. To ensure the result observed is due to the mutation in *α-adaptin* alone and not mutations in other genes, several alleles of *α-adaptin* from other sources that were generated in ways other than by EMS mutagenesis will be obtained and tested for their ability to enhance *auxilin* mutants.

4.2.6.3. Validation of isolated crumbs alleles

One of the genes in the region on the 3rd chromosome uncovered by the deficiency is *crumbs*. Crumbs is an integral membrane protein that functions at adherens junctions and in establishing apical/basal polarity (Grawe et al., 1996). One way to determine the identity of the gene affected by the mutations isolated it to cross the members of the complementation group to other characterized alleles of that gene obtained from other sources. If the members of the complementation group are not alleles of that gene, then the enhancer mutation should complement the characterized allele. If the members of the complementation group are alleles of that gene, then the enhancer mutation should fail to complement the characterized allele. Strong alleles of *crumbs* (crb^1 and crb^2) (Tepass and Knust, 1993; Wodarz et al., 1993) were obtained and failed to complement all four members of complementation group 11. This result suggests that *T297*, *E227*, *D467* and

N508 are alleles of *crumbs* (Table 4). To further confirm they are alleles of *crumbs*, *T297* was sequenced. *T297* is homozygous lethal early during development and is also lethal early in combination with other *crb* alleles. Therefore, larvae of the *crumbs* alleles balanced with *TM6B* were obtained, total RNA was isolated, and RT-PCR was performed to generate cDNA. cDNA was also generated from the screen background and from wild type (w^{1118}) flies to serve as controls. The cDNA was used as the template for PCR with *crumbs* specific primers. However, the primers amplify both the mutant chromosome and the *TM6B* chromosome. The PCR product was run on a gel, the appropriate size band was excised, purified, and sequenced.

The sequence of the *TM6B* chromosome was similar to the wild type (w^{1118}), while the screen background should be distinguishable due to single nucleotide polymorphisms (SNP). *T297* has all of the SNPs present in the screen background sequence as well as any other mutations generated by EMS mutagenesis. *T297* has a point mutation not present in w^{1118}, *TM6B*, or the screen background that changes a C to a T, which changes a CAA glutamine codon to a UAA stop codon. A stop codon at that location would result in truncation of the protein early in the coding region. The other mutations in the complementation group will be sequenced in the same manner in which T297 was sequenced.

4.2.6.4. *crumbs* mutations enhance the *auxilin* mutant photoreceptor defect

Three of the *crumbs* mutations isolated from the screen enhance *auxilin* mutants to lethality, but one *crumbs* mutation, N508, is viable and weakly enhances the screen background rough eye defect. A rough eye defect can be manifested by defects in several types of cells. The slightly rough eye of the screen background is due to aberrant photoreceptor number and patterning (Figure 4.6).

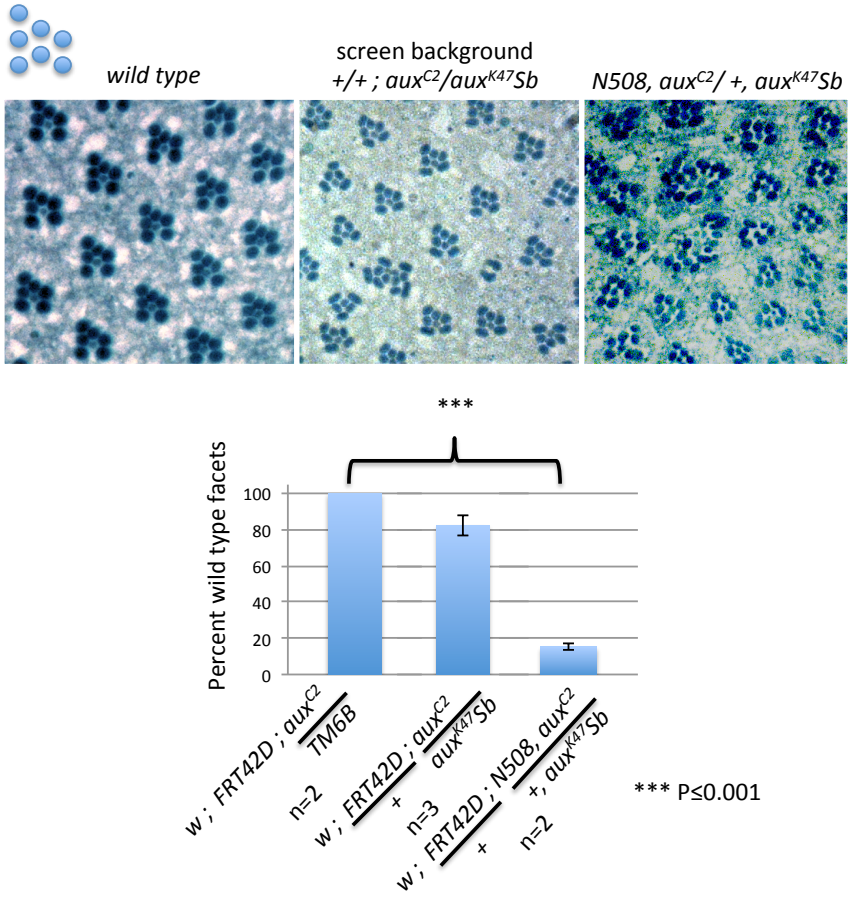

Figure 4.6. *crumbs* mutations enhance the *auxilin* hypomorphic photoreceptor defect

A cartoon depicts the pattern of wild type receptors. Adult eye sections show the wild type photoreceptor pattern, the photoreceptor defects observed in the screen background, and the photoreceptor defects observed in one copy of the *crumbs* mutation in the screen background. Wild type facets were counted in sections of 2 or 3 different eyes of each genotype. The sections appear to show a change in the number of wild type facets. An ANOVA test was performed to determine the significance of the difference among the samples. The difference observed is significant at a P≤0.001.

The data was collected and analyzed in the same manner as *α-adaptin*, which is described in section 4.2.6.2. For the wild type genotype, facets were counted in a single section from two different flies. Of the facets counted, all were wild type. Sections of the screen background slightly rough adult eyes consist mostly of wild type facets with an average of 82±7% wild type facets observed. Non-wild type facets were observed with too many photoreceptors, too few photoreceptors, and defects in facet organization. Sections of adult eyes from flies that have one copy of *N508* in the screen background show a decrease in the number of wild type facets with an average of 15±1% wild type facets observed. Non-wild type facets were observed with too many photoreceptors, too few photoreceptors, symmetry defects, and orientation defects. The percent of wild type facets was determined for each section of a genotype. Within each genotype, the mean number of wild type facets was determined and charted with standard error.

Results were analyzed by an ANOVA test to determine the significance of differences observed among the means of the genotypes (Davenport, 1940). ANOVA analysis revealed the difference observed among the means of the genotypes is highly significant ($p \leq 0.001$). This analysis does not indicate whether or not the difference in means between any two groups is significant.

4.3 DISCUSSION

The screen was performed in an *auxilin* sensitized background and screening ~20,000 F1 ultimately resulted in establishment of 136 stable stocks of enhancers. Complementation groups were identified on both chromosomes. Further identification revealed several positive controls were isolated as enhancers. Identification of the positive controls *Dl*, *lqf*, *faf*, and *Hsc70*, suggests the screen was successful in isolating

enhancers that are closely tied to the Notch signaling pathway or are known to directly interact with Auxilin.

Two components not previously described to play a role during Notch signaling in the developing *Drosophila* eye were identified in the screen. The possible roles α-Adaptin and Crumbs may be playing during Notch signaling will be discussed in the following chapter.

4.3.1. Extent of screen coverage of the genome

The formation of complementation groups suggests that enough flies were screened to isolate multiple mutations in one gene. However, the screen is by no means exhaustive, because many enhancers do not fall into complementation groups. Most of the positive controls identified in the screen were only single alleles. The single enhancers were not studied further due to the difficulty of performing mapping and other means of identification. However, there still could be many interesting results left unidentified. Perhaps more flies can be screened at a later date to isolate more alleles and generate complementation groups.

This screen only isolated enhancers located on the autosomes. However, there are many known Notch signaling components present on the X chromosome, including the Notch receptor, Chc, and Ral. A future screen of X chromosome enhancers could be performed to identify new components involved in Notch signaling.

4.3.2. Possible types of enhancers to be isolated from the screen

The screen was performed in an *auxilin*-sensitized background. Prior to the screen, it was established that Auxilin functions in the signal-sending cells during Notch signaling in the eye (Banks et al., 2011; Eun et al., 2008). Therefore, I expected to isolate components that function during endocytosis of Delta for signaling. The positive

controls identified from the screen (*Dl, lqf, faf, hsc70*) suggests those types of enhancers were isolated. Delta is one of the ligands that binds and initiates activation of Notch (Fehon et al., 1990; Lieber et al., 1993; Rebay et al., 1991). Epsin is essential in recognizing ubiquitinated Delta and targeting it for internalization into the signal-sending cell (Overstreet et al., 2003; Wang and Struhl, 2004). Faf maintains levels of Epsin, which is required in the signal-sending cell for Delta internalization (Cadavid et al., 2000; Chen, 2002). Hsc70 is not explicitly known to function solely in the signal-sending cells to activate Notch signaling. However, it is known to function with Auxilin (Holstein et al., 1996; Jiang et al., 1997), which does function in signal-sending cells to activate Notch signaling (Banks et al., 2011; Eun et al., 2008).

One type of enhancer I expected to isolate in the screen may function in endosomal trafficking of vesicles. For example, a Rab GTPase or other endosomal protein essential in trafficking Delta through the endosomal pathway could have been identified. However, prior results suggested that trafficking or recycling of Delta through canonical endosomal pathways was not required to activate Notch signaling (Banks et al., 2011). All enhancers isolated on the 2nd chromosome were tested against alleles of *rab5*, but none of the enhancers failed to complement. At present no enhancers involved in endosomal vesicle trafficking have been identified, but they could be present in an as yet uncharacterized complementation group or one of the single alleles isolated from the screen.

Another type of enhancer I expected to isolate in the screen may function during endocytosis. Endocytosis of Delta is essential for activation of Notch signaling (Bray, 2006), so endocytic regulatory proteins may be isolated from the screen. Based on prior results, it was possible to almost completely rescue an *auxilin* hypomorphic mutant with overexpression of Chc and Epsin. However, we did not observe complete rescue in the

auxilin hypomorphic mutant and no rescue in an *auxilin* null mutant. These results could suggest a requirement of other endocytic components that are limiting in *auxilin* mutants. Identification of *α-adaptin* as an enhancer may provide further information on regulation of endocytosis, as α-Adaptin is a subunit of the endocytic Clathrin adaptor AP-2 (Kirchhausen et al., 1989). Genes encoding either the σ or μ2 subunits of AP-2 are on the 3rd chromosome and alleles of these genes were tested against the complementation groups. None of the enhancers in complementation groups failed to complement alleles of the σ or μ2 subunits, but the single enhancers were not checked for complementation. Therefore, it is still possible alleles of the σ and μ2 subunits were isolated from the screen. Other components or regulators of endocytosis could have been isolated in other complementation groups or as single alleles, but they have not been identified.

A third type of enhancer I expected to isolate from this screen may function in Actin polymerization/regulation. Actin polymerization is important in formation and internalization of endocytic vesicles (Giardini et al., 2003; Upadhyaya et al., 2003; Yarar et al., 2005). When Notch signaling fails, an Actin defect is observed (Major and Irvine, 2005). However, at this point, no complementation groups or single alleles have been identified as Actin regulators.

Finally, it is possible that enhancers isolated from this screen could be functioning in some other capacity during Notch signaling. For example, an enhancer could be functioning in the signal-sending cells not to positively regulate Delta signaling, but instead to bias the cell by down regulating the Notch receptor. As will be discussed further in chapter 5, AP-2 could also be negatively regulating the Notch receptor in the signal-sending cell. While the discussion of expected results has focused on components functioning in the signal-sending cells, it is possible that proteins functioning in the signal-receiving cell or both could be isolated from the screen. If Notch signaling is

inhibited by reduced Auxilin function in the signal-sending cell, further inhibiting the ability of the signal-receiving cell to activate signaling could enhance the screen background. Crumbs could also be functioning in the signal-sending cells or in the signal-receiving cells. Crumbs has been described having a function at adherens junctions, in establishing apical/basal polarity (Grawe et al., 1996), and may (Herranz et al., 2006; Mitsuishi et al., 2010) or may not (Pardossi-Piquard et al., 2007) play a role in negatively regulating the γ-secretase complex, which functions to cleave the Notch receptor during activation.

4.3.3. Role of α-Adaptin in photoreceptor differentiation

Photoreceptor defects generate the weak rough eye defect observed in the screen background. One copy of either *α-adaptin* mutation in the screen background results in a significant change in the number of wild type facets present in the adult eye. The types of mutant facets that occur more frequently in the enhanced fly eyes include facets that contain extra photoreceptors, too few photoreceptors, symmetry defects and orientation defects. These results suggest that α-Adaptin functions in photoreceptor differentiation during *Drosophila* eye development. Previously, we determined that Auxilin functions in signal-sending cells to activate Notch signaling during photoreceptor differentiation. The photoreceptor defects observed in the enhanced fly eyes are similar to those observed in mutants of other Notch signaling pathway components. However, this assay does not conclusively indicate the photoreceptor defects are due to a functional requirement of α-Adaptin in Notch signaling. Further experiments will be required to show a role for α-adaptin in Notch signaling during photoreceptor differentiation.

Chapter 5: Discussion and future directions

5.1. Discussion

Canonical Notch receptor activation requires not only binding to one of its ligands, but also internalization of that ligand into the signal-sending cell. It is not clear why the ligand must be endocytosed to activate Notch signaling. Experiments described in this dissertation suggest Auxilin functions in signal-sending cells to activate Notch signaling. More specifically Auxilin plays a role in Delta endocytosis by uncoating Clathrin-coated Delta-containing vesicles to generate pools of free Clathrin and Epsin. A screen was performed to identify enhancers of an *auxilin* hypomorphic mutant eye defect. If the isolated enhancers are mutations in Notch signaling pathway components, they may provide information about why Delta must be endocytosed to activate Notch signaling.

5.1.1. Possible role for α-Adaptin in Notch signaling

α-Adaptin has been well characterized as a subunit of the Clathrin-adaptor protein AP-2, which functions at the plasma membrane in Clathrin-dependent endocytosis (Beck and Keen, 1991; Hirst and Robinson, 1998; Keen, 1987; Keen and Beck, 1989; Kirchhausen, 1999). Weak *α-adaptin* mutant alleles do not have a rough eye defect, but do have a wing defect (González-Gaitán and Jäckle, 1999). Strong *α-adaptin* mutant alleles have a tumorigenic defect (Menut et al., 2007; Windler and Bilder, 2010) making it difficult to assess photoreceptor defects. Ectopic activation of the Notch receptor also results in tumorigenic defects in the eye (Brumby and Richardson, 2003; Vaccari and Bilder, 2005). Delta internalization occurs in a Clathrin-dependent manner in the developing *Drosophila* eye. Therefore, it is possible that α-Adaptin is required in signal-sending cells for Delta endocytosis. α-Adaptin was previously investigated as a possible component of Notch signaling in the eye. However, a strong *α-adaptin* mutant allele

failed to dominantly enhance *faf* mutant eyes. The ability of a strong *α-adaptin* mutant allele to dominantly enhance *lqf* hypomorphic mutant eyes was also tested, but the enhancement was weak and variable (Cadavid et al., 2000). These results did not indicate a strong role for AP-2 in Notch signaling in the eye and it was not pursued further.

One possible explanation for isolating *α-adaptin* mutations as enhancers from my screen is that some form of redundancy was removed and it was possible to observe a requirement for α-Adaptin. α-Adaptin may be functioning redundantly with Epsin to recruit Clathrin for Clathrin-dependent endocytosis (Figure 5.0).

The previous experiment to test dominant enhancement by an *α-adaptin* mutation occurred in a background where only the level of Epsin was reduced in the cell and did not directly affect Clathrin levels. In *auxilin* mutants, if Clathrin-coated vesicles are being uncoated inefficiently, then levels of both Epsin and Clathrin become limiting in the cell. It is not until both Epsin and Clathrin are reduced together that dominant enhancement by an *α-adaptin* mutation is observed, suggesting redundancy of these components to endocytose Delta. Reagents are not currently available to specifically test the possibility of redundancy between α-Adaptin and Epsin to act as Clathrin-adaptors. However, genetic interaction experiments can be performed with mutant alleles that reduce levels of α-Adaptin, Epsin, and Chc in combination.

There is some evidence that supports a role for α-Adaptin in signal-sending cells during the Notch signaling event required for SOP development. Notch signaling in SOP cells is classified as a lineage decision Notch signaling event (Figure 1.0). An undifferentiated pI cell undergoes an asymmetric cell division to generate a pIIa cell that upon Notch signaling adopts the epithelial hair/socket cell fate and a pIIb cell that upon Notch signaling adopts the neuron/sheath cell fate. Components of the Notch pathway,

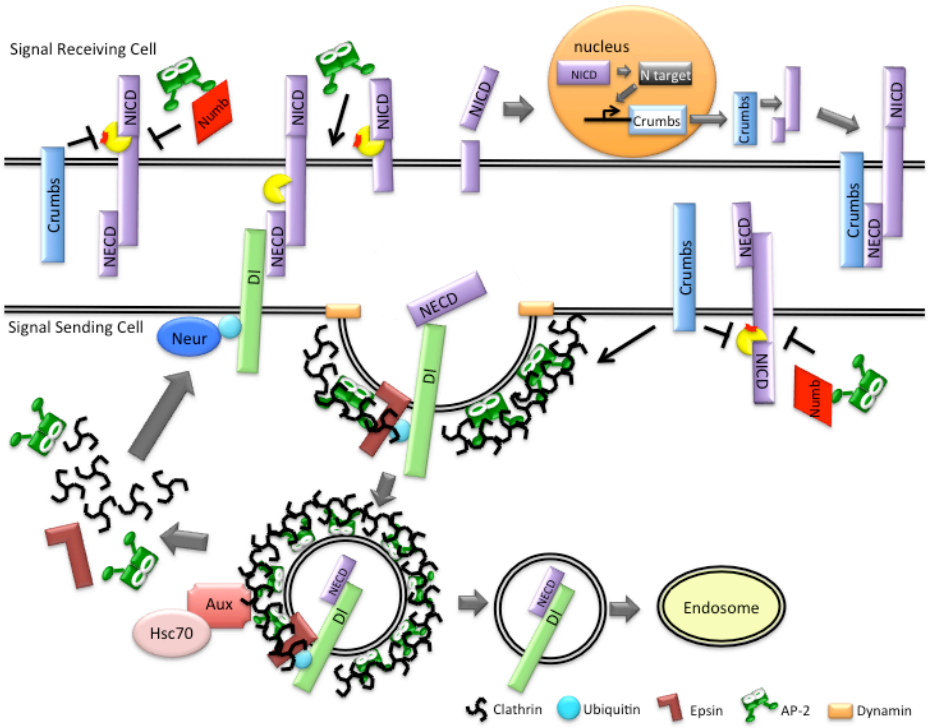

Figure 5.0. New model for Delta endocytosis and Notch signaling

In the signal-receiving cell Notch activation could be inhibited through Crumbs inhibition of γ-secretase complex activity or via Numb/AP-2 inhibition. Notch activation occurs upon binding ligand and results in a series of cleavage events. Endocytosis via an AP-2 dependent mechanism may be required during activation of the Notch receptor. The NICD is released from the rest of the receptor and trafficked to the nucleus where it functions as a transcription factor to affect target gene expression. One indirect downstream target of Notch is the gene *crumbs*, which once expressed can function in trafficking of the Notch receptor to the plasma membrane. It could be interacting with the Notch receptor by directly binding the extracellular domain of the receptor. In the signal-sending cell, Notch receptor could be inhibited by Crumbs and/or Numb/AP-2 as a means to bias the cell to become a signal-sending cell. Crumbs could also be positively regulating Delta endocytosis. The ligand must be endocytosed into the signal-sending cell in a Clathrin-dependent, Epsin-dependent, AP-2 dependent manner to activate signaling. Upon internalization, the Clathrin-coated vesicle is uncoated by Auxilin and Hsc70 to generate pools of free Clathrin, Epsin, AP-2 and other components required for Clathrin-dependent endocytosis. Delta-containing vesicles can fuse with the early endosome and processed through the endosomal pathway.

such as Neur (Lai and Rubin, 2001; Le Borgne and Schweisguth, 2003) and the Notch negative regulator Numb (Guo et al., 1996; Rhyu et al., 1994; Santolini et al., 2000) asymmetrically localize to the pIIb cell during division. One possibility for this asymmetric localization is that Neur is positively regulating Delta while Numb is negatively regulating the Notch receptor to bias the pIIb cell to become the signal-sending cell. α-Adaptin has been shown to bind Numb (Santolini et al., 2000) and is required to properly localize Numb to the pIIb cell (Berdnik et al., 2002; Le Borgne, 2005). Numb may be functioning to inhibit signaling by endocytosing the Notch receptor to down-regulate activation at cytokinesis during asymmetric SOP cell division (Couturier et al., 2012).

Though it is unclear if α-Adaptin has a direct role in Notch signaling for SOP cell differentiation, it is possible that α-Adaptin, like Neur, is functioning to positively regulate Delta endocytosis in the pIIb cell. It is also possible that α-Adaptin functions with Numb to regulate the Notch receptor in the pIIb cell. As endocytosis is required for both functions, α-Adaptin could be required in both capacities to facilitate biasing the cell (Figure 5.0). The type of signaling event described in SOP differentiation is different from those necessary for photoreceptor differentiation. Therefore, it is possible that α-Adaptin is functioning in a different capacity during photoreceptor differentiation than it does during SOP differentiation. Experiments to specifically test for a role of α-Adaptin during Notch signaling to differentiate photoreceptors will be described in the future directions below.

5.1.2. Possible role for Crumbs in Notch signaling

Crumbs has been well characterized as a single-pass transmembrane protein that functions at the adherens junctions and is localized to the apical membrane, establishing

apical/basal polarity within an epithelial cell (Tepass et al., 1990). *crumbs* was first identified in *Drosophila* in a screen to identify other genes that like *Notch* and *Delta* encode proteins with EGF-like repeats (Knust et al., 1987). Humans have three identified Crumbs homologues, CRB1, CRB2, and CRB3. Mutations in *crumbs* have been linked to the human retinopathies retinitis pigmentosa, Leber congenital amaurosis (van den Hurk et al., 2005) and tumorigenesis (Laprise, 2011). The majority of the Crumbs protein is extracellular and consists of four Laminin-A globular-domain-like (Laminin AG-like) repeats and thirty EGF-like repeats that facilitate binding other proteins at the adherens junction. Crumbs has a transmembrane domain and is anchored at the plasma membrane via interaction of the short intracellular portion with the Crumbs complex (Figure 5.1).

The members of the Crumb complex that bind Crumbs are Protein/Pals-1 Associated with Tight Junctions (PATJ), which functions at tight junctions and is important for cell polarity (Roh et al., 2002), and the scaffolding proteins Stardust (Sdt) (Knust 1993,(Grawe et al., 1996)) and Lin-7 (Bachmann et al., 2008). The intracellular portion of Crumbs has also been shown to interact with Par6 (Hurd et al., 2003; Nam, 2003), aPKC (Sotillos et al., 2004; Walther and Pichaud, 2010), Yurt (Laprise et al., 2006), β-spectrin and Moesin (Medina et al., 2002; Pellikka et al., 2002) (Richardson and Pichaud, 2010). One of the *crumbs* mutations isolated as an enhancer introduces a stop codon, which truncates the protein early on in the first grouping of EGF-like repeats.

A role for Crumbs in *Drosophila* photoreceptor morphogenesis and integrity has been identified. *Drosophila* photoreceptors span the entire length of the retina. *crumbs* mutations show a mispositioning of adherens junctions at the apical membrane and loss of the basal connection of the photoreceptor with the basal lamina of the retina (Izaddoost et al., 2002). This decrease in length results in mispositioning of the photoreceptor (Pellikka et al., 2002). It has been shown that exposure to continuous light induces

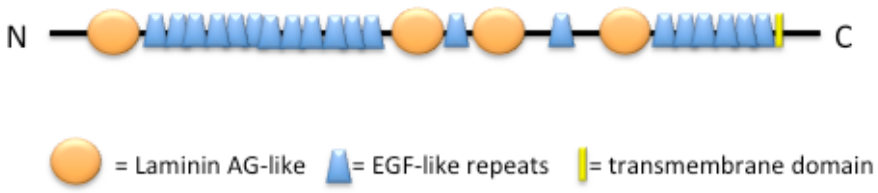

Figure 5.1. Crumbs structure

Crumbs has four Laminin AG-like repeats, thirty EGF-like repeats, and a transmembrane domain. Most of the protein is extracellular. Other proteins that form a Crumbs complex anchor the protein via interaction with the small intracellular region [Modified from (Tepass et al., 1990), (Bulgakova and Knust, 2009)].

apoptosis and results in photoreceptor degeneration in *crumbs* mutants (Johnson et al., 2002). There is some evidence in tissues other than the eye and in cell culture that Crumbs negatively regulates the γ-secretase complex to prevent ectopic Notch activation (Herranz et al., 2006). Other evidence supports these findings using human Crumbs homolog (CRB2) knockdown and overexpression in cell culture (Mitsuishi et al., 2010). However, experiments performed with the human Crumbs homologues in Hek293 cells suggested there was no significant direct interaction between Crumbs and γ-secretase (Pardossi-Piquard et al., 2007). This discrepancy was addressed in Mitsuishi, *et al* 2010 and it was suggested the difference in results could simply be due to the difference in plasmids transfected and type of cell in which the experiment was performed.

Previous experiments looked at the affect on Notch activation when one copy of *crumbs* was removed from R4 photoreceptor cells that were next to wild type R3 photoreceptor cells. *mβ-lacZ* is a Notch activation indicator expressed in R4 cells. R4 cells heterozygous for a *crumbs* mutation showed an increase in β-Gal expression as compared to the level of activation observed in wild type R4 cells. This result suggests that Crumbs is required in R4 cells to limit Notch activation, perhaps via inhibition of the γ-secretase complex (Figure 5.0). Experiments in the ovary and eye disc suggest the increase in Notch activation observed in *crumbs* mutants is ligand dependent and an increase in Delta/NECD positive puncta were observed in cells indicating an increase in Delta endocytosis to activate Notch signaling (Richardson and Pichaud, 2010). Though these experiments did not test a requirement for Crumbs function in signal-sending cells, the observation that the increase in Notch activation occurred due to an increase of Delta endocytosis and activation of Notch signaling would suggest that Crumbs could also have a role in endocytosis of Delta in signal-sending cells.

It has also been suggested that a feedback mechanism is present in developing *Drosophila* gut epithelia. During *Drosophila* gut formation a dorsal/ventral boundary is established via Notch signaling. Notch activation indirectly positively regulates Crumbs expression. Upon indirect Notch-dependent expression, Crumbs is targeted to the apical cell margins and is important in Notch localization to the apical plasma membrane (Figure 5.0) (Fuβ and Hoch, 2002). A co-immunoprecipitation experiment with proteins transfected into 293T cells and experiments done with *in vitro* expression of the proteins indicate the extracellular domains of Crumbs and the Notch receptor can bind one another. Further experiments suggest the interaction is occurring in *cis* to inhibit Notch activation (Ohata et al., 2011).

Based on what is currently known about Crumbs function in Notch signaling, it is not immediately clear exactly why *crumbs* mutations were isolated as enhancers from the screen. If Crumbs is functioning to inhibit the γ-secretase complex, then in *crumbs* mutants, ectopic Notch activation may be occurring. However, if Notch activation is decreased in the *auxilin* mutant background and increased in a *crumbs* mutant, then it is more likely that a *crumbs* mutations would suppress the *auxilin* mutant background, not enhance it. Suppression was observed in genetic interaction experiments removing one copy of *crumbs* in combination with heterozygotes of *Notch, Delta,* and *Serrate* mutant flies (Herranz et al., 2006; Richardson and Pichaud, 2010). It is possible that ectopic Notch activation could result in an increase in Notch signaling, incorrect photoreceptor differentiation, and ultimately produce the enhancement of the eye defect that was observed. However, prior experiments suggest an increase of Notch activation beyond wild type levels would produce tumorigenic defects, which were not observed in the *crumbs* mutants when they were identified in the screen background.

If Crumbs is functioning in a feedback loop with Notch, then it might result in enhancement of the *auxilin* mutant background. Notch signaling is reduced in *auxilin* mutants, which would result in a reduction of *crumbs* expression and ultimately proper localization of Notch at the plasma membrane. The receptor would not be present in the correct position on the plasma membrane and could further reduce Notch activation, making it plausible that *crumbs* mutations would be isolated as enhancers from the screen. The evidence suggesting the extracellular domains of Crumbs and Notch bind in *cis* could support the idea that Crumbs binds the Notch receptor and localizes it to the apical plasma membrane. Experiments to specifically test for a role of Crumbs during Notch signaling to differentiate photoreceptors will be described in the future directions below.

5.2. FUTURE DIRECTIONS

Initial experiments suggest α-Adaptin has a functional role during photoreceptor differentiation. However, further experiments are required to determine a functional role for α-Adaptin specifically during Notch signaling for photoreceptor differentiation. Crumbs has been implicated in the Notch signaling pathway during photoreceptor differentiation, however it is not clear exactly how it is functioning and whether or not it has a role in signal-receiving cells alone or in both signal-sending and signal-receiving cells.

5.2.1. Identification of other enhancers isolated from the screen and testing genetic interactions

There are still several complementation groups and many single alleles that have yet to be identified. Mapping of complementation groups 1 and 2 was attempted, but a region of interest was not identified. Complementation group 3 has two strong enhancer

mutations and complementation group 4 consists of one strong and one weak enhancer mutation. The other complementation groups are not what I refer to as clean. A clean complementation group occurs when every member is lethal in combination with any of the other complementation group members. A complementation group that is not clean occurs when a member is lethal with at least one member of the complementation group, but may not be lethal in combination with every member of the group. For example, complementation group 6 is not clean, because mutations *S2123* and *V16* are lethal in combination with one another, mutations *D339* and *Q417* are lethal in combination with one another, but neither *S2123* nor *V16* is lethal in combination with either *D339* or *Q417*. The screen chromosomes were outcrossed and isogenized twice prior to performing the mutagenesis. Therefore, the complementation groups that are not clean are most likely not due to interactions of mutations present prior to mutagenesis on the chromosomes used for the screen background. One possibility is that the mutations are present in different components of a complex and interactions between the different complex components are observed within the complementation group. Identification of the members of these groups will be more complicated, because multiple enhancer mutations from the group would need to undergo mapping. If they are mutations in different genes, they should map to different locations on the chromosome. If they are mutations in the same gene, they should map to the same location on the chromosome. Even though these groups are complicated and they could be interesting and should be pursued further.

The enhancer mutations should also be tested for failure to complement mutations in genes encoding other proteins that may interact with AP-2 (i.e. Numb) or Crumbs (i.e. Stardust, Moesin, Yurt, etc.). If alleles of these interactors were not isolated from the screen, but other alleles are available, experiments could be performed to test genetic

interactions. These experiments may be helpful in determining which known interactions may be required during photoreceptor development, but the genetic interactions do not explicitly identify a direct role and may not specifically show involvement in Notch signaling. Therefore, more experiments must be performed to identify a role in Notch signaling.

5.2.2. Delta localization in *α-adaptin* mutants

α-Adaptin has been characterized as an adaptor protein important for Clathrin-dependent endocytosis (Keen and Beck, 1989). In the developing *Drosophila* eye, Delta is endocytosed into signal-sending cells in an Epsin-dependent and Clathrin-dependent manner to activate Notch signaling (Banks et al., 2011; Eun et al., 2008; Overstreet et al., 2003; Overstreet et al., 2004; Wang and Struhl, 2004). Therefore, α-Adaptin may be functioning in signal-sending cells to endocytose Delta in a Clathrin-dependent manner. α-Adaptin could be functioning as a Clathrin-adaptor directly and/or interacting with Epsin through its DPW motifs to mediate Clathrin-dependent endocytosis. Reagents are not currently available to test this hypothesis specifically, but it is possible to generally assay a potential role for α-Adaptin in Delta endocytosis. Eye discs mutant for *α-adaptin* will be generated and stained with an anti-Delta antibody as previously described in section 2.2.1. When Notch signaling fails during *Drosophila* eye development, it results in an increase in *Delta* transcription and an increase of Delta present on the membrane at the morphogenetic furrow. If no increase of Delta on the membrane is observed at the morphogenetic furrow, it will indicate that α-Adaptin may not function in Notch signaling during photoreceptor differentiation. However, if Delta is increased at the morphogenetic furrow, it suggests that α-Adaptin may be functioning in Notch signaling. If Delta is increased on the membrane at the morphogenetic furrow, but does not perdure

on the membrane posterior to the morphogenetic furrow, it suggests a function for α-Adaptin in Notch signaling other than endocytosis of Delta. If Delta is increased on the membrane at the morphogenetic furrow and perdures on the membrane, it suggests α-Adaptin is functioning in Notch signaling at least to endocytose Delta. α-Adaptin may also function in Notch signaling in some other capacity. However, this experiment is not sensitive enough to distinguish between multiple roles.

Initially the previously described experiment will be performed in eye discs mutant for *α-adaptin* only. However, due to possible redundancy in the system, it may not be possible to observe a Delta endocytosis defect. Therefore, the experiment may need to be performed in the sensitized screen background. The whole eye disc will be weakly mutant for *auxilin* and clones mutant for *α-adaptin* will be generated using the FLP/FRT system. This experiment will be performed with the *α-adaptin* alleles isolated from the screen as well as other acquired *α-adaptin* mutant alleles. It is necessary to perform the experiment with multiple alleles to ensure any Delta endocytosis defect observed is due to the enhancer mutation and not other mutations present on other locations in the genome.

5.2.3. Function of α-Adaptin during Notch signaling in signal-sending cells, signal-receiving cells or both

An experiment performed at a specific Notch signaling event may be able to specifically identify a role for α-Adaptin in signal-sending cells, signal-receiving cells or both. The specific Notch signaling event chosen for this experiment occurs during the differentiation of photoreceptor R3 from R4. Specification of R3 from R4 results in asymmetric patterning or chirality of the photoreceptors within the facet. R3 and R4 are initially equivalent proneural cells with the presumptive R3 cell positioned more equatorial to the presumptive R4 cell. At the equator the activity of a Planar Cell Polarity

(PCP) signaling receptor Frizzled (Fz) is elevated, but occurs as a decreasing gradient towards the dorsal or ventral poles (Baeg et al., 2004; Fanto et al., 1998; Strutt et al., 1997; Tomlinson et al., 1997; Zheng et al., 1995). The pre-R3 cell is biased by an earlier or more robust Fz activation based on is position relative to R4 and to the equator of the eye disc. Activation of the Fz receptor in the presumptive R3 converts the cell to a pre-R3 fate. The bias could be generated by an increase in Delta and Neur transcription in the pre-R3 cell. It has been proposed that Notch activation in the presumptive R4 may repress Delta and Neur transcription, which biases the cell to assume a pre-R4 fate. Fz activation can also negatively regulate the Notch receptor in the pre-R3 through either Dishevelled (Cooper and Bray, 1999; Fanto and Mlodzik, 1999) or Ral (Cho and Fischer, 2011). The pre-R3 is biased to be the signal-sending cell and the pre-R4 is biased to be the signal-receiving cell. A Notch signaling event between a wild type pre-R3 and pre-R4 would result in Notch activation in the pre-R4 cell, establishing the R4 cell fate and ultimately the R3 cell fate. Removal of a Notch signaling component from both pre-R3 and pre-R4 should result in an inability to send and/or receive a signal, therefore no Notch activation would be observed in a pre-R4 cell. If a Notch signaling pathway component functioning solely in the pre-R3 cell to send a signal is mutant only in pre-R3, wild type pre-R4 will not receive a signal and Notch will not be activated in pre-R4. If a Notch signaling pathway component functioning solely in the pre-R4 cell to receive a signal is mutant only in pre-R4, it will not be able to receive a signal from the wild type pre-R3 and Notch signaling will not be activated in pre-R4. If a Notch signaling pathway component is required in both pre-R3 to send a signal in pre-R4 to receive a signal, then in either situation (mutant pre-R3/wild type pre-R4 or wild type pre-R3/mutant pre-R4) it will result in a failure to activate Notch signaling in the pre-R4 cell

This system can be used to test a requirement for pathway components in signal-sending cells, signal-receiving cells, or both using the *mδ0.5-lacZ* transgene expression as an indicator of Notch activation (Cooper and Bray, 1999; Fanto and Mlodzik, 1999), Seven-up as a marker to differentiate R3 and R4 from other photoreceptors in the developing eye disc (Fanto et al., 1998), and the MARCM technique to generate small mutant clones. The MARCM technique relies on Gal80 repression of *tub-Gal4* driving *UAS-GFP* expression until specific expression of FLP induces recombination at FRT sites. This mitotic recombination event results in some cells that have the parental chromosomes, some cells that have a recombinant chromosome that results in the cell being homozygous for Gal80, or a twin spot of cells that no longer have a copy of Gal80. The clone cells generated by the recombination that lack Gal80 can then express GFP. This system can be used to generate mutant clones if the mutant allele is present on an *FRT* chromosome in trans to the *Gal-80 FRT* containing chromosome. Upon recombination all of the cells that lack Gal80 will be homozygous for the mutant allele and will express GFP (Cho and Fischer, 2011; Lee and Luo, 2001).

Several *α-adaptin* alleles are present on *FRT* chromosomes that can be used in this assay. They will be crossed into the MARCM system and small clones will be generated in developing eye discs using heat shock of larvae for 1 hour at 38°C to drive expression of *hs-FLP*. The eye discs will be dissected from wandering 3rd instar larvae, fixed and stained with anti-Svp and anti-β-Gal antibodies. In facets where both the pre-R3 and the neighboring pre-R4 are wild type, it is expected the pre-R3 will signal to pre-R4 and activate Notch signaling resulting in the presence of β-Gal in pre-R4 cells only. Clones generated that are mutant for *α-adaptin* in pre-R3 and the neighboring pre-R4 cell should show a lack of β-Gal in pre-R4 cells if α-Adaptin function is required for Notch signaling in either cell. Clones generated that are mutant for *α-adaptin* in pre-R3 cells

next to a wild type pre-R4 cells should show a lack of β-Gal in pre-R4 cells if α-Adaptin function is required in the signal-sending cell to activate Notch signaling. If it is not required in signal-sending cells, Notch signaling should not be affected and β-Gal will be observed in the pre-R4 cell. Clones generated that are mutant for *α-adaptin* in pre-R4 cells next to wild type pre-R3 cells should show a lack of β-Gal in pre-R4 cells if α-Adaptin function is required in the signal-receiving cell to activate Notch signaling. If it is not required in signal-receiving cells, Notch signaling should not be affected and β-Gal will be observed in the pre-R4 cell. If α-Adaptin function is required in both signal-sending and signal-receiving cells, then when either pre-R3 is mutant for *α-adaptin* next to a wild type pre-R4 cell or pre-R4 is mutant for *α-adaptin* next to a wild type pre-R3 cell, there should be a lack of β-Gal in pre-R4 cells. This result indicates that Notch was not activated in either situation. Any of the expected results will be informative as to what role if any α-Adaptin plays in Notch signaling during photoreceptor differentiation.

5.2.4. Further experiments to characterize α-Adaptin function in Notch signaling

If α-Adaptin is found to function solely in signal-receiving cells, it will be necessary to determine if it is functioning during endocytosis for activation or with Numb to inhibit signaling (Figure 5.0). A monoclonal antibody against α-Adaptin is available (González-Gaitán and Jäckle, 1997) and a polyclonal antibody against Numb is also available (Uemura et al., 1989) and could be used to look at Numb co-localization with Notch or α-Adaptin in cells and whether or not the localization changes in the absence of α-Adaptin. Monoclonal antibodies directed against the NECD or the NICD are available (Fehon et al., 1991). Recently, transgenes expressing a GFP tagged form of Notch were generated. The transgene can rescue mutants fully and makes it possible to track the whole Notch receptor as well as the NICD within the cell (Couturier et al., 2012).

However, localization studies in photoreceptors can be difficult due to the overall structure and organization of the cell. The nucleus makes up most of the cell volume with little cytoplasmic space and the apical foot, where Notch signaling is thought to occur, is small. Also, when Notch signaling fails it results in aberrant actin organization and cellular structure is disrupted. Therefore, co-localization studies may not be informative.

The pre-R3/pre-R4 MARCM clonal analysis could be performed as described above with available *numb* mutant alleles to determine if Numb is required in signal-receiving cells. If Numb is not functioning in signal-receiving cells, then it would suggest that α-Adaptin cannot be interacting with Numb to inhibit Notch activation. To test a role for α-Adaptin to endocytose Notch, pre-R4 cells that are mutant for *α-adaptin* can be generated and stained with antibodies against the Notch receptor. If Notch is present on the plasma membrane at a higher level in *α-adaptin* mutant pre-R4 cells than observed in wild type pre-R4 cells, it would suggest a failure in endocytosis. If α-Adaptin was identified to solely be functioning in signal-receiving cells it would have been due to the observation of Notch activation occurring in a wild type pre-R4 cell next to a pre-R3 *α-adaptin* mutant cell and a lack of Notch activation in a pre-R4 *α-adaptin* mutant cell next to wild type pre-R3 cell. Therefore, an increase of Notch present on the plasma membrane together with a lack of Notch activation in pre-R4 *α-Adaptin* mutant cells would suggest that α-Adaptin is functioning in signal-receiving cells to endocytose and activate Notch.

If α-Adaptin is found to function solely in signal-sending cells, it could be functioning either to positively regulate Delta endocytosis or to negatively regulate Notch through interaction with Numb. Once again antibodies directed against Numb could be used for co-localization studies with α-Adaptin and Delta, but they may not be

informative as explained above. The experiments proposed to look at Delta endocytosis in *α-adaptin* mutants could provide evidence for or against a role for α-Adaptin in that capacity. The pre-R3/pre-R4 MARCM clonal analysis could be performed as described above with available *numb* mutant alleles to determine if Numb is required in signal-sending cells. These experiments together could determine if α-Adaptin is functioning just to endocytose Delta or just with Numb to inhibit Notch activation or both. A role for α-Adaptin to endocytose Notch or Delta during signal activation could provide more information about how endocytosis in Notch signaling is regulating. If there is another function for α-Adaptin during Notch signaling, it could provide more information about how the cells become biased as a signal-sending cell or a signal-receiving cell for Notch signaling.

5.2.5. Determining a role for Crumbs in Notch signaling during photoreceptor differentiation

Adult eyes from flies that have one mutant copy of *crumbs* in the *auxilin* mutant screen background will be dissected, fixed, embedded and sectioned. Photoreceptor defects will be observed and the percent of wild type facets will be determined. Sections from wild type and *auxilin* mutant screen background flies will serve as controls. ANOVA analysis will be applied to determine if there is a signification difference among the means of those three genotypes. Preliminary results suggest that an enhancement in photoreceptor defects is occurring in a *crumbs* mutant enhanced screen background. However, more samples must be prepared to determine if the enhancement is significant.

A role for Crumbs during Notch signaling is as yet unclear. The experiments presented in sections 5.3.2. and 5.3.3. can also be performed with *crumbs* mutants. The *crumbs* alleles generated from the screen are lethal in combination with one another. To perform the Delta localization experiment, it will be necessary to generate clones with the

crumbs mutants. A *crumbs* mutant allele on a chromosome with *FRT82B* will be crossed to a fly of the genotype *eyFLP;;FRT82B, ubi-GFP*. 3rd instar eye discs will dissected, fixed and stained with an anti-Delta antibody. Wild type cells will be GFP positive and *crumbs* mutant cells will lack GFP. The expected results would be similar to those described in section 5.3.2. However, a result from this experiment that shows defects in Delta endocytosis in *crumbs* mutants does not rule out Crumbs function in some other capacity during Notch signaling. *crumbs* mutants will also be crossed into the MARCM system, as described in section 5.3.3., to determine if Crumbs functions solely in signal-sending cells, solely in signal-receiving cells, or in both.

5.2.6. Further experiments to characterize Crumbs function in Notch signaling

Testing for a role for Crumbs in signal-sending cells with MARCM clones will also provide results that repeat the previous experiments performed to study a role for Crumbs in signal-receiving cells. In pre-R4 cells mutant for *crumbs* next to wild type pre-R3 cells, an increase in Notch activation was observed. Experiments suggested that an increase in Notch activation was ligand dependent and removal of ligand from the neighboring cells resulted in suppression of the Notch activation increase. To test a requirement for ligand to see an increase in Notch activation in *crumbs* mutant signal-receiving cells, *crumbs* mutant clones can be generated in an eye disc that is mutant for one copy of *Delta*. While removal of one copy of Delta will not completely prevent Notch signaling, it should reduce signaling and could suppress the increase in Notch activation observed in *crumbs* mutant cells. If suppression is not observed, it could indicate the increase in Notch activation observed is ligand-independent. A lack of suppression could also be a result of not reducing levels of Delta beyond the minimum threshold to observe a defect. However, suppression experiments were performed with

only one copy of a mutant allele of *Delta* in combination with one copy of a mutant allele of *crumbs* and suppression of the whole eye was observed (Richardson and Pichaud, 2010). The experiments proposed here would look at activation of Notch signaling not in the eye as a whole, but specifically in pre-R4 cells, which will provide more specific information about how suppression could be occurring. If suppression is observed, it could indicate a requirement for ligand-dependent Notch activation in the pre-R4 *crumbs* mutant cells. This result would also shed light on why *crumbs* mutations were isolated from the screen. In the *auxilin* mutant screen background, Delta endocytosis is inhibited and results in a decrease in Notch activation in the pre-R4 cell. One mutant copy of *crumbs* may not result in a defect suppressing increase of Notch activation in pre-R4 cells, because of the lack of ligand-dependent activation of Notch (i.e. Delta endocytosis). However, *crumbs* mutations were isolated as enhancers, indicating that there may be an additional function of Crumbs in inhibition of Notch signaling that could not be observed previously due to the increase in Notch activation masking the function. The results of these experiments all together could provide information about a possible role for Crumbs to regulate Notch signaling.

5.3. NEW MODEL FOR DELTA ENDOCYTOSIS TO ACTIVATE NOTCH SIGNALING

Delta binding the Notch receptor initiates a series of cleavage events that results in the release of the NICD which is trafficked to the nucleus where it functions as a transcription factor to regulate expression of several target genes (Figure 5.0). One of the indirect targets is *crumbs*. Crumbs could be expressed, bind Notch and be trafficked to the apical plasma membrane where Notch can bind Delta for future signaling events. Crumbs could also interact with γ-secretase to ensure Notch is not ectopically activated (Figure 5.0). Regulation of the Notch receptor could occur via AP-2 dependent

endocytosis (Figure 5.0). To activate the Notch receptor it is necessary for Delta to be endocytosed into the-signal-sending cell. For endocytosis of Delta to occur to activate signaling it must first be ubiquitinated by Neur. Ubiquitinated Delta is recognized by Epsin, which together with AP-2 forms a Clathrin-coated Delta-containing vesicle. Dynamin pinches off the fully formed vesicle, which is then uncoated by Auxilin and Hsc70. The Delta-containing vesicles are trafficked through the endosomal pathway (Figure 5.0). The endocytic components such as Clathrin, Epsin and AP-2 that formed the Clathrin-coated vesicle are released and trafficked to the plasma membrane for use in future endocytic events. Faf maintains levels of Epsin in the cell by deubiquitinating non-functional ubiquitinated Epsin. Crumbs and AP-2 could be functioning to inhibit ectopic Notch activation in the signal-sending cell, further biasing it to be a signal-sending cell (Figure 5.0).

Notch signaling occurs in many different tissues and contexts within the tissue. Sometimes Notch signaling events occur multiple times within the same cell population with different outcomes. To ensure the correct signal is being sent in the proper cell at the right moment most likely relies on extremely specific regulation of the pathway. The Notch signaling pathway and more specifically the requirement of the ligand to be endocytosed into the signal-sending cells may have evolved to provide several context specific ways to regulate activation of signaling. In order to send a signal, the receptor must be properly modified and present on the plasma membrane while the ligand must be present in a position on the plasma membrane in a cell adjacent to be able to bind the Notch receptor. However, it is possible that Notch can bind its ligand and not activate signaling, until that ligand is endocytosed. Ligand endocytosis could generate a pulling force changing the receptor confirmation and allowing activation to occur. AP-2 and

Crumbs may ultimately provide information about the Notch signaling pathway and its regulation during *Drosophila* eye development.

Appendices

APPENDIX 1: MATERIALS

A.1.1. *Drosophila* stocks

All *Drosophila* stocks were maintained on standard media. Crosses were performed in the standard manner at 25°C unless otherwise indicated .

Eun, *et al*, 2008 Stocks:

w, eyFLP; FRT42D / CyO, YFP ; aux^{D128} / TM6B

w ; FRT42DgAuxUbi-GFP / CyO, YFP ; aux^{727} / TM6B

Banks, *et al*, 2011 Stocks:

w ; PgChc^{+}/ CyO ; aux^{K47} / TM6B

w ; Pglqf^{tC} / CyO ; aux^{D128} / TM6B

Notch mutant clones

w, N^{5419} / FM6

w, FRT19A

w, N^{5419}FRT19A / FM6

w, ubi-GFP FRT19A ; EGUF

Rab11 mutant clones

w ;; FRT5377Histone::GFP / TM3, Sb obtained from Robert Cohen

w ;; FRT5377Hrb98DE::GFP / TM3, Sb obtained from Robert Cohen

w ;; FRT5377Histone::GFP / TM6B

w ;; FRT5377Hrb98DE::GFP / TM6B

yw, hsflp ;; Rab11$^{\Delta FRT5377}$ / TM3, Sb obtained from Robert Cohen
eyFLP ; Sco / CyO; Rab11$^{\Delta FRT5377}$ / TM6B

Screen stocks

w ; FRT42D ; aux^{C2} / TM6B

w, eyFLP; FRT42D, gAux^{+}, GMR-hid / CyO, YFP ; aux^{K47}, Sb / TM6B

w; Sco / CyO ; aux^{K47}Sb / TM6B

w ;; aux^{K47}, Sb / TM6B

w, PgAux^{+} ;; Sb / TM6B

w ; FRT42D / CyO ; aux^{C2} A287 / TM6B

w ; FRT42D / CyO ; aux^{C2} A534 / TM6B

w ; FRT42D / CyO ; aux^{C2} A541 / TM6B

w ; FRT42D / CyO ; aux^{C2} B337 / TM6B

w ; FRT42D / CyO ; aux^{C2} C428 / TM6B

w ; FRT42D / CyO ; aux^{C2} C529 / TM6B

w ; FRT42D / CyO ; aux^{C2} D270 / TM6B

w ; FRT42D / CyO ; aux^{C2} D292 / TM6B

w ; FRT42D / CyO ; aux^{C2} D339 / TM6B

w ; FRT42D / CyO ; aux^{C2} D467 / TM6B

w ; FRT42D / CyO ; aux^{C2} D529 / TM6B

w ; FRT42D / CyO ; aux^{C2} D534 / TM6B

w ; FRT42D / CyO ; aux^{C2} E227 / TM6B

w ; FRT42D / CyO ; aux^{C2} E414 / TM6B

w ; FRT42D / CyO ; aux^{C2} E504 / TM6B

w ; FRT42D / CyO ; aux^{C2} E511 / TM6B

w ; FRT42D / CyO ; aux^{C2} E514 / TM6B

w ; FRT42D / CyO ; aux^{C2} E534 / TM6B

w ; FRT42D / CyO ; aux^{C2} F6 / TM6B

w ; FRT42D / CyO ; aux^{C2} F8 / TM6B

w ; FRT42D / CyO ; aux^{C2} F9 / TM6B

w ; FRT42D / CyO ; aux^{C2} F311 / TM6B

w ; FRT42D / CyO ; aux^{C2} F401 / TM6B

w ; FRT42D / CyO ; aux^{C2} F459 / TM6B

w ; FRT42D / CyO ; aux^{C2} F474 / TM6B

w ; FRT42D / CyO ; aux^{C2} F508 / TM6B

w ; FRT42D / CyO ; aux^{C2} F546 / TM6B

w ; FRT42D / CyO ; aux^{C2} G4 / TM6B

w ; FRT42D / CyO ; aux^{C2} G438 / TM6B

w ; FRT42D / CyO ; aux^{C2} G512 / TM6B

w ; FRT42D / CyO ; aux^{C2} H403 / TM6B

w ; FRT42D / CyO ; aux^{C2} H405 / TM6B

w ; FRT42D / CyO ; aux^{C2} H430 / TM6B

w ; FRT42D / CyO ; aux^{C2} I3 / TM6B

w ; FRT42D / CyO ; aux^{C2} I10 / TM6B

w ; FRT42D / CyO ; aux^{C2} I225 / TM6B

w ; FRT42D / CyO ; aux^{C2} I230 / TM6B

w ; FRT42D / CyO ; aux^{C2} I519 / TM6B

w ; FRT42D / CyO ; aux^{C2} J3 / TM6B

w ; FRT42D / CyO ; aux^{C2} J340 / TM6B

w ; FRT42D / CyO ; aux^{C2} J525 / TM6B

w ; FRT42D / CyO ; aux^{C2} J549 / TM6B

w ; FRT42D / CyO ; aux^{C2} K238 / TM6B

w ; FRT42D / CyO ; aux^{C2} K263 / TM6B

w ; FRT42D / CyO ; aux^{C2} K413 / TM6B

w ; FRT42D / CyO ; aux^{C2} K473 / TM6B

w ; FRT42D / CyO ; aux^{C2} K504 / TM6B

w ; FRT42D / CyO ; aux^{C2} K530 / TM6B

w ; FRT42D / CyO ; aux^{C2} L14 / TM6B

w ; FRT42D / CyO ; aux^{C2} L357 / TM6B

w ; FRT42D / CyO ; aux^{C2} M10 / TM6B

w ; FRT42D / CyO ; aux^{C2} M411 / TM6B

w ; FRT42D / CyO ; aux^{C2} M458 / TM6B

w ; FRT42D / CyO ; aux^{C2} M502 / TM6B

w ; FRT42D / CyO ; aux^{C2} M522 / TM6B

w ; FRT42D / CyO ; aux^{C2} N32 / TM6B

w ; FRT42D / CyO ; aux^{C2} N75 / TM6B

w ; FRT42D / CyO ; aux^{C2} N79 / TM6B

w ; FRT42D / CyO ; aux^{C2} N435 / TM6B

w ; FRT42D / CyO ; aux^{C2} N508 / TM6B

w ; FRT42D / CyO ; aux^{C2} N514 / TM6B

w ; FRT42D / CyO ; aux^{C2} N522 / TM6B

w ; FRT42D / CyO ; aux^{C2} N539 / TM6B

w ; FRT42D / CyO ; aux^{C2} N564 / TM6B

w ; FRT42D / CyO ; aux^{C2} O37 / TM6B

w ; FRT42D / CyO ; aux^{C2} O114 / TM6B

w ; FRT42D / CyO ; aux^{C2} O116 / TM6B

w ; FRT42D / CyO ; aux^{C2} O563 / TM6B

w ; FRT42D / CyO ; aux^{C2} P2 / TM6B

w ; FRT42D / CyO ; aux^{C2} P7 / TM6B

w ; FRT42D / CyO ; aux^{C2} P36 / TM6B

w ; FRT42D / CyO ; aux^{C2} P314 / TM6B

w ; FRT42D / CyO ; aux^{C2} P509 / TM6B

w ; FRT42D / CyO ; aux^{C2} Q325 / TM6B

w ; FRT42D / CyO ; aux^{C2} Q403 / TM6B

w ; FRT42D / CyO ; aux^{C2} Q417 / TM6B

w ; FRT42D / CyO ; aux^{C2} Q512 / TM6B

w ; FRT42D / CyO ; aux^{C2} R32 / TM6B

w ; FRT42D / CyO ; aux^{C2} R34 / TM6B

w ; FRT42D / CyO ; aux^{C2} R247 / TM6B

w ; FRT42D / CyO ; aux^{C2} R509 / TM6B

w ; FRT42D / CyO ; aux^{C2} R519 / TM6B

w ; FRT42D / CyO ; aux^{C2} R531 / TM6B

w ; FRT42D / CyO ; aux^{C2} R540 / TM6B

w ; FRT42D / CyO ; aux^{C2} S16 / TM6B

w ; FRT42D / CyO ; aux^{C2} S38 / TM6B

w ; FRT42D / CyO ; aux^{C2} S260 / TM6B

w ; FRT42D / CyO ; aux^{C2} S2123 / TM6B

w ; FRT42D / CyO ; aux^{C2} S411 / TM6B

w ; FRT42D / CyO ; aux^{C2} S427 / TM6B

w ; FRT42D / CyO ; aux^{C2} S430 / TM6B

w ; FRT42D / CyO ; aux^{C2} S432 / TM6B

w ; FRT42D / CyO ; aux^{C2} S515 / TM6B

w ; FRT42D / CyO ; aux^{C2} S563 / TM6B

w ; FRT42D / CyO ; aux^{C2} T237 / TM6B

w ; FRT42D / CyO ; aux^{C2} T283 / TM6B

w ; FRT42D / CyO ; aux^{C2} T287 / TM6B

w ; FRT42D / CyO ; aux^{C2} T297 / TM6B

w ; FRT42D / CyO ; aux^{C2} T415 / TM6B

w ; FRT42D / CyO ; aux^{C2} T453 / TM6B

w ; FRT42D / CyO ; aux^{C2} T457 / TM6B

w ; FRT42D / CyO ; aux^{C2} T506 / TM6B

w ; FRT42D / CyO ; aux^{C2} T596 / TM6B

w ; FRT42D / CyO ; aux^{C2} U250 / TM6B

w ; FRT42D / CyO ; aux^{C2} U439 / TM6B

w ; FRT42D / CyO ; aux^{C2} U528 / TM6B

w ; FRT42D / CyO ; aux^{C2} V16 / TM6B

w ; FRT42D / CyO ; aux^{C2} V508 / TM6B

w ; FRT42D / CyO ; aux^{C2} W217 / TM6B

w ; FRT42D / CyO ; aux^{C2} W256 / TM6B

w ; FRT42D / CyO ; aux^{C2} W319 / TM6B

w ; FRT42D / CyO ; aux^{C2} W438 / TM6B

w ; FRT42D / CyO ; aux^{C2} W465 / TM6B

w ; FRT42D / CyO ; aux^{C2} X119 / TM6B

w ; FRT42D / CyO ; aux^{C2} X138 / TM6B

w ; FRT42D / CyO ; aux^{C2} X225 / TM6B

w ; FRT42D / CyO ; aux^{C2} X231 / TM6B

w ; FRT42D / CyO ; aux^{C2} X501 / TM6B

w ; FRT42D / CyO ; aux^{C2} X508 / TM6B

w ; FRT42D / CyO ; aux^{C2} X516 / TM6B

w ; FRT42D / CyO ; aux^{C2} X517 / TM6B

w ; FRT42D / CyO ; aux^{C2} Y49 / TM6B

w ; FRT42D / CyO ; aux^{C2} Y214 / TM6B

w ; FRT42D / CyO ; aux^{C2} Y301 / TM6B

w ; FRT42D / CyO ; aux^{C2} Y507 / TM6B

w ; FRT42D / CyO ; aux^{C2} Y512 / TM6B

w ; FRT42D / CyO ; aux^{C2} Z10 / TM6B

w ; FRT42D / CyO ; aux^{C2} Z54 / TM6B

w ; FRT42D / CyO ; aux^{C2} Z74 / TM6B

w ; FRT42D / CyO ; aux^{C2} Z139 / TM6B

w ; FRT42D / CyO ; aux^{C2} Z402 / TM6B

w ; FRT42D α-ada^{Y49} / CyO, ubi-GFP

w ; FRT42D α-ada^{G512} / CyO, ubi-GFP

w ; FRT42D α-ada^{Y49} / CyO

w ; FRT42D α-ada^{G512} / CyO

w ; FRT42D A534 / CyO

w ; FRT42D D529 / CyO

w ; FRT42D F6 / CyO

w ; FRT42D F474 / CyO

w ; FRT42D F546 / CyO

w ; FRT42D H430 / CyO

w ; FRT42D M548 / CyO

w ; FRT42D M522 / CyO

w ; FRT42D N522 / CyO

w ; FRT42D P314 / CyO

w ; FRT42D U439 / CyO

w ; FRT42D U528 / CyO

w ; FRT42D Y507 / CyO

w ; FRT42D Y512 / CyO

w, Pgaux^{+} ;; aux^{C2} A287 / TM6B

w, Pgaux^{+} ;; aux^{C2} A541 / TM6B

w, Pgaux^{+} ;; aux^{C2} B337 / TM6B

w, Pgaux^{+} ;; aux^{C2} C529 / TM6B

w, Pgaux^{+} ;; aux^{C2} D270 / TM6B

w, Pgaux^{+} ;; aux^{C2} D292 / TM6B

w, Pgaux^{+} ;; aux^{C2} D339 / TM6B

w, Pgaux^{+} ;; aux^{C2} D467 / TM6B

w, Pgaux^{+} ;; aux^{C2} D534 / TM6B

w, Pgaux^{+} ;; aux^{C2} E227 / TM6B

w, Pgaux^{+} ;; aux^{C2} E414 / TM6B

w, Pgaux^{+} ;; aux^{C2} E504 / TM6B

w, Pgaux^{+} ;; aux^{C2} E511 / TM6B

w, Pgaux^{+} ;; aux^{C2} E514 / TM6B

w, Pgaux^{+} ;; aux^{C2} E534 / TM6B

w, Pgaux^{+} ;; aux^{C2} F8 / TM6B

w, Pgaux^{+} ;; aux^{C2} F9 / TM6B

w, Pgaux^{+} ;; aux^{C2} F311 / TM6B

w, Pgaux^{+} ;; aux^{C2} F401 / TM6B

w, Pgaux^{+} ;; aux^{C2} F508 / TM6B

w, Pgaux^{+} ;; aux^{C2} G4 / TM6B

w, Pgaux^{+} ;; aux^{C2} G438 / TM6B

w, Pgaux^{+} ;; aux^{C2} I225 / TM6B

w, Pgaux^{+} ;; aux^{C2} I230 / TM6B

w, Pgaux^{+} ;; aux^{C2} J3 / TM6B

w, Pgaux^{+} ;; aux^{C2} J340 / TM6B

w, Pgaux^{+} ;; aux^{C2} J525 / TM6B

w, Pgaux^{+} ;; aux^{C2} J549 / TM6B

w, Pgaux^{+} ;; aux^{C2} K238 / TM6B

w, Pgaux^{+} ;; aux^{C2} K263 / TM6B

w, Pgaux^{+} ;; aux^{C2} K413 / TM6B

w, Pgaux^{+} ;; aux^{C2} K504 / TM6B

w, Pgaux^{+} ;; aux^{C2} K530 / TM6B

w, Pgaux^{+} ;; aux^{C2} L14 / TM6B

w, Pgaux^{+} ;; aux^{C2} M10 / TM6B

w, Pgaux^{+} ;; aux^{C2} M411 / TM6B

w, Pgaux^{+} ;; aux^{C2} M502 / TM6B

w, Pgaux^{+} ;; aux^{C2} N32 / TM6B

w, Pgaux^{+} ;; aux^{C2} N75 / TM6B

w, Pgaux^{+} ;; aux^{C2} N435 / TM6B

w, Pgaux^{+} ;; aux^{C2} N508 / TM6B

w, Pgaux^{+} ;; aux^{C2} N514 / TM6B

w, Pgaux^{+} ;; aux^{C2} N539 / TM6B

w, Pgaux^{+} ;; aux^{C2} N564 / TM6B

w, Pgaux^{+} ;; aux^{C2} O114 / TM6B

w, Pgaux^{+} ;; aux^{C2} P2 / TM6B

w, Pgaux^{+} ;; aux^{C2} P7 / TM6B

w, Pgaux^{+} ;; aux^{C2} P535 / TM6B

w, Pgaux^{+} ;; aux^{C2} Q325 / TM6B

w, Pgaux^{+} ;; aux^{C2} Q403 / TM6B

w, Pgaux^{+} ;; aux^{C2} Q417 / TM6B

w, Pgaux^{+} ;; aux^{C2} Q512 / TM6B

w, Pgaux^{+} ;; aux^{C2} R32 / TM6B

w, Pgaux^{+} ;; aux^{C2} R34 / TM6B

w, Pgaux^{+} ;; aux^{C2} R308 / TM6B

w, Pgaux^{+} ;; aux^{C2} R426 / TM6B

w, Pgaux^{+} ;; aux^{C2} R509 / TM6B

w, Pgaux^{+} ;; aux^{C2} R531 / TM6B

w, Pgaux^{+} ;; aux^{C2} R540 / TM6B

w, Pgaux^{+} ;; aux^{C2} S38 / TM6B

w, Pgaux^{+} ;; aux^{C2} S2123 / TM6B

w, Pgaux^{+} ;; aux^{C2} S411 / TM6B

w, Pgaux^{+} ;; aux^{C2} S427 / TM6B

w, Pgaux^{+} ;; aux^{C2} S432 / TM6B

w, Pgaux^{+} ;; aux^{C2} S515 / TM6B

w, Pgaux^{+} ;; aux^{C2} S563 / TM6B

w, Pgaux^{+} ;; aux^{C2} T283 / TM6B

w, Pgaux^{+} ;; aux^{C2} T297 / TM6B

w, Pgaux^{+} ;; aux^{C2} T415 / TM6B

w, Pgaux^{+} ;; aux^{C2} T453 / TM6B

w, Pgaux^{+} ;; aux^{C2} T457 / TM6B

w, Pgaux^{+} ;; aux^{C2} T506 / TM6B

w, Pgaux^{+} ;; aux^{C2} T571 / TM6B

w, Pgaux^{+} ;; aux^{C2} T596 / TM6B

w, Pgaux^{+} ;; aux^{C2} V16 / TM6B

w, Pgaux^{+} ;; aux^{C2} W256 / TM6B

w, Pgaux^{+} ;; aux^{C2} W319 / TM6B

w, Pgaux^{+} ;; aux^{C2} W465 / TM6B

w, Pgaux^{+} ;; aux^{C2} X119 / TM6B

w, Pgaux^{+} ;; aux^{C2} X138 / TM6B

w, Pgaux^{+} ;; aux^{C2} X225 / TM6B

w, Pgaux^{+} ;; aux^{C2} X231 / TM6B

w, Pgaux^{+} ;; aux^{C2} X501 / TM6B

w, Pgaux^{+} ;; aux^{C2} X508 / TM6B

w, Pgaux^{+} ;; aux^{C2} X516 / TM6B

w, Pgaux^{+} ;; aux^{C2} X517 / TM6B

w, Pgaux^{+} ;; aux^{C2} Y214 / TM6B

w, Pgaux^{+} ;; aux^{C2} Y301 / TM6B

w, Pgaux^{+} ;; aux^{C2} Z10 / TM6B

w, Pgaux^{+} ;; aux^{C2} Z54 / TM6B

w, Pgaux^{+} ;; aux^{C2} Z74 / TM6B

w, Pgaux^{+} ;; aux^{C2} Z139 / TM6B

w, Pgaux^{+} ;; aux^{C2} Z402 / TM6B

w, Pgaux^{+} ;; aux^{C2} C428 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} F459 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} H403 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} H405 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} I519 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} K473 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} L357 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} N522 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} O37 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} O116 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} P36 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} P509 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} R247 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} R519 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} S260 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} S430 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} T237 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} V508 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} W217 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} W438/ TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} I3 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} T571 / TM6B *homozygous viable*

w, Pgaux^{+} ;; aux^{C2} S16 / TM6B *homozygous viable*

Bloomington Stock Center deficiency kits

Exelixis deficiency lines

BSC deficiency lines

DrosDel deficiency lines

Dl^{9P39} / TM6B

w ;; stlqf^{FDD9} / TM6B

w ;; lqf^{ARI} FRT80B / TM6B

w ;; lqf^{L71} FRT80B / TM6B

w ;; FRT82B, Hsc70$^{4\text{-}R447H}$ / TM6B

w ;; FRT82B, faf^{FO8} / TM6B

w ;; FRT82B, neur^{1} / TM6B

cn^{1}, P{PZ}α-ada^{06694} /CyO ; ry^{506} obtained from Bloomington Stock Center

yw, Ubxflp ; αada^{ear4}FRT40A / CyO ; ki / TM6y^{+} obtained from Fabrice Roegiers

yw ubx-FLP; Gal80 FRT40A; neurGal4, Kgv, UASmCD8-GFP/TM6,Ubx, y+
obtained from Fabrice Roegiers

yw ubx-FLP;scaGal4, UAS-Pon-GFP, UAS-Tau-GFP; FRT82b Gal80
obtained from Fabrice Roegiers

AP2a40-31FRT40A / CyO, Twist-Gal4>UAS-GFP obtained from David Bilder

Ada^{3}FRT / CyO, Actin-GFP JMR1 obtained from Marcos González-Gaitán

6694# cn/ CyO, hbB Ada^{3} cn / CyO obtained from Marcos González-Gaitán

ru^{1}h^{1}th^{1}st^{1}cu^{1}sr^{1}e^{s}crb^{2}ca^{1} / TM6B, Sb^{1}, ser^{1} obtained from Bloomington Stock Center

ru^{1}h^{1}th^{1}st^{1}cu^{1}sr^{1}e^{s}crb^{1} / TM6B, Sb^{1} obtained from Bloomington Stock Center

w ; P{w^{+}mC=UAScrb.w^{+}}30.12E obtained from Bloomington Stock Center

FRT82crb$^{82.04}$ / TM6B obtained from Duojia Pan

mδ0.5-lacZ on 2 obtained from Sarah Bray

mδ0.5-lacZ on 3 obtained from Sarah Bray

hs-flp, tub-Gal4, UAS-nGFP ; tub-Gal80, FRT40A / CyO, ubi-GFP

w ; αada^{ear4}FRT40A / CyO, ubi-GFP ; mδ0.5-lacZ / TM6B

hs-flp, tub-Gal4, UAS-nGFP ; tub-Gal80, FRT40A / CyO, ubi-GFP ; aux^{K47}, Sb

w ; αada^{ear4}FRT40A / CyO, ubi-GFP ; aux^{C2}, mδ0.5-lacZ / TM6B

w ; mδ0.5-lacZ / CyO, ubi-GFP ; FRT82crb$^{82.04}$ / TM6B

A.1.2. Antibodies

Primary antibodies were obtained from the Developmental Studies Hybridoma Bank: rat anti-Elav used at a 9:1 dilution, mouse anti-Dl used at a 1:10 dilution, mouse anti-βgal used at a 1:50 dilution. Secondary antibodies were obtained from Molecular Probes: 568 Alexa-fluor goat anti-mouse used at a 1:200 dilution, 647 Alexa-fluor goat anti-rat used at a 1:300 dilution.

A.1.4. PCR Primers

All primers were designed using MacVector.

Detection of FRT:

Primer pair #2

Forward 5’ CGCTTTCTCAATGCTCACGC 3’

Reverse 5’ TTATCTACACGACGGGGAGTCAAG 3’

Primer pair #3

Forward 5’ GCTTCCTCGTGCTTTACGGTATC 3’

Reverse 5’ GCGTCATCAACTCCATCACTGTC 3’

Primer pair #4

Forward 5’ TTCAAGCACTTCCTCTACCCGC 3’

Reverse 5’ CCGCCACAATGTTGGATTTTTC 3’

Detecting LacZ:

Forward 5’ CTGGCGTAATAGCGAAGACC 3’

Reverse 5’ ATGTTGAACTGGAACTCGCC 3’

Detecting Gal80:

Forward 5’ AGGATGGGCAATCAAGACAC 3’

Reverse 5’ TCTACACCGTTCCCGATTTC 3’

RT PCR primers:

α-adaptin RA:

Forward 5’ GCAGCGAGAGCGAAGAAAAAT 3’

Reverse 5’ GAAAATCGTCAATGGATGGCG3’

crumbs

Forward 5’ CCTGTTCTAAGCCAAGTGCC 3’

Reverse 5’ ATTTGATGGGCTTCCTTGTG 3’

Sequencing primers:

α-adaptin

CDS-F 5’ GCTGAAGGGTGTGAAGTCGT 3’

CDS-F1 5’ TTCCAACGAAATACCCAAGC 3’

CDS-F2 5’GCTATGCCGCAAAGACTGTT 3’

CDS-F3 5’ ATCGGTGTGAAGAGCGAGTT 3’

crumbs

F1 5’ TCACTCGGCGATTAGTTTCC 3’

F2 5’ AATGTGGATGAGTGCGACAA 3’

F3 5’ ATGTGGACGAATGCCTTAGC 3’

F4 5’ AAATGCGAGAAGACCACCAC 3’

F5 5’ GAAGACACAACAGTGCCCAA 3’

F6 5’ ACAACAGCAAACTGGAGGGT 3’

F7 5’ ACCCTATTGCGGAGCCTACT 3’

APPENDIX 2: METHODS

A.2.1. Eye disc dissection

Eye Discs were dissected in 1X PBS then placed in fix solution (paraformaldehyde in 2XPEMS) for 30 minutes. Next the discs were washed in 1X PBST for 20 minutes. The eye discs were placed in a well of a Terasaki plate with primary antibody at appropriate dilution and incubated at 4°C overnight. Three wash steps were performed for 5 minutes each in 1X PBST. The eye discs were placed in secondary antibody at the appropriate dilution, the plate was wrapped in foil, and incubated at 25°C for 2 hours. Three wash steps were performed for 5 minutes each in 1X PBST. The eye discs were mounted on Fisher brand Superfrost Plus slides with Vectashield Mounting Media for Fluorescence. All imaging was performed on either a Leica TCSSP2 or SP2AOBS confocal microscope. Images were processed using Adobe Photoshop.

A.2.2. EMS mutagenesis

50 1-2 day old males were collected and dehydrated 30 minutes. Kimwipes were placed in the bottom of vials without food. A solution of 0.025M EMS in 1% sucrose was added to the Kimwipe so that it was saturated, but not flooded. The males were knocked into the EMS containing vial and left at room temperature for 24 hours. The

next day the flies were knocked into a vial containing food for 30 minutes. 50 virgin females were then combined with the mutagenized males in a bottle. The flies were knocked every 24 hours into a new bottle for four days. After 4 days, the parents were discarded.

A.2.3. RNA isolation and RT PCR

Five 3rd instar wandering larvae were homogenized in RLT buffer with DTT. The sample was then centrifuged for 3 minutes and 250µl of sample was combined with ethanol and applied to a Qiagen RNeasy column. The Qiagen RNeasy kit protocol is followed. The resulting RNA sample was resuspended in 30µl RNAse free water.
RT-PCR was performed using the Invitrogen Superscript II RT-PCR kit.

References

Acar, M., H. Jafar-Nejad, H. Takeuchi, A. Rajan, D. Ibrani, N.A. Rana, H. Pan, R.S. Haltiwanger, and H.J. Bellen. 2008. Rumi Is a CAP10 Domain Glycosyltransferase that Modifies Notch and Is Required for Notch Signaling. *Cell*. 132:247-258.

Aguilar, R.C., H.A. Watson, and B. Wendland. 2003. The yeast Epsin Ent1 is recruited to membranes through multiple independent interactions. *The Journal of Biological Chemistry*. 278:10737-10743.

Ahle, S., and E. Ungewickell. 1990. Auxilin, a Newly Identified Clathrin-associated Protein in Coated Vesicles from Bovine Brain. *The Journal of Cell Biology*. 111:19-29.

Artavanis-Tsakonas, S. 1999. Notch Signaling: Cell Fate Control and Signal Integration in Development. *Science*. 284:770-776.

Artavanis-Tsakonas, S., M.E. Fortini, and K. Matsuno. 1995. Notch signaling. *Science*. 268:225-232.

Bachmann, A., F. Grawe, K. Johnson, and E. Knust. 2008. Drosophila Lin-7 is a component of the Crumbs complex in epithelia and photoreceptor cells and prevents light-induced retinal degeneration. *European Journal of Cell Biology*. 87:123-136.

Baeg, G.H., E.M. Selva, R.M. Goodman, R. Dasgupta, and N. Perrimon. 2004. The Wingless morphogen gradient is established by the cooperative action of Frizzled and Heparan Sulfate Proteoglycan receptors. *Developmental Biology*. 276:89-100.

Bai, T., J.L. Seebald, K.-E. Kim, H.-M. Ding, D.P. Szeto, and H.C. Chang. 2010. Disruption of zebrafish cyclin G-associated kinase (GAK) function impairs the expression of Notch-dependent genes during neurogenesis and causes defects in neuronal development. *BMC Developmental Biology*. 10:7.

Baker, N.E., and S.-Y. Yu. 1997. Proneural function of neurogenic genes in the developoing *Drosophila* eye. *Current Biology*. 7:122-132.

Banks, S.M.L., B. Cho, S.H. Eun, J.-H. Lee, S.L. Windler, X. Xie, D. Bilder, and J.A. Fischer. 2011. The Functions of Auxilin and Rab11 in Drosophila Suggest That

the Fundamental Role of Ligand Endocytosis in Notch Signaling Cells Is Not Recycling. *PLoS ONE*. 6:e18259.

Bazinet, C., A.L. Katzen, M. Morgan, A.P. Mahowald, and S.K. Lemmon. 1993. The Drosophila clathrin heary chain gene: clathrin function is essential in a multicellular organism. *Genetics*. 134:1119-1134.

Beck, K.A., and J.H. Keen. 1991. Interaction of Phosphoinositide Cycle Intermediates with the Plasma Membrane-associated Clathrin Assembly Protein AP-2. *The Journal of Biological Chemistry*. 266:4442-4447.

Benhra, N., F. Vignaux, A. Dussert, F. Schweisguth, and R. Le Borgne. 2010. Neuralized Promotes Basal to Apical Transcytosis of Delta in Epithelial Cells. *Molecular Biology of the Cell*. 21:2078-2086.

Benmerah, A., B. Bègue, A. Dautry-Varsat, and N. Cerf-Bensussan. 1996. The Ear of α-Adaptin Interacts with the COOH-terminal Domain of the Eps15 Protein. *The Journal of Biological Chemistry*. 271:12111-12116.

Benmerah, A., C. Lamaze, B. Bègue, S.L. Schmid, A. Dautry-Varsat, and N. Cerf-Bensussan. 1998. AP-2/Eps15 Interaction Is Required for Receptor-mediated Endocytosis. *The Journal of Cell Biology*. 140:1055-1062.

Berdnik, D., T. Torok, M. González-Gaitán, and J.A. Knoblich. 2002. The endocytic protein alpha-adaptin is required for numb-mediated asymmectric cell division in Drosophila. *Developmental Cell*. 3:221-231.

Bettenhausen, B., M. Hrabě de Angelis, S. Dominique, J.-L. Guénet, and A. Gossler. 1995. Transient and restricted expression during mouse embryogenesis of *Dll1,* a murine gene closely related to *Drosophila Delta*. *Development*. 121:2407-2418.

Bickel, P.E., P.E. Scherer, J.E. Schnitzer, P. Oh, M.P. Lisanti, and H.F. Lodish. 1997. Flotillin and Epidermal Surface Antigen Define a New Family of Caveolae-associated Integral Membrane Proteins. *The Journal of Biological Chemistry*. 272:13793-13802.

Bland, C.E., P. Kimberly, and M.D. Rand. 2003. Notch-induced proteolysis and nuclear localization of the Delta ligand. *The Journal of Biological Chemistry*. 278:13607-13610.

Bray, S.J. 2006. Notch signalling: a simple pathway becomes complex. *Nature Reviews Molecular Cell Biology*. 7:678-689.

Bray, S.J., and M. Furriols. 2001. Notch pathway: Making sense of Suppressor of Hairless. *Current Biology*. 11:R217-E221.

Brückner, K., L. Perez, H. Clausen, and S. Cohen. 2000. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature*. 406:411-415.

Brumby, A.M., and H.E. Richardson. 2003. *scribble* mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in *Drosophila*. *The EMBO Journal*. 22:5769-5779.

Bulgakova, N.A., and E. Knust. 2009. The Crumbs complex: from epithelial-cell polarity to retinal degeneration. *Journal of Cell Science*. 122:2587-2596.

Burgos, P.V., G.A. Mardones, A.L. Rojas, L.L. daSilva, Y. Prabhu, J.H. Hurley, and J.S. Bonifacino. 2010. Sorting of the Alzheimer's disease amyloid precursor protein mediated by the AP-4 complex. *Developmental Cell*. 18:425-436.

Buss, F., S.D. Arden, M. Lindsay, J.P. Luzio, and J. Kendrick-Jones. 2001. Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis. *The EMBO Journal*. 20:3676-3684.

Cadavid, A.L.M., A. Ginzel, and J.A. Fischer. 2000. The function of the Drosophila Fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. *Development*. 127:1727-1736.

Chang, H.C. 2004. The J-domain protein Rme-8 interacts with Hsc70 to control clathrin-dependent endocytosis in Drosophila. *The Journal of Cell Biology*. 164:1055-1064.

Chao, M.Y., J. Larkins-Ford, T.M. Tucey, and A.C. Hart. 2005. lin-12 Notch functions in the adult nervous system of C. elegans. *BMC Neurosci*. 6:45.

Chappell, T.G., W.J. Welch, D.M. Schlossman, K.B. Palter, M.J. Schlesinger, and J.E. Rothman. 1986. Uncoating ATPase Is a Member of the 70 Kilodalton Family of Stress Proteins. *Cell*. 45:3-13.

Chen, H., S. Fre, V.I. Slepnev, M.R. Capua, K. Takei, M.H. Butler, P.P. Di Fiore, and P. De Camilli. 1998a. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature*. 394:793-797.

Chen, H., V.I. Slepnev, P.P. Di Fiore, and P. De Camilli. 1999. The Interaction of Epsin and Eps15 with the Clathrin Adaptor AP-2 Is Inhibited by Mitotic Phosphorylation and Enhnaced by Stimulation-dependent Dephosphorylation in Nerve Terminals. *The Journal of Biological Chemistry*. 274:3257-3260.

Chen, M.S., R.A. Obar, C.C. Schroeder, T.W. Austin, C.A. Poodry, S.C. Wadsworth, and R.B. Vallee. 1991. Multiple forms of dynamin are encoded by *shibire* a *Drosophila* gene involved in endocytosis. *Nature*. 351:583-586.

Chen, N., and I. Greenwald. 2004. The Lateral Signal for LIN-12/Notch in *C. elegans* Vulval Development Comprises Redundant Secreted and Transmembrane DSL Proteins. *Developmenal Cell*. 6:183-192.

Chen, W., Y. Feng, D. Chen, and A. Wandinger-Ness. 1998b. Rab11 Is Required for Trans-Golgi Network-to-Plasma Membrane Transport and a Preferential Target for GDP Dissociation Inhibitor. *Molecular Biology of the Cell*. 9:3241-3257.

Chen, X. 2002. A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. *Genes & Development*. 16:289-294.

Cho, B., and J.A. Fischer. 2011. Ral GTPase promotes asymmetric Notch activation in the Drosophila eye in response to Frizzled/PCP signaling by repressing ligand-independent receptor activation. *Development*. 138:1349-1359.

Cho, K.-O., and K.-W. Choi. 1998. Fringe is essential for mirror symmetry and morphogenesis in the *Drosophila* eye. *Nature*. 396:272-276.

Cooper, M.T.D., and S.J. Bray. 1999. Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. *Nature*. 397:526-530.

Cousin, M.A. 2009. Activity-dependent bulk synaptic vesicle endocytosis--a fast, high capacity membrane retrieval mechanism. *Mol Neurobiol*. 39:185-189.

Couturier, L., N. Vodovar, and F. Schweisguth. 2012. Endocytosis by Numb breaks Notch symmetry at cytokinesis. *Nature Cell Biology*. 14:131-139.

Dang, D.T., and N. Perrimon. 1992. Use of a yeast site-specific recombinase to generate embryonic mosaics in *Drosophila*. *Develpmental Genetics*. 13:367-375.

Davenport, C.B. 1940. Analysis of Variance Applied to Human Genetics. *Proceedings of the National Academy of Sciences*. 26:1-3.

De Camilli, P., H. Chen, J. Hyman, E. Panepucci, A. Bateman, and A.T. Brunger. 2002. The ENTH domain. *FEBS Letters*. 513:11-18.

De Camilli, P., K. Takei, and P.S. McPherson. 1995. The function of dynamin in endocytosis. *Current Opinion in Neurobiology*. 5:559-565.

Dell'Angelica, E.C., H. Ohno, C.E. Ooi, E. Rabinovich, K.W. Roche, and J.S. Bonifacino. 1997. AP-3: an adaptor-like protein complex with ubiquitous expression. *The EMBO Journal*. 16:917-928.

Deng, W.-M., C. Althauser, and H. Ruohola-Baker. 2001. Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in *Drosophila* follicle cells. *Development*. 128:4737-4746.

Dexter, J.S. 1914. The Analysis of a Case of Continuous Variation in *Drosophila* by a Study of Its Linkage Relations. *American Society of Naturalists*. 48:712-758.

Doherty, G.J., and H.T. McMahon. 2009. Mechanisms of Endocytosis. *Annual Review of Biochemistry*. 78:857-902.

Domínguez, M., and J.F. de Celis. 1998. A dorsal/ventral boundary established by Notch controls growth and polarity in the *Drosophila* eye. *Nature*. 396:276-278.

Domínguez, M., J.D. Wasserman, and M. Freeman. 1998. Multiple functions of the EGF receptor in *Drosophila* eye development. *Current Biology*. 8:1039-1048.

Doray, B., P. Ghosh, J. Griffith, H.J. Geuze, and S. Kornfeld. 2002. Cooperation of GGAs and AP-1 in packaging MPRs at the trans-Golgi network. *Science*. 297:1700-1703.

Dunwoodie, S.L., D. Henrique, S.M. Harrison, and R.S.P. Beddington. 1997. Mouse *Dll3*: a novel divergent *Delta* gene which may complement the function of other *Delta* homologues during early pattern formation in the mouse embryo. *Development*. 124:3065-3076.

Eisenberg, E., and L.E. Greene. 2007. Multiple Roles of Auxilin and Hsc70 in Clathrin-Mediated Endocytosis. *Traffic*. 8:640-646.

Ellisen, L.W., J. Bird, D.C. West, A.L. Soreng, T.C. Reynolds, S.D. Smith, and J. Sklar. 1991. *TAN-1*, the Human Homolog of the *Drosophila Notch* Gene, Is Broken by Chromosomal Translocations in T Lymphoblastic Neoplasms. *Cell*. 66:649-661.

Emery, G., A. Hutterer, D. Berdnik, B. Mayer, F. Wirtz-Peitz, M.G. Gaitan, and J.A. Knoblich. 2005. Asymmetric Rab11 Endosomes Regulate Delta Recycling and Specify Cell Fate in the Drosophila Nervous System. *Cell*. 122:763-773.

Eun, S.H., S.M.L. Banks, and J.A. Fischer. 2008. Auxilin is essential for Delta signaling. *Development*. 135:1089-1095.

Eun, S.H., K. Lea, E. Overstreet, S. Stevens, J.H. Lee, and J.A. Fischer. 2007. Identification of Genes That Interact With Drosophila liquid facets. *Genetics*. 175:1163-1174.

Fanto, M., C.A. Mayes, and M. Mlodzik. 1998. Linking cell-fate specification to planar polarity: determination of the R3/R4 photoreceptors is a prerequisite for the interpretation of the Frizzled mediated polarity signal. *Mechanisms of Development*. 74:51-58.

Fanto, M., and M. Mlodzik. 1999. Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the Drosophila eye. *Nature*. 397:523-526.

Fehon, R.G., K. Johansen, I. Rebay, and S. Artavanis-Tsakonas. 1991. Complex cellular and subcellular regulation of Notch expression during embryonic and imaginal development of Drosophila: implications for Notch function. *The Journal of Cell Biology*. 113:657-669.

Fehon, R.G., P.J. Kooh, I. Rebay, C.L. Regan, T. Xu, M.A.T. Muskavitch, and S. Artavanis-Tsakonas. 1990. Molecular Interactions between the Protein Products of the Neurogenic Loci *Notch* and *Delta*, Two EGF-Homologous Genes in Drosophila. *Cell*. 61:523-534.

Feng, Y., B. Press, and A. Wandinger-Ness. 1995. Rab7: An Important Regulator of Late Endocytic Membrane Traffic. *The Journal of Cell Biology*. 131:1435-1452.

Fischer, J.A., S.H. Eun, and B.T. Doolan. 2006. Endocytosis, Endosome Trafficking, and the Regulation ofDrosophilaDevelopment. *Annual Review of Cell and Developmental Biology*. 22:181-206.

Fischer, J.A., S.K. Leavell, and Q. Li. 1997. Mutagenesis Screens for Interacting Genes Reveal Three Roles for *fat facets* During *Drosophila* Eye Development. *Developmenal Genetics*. 21:167-174.

Fleming, R.J. 1998. Structural conservation of Notch receptors and ligands. *Seminars in Cell & Developmental Biology*. 9:599-607.

Fleming, R.J., Y. Gu, and N.A. Hukriede. 1997. *Serrate*-mediated activation of *Notch* is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development*. 124:2973-2981.

Fleming, R.J., T.N. Scottgale, R.J. Diederich, and S. Artavanis-Tsakonas. 1990. The gene Serrate encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in Drosophila melanogaster. *Genes & Development*. 4:2188-2201.

Flores, G.V., H. Duan, H. Yan, R. Nagaraj, W. Fu, Y. Zou, M. Noll, and U. Banerjee. 2000. Combinatorial signaling in the specification of unique cell fates. *Cell*. 103:75-85.

Fogel, S., and D.D. Hurst. 1967. Meiotic Gene Conversion in Yeast Tetrands and the Theory of Recombination. *Genetics*. 57:455-481.

Ford, M.G., I.G. Mills, B.J. Peter, Y. Wallis, G.J.K. Praefcke, P.R. Evans, and H.T. McMahon. 2002. Curvature of clathrin-coated pits driven by epsin. *Nature*. 419:361-366.

Ford, M.G., B.M. Pearse, M.K. Higgins, Y. Vallis, D.J. Owen, A. Gibson, C.R. Hopkins, P.R. Evans, and H.T. McMahon. 2001. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science*. 291:1051-1055.

Fortini, M.E. 2002. Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. *Nat Rev Mol Cell Biol*. 3:673-684.

Fotin, A., Y. Chang, N. Grigorieff, T. Walz, S.C. Harrison, and T. Kirchhausen. 2004. Structure of an auxilin-bound clathrin coat and its implications for the mechanism of uncoating. *Nature*. 432:649-653.

Fryer, C.J., E. Lamar, I. Turbachova, C. Kintner, and K.A. Jones. 2002. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes & Development*. 16:1397-1411.

Fuβ, B., and M. Hoch. 2002. Notch Signaling Controls Cell Fate Specification along the Dorsoventral Axis of the *Drosophila* Gut. *Current Biology*. 12:171-179.

Gaidarov, I., Q. Chen, J.R. Falck, K. Reddy, and J.H. Keen. 1996. A Functional Phosphatidylinositol 3,4,5-Trisphosphate/Phosphoinositide Binding Domain in the Clathrin Adaptor AP-2 α Subunit. *The Journal of Biological Chemistry*. 271:20922-20929.

Galbiati, F., D. Volonté, J.S. Goltz, Z. Steele, J. Sen, J. Jurcsak, D. Stein, S. Leslie, and M.P. Lisanti. 1998. Identification, sequence and developmental expression of invertebrate flotillins from *Drosophila melanogaster*. *Gene*. 210:229-237.

Gall, W.E., M.A. Higginbotham, C.-Y. Chen, M.F. Ingram, D.M. Cyr, and T.R. Graham. 2000. The auxilin-like phophoprotein Swa2p is required for clathrin function in yeast. *Current Biology*. 10:1349-1358.

Gallusser, A., and T. Kirchhausen. 1993. The β1 and β2 subunits of the AP complexes are the clathrin coat assembly components. *The EMBO Journal*. 12:5237-5244.

Giardini, P.A., D.A. Fletcher, and J.A. Theriot. 2003. Compression forces generated by actin comet tails on lipid vesicles. *Proc Natl Acad Sci U S A*. 100:6493-6498.

Glittenberg, M., C. Pitsouli, C. Garvey, C. Delidakis, and S.J. Bray. 2006. Role of conserved intracellular motifs in Serrate signalling, cis-inhibition and endocytosis. *The EMBO Journal*. 25:4697-4706.

González-Gaitán, M. 2003. Endocytic trafficking during Drosophila development. *Mechanisms of Development*. 120:1265-1282.

González-Gaitán, M., and H. Jäckle. 1997. Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. *Cell*. 88:767-776.

González-Gaitán, M., and H. Jäckle. 1999. The range of *spalt*-activating Dpp signalling is reduced in endocytosis-defective *Drosophila* wing discs. *Mechanisms of Development*. 87:143-151.

Gordon, W.R., D. Vardar-Ulu, G. Histen, C. Sanchez-Irizarry, J.C. Aster, and S.C. Blacklow. 2007. Structural basis for autoinhibition of Notch. *Nature Structural & Molecular Biology*. 14:295-300.

Grant, B.D., and J.G. Donaldson. 2009. Pathways and mechanisms of endocytic recycling. *Nature Reviews Molecular Cell Biology*. 10:597-608.

Grawe, F., A. Wodarz, B. Lee, E. Knust, and H. Skaer. 1996. The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions. *Development*. 122:951-959.

Greener, T., B. Grant, Y. Zhange, X. Wu, L.E. Greene, D. Hirsh, and E. Eisenberg. 2001. *Caenorhabditis elegans* auxilin: a J-domain protein essential for clathrin-mediated endocytosis *in vivo*. *Nature Cell Biology*. 3:215-219.

Greener, T., X. Zhao, H. Nojima, E. Eisenberg, and L.E. Greene. 2000. Role of cyclin g-associated kinase in uncoating clathrin-coated vesicles from non-neuronal cells. *The Journal of Biological Chemistry*. 275:1365-1370.

Greenwald, I.S., P.W. Sternberg, and H.R. Horvitz. 1983. The *lin-12* Locus Specifies Cell Fates in Caenorhabditis elegans. *Cell*. 34:435-444.

Guo, M., L.Y. Jan, and Y.N. Jan. 1996. Control of Daughter Cell Fates during Asymmetric Division: Interaction of Numb and Notch. *Neuron*. 17:27-41.

Hagedorn, E.J. 2006. Drosophila melanogaster auxilin regulates the internalization of Delta to control activity of the Notch signaling pathway. *The Journal of Cell Biology*. 173:443-452.

Heberlein, U., T. Wolff, and G.M. Rubin. 1993. The TGFβ Homolog *dpp* and the Segment Polarity Gene *hedgehog* Are Required for Propagation of a Morphogenetic Wave in the *Drosophila* Retina. *Cell*. 75:913-926.

Heitzler, P., M. Bourouis, L. Ruel, C. Carteret, and P. Simpson. 1996. Genes of the *Enhancer of split* and *achaete-scute* complexes are required for a regulatory loop between *Notch* and *Delta* during lateral signalling in *Drosophila*. *Development*. 122:161-171.

Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends in Biochemical Sciences*. 8:245-250.

Henley, J.R., E.W.A. Krueger, B.J. Oswald, and M.A. McNiven. 1998. Dynamin-mediated Internalization of Caveolae. *The Journal of Cell Biology*. 141:85-99.

Hermann, G.J., B. Leung, and J.R. Priess. 2000. Left-right asymmetry in *C. elegans* intestine organogenesis involves a LIN-12/Notch signaling pathway. *Development*. 127:3429-3440.

Herranz, H., E. Stamataki, F. Feiguin, and M. Milan. 2006. Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of gamma-secretase activity. *EMBO reports*. 7:297-302.

Heuser, J.E., and J. Keen. 1988. Deep-Etch Visualization of Proteins Involved in Clathrin Assembly. *The Journal of Cell Biology*. 107:877-886.

Heymann, J.B. 2005. Visualization of the Binding of Hsc70 ATPase to Clathrin Baskets: IMPLICATIONS FOR AN UNCOATING MECHANISM. *Journal of Biological Chemistry*. 280:7156-7161.

Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annual Review of Cell and Developmental Biology*. 19:141-172.

Hinshaw, J.E., and S.L. Schmid. 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature*. 374:190-192.

Hirota, Y., T. Kuronita, H. Fujita, and Y. Tanaka. 2007. A role for Rab5 activity in the biogenesis of endosomal and lysosomal compartments. *Biochemical and Biophysical Research Communications*. 364:40-47.

Hirst, J., L.D. Barlow, G.C. Francisco, D.A. Sahlender, M.N.J. Seaman, J.B. Dacks, and M.S. Robinson. 2011. The Fifth Adaptor Protein Complex. *PLoS Biology*. 9:e1001170.

Hirst, J., and M.S. Robinson. 1998. Clathrin and adaptors. *Biochimica et Biophysica Acta*. 1404:173-193.

Holstein, S.E.H., H. Ungewickell, and E.J. Ungewickell. 1996. Mechanism of clathrin basket dissociation: separate functions of protein domains of the DnaJ homologue auxilin. *The Journal of Cell Biology*. 15:925-937.

Hubbard, E.J.A., G. Wu, J. Kitajewski, and I. Greenwald. 1997. sel-10, a negative regulator of lin-12 activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. *Genes & Development*. 11:3182-3193.

Hurd, T.W., L. Gao, M.H. Roh, I.G. Macara, and B. Margolis. 2003. Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nature Cell Biology*. 5:137-142.

Hutterer, A., and J.A. Knoblich. 2005. Numb and α-Adaptin regulate Sanpodo endocytosis to specify cell fate in Drosophila external sensory organs. *EMBO reports*. 6:836-842.

Irvine, K.D., and T.F. Vogt. 1997. Dorsal-ventral signaling in limb development. *Current Opinion in Cell Biology*. 9:867-876.

Ito, T., R.M. Allen, W.F.t. Carson, M. Schaller, K.A. Cavassani, C.M. Hogaboam, N.W. Lukacs, A. Matsukawa, and S.L. Kunkel. 2011. The critical role of Notch ligand Delta-like 1 in the pathogenesis of influenza A virus (H1N1) infection. *PLoS Pathog*. 7:e1002341.

Itoh, M., C.-H. Kim, G. Palardy, T. Oda, Y.-J. Jiang, D. Maust, S.-Y. Yeo, K. Lorick, G.J. Wright, L. Ariza-McNaughton, A.M. Weissman, J. Lewis, S.C. Chandrasekharappa, and A.B. Chitnis. 2003. Mind Bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Developmental Cell*. 4:67-82.

Itoh, T., K.S. Erdmann, A. Roux, B. Habermann, H. Werner, and P. De Camilli. 2005. Dynamin and the actin cytoskeleton cooperatively regulate plasma membrane invagination by BAR and F-BAR proteins. *Developmental Cell*. 9:791-804.

Itoh, T., S. Koshiba, T. Kigawa, A. Kikuchi, S. Yokoyama, and T. Takenawa. 2001. Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science*. 291:1047-1051.

Izaddoost, S., S.-C. Nam, M. Bhat, H.J. Bellen, and K.-W. Cho. 2002. *Drosophila* Crumbs is a positional cue in photoreceptor adherens junctions and rhabdomeres. *Nature*. 416:178-182.

Jafar-Nejad, H., H.K. Andrews, M. Acar, V. Bayat, F. Wirtz-Peitz, S.Q. Mehta, J.A. Knoblich, and H.J. Bellen. 2005. Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of Drosophila sensory organ precursors. *Developmental Cell*. 9:351-363.

Jenkins, J.B. 1967. Mutagenesis at a Complex Locus in *Drosophila* with the Monofunctional Alkylating Agent, Ethyl Methanesulfonate. *Genetics*. 57:783-793.

Jennings, B., A. Preiss, C. Dlidakis, and S.J. Bray. 1994. The Notch signalling pathway is required for *Enhancer of split* bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development*. 120:3537-3548.

Jiang, R.-F., T. Greener, W. Barouch, L.E. Greene, and E. Eisenberg. 1997. Interaction of auxilin with the molecular chaperone, Hsc70. *The Journal of Biological Chemistry*. 272:6141-6145.

Johnson, K., F. Grawe, N. Grzeschik, and E. Knust. 2002. *Drosophila* Crumbs Is Required to Inhibit Light-Induced Photoreceptor Degeneration. *Current Biology*. 12:1675-1680.

Johnson, S.E., M.X. Ilagan, R. Kopan, and D. Barrick. 2010. Thermodynamic analysis of the CSL x Notch interaction: distribution of binding energy of the Notch RAM region to the CSL beta-trefoil domain and the mode of competition with the viral transactivator EBNA2. *The Journal of Biological Chemistry*. 285:6681-6692.

Jost, M., F. Simpson, J.M. Kavran, M.A. Lemmon, and S.L. Schmid. 1998. Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Current Biology*. 8:1399-1402.

Kageyama, R., and T. Ohtsuka. 1999. The Notch-Hes pathway in mammalian neural development. *Cell Research*. 9:179-188.

Kanaoka, Y., S.H. Kimura, I. Okazaki, M. Ikeda, and H. Nojima. 1997. GAK: a cyclin G associated kinase contains a tensin/auxilin-like domain. *FEBS Letters*. 402:73-80.

Keen, J.H. 1987. Clathrin Assembly Proteins: Affinity Purification and a Model for Coat Assembly. *The Journal of Cell Biology*. 105:1989-1998.

Keen, J.H., and K.A. Beck. 1989. Identification of the Clathrin-binding Domain of Assembly Protein AP-2. *Biochemical and Biophysical Research Communications*. 158:17-23.

Kelly, D.F., R.J. Lake, T.C. Middlekoop, H.-Y. Fan, S. Artavanis-Tsakonas, and T. Walz. 2010. Molecular Structure and Dimeric Organization of the Notch Extracellular Domain as Revealed by Electron Microscopy. *PLoS ONE*. 5:e10532.

Kidd, S., M.R. Kelley, and M.W. Young. 1986. Sequence of the Notch Locus of *Drosophila melanogaster*: Relationship of the Encoded Protein to Mammalian Clotting and Growth Factors. *Molecular and Cellular Biology*. 6:3094-3108.

Kimura, S.H., H. Tsuruga, N. Yabuta, Y. Endo, and H. Nojima. 1997. Structure, Expression, and Chromosomal Localization of Human GAK. *Genomics*. 44:179-187.

Kirchhausen, T. 1999. Adaptors for clathrin-mediated traffic. *Annual Review of Cell and Developmental Biology*. 15:705-732.

Kirchhausen, T. 2000. Clathrin. *Annual Review of Biochemistry*. 69:699-727.

Kirchhausen, T., J.S. Bonifacino, and H. Riezman. 1997. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. *Current Opinion in Cell Biology*. 9:488-495.

Kirchhausen, T., K.L. Nathanson, W. Matsui, A. Vaisberg, E.P. Chow, C. Burne, J.H. Keen, and A.E. Davis. 1989. Structural and functional division into two domains of the large (100 to 115-kDa) chains of the clathrin-associated protein complex AP-2. *Proceedings of the National Academy of Sciences*. 86:2612-2616.

Kiss, A.L., and E. Botos. 2009. Endocytosis via caveolae: alternative pathway with distinct cellular compartments to avoid lysosomal degradation? *Journal of Cellular and Molecular Medicine*. 13:1228-1237.

Klueg, K.M., T.R. Parody, and M.A.T. Muskavitch. 1998. Complex Proteolytic Processing Acts on Delta, a Transmembrane Ligand for Notch, during *Drosophila* Development. *Molecular Biology of the Cell*. 9:1709-1723.

Knust, E., U. Dietrich, U. Tepass, K.A. Bremer, D. Weigel, H. Vässin, and J.A. Campos-Ortega. 1987. EGF homologous sequences encoded in the genome of *Drosophila melanogaster,* and their relation to neurogenic genes. *The EMBO Journal*. 6:761-766.

Koundakjian, E.J., D.M. Cowan, R.W. Hardy, and A.H. Becker. 2004. The Zucker Collection: A Resource for the Analysis of Autosomal Gene Function in *Drosophila melanogaster*. *Genetics*. 167:203-206.

Krieg, D.R. 1962. Ethyl Methanesulfonate-Induced Reversion of Bacteriophage *T4rII* Mutants. *Genetics*. 48:561-580.

Kuang, B., S.C.-Y. Wu, Y.-A. Shin, L. Luo, and P. Kolodziej. 2000. split ends encodes large nuclear proteins that regulate neuronal cell fate and axon extension in the Drosophila embryo. *Development*. 127:1517-1529.

Kübler, E., and H. Riezman. 1993. Actin and fimbrin are required for the internalization step of endocytosis in yeast. *The EMBO Journal*. 12:2855-2862.

Kurooka, H., K. Kuroda, and T. Honjo. 1998. Roles of the ankyrin repeats and C-terminal region of the mouse Notch1 intracellular region. *Nucleic Acids Research*. 26:5448-5455.

Lai, E.C. 2005. Pervasive regulation of Drosophila Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. *Genes & Development*. 19:1067-1080.

Lai, E.C., G.A. Deblandre, C. Kintner, and G.M. Rubin. 2001. *Drosophila* Neuralized Is a Ubiquitin Ligase that Promotes the Internalization and Degradation of Delta. *Developmenal Cell*. 1:783-794.

Lai, E.C., and G.M. Rubin. 2001. neuralized functions cell-autonomously to regulate a subset of notch-dependent processes during adult Drosophila development. *Developmental Biology*. 231:217-233.

Laprise, P. 2011. Emerging role for epithelial polarity proteins of the Crumbs family as potential tumor suppressors. *J Biomed Biotechnol*. 2011:868217.

Laprise, P., S. Beronja, N.F. Silva-Gagliardi, M. Pellikka, A.M. Jensen, C.J. McGlade, and U. Tepass. 2006. The FERM protein Yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size. *Developmental Cell*. 11:363-374.

LaVoie, M.J., and D.J. Selkoe. 2003. The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. *The Journal of Biological Chemistry*. 278:34427-34437.

Le Borgne, R. 2005. The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development*. 132:1751-1762.

Le Borgne, R., and F. Schweisguth. 2003. Unequal Segregation of Neuralized Biases Notch Activation during Asymmetric Cell Division. *Developmental Cell*. 5:139-148.

Lee, C.Y., K.M. Vogeli, S.-H. Kim, S.-W. Chong, Y.-J. Jiang, D.Y.R. Stainier, and S.-W. Jin. 2009. Notch Signaling Functions as a Cell-Fate Switch between the Endothelial and Hematopoietic Lineages. *Current Biology*. 19:1616-1622.

Lee, T., and L. Luo. 2001. Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *TRENDS in Neurosciences*. 24:251-254.

Lemmon, S.K. 2001. Clathrin-uncoating: Auxilin comes to life. *Current Biology*. 11:R49-R52.

Levitan, D., and I. Greenwald. 1995. Facilitation of *lin-12*-mediated signalling by *sel-12*, a *Caenorhabditis elegans S182* Alzheimer's disease gene. *Nature*. 377:351-354.

Li, Y., and N.E. Baker. 2001. Proneural enhancement by Notch overcomes Supressor-of-Hairless repressor function in the developing Drosophila eye. *Current Biology*. 11:330-338.

Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M.W. Young. 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes & Development*. 7:1949-1965.

Ligoxygakis, P., S.-Y. Yu, C. Delidakis, and N.E. Baker. 1998. A subset of Notch functions during Drosophila eye development require Su(H) and the E(spl) gene complex. *Development*. 125:2893-2900.

Lin, H.C., and A.G. Gilman. 1996. Regulation of Dynamin I GTPase Activity by G Protein βγ Subunits and Phosphatidylinositol 4,5-Bisphosphate. *The Journal of Biological Chemistry*. 271:27979-27982.

Lindner, R., and E. Ungewickell. 1991. Light-Chain-Independent Binding of Adaptors, AP180, and Auxilin to Clathrin. *Biochemistry*. 30:9097-9101.

Lindsell, C.E., C.J. Shawber, J. Boulter, and G. Weinmaster. 1995. Jagged: A Mammalian Ligand That Activates Notch1. *Cell*. 80:909-917.

Logeat, F., C. Bessia, C. Brou, O. LeBail, S. Jarriault, N.G. Seidah, and A. Israël. 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proceedings of the National Academy of Sciences*. 95:8108-8112.

Major, R.J., and K.D. Irvine. 2005. Influence of Notch on dorsoventral compartmentalization and actin organization in the Drosophila wing. *Development*. 132:3823-3833.

Medina, E., J. Williams, E. Klipfell, D. Zarnescu, G. Thomas, and A. Le Bivic. 2002. Crumbs interacts with moesin and beta(Heavy)-spectrin in the apical membrane skeleton of Drosophila. *The Journal of Cell Biology*. 158:941-951.

Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the Endocytic and Exocytic Pathways. *Annual Review of Biochemistry*. 55:663-700.

Menut, L., T. Vaccari, H. Dionne, J. Hill, G. Wu, and D. Bilder. 2007. A mosaic genetic screen for Drosophila neoplastic tumor suppressor genes based on defective pupation. *Genetics*. 177:1667-1677.

Mishra-Gorur, K. 2002. Down-regulation of Delta by proteolytic processing. *The Journal of Cell Biology*. 159:313-324.

Mitsuishi, Y., H. Hasegawa, A. Matsuo, W. Araki, T. Suzuki, S. Tagami, M. Okochi, M. Takeda, R. Roepman, and M. Nishimura. 2010. Human CRB2 inhibits gamma-secretase cleavage of amyloid precursor protein by binding to the presenilin complex. *The Journal of Biological Chemistry*. 285:14920-14931.

Moloney, D.J., V.M. Panin, S.H. Johnston, J. Chen, L. Shao, R. Wilson, Y. Wang, P. Stanley, K.D. Irvine, R.S. Haltiwanger, and T.F. Vogt. 2000. Fringe is a glycosyltransferase that modifies Notch. *Nature*. 406:369-375.

Moreau, V., J.-M. Galan, G. Devilliers, R. Haguenauer-Tsapis, and B. Winsor. 1997. The Yeast Actin-related Protein Arp2p Is Required for the Internalization Step of Endocytosis. *Molecular Biology of the Cell*. 8:1361-1375.

Morgan, J.R. 2003. Eps15 Homology Domain-NPF Motif Interactions Regulate Clathrin Coat Assembly during Synaptic Vesicle Recycling. *Journal of Biological Chemistry*. 278:33583-33592.

Morris, S.A., A. Mann, and E. Ungewickell. 1990. Analysis of 100-180-kDa Phosphoproteins in Clathrin-coated Vesicles from Bovine Brain. *The Journal of Biological Chemistry*. 265:3354-3357.

Muller, H.J. 1928. The Production of Mutations by X-rays. *Proceedings of the National Academy of Sciences*. 14:714-726.

Musse, A., L. Meioty-Kapella, and G. Weinmaster. 2012. Notch ligand endocytosis: Mechanistic basis of signaling activity. *Seminars in Cell & Developmental Biology*.

Nakayama, K., H. Nagase, C.S. Koh, and T. Ohkawara. 2011. gamma-Secretase-regulated mechanisms similar to notch signaling may play a role in signaling events, including APP signaling, which leads to Alzheimer's disease. *Cell Mol Neurobiol*. 31:887-900.

Nam, S.C. 2003. Interaction of Par-6 and Crumbs complexes is essential for photoreceptor morphogenesis in Drosophila. *Development*. 130:4363-4372.

Nelson, B.R., Y. Ueki, S. Reardon, M.O. Karl, S. Georgi, B.H. Hartman, D.A. Lamba, and T.A. Reh. 2011. Genome-Wide Analysis of Müller Glial Differentiation Reveals a Requirement for Notch Signaling in Postmitotic Cells to Maintain the Glial Fate. *PLoS ONE*. 6:e22817.

Nichols, J.T., A. Miyamoto, S.L. Olsen, B. D'Souza, C. Yao, and G. Weinmaster. 2007. DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *The Journal of Cell Biology*. 176:445-458.

Ohata, S., R. Aoki, S. Kinoshita, M. Yamaguchi, S. Tsuruoka-Kinoshita, H. Tanaka, H. Wada, S. Watabe, T. Tsuboi, I. Masai, and H. Okamoto. 2011. Dual roles of Notch in regulation of apically restricted mitosis and apicobasal polarity of neuroepithelial cells. *Neuron*. 69:215-230.

Okamoto, K., and D. Beach. 1994. Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *The EMBO Journal*. 13:4816-4822.

Overstreet, E., X. Chen, B. Wendland, and J.A. Fischer. 2003. Either Part of a Drosophila Epsin Protein, Divided after the ENTH Domain, Functions in Endocytosis of Delta in the Developing Eye. *Current Biology*. 13:854-860.

Overstreet, E., E. Fitch, and J.A. Fischer. 2004. Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. *Development*. 131:5355-5366.

Owen, D.J., B.M. Collins, and P.R. Evans. 2004. ADAPTORS FOR CLATHRIN COATS: Structure and Function. *Annual Review of Cell and Developmental Biology*. 20:153-191.

Owen, D.J., Y. Vallis, M.E.M. Noble, J.B. Hunter, T.R. Dafforn, P.R. Evans, and H.T. McMahon. 1999. A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. *Cell*. 97:805-815.

Pan, D., and G.M. Rubin. 1997. Kuzbanian Controls Proteolytic Processing of Notch and Mediates Lateral Inhibition during *Drosophila* and Vertebrate Neurogenesis. *Cell*. 90:271-280.

Panin, V.M., V. Papayannopoulous, R. Wilson, and K.D. Irvine. 1997. Fringe modulates Notch-ligand interactions. *Nature*. 387:908-912.

Panin, V.M., L. Shao, L. Lei, D.J. Moloney, K.D. Irvine, and R.S. Haltiwanger. 2002. Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *The Journal of Biological Chemistry*. 277:29945-29952.

Pardossi-Piquard, R., F. Chen, N.F. Silva-Gagliardi, M. Szego, R. McInnes, C.J. McGlade, P. St George-Hyslop, and P.E. Fraser. 2007. Overexpression of Human CRB1 or Related Isoforms, CRB2 and CRB3, Does Not Regulate the Human Presenilin Complex in Culture Cells. *Biochemistry*. 46:13704-13710.

Parks, A.L., K.M. Klueg, J.R. Stout, and M.A.T. Muskavitch. 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development*. 127:1373-1385.

Parks, A.L., J.R. Stout, S.B. Shepard, K.M. Klueg, A.A. Dos Santos, T.R. Parody, M. Vaskova, and M.A. Muskavitch. 2006. Structure-function analysis of delta trafficking, receptor binding and signaling in Drosophila. *Genetics*. 174:1947-1961.

Parks, A.L., F.R. Turner, and M.A.T. Muskavitch. 1995. Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. *Mechanisms of Development*. 50:201-216.

Pellikka, M., G. Tanentzapf, M. Pinto, C. Smith, C.J. McGlade, D.F. Ready, and U. Tepass. 2002. Crumbs, the *Drosophila* homologue of human CRB1/RP12, is essential for photoreceptor morphogenesis. *Nature*. 416:143-149.

Phng, L.K., and H. Gerhardt. 2009. Angiogenesis: a team effort coordinated by notch. *Developmental Cell*. 16:196-208.

Polo, S., S. Sigismund, M. Faretta, M. Guidi, M.R. Capua, G. Bossi, H. Chen, P. De Camilli, and P.P. Di Fiore. 2002. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature*. 416:451-455.

Poulson, D.F. 1937. Chromosomal Deficiencies and the Embryonic Development of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*. 23:133-137.

Qi, H. 1999. Processing of the Notch Ligand Delta by the Metalloprotease Kuzbanian. *Science*. 283:91-94.

Rajan, A., A.-C. Tien, C.M. Haueter, K.L. Schulze, and H.J. Bellen. 2009. The Arp2/3 complex and WASp are required for apical trafficking of Delta into microvilli during cell fate specification of sensory organ precursors. *Nature Cell Biology*. 11:815-824.

Rand, M.D., L.M. Grimm, S. Artavanis-Tsakonas, V. Patriub, S.C. Blacklow, J. Sklar, and J.C. Aster. 2000. Calcium Depletion Dissociates and Activates Heterodimeric Notch Receptors. *Molecular and Cellular Biology*. 20:1825-1835.

Rapoport, I., M. Miyazaki, W. Boll, B. Duckworth, L.C. Cantley, S. Shoelson, and T. Kirchhausen. 1997. Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes. *The EMBO Journal*. 16:2240-2250.

Rebay , I., R.G. Fehon, and S. Artavanis-Tsakonas. 1993. Specific Truncations of *Drosophila* Notch Define Dominant Activated and Dominant Negative Forms of the Receptor. *Cell*. 74:319-329.

Rebay , I., R.J. Fleming, R.G. Fehon, L. Cherbas, L. Cherbas, and S. Artavanis-Tsakonas. 1991. Specific EGF Repeats of Notch Mediate Interactions with Delta and Serrate: Implications for Notch as a Multifunctional Receptor. *Cell*. 67:687-699.

Rhyu, M.S., L.Y. Jan, and Y.N. Jan. 1994. Asymmetric Distribution of Numb Protein during Division of the Sensory Organ Precursor Cell Confers Distinct Fates to Daughter Cells. *Cell*. 76:477-491.

Richardson, E.C., and F. Pichaud. 2010. Crumbs is required to achieve proper organ size control during Drosophila head development. *Development*. 137:641-650.

Robbins, J., B.J. Blondel, D. Gallahan, and R. Callahan. 1992. Mouse Mammary Tumor Gene *int-3*: a Member of the *notch* Gene Family Transforms Mammary Epithelial Cells. *Journal of Virology*. 66:2594-2599.

Robinow, S., and K. White. 1991. Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. *The Journal of Neurobiology*. 22:443-461.

Robinson, M.S. 1992. Adaptins. *TRENDS in Cell Biology*. 2:293-297.

Robinson, M.S. 1993. Assembly and Targeting of Adaptin Chimeras in Transfected Cells. *The Journal of Cell Biology*. 123:67-77.

Robinson, M.S., D.A. Sahlender, and S.D. Foster. 2010. Rapid Inactivation of Proteins by Rapamycin-Induced Rerouting to Mitochondria. *Developmenal Cell*. 18:324-331.

Roehl, H., and J. Kimble. 1993. Control of cell fate in *C. elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. *Nature*. 364:632-635.

Roh, M.H., O. Makarova, C.J. Liu, K. Shin, S. Lee, S. Laurinec, M. Goyal, R. Wiggins, and B. Margolis. 2002. The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *The Journal of Cell Biology*. 157:161-172.

Rohde, G., D. Wenzel, and V. Haucke. 2002. A phosphatidylinositol (4,5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis. *The Journal of Cell Biology*. 158:209-214.

Rothberg, K.G., J.E. Heuser, W.C. Donzell, Y.-S. Ying, J.R. Glenney, and R.G.W. Anderson. 1992. Caveolin, a Protein Component of Caveolae Membrane Coats. *Cell*. 68:673-682.

Roux, A., K. Uyhazi, A. Frost, and P. De Camilli. 2006. GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. *Nature*. 441:528-531.

Roy, M., W.S. Pear, and J.C. Aster. 2007. The multifaceted role of Notch in cancer. *Curr Opin Genet Dev*. 17:52-59.

Sakamoto, K., W.S. Chao, K. Katsube, and A. Yamaguchi. 2005. Distinct roles of EGF repeats for the Notch signaling system. *Experimental Cell Research*. 302:281-291.

Salcini, A.E., S. Confalonieri, M. Doria, E. Santolini, E. Tassi, O. Minenkova, G. Cesareni, P.G. Pelicci, and P.P. Di Fiore. 1997. Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. *Genes & Development*. 11:2239-2249.

Santolini, E., C. Puri, A.E. Salcini, M.C. Gagliani, P.G. Pelicci, C. Tacchetti, and P.P. Di Fiore. 2000. Numb is an Endocytic Protein. *The Journal of Cell Biology*. 151:1345-1351.

Schweisguth, F. 2004. Regulation of Notch Signaling Activity. *Current Biology*. 14:R129-R138.

Seugnet, L., P. Simpson, and M. Haenlin. 1997. Requirement for dynamin during Notch signaling in Drosophila neurogenesis. *Developmental Biology*. 192:585-598.

Shawber, C., J. Boulter, C.E. Lindsell, and G. Weinmaster. 1996. *Jagged2*: A Serrate-like Gene Expressed during Rat Embryogenesis. *Developmental Biology*. 180:370-376.

Shelly, L.L., C. Fuchs, and L. Miele. 1999. Notch-1 Inhibits Apoptosis in Murine Erythroleukemia Cells and Is Necessary for Differentiation Induced by Hybrid Polar Compounds. *Journal of Cellular Biochemistry*. 73:164-175.

Shih, W., A. Gallusser, and T. Kirchhausen. 1995. A Clathrin-binding Site in the Hinge of the β2 Chain of Mammalian AP-2 Complexes. *The Journal of Biological Chemistry*. 270:31083-31090.

Shutter, J.R., S. Scully, W. Fan, W.G. Richards, J. Kitajewski, G.A. Deblandre, C.R. Kintner, and K.L. Stark. 2000. *Dll4*, a novel Notch ligand expressed in arterial endothelium. *Genes & Development*. 14:1313-1318.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P.P. Di Fiore, and S. Polo. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. *Proc Natl Acad Sci U S A*. 102:2760-2765.

Simpson, F., A.A. Peden, L. Christopoulou, and M.S. Robinson. 1997. Characterization of the Adaptor-related Protein Complex, AP-3. *The Journal of Cell Biology*. 137:835-845.

Slepnev, V.I., and P. De Camilli. 2000. Accessory Factors in Clathrin-dependent Synapic Vesicle Endocytosis. *Nature Reviews Neuroscience*. 1:161-172.

Sorkin, A. 2000. The endocytosis machinery. *The Journal of Cell Science*. 113:4375-4376.

Sotillos, S., M.T. Diaz-Meco, E. Caminero, J. Moscat, and S. Campuzano. 2004. DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in Drosophila. *The Journal of Cell Biology*. 166:549-557.

Spencer, F.A., F.M. Hoffmann, and W.M. Geibart. 1982. Decapentaplegic: A Gene Complex Affecting Morphogenesis in *Drosophila melanogaster*. *Cell*. 28:451-461.

Stenmark, H. 2009. Rab GTPases as coordinators of vesicle traffic. *Nature Reviews Molecular Cell Biology*. 10:513-525.

Struhl, G. 1998. Nuclear Access and Action of Notch *in vivo*. *Cell*. 93:649-660.

Struhl, G., and I. Greenwald. 1999. Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature*. 398:522-525.

Strutt, D.I., U. Weber, and M. Mlodzik. 1997. The role of RhoA in tissue polarity and Frizzled signalling. *Nature*. 387:292-295.

Sun, X., and S. Artavanis-Tsakonas. 1997. Secreted forms of Delta and Serrate define antagonists of Notch signaling in *Drosophila*. *Development*. 124:3439-3448.

Takei, K., P.S. McPherson, S.L. Schmid, and P. De Camilli. 1995. Tubular membrane invaginations coated by dynamin rings are induced by GTP-γS in nerve terminals. *Nature*. 374:186-190.

Tax, F.E., J.J. Yeargers, and J.H. Thomas. 1994. Sequence of *C. elegans lag-2* reveals a cell-signalling domain shared with *Delta* and *Serrate* of *Drosophila*. *Nature*. 368:150-154.

Tebar, F., S.K. Bohlander, and A. Sorkin. 1999. Clathrin Assembly Lymphoid Myeloid Leukemia (CALM) Protein: Localization in Endocytic-coated Pits, Interactions with Clathrin, and the Impact of Overexpression on Clathrin-mediated Traffic. *Molecular Biology of the Cell*. 10:2687-2702.

Tepass, U., and E. Knust. 1993. *crumbs* and *stardust* Act in a Genetic Pathway That Controls the Organization of Epithelia in *Drosophila melanogaster*. *Developmental Biology*. 159:311-326.

Tepass, U., C. Theres, and E. Knust. 1990. *crumbs* Encodes an EGF-like Protein Expressed on Apical Membranes of *Drosophila* Epithelial Cells and Required for Organization of Epithelia. *Cell*. 61:787-799.

Thomas, U., S.A. Speicher, and E. Knust. 1991. The *Drosophila* gene *Serrate* encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. *Development*. 111:749-761.

Tomlinson, A., and D.F. Ready. 1987. Cell Fate in the *Drosophila* Ommatidium. *Developmental Biology*. 123:264-275.

Tomlinson, A., W.R. Strapps, and J. Heemskerk. 1997. Linking Frizzled and Wnt signaling in *Drosophila* development. *Development*. 124:4515-4521.

Uemura, T., S. Shepherd, L. Ackerman, L.Y. Jan, and Y.N. Jan. 1989. *numb*, a Gene Required in Determination of Cell Fate during Sensory Organ Formation in *Drosophila* Embryos. *Cell*. 58:349-360.

Umeda, A. 2000. Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. *European Journal of Cell Biology*. 79:336-342.

Ungewickell, E. 1985. The 70-kd mammalian heat shock proteins are structurally and functionally related to the uncoating protein that release clathrin triskelia from coated vesicles. *The EMBO Journal*. 4:3385-3391.

Ungewickell, E.J., H. Ungewickell, S.E.H. Holstein, R. Lindner, K. Prasad, W. Barouch, B. Martin, L.E. Greene, and E. Eisenberg. 1995. Role of auxilin in uncoating clathrin-coated vesicles. *Nature*. 378:632-635.

Upadhyaya, A., J.R. Chabot, A. Andreeva, A. Samadani, and A. van Oudenaarden. 2003. Probing polymerization forces by using actin-propelled lipid vesicles. *Proc Natl Acad Sci U S A*. 100:4521-4526.

Vaccari, T., and D. Bilder. 2005. The Drosophila tumor suppressor vps25 prevents nonautonomous overproliferation by regulating notch trafficking. *Developmental Cell*. 9:687-698.

van den Hurk, J.A.J.M., P. Rashbass, R. Roepman, J. Davis, K.E.J. Voesenek, M.L. Arends, M.N. Zonneveld, M.H.G. van Roekel, K. Cameron, K. Rohrschneider, J.R. Heckenlively, R.K. Koenekoop, C.B. Hoyng, F.P.M. Cremers, and A.I. den Hollander. 2005. Characterization of the *Crumbs homolog 2 (CRB2)* gene and analysis of its role in retinitis pigmentosa and Leber congenital amaurosis. *Molecular Vision*. 11:263-273.

van der Bliek, A.M., and E.M. Meyerowitz. 1991. Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature*. 351:411-414.

Walther, R.F., and F. Pichaud. 2010. Crumbs/DaPKC-dependent apical exclusion of Bazooka promotes photoreceptor polarity remodeling. *Curr Biol*. 20:1065-1074.

Wang, L.-H., T.C. Südhof, and R.G.W. Anderson. 1995. The Appendage Domain of α-Adaptin Is a High Affinity Binding Site for Dyanmin. *The Journal of Biological Chemistry*. 270:10079-10083.

Wang, W. 2005. Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. *Development*. 132:2883-2894.

Wang, W., and G. Struhl. 2004. Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. *Development*. 131:5367-5380.

Wang, Y., L. Shao, S. Shi, R.J. Harris, M.W. Spellman, P. Stanley, and R.S. Haltiwanger. 2001. Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *The Journal of Biological Chemistry*. 276:40338-40345.

Weinmaster, G., V.J. Roberts, and G. Lemke. 1992. *Notch2*: a second mammalian *Notch* gene. *Development*. 116:931-941.

Weissman, A.M. 2001. Themes and Variations on Ubiquitylation. *Nature Reviews Molecular Cell Biology*. 2:169-178.

Wharton, K.A., K.M. Johansen, T. Xu, and S. Artavanis-Tsakonas. 1985. Nucleotide Sequence from the Neurogenic Locus Notch Implies a Gene Product That Shares Homology with Proteins Containing EGF-like Repeats. *Cell*. 43:567-581.

Windler, S.L., and D. Bilder. 2010. Endocytic Internalization Routes Required for Delta/Notch Signaling. *Current Biology*. 20:538-543.

Wodarz, A., F. Grawe, and E. Knust. 1993. CRUMBS is involved in the control of apical protein targeting during *Drosophila* epithelial development. *Mechanisms of Development*. 44:175-187.

Wolff, T., and D.F. Ready. 1993. Pattern formation in the *Drosophila* retina. *In* The Development of *Drosophila melanogaster*. Vol. 2. M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 1277-1325.

Xie, X., B. Cho, and J.A. Fischer. 2012. Drosophila Epsin's role in Notch ligand cells requires three Epsin protein functions: the lipid binding function of the ENTH domain, a single Ubiquitin interaction motif, and a subset of the C-terminal protein binding modules. *Developmental Biology*. 363:399-412.

Xu, T., I. Rebay, R.J. Fleming, T.N. Scottgale, and S. Artavanis-Tsakonas. 1990. The Notch locus and the genetic circuitry involved in early Drosophila neurogenesis. *Genes & Development*. 4:464-475.

Xu, T., and G.M. Rubin. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development*. 117:1223-1237.

Yarar, D., C.M. Waterman-Storer, and S.L. Schmid. 2005. A Dynamic Actin Cytoskeleton Functions at Multiple Stages of Clathrin-mediated Endocytosis. *Molecular Biology of the Cell*. 16:964-975.

Zhang, J., K.L. Schulze, P.R. Hiesinger, K. Suyama, S. Wang, M. Fish, M. Acar, R.A. Hoskins, H.J. Bellen, and M.P. Scott. 2007. Thirty-One Flavors of Drosophila Rab Proteins. *Genetics*. 176:1307-1322.

Zheng, J., S.M. Cahill, M.A. Lemmon, D. Fushman, J. Schlessinger, and D. Cowburn. 1996. Identification of the Binding Site for Acidic Phospholipids on the PH

Domain of Dynamin: Implications for Stimulation of GTPase Activity. *Journal of Molecular Biology*. 255:14-21.

Zheng, L., J. Zhang, and R.W. Carthew. 1995. *frizzled* regulates mirror-symmetric pattern formation in the *Drosophila* eye. *Development*. 121:3045-3055.

Vita

Susan Banks graduated from The University of Kansas with a B.A. in Biology in 2004. She began her graduate studies at The University of Texas at Austin in 2004 and joined Janice Fischer's laboratory in 2005. During her time at The University of Texas at Austin, Susan was recognized for her work as a teaching assistant and received several travel awards. In 2010 and again in 2011, she was awarded a summer fellowship to support her research. Susan earned her degree of Doctor of Philosophy in May of 2012.

Permanent address (or email): smlbanks@utexas.edu

This dissertation was typed by Susan Banks.