THE UNIVERSITY OF CHICAGO

REGULATION OF NOTCH TRAFFICKING AND SIGNALING BY ABELSON KINASE

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF BIOLOGICAL SCIENCES
AND THE PRITZKER SCHOOL OF MEDICINE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

INTERDISCIPLINARY SCIENTIST TRAINING PROGRAM:
DEVELOPMENT, REGENERATION AND STEM CELL BIOLOGY

BY
NICELIO SANCHEZ-LUEGE

CHICAGO, ILLINOIS
DECEMBER 2018

Table of contents

List of figures

List of tables

Acknowledgements

First and foremost, I especially thank Ilaria Rebay for her endless patience and generosity as a mentor. Ilaria taught me how to think and work like a scientist. She challenged me to do my best while giving me independence to learn on my own. This work would surely not be possible without her support.

Secondly, I am forever indebted to my parents. Whatever I have and will accomplish in life is due to their love and support.

Finally, I would like to thank my friends in the Rebay lab that created a stimulating and supportive environment. I feel particular gratitude towards the senior graduate students and post-docs who were invaluable mentors throughout my journey: Charlene Hoi, Trevor Davis, Matt Hope, Jemma Webber, and Jean-Francois Lachance.

Abstract

Development relies on molecular signaling pathways to coordinate complex cellular processes. Notch signaling during development coordinates processes such as proliferation, cell fate decision making, and morphogenesis. While the core molecular and cellular events of Notch signaling have been elucidated, a complete understanding of how Notch signaling is regulated remains elusive. Here I dissect the regulation of Notch trafficking and signaling by Abelson kinase (Abl). Using a combination of *Drosophila* genetics and cell biology, I show that Abl prevents the accumulation of Notch receptor in endosomal compartments. In addition, Abl promotes Notch signaling during retina and wing development. In cell culture, Abl prevents Notch accumulation and promotes Notch signaling in a kinase-dependent way. Mechanistically, Abl phosphorylates Notch *in vitro*. One of the Abl-target tyrosine residues in Notch is required for repression of Notch signaling, revealing that Abl may also have repressive activity on Notch. This work elucidates a novel mechanism to regulate Notch trafficking and signaling. Given that Notch and Abelson play important roles in human cancers, the findings of this work are of potential relevance to human health.

Chapter 1: Introduction and background

Introduction

All animals originate from a single-celled zygote that is produced by the fusion of the maternal and paternal haploid gametes. Directed primarily from the zygotic genome, perfectly choreographed sequences of proliferation to generate a sufficient pool of uncommitted progenitor cells, tissue movements to set up the three-dimensional body plan and organ structures, cell fate specification to pattern cell types across a tissue, and terminal differentiation to sculpt the final form of each tissue culminate in the formation of a properly patterned multicellular organism. Mirroring the path taken by the zygote, each organ primordium undergoes an analogous sequence of cell proliferation, fate specification and morphogenesis to produce the cell types and tissue shapes required to fulfill the specific functions of the mature organ.

Two general strategies for coordinating these cellular events during development have been observed: mosaic development and regulative development (Gilbert, 2013). In mosaic development, determinant molecules are spatially separated and organized within an initial cell. Stereotyped cleavage events ensure that cells differentially inherit these molecules as the tissue subdivides into multiple cells. This inherited difference in molecular content between cells instructs developmental transitions across the tissue. Cell-cell interaction is not required beyond the inheritance of molecular determinants from a parent cell, and cellular process occur autonomously according to cell lineage. In regulative development, developmental events are instructed by interactions between cells, rather than factors inherited during cell division. Interactions can occur directly between neighboring cells or at a distance using secreted factors. Regulative mechanisms prominently govern the development of complex organisms, including vertebrates. Therefore, this dissertation will focus on regulative development.

Regulative development relies primarily on cell-cell interactions to coordinate developmental events. At a molecular level, cell-cell interactions typically occur between a ligand molecule, expressed by a signal-sending cell, and a receptor protein, expressed on a signal-receiving cell. Ligands can be exposed on the cell surface or secreted by the signal-sending cell. Once a receptor binds and recognizes the ligand, the signal is transduced intracellularly among effector proteins in the cytoplasm. These signaling pathways can ultimately regulate nuclear events, such as target gene transcription, or cytoplasmic events, such as cytoskeletal remodeling, to carry out a biological function during development. In a saturating genetic screen for mutants involved in patterning and differentiation of *Drosophila* embryos (Nüsslein-Volhard et al., 1984; Wieschaus and Nüsslein-Volhard, 2016), researchers captured almost all the major signaling pathways that underly cell-cell interactions in development, namely Epidermal growth factor (EGF), Hedgehog, Bone morphogenic protein (BMP), Wnt, and Notch. The development of such diverse organs as a brain and a heart involve fundamental events at the cellular level, such as proliferation, cell type specification, and morphogenesis. Therefore, this limited number of signaling pathways is reiteratively deployed in both embryonic development and imaginal tissues that form adult organs in *Drosophila*. Furthermore, these signaling pathways are evolutionarily conserved in vertebrates and mammals, a testament to the fundamental nature of developmental processes across species despite striking phenotypic diversity.

Among these pathways, the Notch signaling pathway plays critical roles in a multitude of developmental processes from worms to humans (Greenwald, 2012). The structure, function, and regulation of Notch has inspired inquiry for over 100 years. However, questions remain about how Notch is trafficked and regulated within the cell. This chapter will first

provide a brief history of Notch biology. Then it will summarize the present day understanding of how Notch functions in *Drosophila* development and human disease. My work focuses on Abelson kinase as a regulator of Notch trafficking and signaling. Therefore, I have included a deeper treatment of Notch trafficking. Finally, I provide a summary of Abelson kinase biology with an emphasis on how it relates to Notch.

A brief history of Notch biology

Notch is one of the oldest developmental signaling pathways to be studied, with the history of its discovery and characterization predating the dawn of molecular genetics. Sometime before 1914, John Dexter found a single female *Drosophila* with both wings having a "notched" phenotype, noting that they appeared as if they had been cut along the margin (Dexter, 1914). He named the founding matriarch of the notched wing strain "Perfect Notched", and found that the phenotype was passed from mothers to daughters, indicative of dominant sex-linked inheritance. Similar mutants arose independently several times during the 1910s, and eventually Thomas Hunt Morgan established various *Notch* stocks. Intriguingly, *Notch* was initially used just as a marker gene to establish the pattern of dominant-phenotype, recessive-lethal heredity (Morgan, 1917) and it was not until much later in the twentieth century that the focus of research shifted to understanding its central roles in organizing Drosophila development.

Donald Paulson at Yale brought forth the next chapter of *Notch* history when he described the lethal phenotype of *Notch* homozygous embryos (Poulson, 1940). In the embryo, neural precursor cells normally delaminate from the ectodermal epithelium that goes on to form the epidermis. Poulson found that embryos lacking *Notch* exhibit a neurogenic phenotype in

which excessive specification and delamination of neural precursors produces a massively hypertrophied nervous system at the expense of epidermal tissue.

Poulson's work marked the start of developmental genetics as a field. During much of the twentieth century, the now obvious connection between gene function and developmental progression was largely overlooked by both geneticists and developmental biologists. It was the pioneering, and ultimately Nobel-prize winning, work of Christiane Nusslein-Volhard and Eric Wieschaus that formalized this connection (Nüsslein-Volhard et al., 1984). These researchers realized that recessive lethality often results from embryonic catastrophes, and, even more importantly, that because the dead embryos contain the phenotypic record of complete gene loss, embryonic phenotypes can provide a direct readout of biological function. Dead embryos are difficult to examine under a microscope, so the researchers chose to analyze larval cuticle preps that are secreted by the embryo. The larval cuticle contains stereotypic structures that reflect the patterning of the larval body plan during embryogenesis. This allowed them to perform a large-scale saturating mutagenesis screen in which they discarded embryonic viable and embryonic lethal but normal-cuticle mutants. They kept and isolated mutants with phenotypes affecting cuticular patterning. From this effort they identified about 200 zygotically acting and essential genes, that when mutated resulted in a huge array of defects in normal cuticular patterning.

While the most publicized discovery of this screen was the identification of a cascade of 15 zygotically acting genes that sequentially refine the pattern of the anterior-posterior axis of the embryo, as mentioned above, the screen also identified key components of most of the major signaling pathways that we now know orchestrate many facets of development. Among these were five loci with neurogenic phenotypes similar to those Poulson had identified for *Notch*:

Delta, *mastermind*, *Enhancer of split (E(spl))*, *neutralized*, and *big brain* (Lehmann et al., 1983). Once the core neurogenic genes were identified, efforts naturally shifted towards molecular cloning and characterization of the protein products.

Efforts to molecularly clone *Notch* started during a nascent period of modern molecular genetics, in which the conservation of developmental genes was merely a speculative hypothesis. With the emergence of the *Caenorhabditis elegans* worm as a genetic model organism, *C. elegans* researchers had isolated mutants in a gene called *lin-12* that would later be revealed to encode an ortholog of fly *Notch* (Greenwald et al., 1983). Although *lin-12* mutants exhibited embryonic cell fate defects, particularly in the few cell fate decisions in *C. elegans* that require cell-cell interaction, there were no phenotypic hints that it might encode a Notch homolog. Working independently, researchers from both the fly and worm camps embarked on molecular cloning of their respective genes using established mutants. Upon publishing of the protein sequences, it became clear that Notch is conserved between flies and worms (Wharton et al., 1985a; Yochem et al., 1988). The sequence also revealed that Notch shares repeated motifs with the human Epidermal Growth Factor (EGF) protein (Figure 1.1). Notch became an early example of the profound finding that genes are largely conserved across animals, linking Darwinian shared ancestry with molecular biology.

The molecular cloning of another neurogenic locus, *Delta*, proved significant for understanding Notch as a receptor. The locus was revealed to also encode a transmembrane protein with EGF-like repeats in the extracellular domain, but with a short 214 amino acid intracellular domain (Kopczynski et al., 1988) relative to that of Notch, which contains 937 amino acids. At the time, proteins with EGF-like repeats were known to bind each other in the

blood coagulation cascade (Furie and Furie, 1988), therefore it was hypothesized that EGF-repeats mediated protein-protein interactions. Given that Notch and Delta both contained EGF-like repeats, and that both proteins are encoded by neurogenic loci, the possibility was raised that Notch and Delta directly interact to regulate embryonic neurogenesis. This hypothesis was supported by the finding that Delta-expressing cells aggregate with Notch-expressing cells in culture (Fehon et al., 1990). Furthermore, Notch and Delta concentrate at the contact points

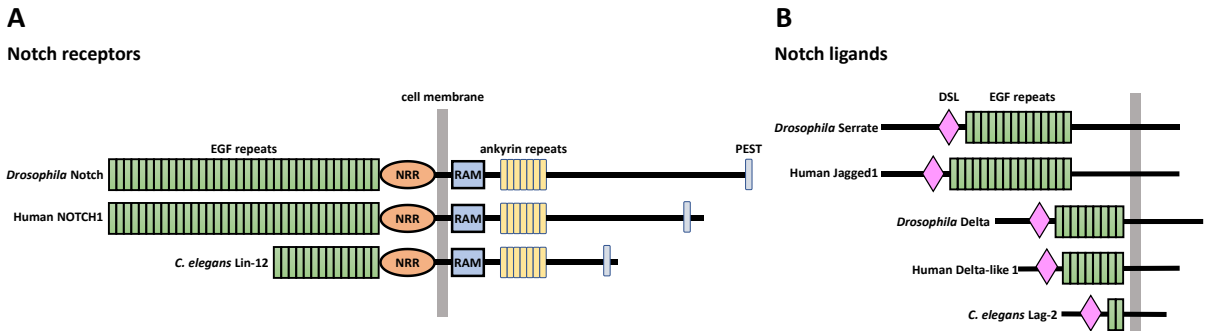


Figure 1.1 Protein domain organization of Notch and its ligands
(A) Notch receptors from *Drosophila*, human, and *C. elegans* (B) Notch ligands from *Drosophila*, human, and *C. elegans* Adapted from Gordon, et al. 2008

between these cells. Finally, Notch and Delta coimmunoprecipitate from cells expressing both proteins. These results supported the notion that Notch and Delta could directly interact in cell-cell interactions *in vivo.*

Once Notch and Delta were identified as transmembrane proteins with EGF repeat homology, natural questions about their function in mediating cell-cell interactions emerged. One hypothesis proposed that Notch acts as a cell surface receptor that transduces Delta-initiated signaling events to the nucleus to repress neural fates within the embryonic epidermis (Fehon et al., 1990; Lyman and Young, 1993). The countering hypothesis was that Notch works as a homophilic adhesion molecule to mediate cell contacts within the embryonic epidermis that are important for maintaining epidermal fate (Hoppe and Greenspan, 1986, 1990). Both models

provided a plausible explanation for the *Notch* neurogenic phenotype, although the extensive genetic interactions that had been demonstrated between many of the neurogenic loci made the idea the Notch and the other neurogenic genes, including *Delta*, might participate in a common signaling pathway to specify neuronal versus epidermal fates particularly appealing (Kelley et al., 1987; Xu et al., 1990). Focusing on work performed in *Drosophila*, what follows is a summary of the most important genetic and biochemical experiments whose results resolved the signaling receptor versus adhesion molecule debate.

One prediction of a signaling receptor is that it functions in a cell autonomous way. It is genetically required in the cells that carry out the relevant cell fate decision, independent of neighboring cell genotype. The adult thoracic epidermis proved a useful biological context to test this genetic prediction of Notch as a receptor. The adult thoracic epidermis elaborates mechanosensory bristles that are derived from neural precursor cells (Hartenstein and Posakony, 1989). The primordial thoracic epithelium segregates epidermal and neural precursor cells into an evenly spaced pattern using lateral inhibition (Figure 1.2), whereby neural precursors prevent neighboring epidermal cells from taking on a neural fate (Simpson, 1990). Neural precursor cells divide twice to produce one mechanosensory neuron, which delaminates from the epithelia as in the embryonic neurectoderm, and three supporting cells which form the bristle organ (Hartenstein and Posakony, 1989). A test of cell autonomy using mosaic $Notch^{-/-}$ clones in the thoracic epidermis supported the receptor model (Heitzler and Simpson, 1991). The study found that *Notch* is required to repress neural fate specification in the thoracic epithelia, and therefore bristles on the fly notum. Phenotypically, $Notch^{-/-}$ clones differentiate an excess number of bristles (Figure 1.2). Importantly, these clones differentiate ectopic bristles precisely at clonal borders with neighboring $Notch^{+}$ cells. Therefore, *Notch* is required for repression even when a

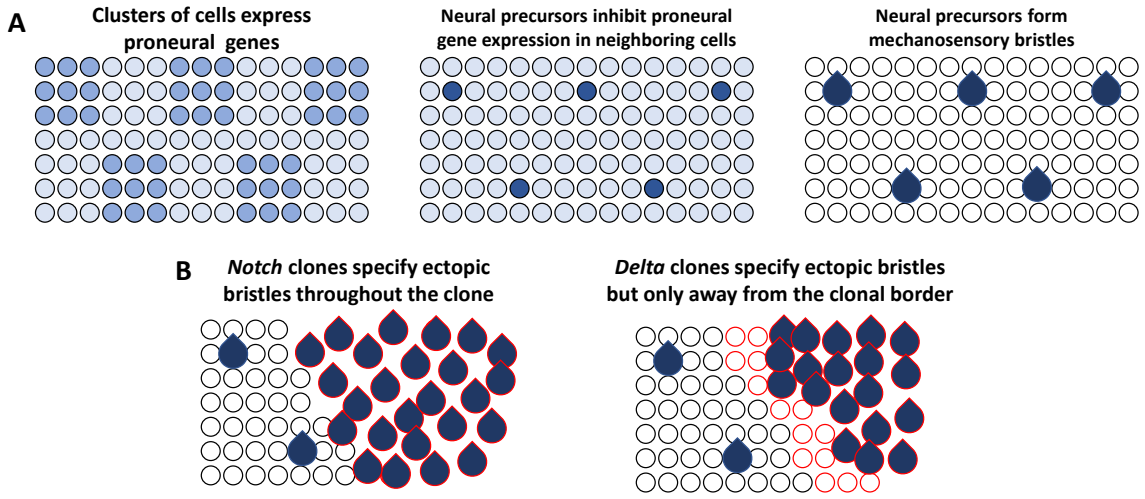


Figure 1.2 *Notch* and *Delta* regulate the neuronal-epidermal cell fate decision in the *Drosophila* peripheral nervous system
(A) The steps of mechanosensory bristle specification in the thoracic epithelium (B) The effects of *Notch* and *Delta* on neuronal-epidermal cell fate choice (red border denotes a mutant cell)

cell is in contact with $Notch^{+}$ cells. As such, *Notch* behaves cell autonomously, as would be predicted for a signal transducing receptor molecule.

Another prediction of a cell-surface signaling receptor is that it interacts with a secreted or cell surface ligand to carry out a cell-cell interaction. *Delta*, one of the neurogenic loci, provided an intriguing candidate. A ligand gene is predicted to act cell non-autonomously. That is, cells are sensitive to neighboring cell genotypes, and act independently of their own. $Delta^{-/-}$ clones also differentiate ectopic bristles in the notum, yet they do so with a distinct pattern as compared to $Notch^{-/-}$ clones (Heitzler and Simpson, 1991). $Delta^{-/-}$ clones only differentiate ectopic bristles in the center of clones, away from clonal borders (Figure 1.2). The phenotype is suppressed near clonal borders where cells contact neighboring $Delta^{+/+}$, indicating mutant cells that lack *Delta* but have *Notch* are rescued from ectopic bristle differentiation by neighboring wildtype *Delta* expressing cells. Therefore, *Delta* behaves consistent with the predictions of a ligand gene.

Once Delta was found to behave as a Notch ligand, the field sought to understand how the Notch signal is transduced in the signal-receiving cell. One possibility was that other neurogenic loci act as a cytoplasmic transduction pathway downstream of ligand recognition. However, molecular cloning of *mastermind* and *E(spl)* revealed nuclear proteins (Smoller et al., 1990; Delidakis et al., 1991), which ruled out their participation in a cytoplasmic pathway. The other neurogenic loci, *big brained* and *neutralized*, failed to modify the *Notch* neurogenic phenotype when genetically duplicated, unlike *Delta* and *E(spl)* (de-la-Concha et al., 1988). Therefore, they were not considered Notch pathway candidates (Artavanis-Tsakonas and Simpson, 1991). The nature of Notch transduction remained elusive.

The finding that a human *Notch* homolog is recurrently rearranged in T cell lymphoblastic leukemias was an early breakthrough in the effort to understand Notch signal transduction (Ellisen et al., 1991; Greenwald, 2012). The genomic rearrangement causes expression of a truncated Notch that lacks the extracellular domain, which raised the possibility that this truncated form acts as a gain of function mutant. This finding inspired researchers to explore the genetic behavior of various Notch truncations in search for insights into Notch signal transduction.

Two studies, published in the same issue of Cell, tested the consequence of overexpressing various truncated forms of Notch. Struhl et al found that overexpressing the Notch intracellular domain (NICD) causes a loss of neuronal specification during embryogenesis, the opposite phenotype of Notch loss (Struhl et al., 1993). Furthermore, the loss of neurons under NICD overexpression is epistatic to the neurogenic phenotype seen in *Notch* and *Delta* backgrounds. Finally, Struhl et al used an antibody against the NICD to compare the

subcellular localization of Notch and overexpressed NICD. While Notch endogenously localizes to the cell membrane, overexpressed NICD was strikingly found in the nucleus. This finding was consistent with a previous study of the endogenous Notch pattern, which only found Notch at the cell membrane using the same anti-NICD antibody (Fehon et al., 1991). These results motivated the interpretation that the NICD acts as an activated form of the receptor to mediate events in the nucleus, with the caveat that it is endogenously turned over as to not be detectable in the nucleus at wildtype levels.

The second study by Rebay et al tested the effect of overexpressing various Notch truncations (Rebay et al., 1993). The study added important insights by testing the effect of overexpressing a form of the Notch extracellular domain (NECD) that lacks the NICD. Overexpressed NECD was found to act as a dominant negative Notch, producing wing notching as seen in the classical *Notch* background. This further supported that Notch acts as a signaling receptor, since deleting intracellular domains in receptors typically produces dominant negative forms, presumably due to titration of available ligand. A follow up study then recapitulated the embryonic Struhl results in the eye (Fortini et al., 1993). Overexpression of NICD was found to repress neuronal specification in the eye, and the NICD was found in the nucleus. These early studies laid the foundation of the "cleavage model", in which the NICD is cleaved during activation and directly enters the nucleus to mediate transcriptional events.

The cleavage model was ultimately validated by *in vivo* studies that tested whether the NICD is transcriptionally active (Struhl and Adachi, 1998; Lecourtois and Schweisguth, 1998). The studies took the approach of inserting GAL4 activating domains, which drive expression at UAS elements, into the intracellular domain of Notch transgenes. These transgenes were found

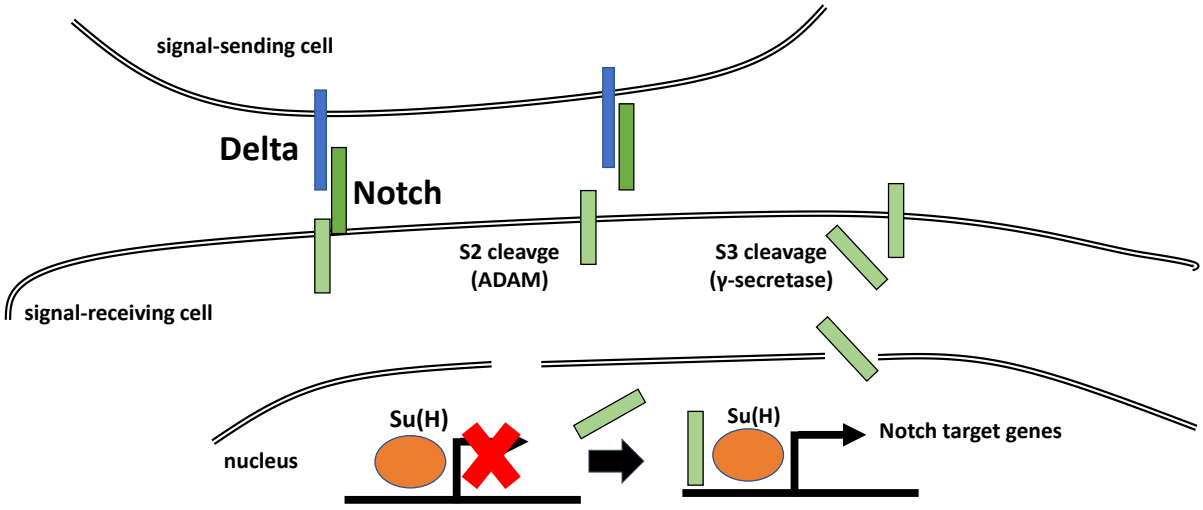


Figure 1.3 The Notch signaling events
The key molecular events involved in Notch signaling include: ligand recognition, S2 cleavage, S3 cleavage, entry into the nucleus, and conversion of Su(H) into an activating transcriptional cofactor.

to drive UAS-LacZ expression in the embryonic neuroectoderm, and this expression is Delta dependent. These results suggest that *in vivo* Notch activation involves entry of the NICD into the nucleus and participation in transcriptionally activating complexes.

Molecular and cellular biology of Notch signaling

Notch is a transmembrane protein containing an N-terminal extracellular (NECD) and C-terminal intracellular domain (NICD) (Bray, 2006; Hori et al., 2013). The key events of Notch signaling are ligand recognition by the NECD, which results in a series of proteolytic cleavages that eventually release the NICD to affect changes in transcription (Figure 1.3). Under normal conditions, the NECD is exposed on the cell surface as a heterodimer, which results from a cleavage event in the Golgi apparatus deemed S1 cleavage. Notch recognizes and directly binds ligands exposed on the surface of neighboring cells. Ligand recognition triggers S2 cleavage in the NECD, catalyzed by Kuzbanian and Presenilin. Recent structural analyses suggest that Notch forms a “catch bond” with its ligands (Luca et al., 2017). A catch bond lifetime is proportional to

tensile force. Therefore, the Notch-ligand complex is highly tolerant of increased tension. S2 cleavage promotes S3 cleavage by γ-secretase, which releases the NICD into the cytoplasm. NICD cleavage is the critical signaling event, since it is then free to mediate transcriptional events in the nucleus. NICD forms a transcriptional complex with Suppressor of Hairless (Su(H)) and Mastermind that drives expression of target genes (Wilson and Kovall, 2006). Su(H) forms repressive complexes at target loci in the absence of signaling, providing a switch-like behavior. The *Enhancer of split* family of genes are commonly activated by Notch signaling in various developmental contexts (Housden et al., 2014). They encode bHLH family transcriptional repressors that silence context-specific genes.

Several distinct protein domains within the NECD have critical roles in Notch signaling or function (Gordon et al., 2008; Hori et al., 2013). The many Notch orthologs found throughout animals contain variable numbers of Epidermal growth factor (EGF) repeats within the NECD (Figure 1.1). *Drosophila* Notch has 36 EGF repeats (Wharton et al., 1985b). LIN-12 and GLP1, the *C. Elegans* Notch homologs, are smaller with 14 and 11 EGF repeats, respectively (Yochem and Greenwald, 1989). The four Human homologs have between 29-36 EGF repeats (Weinmaster et al., 1992; Uyttendaele et al., 1996). The NECD also contains a negative regulatory region, which is required to prevent constitutive NICD cleavage. The negative regulatory region conceals the S2 cleavage site prior to ligand recognition (Gordon et al., 2007).

Both cell culture and structural analyses have interrogated the essential domain interactions for receptor-ligand binding. An early cell culture study assayed the interaction between Notch and Delta/Serrate by measuring the percentage of cells found in clusters of Notch-expressing and ligand-expressing cells (Rebay et al., 1991). Notch-expressing cells

aggregate with Delta- and Serrate-expressing cells due to Notch-ligand affinity (Fehon et al., 1990). The Notch EGF repeats 11 and 12 were found to be necessary and sufficient for association with Delta and Serrate (Rebay et al., 1991). Similar to Notch itself, Notch ligands contain extracellular EGF repeats (Fleming, 1998). They also contain an N-terminal C2 domain, which binds lipid bilayers (Chillakuri et al., 2013), and a Delta-Serrate-Lag2 domain, which is required for Notch binding (Shimizu et al., 1999). A structural analysis of a crystal structure containing Notch and Delta attests to the importance of the Notch EGF repeats 11-12. In the solved structure, Notch EGF repeats 11 and 12 interact with the Delta-Serrate-Lag2 and C2 domain, respectively (Luca et al., 2015). Interestingly, a recent structure shows that the Notch EGF repeats 8 and 12 interact with EGF repeat 3 and the C2 domain of the human Serrate homolog Jagged, respectively (Luca et al., 2017). Therefore, it is hypothesized that Notch EGF repeats other than 11 and 12 also contribute to ligand recognition.

The NICD contains a RAM (recombination binding protein-Jκ-associated molecule) domain, ankyrin repeat domains, and C-terminal a trans-activating domain. Providing an early clue of NICD's biochemical function, Notch RAM domain was identified as a positive hit in a yeast two-hybrid assay that used the murine Su(H) as bait (Tamura et al., 1995). More recent crystallography of a NICD-Su(H)-Mastermind complex bound to DNA shows that the NICD RAM domain interacts with Su(H) (Wilson and Kovall, 2006). The ankyrin repeat domains in NICD mediate Mastermind recruitment to the tripartite complex *in vitro* (Nam et al., 2003). Interestingly, the crystal structure reveals that C-terminus of Su(H) interacts with the interface between the NICD ankyrin repeats and Mastermind (Wilson and Kovall, 2006), showing interaction among all three members. The NICD trans-activating domain was identified in a study that analyzed the transcriptional activation of various murine NICD truncations (Kurooka

et al., 1998). Researchers performed GAL4-sensitive luciferase assays after transfecting various GAL4-fusion NICD mutants into mammalian cell culture. The trans-activating domain was required and sufficient to produce transcriptional activation in the assay. Therefore, it is posited that the trans-activating domain recruits cofactors to the core NICD-Su(H)-Mastermind complex.

Once Notch is activated and cleaved, NICD shuttles to the nucleus where it mediates transcriptional activation of target genes. Because NICD alone does not possess sequence-specific DNA binding ability, interactions with the transcriptional coactivator Su(H) provide critical off-on switch-like regulation of target gene transcription. Thus, in the absence of Notch signaling, Su(H) interacts with various repressors, including Hairless (Morel et al., 2001) and Groucho (Barolo et al., 2002), to silence Notch target gene expression in the absence of signaling. Activation of Notch signaling enables nuclear NICD to bind Su(H) via the RAM domain and ankyrin repeats, as described above. This disrupts the repressive complex, and recruits Mastermind to the NICD ankyrin repeats – Su(H) interface to build the activating complex.

Notch activates expression of general and context-specific targets. For example, Notch drives expression of the *E(spl)* family of genes across *Drosophila* tissues, including the embryo and imaginal discs (Bailey and Posakony, 1995). Strikingly, *E(spl)* homologs are activated downstream of Notch signaling in mammalian cells (Jarriault et al., 1995), attesting to evolutionary conservation of the Notch transcriptional circuitry in addition to the core pathway. Notch also drives context-specific target expression. For example, Notch drives expression of *cut* to specify wing margin fate in the developing *Drosophila* wing (de Celis et al., 1996). It remains poorly understood how a diversity of Notch targets is produced across tissues. One prevailing

theory is that the core NICD-Su(H)-Mastermind complex recruits other cofactors that effect target selection. For example, the SAGA histone acetyltransferase complex interacts in genetic synergy with Notch pathway components and colocalizes with Mastermind on large polytene chromosomes within the larval salivary glands (Gause et al., 2006). It is possible that the availability of different cofactors, including chromatin modifiers, dictates Notch target identity in different developmental contexts.

Notch signaling in *Drosophila* development

Notch is a highly pleiotropic signaling pathway, and is consistently deployed across many contexts during development. As examples, Notch 1) represses neuronal specification in the neuron-epidermis cell fate decision during embryogenesis and mechanosensory organ development (Poulson, 1940; Heitzler and Simpson, 1991) 2) promotes programmed cell death during eye development (Miller and Cagan, 1998) 3) and represses vein specification during wing development (Celis et al., 1997). Its function is defined by the spatiotemporal patterning of the developing tissue in question. For the purposes of this dissertation, I will focus on Notch signaling in two of the best characterized contexts, namely the *Drosophila* eye and wing.

The Drosophila compound eye is composed of repeating multicellular subunits called ommatidia, each of which contains eight photoreceptor neurons, four cone cells, and approximately 10 pigment cells that protect and support the photoreceptors (Ready et al., 1976). The cone cells are the most apical cell type, and secrete the lens material that ultimately forms the cornea of the eye. Two primary pigment cells surround the cone cells and sit apically above the photoreceptors. They enwrap the ommatidia and produce pigment granules to focus light into the center of the ommatidia (Shoup, 1966). The photoreceptors are stereotypically arranged

in the center of each ommatidia. Additional pigment cells, deemed interommatidial pigment (IOP) cells, localize to the junctions between ommatidia. They also produce pigment granules to prevent optic contamination between ommatidia. In addition, there are four-cell mechanosensory bristle complexes arrayed within the interommatidial space (Carthew, 2007). Following specification in the late third instar disc, photoreceptors undergo an elaborate morphogenic program, whereby axons are projected into the brain, the apical membrane domain involutes towards the center of the ommatidia, expanding and elaborating to form light-sensing rhabdomeres (Arikawa et al., 1990; Longley and Ready, 1995; Knust, 2007).

The *Drosophila* eye and antenna originate as a tiny epithelial sac during embryogenesis, called the eye-antennal disc (Morata and Lawrence, 1979; Haynie and Bryant, 1986; Kumar, 2011). This disc proliferates during larval development while dramatically increasing in size, and concomitantly specifies eye and antennal-head fields. Proliferation is organized into two waves. The first mitotic wave lasts for several days, and involves asynchronous cell division until a cell number of about 50,000 is reached in the late third instar. At this point, when the eye-antennal disc has reached proper size and cell number, cells at the posterior edge of the disc stop dividing, arrest in G1, and initiate a sequence of signaling events that induces a wave of differentiation to sweep anteriorly across the eye field from posterior to anterior (Wolff and Ready, 1991a). The morphogenetic furrow, a visible epithelial crease caused by apical constriction, marks the anterior front of this wave. The retinal cell types, starting with the photoreceptors, are specified in the wake of the morphogenic furrow. After specification of the first five photoreceptors, all uncommitted cells undergo a final synchronized cell division known as the second mitotic wave; this ensures an adequate progenitor pool for specification of the remaining photoreceptors and other retinal cell types.

Once the eye-antennal disc reaches proper size and cell number, a wave of differentiation begins to sweep across the eye field from posterior to anterior (Wolff and Ready, 1991a). The morphogenic furrow, a visible epithelial crease caused by apical constriction, marks the anterior front of this wave. The retinal cell types, starting with the photoreceptors, are specified in the wake of the morphogenic furrow. But first, the cell cycle is arrested, and proliferation temporarily ceases. R8 is the first photoreceptor subtype to be specified in each ommatidial cluster. Notch enforces the spacing of initial R8 cells by repressing expression of the pronerual gene *atonal* (Jarman et al., 1994; Baker et al., 1996). Atonal+ R8 cells signal to neighboring cells to repress neurogenesis and ensure that R8 cells are evenly spaced, a mechanism called lateral inhibition. Interestingly, this recapitulates the role of Notch to mediate lateral inhibition in the embryonic neuroectoderm to regulate the spacing of neuroblasts (Cabrera, 1990). R8 acts as a founder cell, initiating the sequential recruitment of unspecified precursors as the R2-R5 photoreceptors into the ommatidial cluster (Figure 1.4). In this process, EGF signaling promotes neuronal recruitment (Freeman, 1996), while Notch signaling represses neuronal recruitment to prevent precocious photoreceptor recruitment (Cagan and Ready, 1989; Doroquez and Rebay, 2006).

Notch signaling also regulates the recruitment of non-photoreceptor cell types, including cone cells and primary pigment cells (Cagan and Ready, 1989). Time shift experiments, whereby Notch is temporally inactivated using a temperature sensitive *Notch*^{ts} allele, reveal that Notch represses cone cell and primary pigment cell recruitment. Loss of Notch during cone cell and

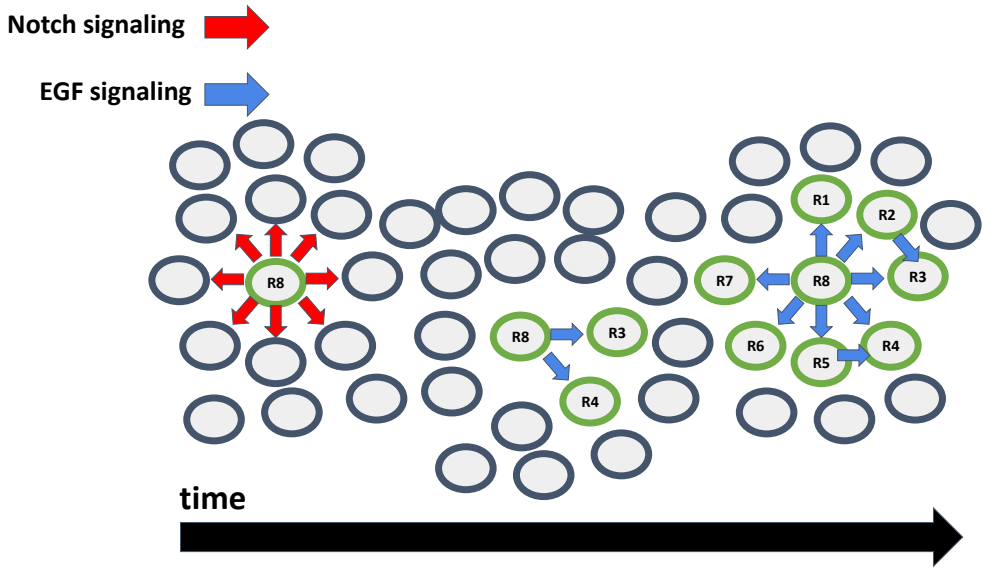


Figure 1.4 Regulation of photoreceptor recruitment by Notch and EGF signaling
R8 founder cells repress neuronal specification of neighboring cells via Notch signaling. EGF signaling then iteratively recruits photoreceptors into each ommatidial cluster.

primary pigment cell recruitment produces excess cone cells and primary pigment cells, respectively.

Notch signaling also controls cell adhesion between cone and primary pigment cells (Bao, 2014). Cone cells express Delta to activate Notch in primary pigment cells. Notch activates expression of *hibris* and *sticks-and-stones*, two Nephrin-encoding genes, in primary pigment cells. Nephrins mediate heterophilic interactions with Neph1 proteins expressed by IOPs. Therefore, primary pigment cells fail to enwrap cone cells in the absence of Notch, because they fail to express Nephrins that are essential for proper cell contains with IOPS.

Notch signaling also controls a programmed cell death decision made by the IOPs during pupal development(Wolff and Ready, 1991b; Miller and Cagan, 1998). Vision requires a precise ommatidial lattice with one-cell layer of IOPS between each ommatidia. To achieve this, excess IOPs are instructed to undergo programmed cell death via Notch signaling (Figure 1.5). IOPs

express both Delta and Notch (Fehon et al., 1991; Parks et al., 1995), and Notch signaling among them promotes cell death (Miller and Cagan, 1998). As expected, loss of Notch signaling during pupal development causes a decrease in programmed cell death and an excess of IOPs to remain in the retina (Miller and Cagan, 1998). EGF signaling from cones and photoreceptors provides a survival signal that overrides the cell death instruction. EGF signaling represses expression of Hid (Yu et al., 2002), which is required for programmed cell death (Kurada and White, 1998). The increased cell death in EGF receptor mutants is epistatic to the decreased cell death seen in Notch mutants (Yu et al., 2002). Therefore, Notch is thought to activate unknown targets that inhibit EGF signaling to promote programmed cell death.

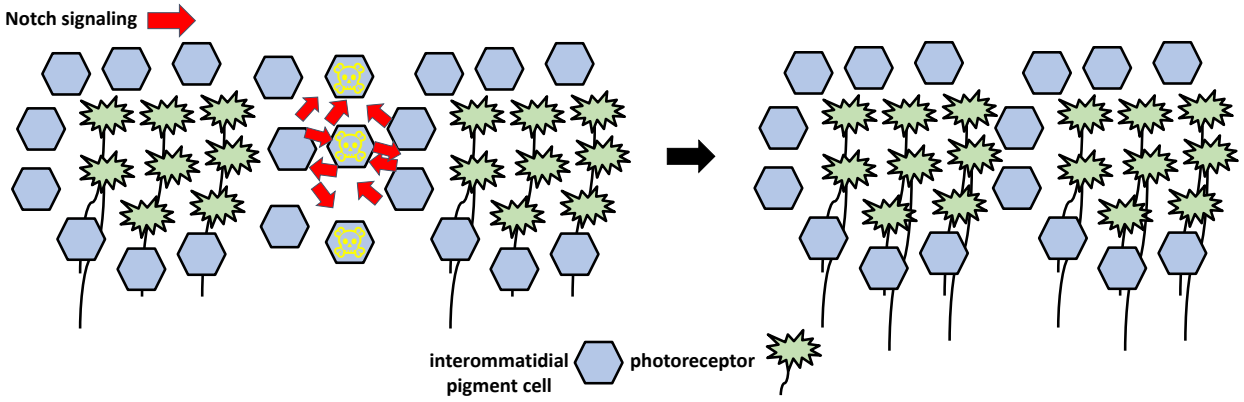


Figure 1.5 Notch signaling regulates programmed cell death in IOPs
Notch signaling promotes programmed cell death to cull IOPs and establish a single-celled border around each ommatidium.

Roles for Notch in pupal development after the IOP cell death decision remain poorly understood. The IOP cell death decision occurs around 32 hours after the start of pupation. Repressive temperature shifts of *Notch*^{ts} between 40 and 58 hours after pupation produce only minor and very infrequent cell fate defects (Cagan and Ready, 1989). However, roles for Notch signaling in late pupal development and adult physiology of the *Drosophila* retina have not been reported.

Notch signaling in wing development

The *Drosophila* wing exhibits a stereotypical blade shape with six proximal-distal longitudinal veins, and two anterior-posterior cross veins. The margin of the wing contains sensory bristles, along with non-innervated hairs. Similar to the eye, the wing is derived from an epithelial sac, called the wing imaginal disc (Garcia-Bellido and Merriam, 1971). The wing disc contains an elliptical pouch from which the wing proper is derived. The tissue surrounding the pouch forms the hinge that connects the adult wing to the thorax. As in eye development, Notch signaling is involved at various steps of wing development.

As seen in the eye, Notch is activated along the D/V boundary of the wing pouch (de Celis et al., 1996). In contrast to the eye, the expression patterns of Delta and Serrate are switched relative to the D/V boundary. Serrate is expressed in the dorsal domain, while Delta is expressed in two stripes flanking the D/V boundary where Notch is active. Notch directly drives expression of *wingless* and *cut* along the D/V boundary of the wing pouch (Rafel and Milan, 2008). Wingless acts as a long-range signal that drives patterning and growth in the wing disc. Specifically, Wingless drives expression of *vestigial*, which is required for wing growth and proliferation. Notch also drives *cut* expression at the D/V boundary to specify margin fate (Micchelli et al., 1997). Cut is a homeobox transcription factor that is required and sufficient to specify margin fate. Notch activation of *cut* is highly dose-sensitive, which explains why *Notch* heterozygotes exhibit the wing notching phenotypes that were first observed by John Dexter and Thomas Hunt Morgan.

Notch signaling also regulates wing vein patterning during pupal development (Celis, 2003). The primordial veins, known as proveins, and intervein regions are first specified in the

wing imaginal disc. The longitudinal proveins, marked by *blistered* expression¸ stripe the wing pouch along the anterior-posterior axis. A variety of signaling factors, including Hedgehog, Wingless, Decapentaplegic, and EGF, pattern the provein-intervein subdivisions of the wing disc. However, Notch is involved in refining the provein field during pupal development. Delta is expressed in the provein regions, and activates Notch in stripes adjacent to the provein. Notch drives expression of an *Enhancer of split* gene, *E(spl)mβ*. *E(spl)mβ* represses expression of EGF pathway members, namely *rhomboid* and *Star*. EGF signaling promotes provein expansion, therefore, Notch signaling confines EGF activity to the provein and prevents vein thickening. Interestingly, EGF signaling drives expression of Delta in the provein, creating a stabilizing feedback loop that maintains the provein field. Increasing Notch activation causes a loss of wing vein material (Zacharioudaki and Bray, 2014a). For example, the gain of function Notch mutant *Abruptex* is so named because it dominantly causes abrupt truncation of the longitudinal veins. Loss of Notch signaling causes vein thickening, the dominant phenotype seen in *Delta* mutants, and ectopic veinlet formation.

Notch in human disease

The relevance of fly genes to human disease was a major triumph of developmental genetics, and *Notch* is no exception. Human *NOTCH* mutations cause developmental disorders with a broad variety of clinical presentation. In addition, *NOTCH* can act in cancer as both a tumor suppressor and oncogene, depending on the specific disease context.

Humans have four Notch receptor genes, each with different expression patterns during development. Therefore, a wide variety of clinical presentations can occur simply due to the possibility of mutating different *NOTCH* paralogs (Mašek and Andersson, 2017). While a variety

of developmental disorders have been linked to Notch, two stand out as having both a rich clinical history and a well understood biological basis: Adams-Oliver syndrome and Alagille syndrome.

Adams-Oliver syndrome is characterized by skin lesions, a thin skull, and missing or fused digits (Adams and Oliver, 1945). Several Notch pathway genes cause Adams-Oliver syndrome. Notably, loss of function mutations in *NOTCH1* and *DLL4*, a *Delta* homolog, dominantly cause the disease (Stittrich et al., 2014; Meester et al., 2015). *NOTCH1* cases additionally present with congenital heart defects, such as tetralogy of Fallot (Garg et al., 2005). The symptoms that accompany *NOTCH1* mutations are consistent with mouse models of *Notch1* function. *Notch1* is required for vascular development in mice, and loss of Notch signaling leads to bleeding in the head from underdeveloped vasculature (Krebs et al., 2000). Loss of *Notch1* also causes digital fusion and loss.

Alagille syndrome results from heterozygosity of the ligands *JAG1/2* or *NOTCH2* (Li et al., 1997; McDaniell et al., 2006) . The symptoms include bile duct paucity, congenital heart defects, and corneal lesions known as “posterior embryotoxon”. As in Adams-Oliver syndrome, each of these symptoms reflects a known role for Notch signaling in the respective mouse model context. For example, Notch2 signaling drives bile duct initiation and morphogenesis in mice. The broad set of symptoms resulting from *NOTCH* loss in humans emphasizes how widely Notch signaling is deployed in human development.

An immense literature also describes both cell autonomous and cell non-autonomous roles for Notch signaling in human cancers (Aster et al., 2017). Notch can take on both tumor suppressive and oncogenic roles. Cancer sequencing has identified three prominent patterns of

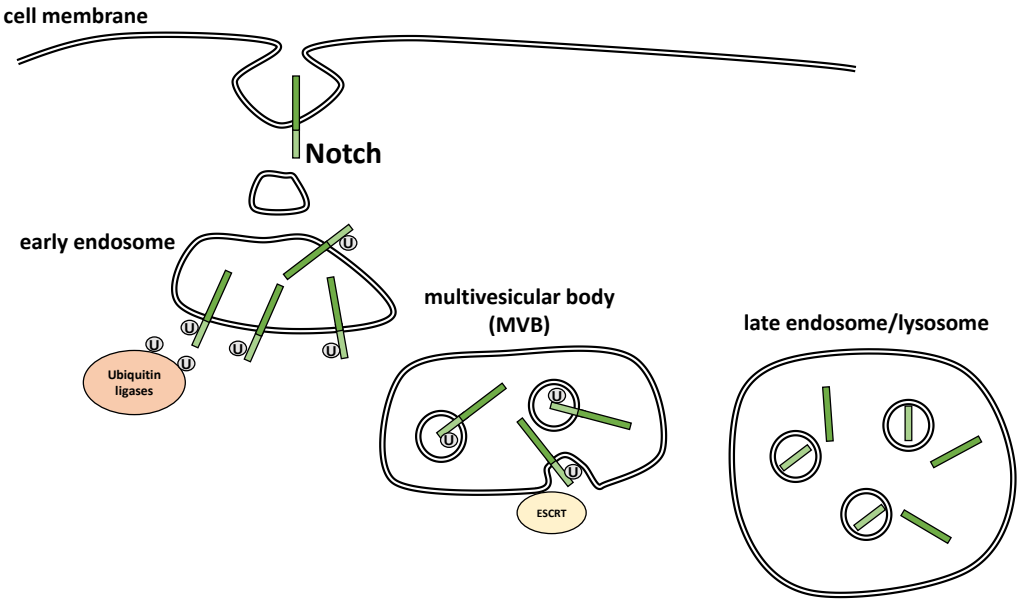


Figure 1.6 Notch trafficking within the endocytic pathway
Notch is continuously endocytosed into the early endosome. The Notch intracellular domain is ubiquitinated, which triggers internalization into the multivesicular body by the ESCRT pathway. Notch then enters the late endosome and is degraded in the lysosome.

mutation. The first pattern, seen most commonly in T-cell acute lymphoblastic leukemia, results from mutations that cause strong gain-of-function phenotypes. While Notch can drive oncogenesis with a variety of target genes, the growth-promoting gene *Myc* commonly promotes oncogenesis downstream of Notch activation in cancers. The second pattern of mutation results in loss of the Notch intracellular PEST domain. These mutants remain ligand-dependent, yet produce increased signaling, since the PEST domain limits Notch stability within the cell. B-cell leukemias typically exhibit PEST domain mutations. Finally, the third pattern of mutation results in Notch loss of function. Squamous cell cancers typically exhibit Notch loss of function mutations. These mutants occur in contexts where Notch has a tumor suppressive effect. A complete accounting of Notch function in human cancers is beyond the scope of this dissertation. However, it should be noted that Notch affects a multitude of cancer processes, including survival, metabolism, immune evasion, inflammation, and metastasis (Aster et al., 2017).

Notch in the endocytic trafficking pathway

More recent developments in the study of Notch biology have elucidated the importance of endocytic trafficking on signaling (Bray, 2006; Palmer and Deng, 2015). Notch is continuously endocytosed and trafficked within cells (Vaccari et al., 2008; Xiong et al., 2013). The destination of Notch within the cell can potentiate or repress signaling. Therefore, the fundamental aspects of endocytosis and molecular sorting (Figure 1.6) provide a context in which to interpret the Notch trafficking perturbations described in Chapter 2 of this dissertation.

Endocytosis of integral membrane proteins typically involves their specific recognition by the cytoplasmic AP-2 adapter complex(Mellman, 1996; Simpson et al., 1996). AP-2 is a heterotetramer consisting of four protein chains that form a bilaterally symmetric structure. AP-2 initiates the formation of Clathrin-coated pits in the plasma membrane, which invaginate and bud off to form Clathrin-coated vesicles (CCVs) (Pearse and Robinson, 1990). CCVs are coated in polygonal lattices of light and heavy Clathrin chains. Eps15 is an important AP-2 interactor and mediator of CCV formation (Benmerah et al., 1998). Eps15 was first identified as a substrate of the EGF receptor kinase in murine 3T3 cells (Fazioli et al., 1993). The protein was later found to bind AP-2 components in plasma membrane fractions of cellular extracts and to colocalize with AP-2 and Clathrin in living cells. Electron microscopy reveals that Eps15 localizes to the edge of budding CCVs (Tebar et al., 1996). Finally, expression of a dominant-negative Eps15 fragment in HeLa cells blocks endocytosis of transferrin and epidermal growth factor receptors (Benmerah et al., 1999). Therefore, AP-2, Clathrin, and Eps15 mark early endocytic CCVs and promote their function in receptor endocytosis.

A genetic analysis of the Drosophila eye suggests that AP-2 and Clathrin drive the early steps of Notch endocytosis (Windler and Bilder, 2010). Nonsense mutations in AP-2 component genes cause Notch accumulation in cell surface puncta, suggesting that Notch fails to internalize in these mutants. Pulse-chase experiments, whereby surface Notch is marked with anti-NECD antibody and stained five hours after labeling, show that Clathrin heavy chain mutants accumulate Notch at the cell surface, similar to AP-2 mutants. Some internal Notch puncta are seen in Clathrin mutants, indicating that a relatively small population of Notch can be internalized in a Clathrin-independent way. The results suggest that Notch is continuously endocytosed into CCVs for further processing in the endocytic pathway.

After receptors are internalized into CCVs, various organelles sort and process this cargo within the endocytic pathway (Mellman, 1996). Three organelles prominently coordinate endocytic trafficking: early endosomes, late endosomes, and lysosomes. Early endosomes are the first organelles encountered by CCVs coming from the cell surface and can be identified and distinguished by specific protein markers. For example, the Rab5 GTPase and Avalanche mark early endosomes (Prekeris et al., 1999). Early endosomes can generally sort internalized receptors towards degradation in downstream compartments or for recycling back to the cell surface. Late endosomes receive and process cargo that has passed through early endosomes. The Rab7 GTPase marks late endosomes, and functions in their maturation into lysosomes (Vitelli et al., 1997). Lysosomes maintain an acidic internal environment to degrade incoming cargo. Proton pumps, such as the vacuolar ATPase, expend cellular energy to maintain a pH of approximately 5 within the lysosomal lumen (Stevens and Forgac, 1997). In addition, lysosomal proteases can degrade proteins within the lysosome (Mellman, 1996). While early endosomes,

late endosomes, and lysosomes form the core endocytic pathway, significant sorting events can also occur in compartments that mediate trafficking between these organelles.

Aside from the core organelles, multivesicular bodies (MVBs) are among the most prominent and well-understood structures within the endocytic pathway (Gruenberg and Stenmark, 2004). MVBs were first identified microscopically as compartments with numerous internal vesicles within the lumen of the limiting membrane (Trump et al., 1965). In addition, images show invagination of the limiting membrane, exhibiting the presumptive formation of internal vesicles. In a landmark pulse-chase study by Harry Haigler et al, ferritin-tagged epidermal growth factor was exposed to receptors on the surface of human fibroblasts (Haigler et al., 1979). Receptors on the cell surface recognize the tagged ligand, which triggers endocytosis. Therefore, the ferritin tag traces the route of endocytosed receptors within the cell. The authors found that receptors are internalized into internal vesicles of the MVB prior to late endosome entry. Internalization into the MVB was thus identified as an important intermediate step in receptor processing between the early and late endosome. In recent years, more molecular details have emerged about how receptor internalization is regulated in the MVB.

Post-translational modification of receptors by ubiquitination regulates internalization into the MVB (Hershko and Ciechanover, 1998). Ubiquitin is a peptide tag that is conjugated to lysine residues within target proteins. Since ubiquitin contains internal lysine residues, ubiquitin monomers can be linked to form polyubiquitin chains. Ubiquitination occurs by the action of three classes of enzymes: E1, E2, and E3. The single E1 enzyme is conserved across eukaryotes. E1 binds ubiquitin and prepares it for transfer to E2 enzymes. E2 enzymes bind ubiquitin and present it to specific E3 enzymes. E3 enzymes recognize targets and transfer the ubiquitin chain

from E2 enzymes to lysine residues within the target protein. Ubiquitination can mediate various processing events, including internalization into the MVB.

The ESCRT (endosomal sorting complex required for transport) complexes play critical roles in recognizing ubiquitinated transmembrane proteins and driving MVB internalization. (Henne et al., 2011). Four ESCRT complexes, 0-III, mediate ubiquitin recognition and membrane remodeling to internalize cargo-containing vesicles into MVBs. ESCRT-0 initiates internalization by binding ubiquitinated proteins at the limiting membrane of the early endosome. ESCRT-I acts as an intermediary complex that binds ESCRT-0 and recruits ESCRT-II. ESCRT-II initiates assembly of ESCRT-III, which drives membrane invagination. Finally, Vps4 disassembles the ESCRT-III complex to pinch off the budding vesicle into the lumen of the MVB.

The ESCRT complexes regulate the internalization of a variety of signaling receptors, including Notch. A genetic analysis by the Bilder group elucidated the role of ESCRT factors in regulating Notch signaling (Vaccari et al., 2008). Vaccari et al studied the effect on Notch trafficking and signaling caused by mutating key factors along the endocytic pathway. To study Notch trafficking, they used *in vivo* pulse chase experiments in which surface Notch is labeled with an anti-NECD antibody and then visualized with indirect immunofluorescence in fixed tissue five hours after the initial pulse. Normally, surface Notch is endocytosed and degraded within that time frame. However, expression of a dominant-negative form of dynamin, which is involved in CCV budding and early endosomal entry, causes Notch accumulation near the cell surface. Mutants in other factors required for entry of CCVs to the early endosome cause a similar phenotype. The ESCRT-0 mutant, *hrs* (Hepatocyte growth factor-regulated tyrosine

kinase substrate), causes Notch accumulation in early endosomal compartments marked by Avalanche. Mutants of ESCRT I-III cause Notch accumulation in Hrs+ compartments. Finally, mutating Fab1, which functions in late endosomal maturation following MVB formation, causes Notch accumulation in unmarked Hrs- compartments. These results indicate that abrogation of any step in the endocytic pathway causes Notch accumulation in upstream compartments.

Vacarri et al, then asked how accumulation of Notch in the different compartments in these mutants impacted signaling. A a Notch-sensitive reporter transgene derived from regulatory sequence at a Notch target locus, *E(spl)mβ*, was used to measure downstream transcriptional output. An unexpectedly complicated pattern emerged: 1) mutants that cause accumulation near the cell surface, such as *Rab5*, result in decreased Notch signaling; 2) the *hrs* mutant, which causes accumulation in Avalanche+ early endosome compartments, has no effect on Notch signaling; 3) ESCRT complex mutants that cause accumulation in Hrs+ compartments, increased reporter activity; and 4) the late endosomal *fab1*mutant has no effect on Notch signaling. These results indicate that Notch signaling can be promoted or inhibited depending on the specific endocytic compartments. Interestingly, the ESCRT-I mutant causes ectopic Notch activation in the ovary at a stage where ligand is not expressed. Therefore, ESCRT mutants may cause ligand-independent Notch activation. It remains unknown whether ligand-independent activation has an endogenous function, since it only has been described under experimental conditions.

The loss of signaling caused by upstream endocytic mutants suggest a requirement of endocytosis for Notch activation. Various genetic analyses support a model in which endocytosis regulates Notch signaling events (Hori et al., 2013). The first indication that endocytosis may

carry an important role in Notch signaling comes from a genetic analysis of *shibire* mutants. The *shibire* locus encodes the *Drosophila* Dynamin homolog, an important factor for vesicle budding during endocytosis (Hinshaw and Schmid, 1995). Researchers found that *shibire* mutant clones produce an excess of mechanosensory bristles in the adult thorax, a phenotype suggestive of Notch signal decrease (Seugnet et al., 1997). Overexpression of NICD in the background of these clones causes a complete loss of mechanosensory bristles, indicated that NICD overexpression is epistatic to *shibire* and that endocytosis is important for Notch signaling upstream of NICD cleavage. A later analysis using *shibire* mutants found that trans-endocytosis of the Delta-NECD complex is required for Notch cleavage and signaling (Parks et al., 2000). In wildtype imaginal tissues, NECD colocalizes with Delta and does not colocalize with NICD, suggesting that NECD is internalized with Delta following NICD cleavage. In *shibire* mutants, NECD, NICD, and Delta colocalize and signaling is reduced. This suggests that Notch recognizes Delta under reduced endocytosis, but that Notch fails to cleave and ultimately signal. Thus, trans-endocytosis of the NECD-ligand complex is an important step for Notch cleavage *in vivo*. Finally, a more recent analysis of mechanosensory bristle precursor cells found that Notch is cleaved and activated in Rab5+ endosomes that contain Notch, Delta, and γ-secretase (Coumailleau et al., 2009). Therefore, endocytosis of a Notch-Delta complex in the signal-receiving cell may also be important for signaling.

Recently, two key regulators of Notch trafficking and signaling within the endocytic network have emerged, Deltex and Supressor of Deltex (Su(dx) (Figure 1.7). Deltex and Su(dx) are both E3 ubiquitin ligases that regulate Notch trafficking and signaling. Their discovery has introduced another layer of Notch regulation beyond the core endocytic trafficking factors such as ESCRT.

Deltex is the first described regulator of ligand-independent Notch signaling (Gorman and Girton, 1992; Xu and Artavanis-Tsakonas, 1990a). The *deltex* mutation was identified as a suppressor of *Abruptex*, a gain of function Notch allele that causes recessive lethality. The mutant is named *deltex* because although it is viable, homozygotes have a recessive phenotype similar to the dominant *Delta* phenotype, which is thickening of the wing veins. Consistent with the logic of the screen, *deltex* behaves in synergy with the Notch pathway. For example, heterozygosity for the neurogenic loci *Notch, Delta,* and *mastermind* causes dominant lethality in a homozygous *deltex* background. Furthermore, *deltex* loss dominantly enhances the dominant *Notch* phenotype at the wing margin.

Molecular cloning of *deltex* allowed the elucidation of its molecular function as a regulator of Notch trafficking (Diederich et al., 1994; Matsuno et al., 1995). Deltex is a RING-family E3 ubiquitin ligase that localizes to the cytoplasm. With its catalytic activity, Deltex transfers ubiquitin to lysine residues on Notch, although the specific pattern of ubiquitination remains elusive. Deltex overexpression causes Notch to accumulate in the limiting membrane of Rab7+ late endosomal compartments and promotes signaling activity (Hori et al., 2011). Importantly, genetic analysis reveals that this increased Notch activation is ligand-independent, since loss of Delta and Serrate does not alter the signaling induction caused by Deltex. Further genetic tests support the model that Notch is activated from the late endosome under Deltex overexpression. For example, mutants of complexes that support late endosomal maturation, such as Rab7, AP-3 and HOPS, block the increased activation (Shimizu et al., 2014). Interestingly, ligand-induced signaling is Rab7-independent in S2 cell reporter assays, suggesting that Deltex induces signaling in a different endocytic context than ligand recognition.

The specific factors and chemistries that promote late endosomal activation are not well understood. Adding further complexity, genetic analysis of various developmental contexts suggests that the efficiency of late endosomal activation varies between contexts (Shimizu et al., 2014). In the wing, Deltex activates Notch signaling, as described. However, in leg development, Deltex overexpression causes a variable gain or loss of Notch signaling. This result suggests that Deltex can even act repressively in contexts with a low efficiency of late endosomal activation. When endosomal maturation is promoted with co-expression of active Rab7, Deltex completely silences Notch signaling during leg development. This suggests that Deltex can titrate away functional Notch in a regime where late endosomal activation is low.

Suppressor of Deltex (Su(dx)), the second key regulator of Notch trafficking and signaling within the endocytic network , was identified as a spontaneous genetic modifier of *deltex* (Fostier et al., 1998). Su(dx) is a quintessential member of Nedd4 family of ubiquitin ligases (Ingham et al., 2004a). Su(dx) contains a C2 lipid-binding domain, for membrane localization, four WW domains, for substrate recognition, and a C-terminal catalytic HECT ubiquitin ligase domain. WW domains recognize PPxY motifs (cite). And indeed, NMR-based structural studies have shown that the fourth WW motif in Su(dx) recognizes the PPxY motif in NICD (Jennings et al., 2007).

Consistent with its initial identification as a genetic suppressor of *deltex* phenotypes, Su(dx) behaves genetically as a negative regulator of Notch signaling (Fostier et al., 1998; Sakata et al., 2004). Loss of Su(dx) dominantly suppresses dominant phenotypes of Notch pathway components, such as the characteristic wing notching in *Notch* heterozygotes. Overexpression of Su(dx) lowers Notch signaling, promotes Notch endocytosis, and localizes

Notch within the lumen of an Hrs+ endosomal compartment (Wilkin et al., 2004). Hrs marks a mature sub-compartment of the early endosome, where membrane proteins are eventually routed to the Rab7+ late endosome (Raiborg et al., 2002) and indeed, Su(dx) overexpression reroutes Notch into the lumen of Hrs+ endosomes.

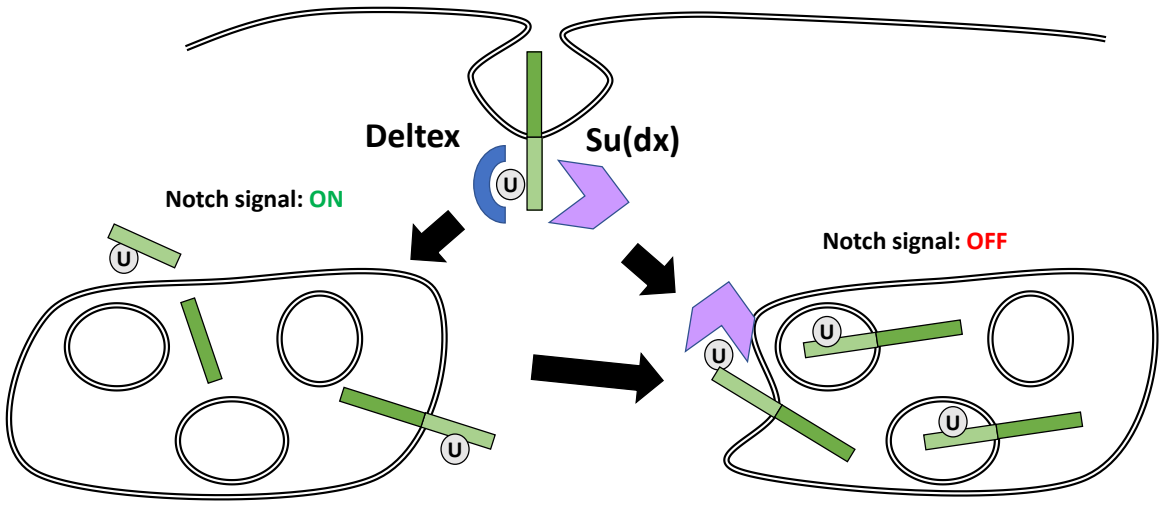


Figure 1.7 Regulation of Notch trafficking and signaling by Deltex and Su(dx)
The E3 ubiquitin ligases Deltex and Su(dx) promote distinct destinations within the endocytic pathway. Deltex ubiquitination activity drives Notch endocytosis and promotes Notch localization to the limiting membrane of the MVB, thus allowing for ligand-independent signaling. Su(dx) promotes Notch endocytosis in a ubiquitin-independent way. Su(dx) ubiquitination activity promotes internalization of Notch into the MVB, thus silencing ligand-independent signaling. Su(dx) activity can override Deltex activity and prevent Deltex-induced ligand-independent signaling.

Together these results support a model in which Su(dx) regulates Notch by promoting internalization of Notch into internal vesicles within the multivesicular body (MVB) (Shimizu et al., 2014). Internalization topologically leads to negative regulation, because NICD is sequestered from the nucleus even if a cleavage event occurs within the MVB. Furthermore, Su(dx) can divert Deltex-induced Notch flux towards internalization. A recent analysis provides more a more nuanced view of Su(dx) function (Shimizu et al., 2014). Su(dx) has two independent biochemical activities. The first promotes Notch endocytosis, and the second, as

described, promotes MVB internalization. The endocytic activity is ubiquitination independent, while the internalization activity depends on a functional HECT domain. Overexpression of a catalytically deficient Su(dx) drives endocytosis, yet this mutated Su(dx) increases basal signaling and positions Notch at the periphery of Hrs+ compartments. Therefore, Notch can also be activated from the limiting membrane of Hrs+ endosomal compartments. It should be noted that Notch can be activated both from the late endosome, under Deltex induction, and from Hrs+ compartments when internalization into the MVB is impaired.

As the function and biochemical activities of the Deltex/Su(dx) network have emerged, the factors that regulate its activity remain elusive. Signaling can occur with both ligand-dependent and ligand-independent mechanisms, and different cellular compartments are capable of promoting activation. Therefore, a broader set of factors likely regulates this network to ensure that Notch is appropriately trafficked. A key candidate that has emerged to link these realms is Abelson kinase (Abl). Abl is an attractive candidate because it localizes to the cytoplasm, where regulation of Deltex and Su(dx) is expected to occur, and is known to interact genetically with Notch. This dissertation focuses on Abl as a potential regulator of Notch trafficking and signaling, particularly in interaction with Su(dx). What follows is an overview of Abl and its connections to Notch.

Abelson kinase as a candidate Notch regulator

Work on Abl began with the isolation of the Abelson murine leukemia virus (Colicelli, 2010). The Abelson virus is a retrovirus that has transferred a cellular gene fragment, *c-abl*, into its viral genome. Molecular cloning of the resulting *v-abl* gene revealed a protein tyrosine kinase, and allowed molecular cloning of the murine *Abl1* gene. Abl is conserved among animals, and

encodes a large, cytoplasmic polypeptide with several distinct characterized protein domains. The N-terminus contains conserved Src-homology domains (SH2, SH3) and a tyrosine kinase domain. SH3 domains bind proline-rich targets, and SH2 domains bind phosphorylated tyrosine targets. The Abl C-terminus contains an F-actin binding domain, which localizes Abl to actin fibers.

Abl came to further prominence when it was discovered that a mutation at the *ABL1* locus causes chronic myeloid leukemia (CML), a human disease that causes hyperproliferation of myeloid-lineage blood cells (Hunter, 2007). CML was first characterized by a chromosomal translocation between chromosomes 9 and 22, deemed the Philadelphia chromosome. Molecular cloning revealed that the Philadelphia chromosome results in the in-frame fusion of the *BCR* and *ABL1* loci, producing a chimeric BCR-ABL protein. BCR-ABL acts as a constitutively active Abl kinase, and its activity is necessary and sufficient for CML. The fusion confers transformation activity in two ways. 1) The BCR portion contains a coiled-coil motif that acts in oligomerization to drive transformation activity (McWhirter et al., 1993). 2) The fusion removes an autoinhibitory N-terminal cap from Abl, thus increasing kinase activity (Hantschel et al., 2003). Imatanib, a 2-phenylaminopyrimidine derived compound, selectively inhibits Abl kinase activity *in vitro* and *in vivo*. Therefore, clinicians routinely administer imatinib-derived compounds as drugs that treat CML with high efficacy.

The *Drosophila* Abl homolog was first identified by cross-species hybridization of mammalian *c-abl* fragments to polytene chromosomes in the *Drosophila* larval salivary glands (Hoffmann et al., 1983). Later, a deficiency mutant spanning the *abl* locus was isolated, which allowed for production of *abl* mutants (Henkemeyer et al., 1987). Loss of Abl was found to

cause embryonic, larval and pupal lethality, with 3% of animals reaching adulthood (Henkemeyer et al., 1990). Escapers had rough eyes with missing photoreceptors. While this early genetic analysis did not provide much further detail in to Abl function, it identified an important Abl interactor, *Disabled*. *Disabled* was identified by virtue of the fact that the *Disabled* locus is very near *abl*, and deficiencies spanning the *Disabled* locus dominantly enhanced the lethality of *abl* mutants by shifting the lethality to embryonic and early larval stages (Henkemeyer et al., 1987; Gertler et al., 1989). This shift towards early lethality is caused by gaps in the axonal tracks between neurons in the embryonic central nervous system. Abl also shares an important interaction with Enabled, an actin-associated factor that promotes actin bundle elongation (Gertler et al., 1995; Winkelman et al., 2014). *Enabled* mutations dominantly suppress Abl lethality, along with neuronal and epithelial phenotypes. Abl biochemically binds and phosphorylates Enabled (Comer et al., 1998). Therefore, Abl in part functions to antagonize Enabled function, perhaps through phosphorylation.

Recently, more studies have elucidated biological roles for Abl in development. For example, an early study found that Abl regulates actin organization during embryonic cellularization, during which cell membranes simultaneously encompass individual nuclei to form epithelial cells (Grevengoed et al., 2003). In *abl* mutant embryos, actin accumulates in the apical cortex of the epidermal epithelia, while actin is lost from the basolateral furrows that mark the front of cellularization. Enabled also accumulates in the apical cortex at the expense of the cellularization furrows in these mutants. This suggests that Abl regulates Enabled localization to organize the balance between of cortical and basolateral actin during cellularization.

Abl also organizes adherens junctions and maintains epithelial integrity in the developing retina (Xiong and Rebay, 2011). Photoreceptors initially express neuronal markers under Abl loss, suggesting that Abl is not required for the specification of photoreceptor fate (Xiong et al., 2013). In line with this interpretation, the adherens junctions between *abl* photoreceptors show a stereotyped apical localization shortly after specification (Xiong and Rebay, 2011). During normal morphogenesis, the apical surfaces of photoreceptors expand and specialize to form rhabdomeres, the light-sensing organelles necessary for vision. However, adherens junction and apical domain markers collapse basally in *abl* photoreceptors by 48 hours after pupation, suggesting a failure to properly elaborate the apical domain. Mutant photoreceptor cell bodies also drop basally and ultimately reside in the lamina layer of the brain. This finding will be elaborated in the appendix of this dissertation.

A genetic analysis of *Notch* and *abl* found that these loci behave synergistically in the context of axonal patterning in the embryonic central nervous system (Giniger, 1998). Loss of Notch causes gaps in the axons between neurons in the embryonic central nervous system (CNS) (Giniger et al., 1993). This role for Notch was also revealed in a genetic interaction with *abl* in the same CNS context (Giniger, 1998). Heterozygosity of a null *Notch* allele strongly enhances the lethality seen in *abl* mutants, similar to the effect of *Disabled*. In further similarity with *Disabled*, *Notch* heterozygosity in an *abl* background reveals the CNS axon track defect. This led to the hypothesis that perhaps Notch acts in a pathway with Abl and Disabled. Notch binds Disabled *in vitro*, supporting the hypothesis that an Abl-Disabled-Notch complex functions *in vivo* (Giniger, 1998). This function of Notch is likely independent of canonical transcriptional signaling, because a Notch mutant that lacks Su(H) binding but preserves Disabled binding is able to properly establish the CNS axon tracts (Le Gall et al., 2008). Nevertheless, evidence of a

genetic and biochemical interaction between Notch and Abl raises the possibility that Abl regulates Notch signaling.

The most direct support that Abl regulates Notch comes from a relatively recent cell biological and genetic analysis of *abl* in the developing retina (Xiong et al., 2013). Along with the aforementioned morphogenic defects, loss of Abl causes a progressive accumulation of Notch in *abl* mutant tissue. Pulse chase experiments using anti-NECD antibodies reveals that Notch is endocytosed normally prior to accumulation in *abl* clones. Furthermore, the photoreceptor loss phenotype is dominantly suppressed by *Su(H)* and *Notch*, reflecting a genetic interaction between Abl and Notch pathway components in the developing eye. This work motivated a more detailed cell biological, genetic, and biochemical analysis of the Abl-Notch interaction, which is presented in the subsequent chapter.

Chapter 2: Abelson kinase regulates Notch trafficking and signaling

Note: Roy Morgan performed the experiment shown in panels D and E of Figure 2.3.

Introduction

Animal development depends on precisely orchestrated patterns of cell survival, death, proliferation, fate specification, migration, and morphogenesis (Gilbert, 2013). Despite this inherent complexity, a surprisingly limited set of signaling pathways suffices to coordinate the specific patterns of gene expression needed to initiate and maintain distinct developmental transitions. Emphasizing the need for exquisite regulatory precision, a vast literature describes how even two-fold changes in expression or activity of core pathway components and key regulators can lead to developmental defects in model organisms like Drosophila and disease in humans. While the inductive events that trigger a particular cell fate transition have been intensively studied, the regulatory mechanisms that act over longer time scales are less well understood. For example, the signaling state of a cell initiates the appropriate response to an inductive cue and then must be maintained to sustain the new pattern over a longer time scale. Therefore, regulatory mechanisms must exist to stabilize and coordinate signaling activity and cellular responses across developmental time scales and with context-specific precision.

The Notch pathway provides an ideal system to investigate the mechanisms that regulate the initiation, strength and duration of signaling during development (Hori et al., 2013). Animals express Notch receptor, for which the pathway is named, broadly during development and spatiotemporal Notch activation drives a plethora of cellular transitions, ranging from neurogenesis to programmed cell death. Notch activation must be precisely regulated, with both too much or too little signaling producing cell fate specification defects in animals ranging from mammals to *Drosophila* (Aster et al., 2017; Zacharioudaki and Bray, 2014b). Of particular clinical relevance, aberrant or excessive signaling can drive oncogenic transformation while reduced signaling is associated with Alagille syndrome, a disease associated with multi-organ

defects in humans (Li et al., 1997). Further emphasizing the need for regulatory mechanisms that fine-tune signaling output, in both flies and humans, Notch and its two ligands Delta and Serrate/Jagged are all haploinsufficient and dose-sensitive to genetic changes that increase or decrease signaling through the pathway (Mašek and Andersson, 2017; Zacharioudaki and Bray, 2014b).

The basic molecular events of Notch signaling are relatively well defined and conserved across species (Hori et al., 2013). Notch is an integral membrane protein with a ligand-binding extracellular domain and a signal transducing intracellular domain (Wharton et al., 1985a). Proteolytic cleavage of the Notch intracellular domain (NICD) marks the fundamental molecular signaling event (Hori et al., 2013; Lieber et al., 1993; Rebay et al., 1993). Free NICD enters the nucleus and forms a transcriptional complex with Suppressor of Hairless (Su(H)) and Mastermind, which drives target gene expression (Bailey and Posakony, 1995; Wilson and Kovall, 2006; Wu et al., 2000). In ligand-dependent activation, Notch receptor binds Delta/Serrate/Lag2-family ligands exposed on neighboring cells (Gordon et al., 2008). Ligand recognition triggers cleavage of the Notch extracellular domain (NECD) by the proteinase ADAM, a process called S2 cleavage (Brou et al., 2000). Following S2 cleavage, another cleavage site is exposed which triggers cleavage and release of NICD by γ-secretase and presenilin, deemed S3 cleavage (Strooper et al., 1999). These events occur within endocytic vesicles following ligand-mediated endocytosis (Yamamoto et al., 2010). In support of this, the key endocytic factor Dynamin is required for Notch signaling in *Drosophila* (Seugnet et al., 1997).

In *Drosophila*, the spatially restricted expression of Notch ligands instructs specific patterns of Notch activity to direct developmental decisions in the ovary, embryo and imaginal

discs (Housden et al., 2014). For example, cells in the third instar wing imaginal disc express the ligand Delta in two stripes flanking the D-V (D-V) boundary (de Celis et al., 1996). The ensuing activation of Notch in adjacent cells is critical to specifying wing margin structures. As a result, insufficient Notch signaling within this domain produces the wing margin "notches" for which the gene is named (Dexter, 1914).

Across tissues, Notch is broadly expressed beyond the specific spatial patterns where its strong activation is observed (Fehon et al., 1991). Continuing the example of the third instar wing imaginal disc, Notch is expressed throughout the tissue, with ligand-dependent activation occurring at the D/V boundary (de Celis et al., 1996). Pulse chase experiments have shown that all cells basally traffic Notch from the cell surface into endocytic compartments (Maitra et al., 2006). Endocytosed Notch can either be recycled back to the surface, or degraded within the endocytic pathway. (Yamamoto et al., 2010). When degraded, Notch is sorted through early endosomes, multivesicular bodies, late endosomes, and the lysosome (Vaccari et al., 2008; Yamamoto et al., 2010). Mutating ESCRT complex factors, which regulate internalization of proteins into the multivesicular body (Henne et al., 2011), causes Notch accumulation and activation in the absence of detectable ligand expression (Vaccari et al., 2008). Therefore, Notch trafficking is actively regulated to prevent ligand-independent activation.

Notch trafficking is regulated by ubiquitination (Hori et al., 2013; Shimizu et al., 2014), a post-translational regulatory mechanism that modulates endocytic trafficking and degradation of many receptors (Hershko and Ciechanover, 1998). Key insights have come from studying two genetic modifiers of Notch that both encode ubiquitin ligases: *deltex* (*dx*) and *Suppressor of dx* (*Su(dx)*). Genetic and molecular studies implicate Dx as a positive regulator and Su(dx) as a negative regulator of Notch signaling, although more recent studies hint to greater context-

specific complexity (Fostier et al., 1998; Fuwa et al., 2006; Shimizu et al., 2014). As summarized below, the emerging picture is that both Dx and Su(dx) influence the early steps of Notch endocytosis via ubiquitination-independent mechanisms and the later steps via ubiquitination-dependent mechanisms.

dx was originally identified as a spontaneous mutant that both recapitulates and strongly enhances Notch loss-of-function phenotypes (Xu and Artavanis-Tsakonas, 1990b). Subsequent work showed that whereas loss of *dx* reduces Notch endocytosis, its overexpression can promote endocytosis and ligand-independent activation (Hori et al., 2011; Yamada et al., 2011; Shimizu et al., 2014). Molecularly, Dx operates as a RING family ubiquitin ligase that binds the NICD, facilitates its ubiquitination and directs endocytosed Notch to signaling-promoting rather than to degradative compartments (Hori et al., 2004; Wilkin et al., 2008). The initial role of Dx in recruiting Notch to clathrin-coated pits requires only the dimerization function, but not the catalytic activity, of the RING finger motif (Matsuno et al., 2002). Dx-mediated activation of Notch signaling can be suppressed by expression of a dominant negative form of Rab5, which blocks early endosome fusion, suggesting that the ubiquitinatin-dependent role of Dx targets Notch that has been trafficked to the late-endosomal limiting membrane (Hori et al., 2004; Wilkin et al., 2008).

Su(dx) was discovered as a spontaneous suppressor of the Notch-like deltex phenotypes (Fostier et al., 1998) and encodes a member of the Nedd4 family HECT domain ubiquitin ligases (Cornell et al., 1999). *Su(dx)* mutants show wing vein gap phenotypes reminiscent of those associated with gain of Notch function, further implicating it as a Notch pathway antagonist. Consistent with this, overexpression studies show that Su(dx) can dampen Notch signaling output in either a wild type or a Dx overexpression background (Mazaleyrat et al., 2003).

Mechanistically, Su(dx) has two distinct and molecularly separable roles in Notch regulation. First, Su(dx) promotes the basal endocytosis of Notch into a sterol-rich endocytic network via a ubiquitination-independent mechanism (Shimizu et al., 2014). This isolated activity can drive ligand-independent activation. Second, via an interaction between the WW domains of Su(dx) and a proline-rich PPXY motif in the NICD, Su(dx) ubiquitinates Notch to promote internalization into the multivesicular body (MVB) (Sakata et al., 2004). This topologically restricts cleaved NICD from accessing the nucleus and thus represses signaling.

Given that Notch is continuously endocytosed in wild type tissues and that ligand-independent activation is possible, ligand-independent signaling could be important for setting basal signaling levels to a state favorable for ligand-dependent signaling, for maintaining tissue homeostasis subsequent to ligand-dependent signaling, or for providing context-specific feedback mechanisms that fine-tune ligand-dependent signaling and confer robustness. However, ligand-independent signaling has only been described under conditions of experimental perturbation, making it formally possible that it does not contribute to endogenous regulation (Palmer and Deng, 2015). Identification of novel sensitized genetic backgrounds that can provide further insight into the mechanisms that regulate Notch trafficking and signaling will undoubtedly prove pivotal in resolving this debate.

Previous work from our lab studying the function of the Drosophila Abelson (Abl) nonreceptor tyrosine kinase during photoreceptor morphogenesis serendipitously identified it as a candidate regulator of Notch trafficking and signaling. Similar to Notch, Abl is broadly expressed in developing embryonic and imaginal tissues, where it coordinates many aspects of epithelial and neuronal morphogenesis (Gertler et al., 1989; Grevengoed et al., 2001). Our study of Abl function during pupal eye development uncovered a defect in Notch passage through the

endocytic pathway (Xiong et al., 2013). Thus in pulse-chase experiments in live 24h after puparium formation (APF) eye imaginal discs, although starting cell surface levels of Notch appear comparable in wild type and *abl* mutant tissue, in the absence of Abl, endocytosed Notch accumulated into large punctal structures rather than being rapidly degraded as was the case in adjacent wild type tissue (Xiong et al., 2013). Although we hypothesized that the increase in intracellular Notch levels might activate ectopic ligand-independent signaling, how Abl influences Notch trafficking and whether it promotes and/or dampens signaling remain to be elucidated.

In the following section we present evidence showing that Abl regulates Notch trafficking within endosomal compartments to promote signaling *in vivo*. We find that Abl phosphorylates Notch *in vitro* at the PPxY motif, which is a known recognition site for Su(dx) activity (Jennings et al., 2007; Sakata et al., 2004). Mutating the PPxY tyrosine unexpectedly confers higher signaling activity, suggesting that Abl promotes Su(dx) activity. Therefore, Abl must promote Notch signaling by a different mechanism. Interestingly, we find that Abl overexpression decreases Notch activity in a kinase-dependent manner, suggesting that the Su(dx) promoting activity can override the signal-promoting role. Our results establish Abl as a novel negative regulator of Notch activity. We include Abl in a broader network of factors that regulate basal Notch trafficking and signaling during development.

Results

Abl prevents Notch accumulation in Hrs+ compartments

A previous analysis of Abl function during pupal eye development revealed that Abl promotes progression of Notch through the endocytic pathway (Xiong et al., 2013). Thus, pulse-chase experiments in live 24h after puparium formation (APF) eye imaginal discs show that

starting cell surface levels of Notch appear comparable in wild type and *abl* mutant tissue. However, in the absence of Abl, endocytosed Notch accumulates into large punctal structures rather than being rapidly degraded (Xiong et al., 2013). Quantification in fixed tissue at 48h APF confirmed that mitotic clones homozygous for the null abl^2 allele exhibit a larger cross-sectional area of Notch puncta as compared to their wildtype counterparts (Figure 2.1 A, B).

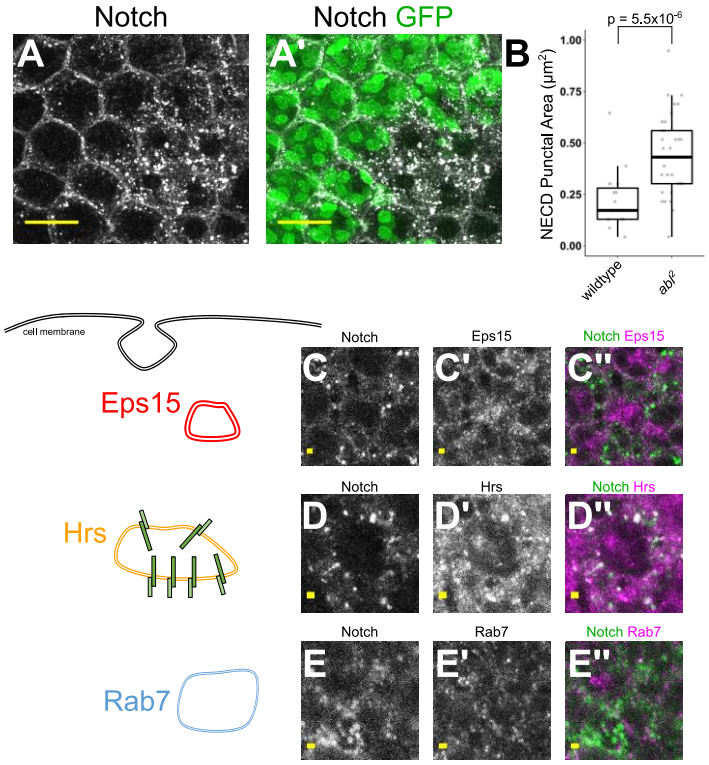


Figure 2.1 Abl prevents Notch accumulation in Hrs+ compartments
(A) Panel A shows a maximal confocal projection from a 48h APF retina containing an abl^2 mutant mitotic clone. Lack of GFP marks *abl* mutant clones in this figure. Scale bar = 10 µm. *abl* clones contain higher Notch punctation intensity, including larger puncta than their wildtype counterparts. (B) Quantification of the data in A, calculated as the maximal cross-sectional area of Notch puncta within each genetic category. Gray circles represent individual data points. Box plots indicate population quadrants. T-test p-values are indicated. (C-E) The data in panels C, D, and E are presented with a corresponding schematic indicating the approximate relative order of compartment markers in the endocytic pathway. All panels show a single confocal slice

Figure 2.1, continued: containing several *abl* mutant retinal cells from 48h APF retinas. Retinas were stained for Notch along with the indicated compartment marker. Scale bar = 1μm. (C) Notch puncta show weak
correspondence with Eps15+ puncta. (D) Notch puncta show strong correspondence with Hrs+ puncta (E) Notch puncta show weak correspondence with Rab7+ puncta.

We first sought to identify the compartment in which Notch accumulates in the absence of Abl by assessing colocalization with three well-characterized markers of early, mid and late endocytic compartments: Eps15, a marker of upstream endocytic compartments (Tebar et al., 1996), Hrs, a marker of endosomal compartments (Raiborg et al., 2002), and Rab7, a late endosomal marker (Bucci et al., 2000). Notch puncta colocalized with Hrs in *abl* clones (Figure 2.1 D), but did not colocalize with Eps15 and only sparingly colocalized with Rab7 (Figure 2.1 C, E). The overall intensity and punctal nature of all three markers was the same in *abl* mutant and wild type tissue (Figure 2.2), arguing that Abl loss does not grossly disrupt endocytosis. Consistent with this, prior work showed normal internalization of fluorescent dextran and normal levels and localization of other cell surface proteins in *abl* mutant clones (Xiong et al., 2013). We conclude that, in the absence of Abl, Notch accumulates in Hrs+ endocytic compartments.

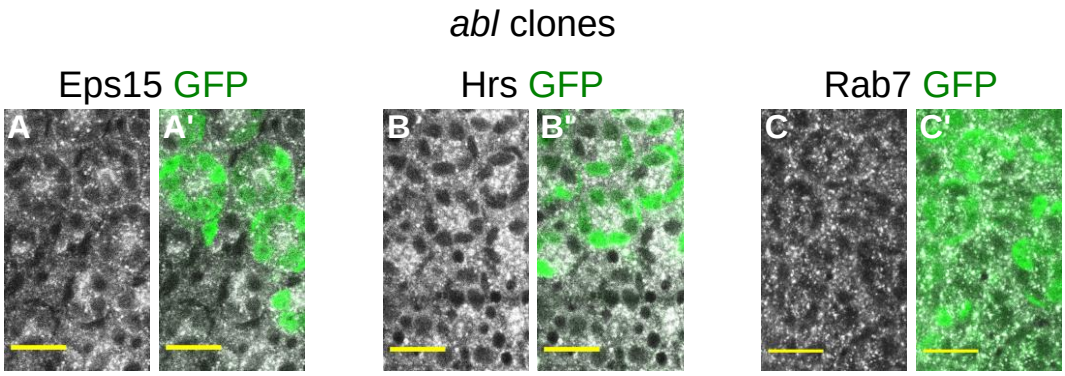


Figure 2.2 Abl loss does not perturb general endocytic trafficking.
(A-C) Panels A-C show single confocal slices from 48h APF retinas containing *abl*^{2} mutant mitotic clones. Lack of GFP marks *abl* mutant clones in this figure. Scale bar = 10 µm.

Given that Notch accumulates in Hrs+ compartments under Abl loss, we asked whether this results in an increase or decrease in Notch signaling. A previous genetic analysis found that mutating ESCRT complex components, which internalize ubiquitinated transmembrane proteins into the lumen of MVBs (Henne et al., 2011), causes Notch accumulation in Hrs+ compartments along with increased signaling (Vaccari et al., 2008). Therefore, it was possible that Abl loss causes a similar increase in signaling. Another possibility is that Notch is sequestered in a signaling-incompetent Hrs+ compartment under Abl loss, resulting in a decrease in signaling.

Abl regulates Notch signaling *in vivo*

To test this, we compared expression of the transgenic Notch-responsive element (NRE) GFP reporter (Zacharioudaki and Bray, 2014b) in wildtype versus *abl* mutant eye discs. The reporter carries tandem Su(H) binding sites, a binding site for the broadly expressed activator Grainy head and a minimal hsp70 promoter. This configuration allows activation in response to Notch signaling and repression in the absence of signaling, resulting in sensitive detection of Notch pathway activity in a range of embryonic and imaginal tissues. In wildtype 48hr APF retinas, Notch signaling is activated in primary pigment cells, which express Abl at 48h APF, by

Delta expression in neighboring lens-secreting cone cells (Bao, 2014). Therefore, *NRE* expression is detected in these two cells. Anterior primary pigment cells exhibit higher Notch activation than posterior pigment cells (Bao, 2014). NRE-GFP shows visibly reduced expression in abl^{1}/abl^{2} retinas at 48h APF (Figure 2.3 A, B). Quantification of *NRE* expression per ommatidium shows approximately a 20% reduction in *abl* retinas (Figure 2.3 C). Therefore, Abl is required for normal Notch signaling levels in primary pigment cells at 48h APF.

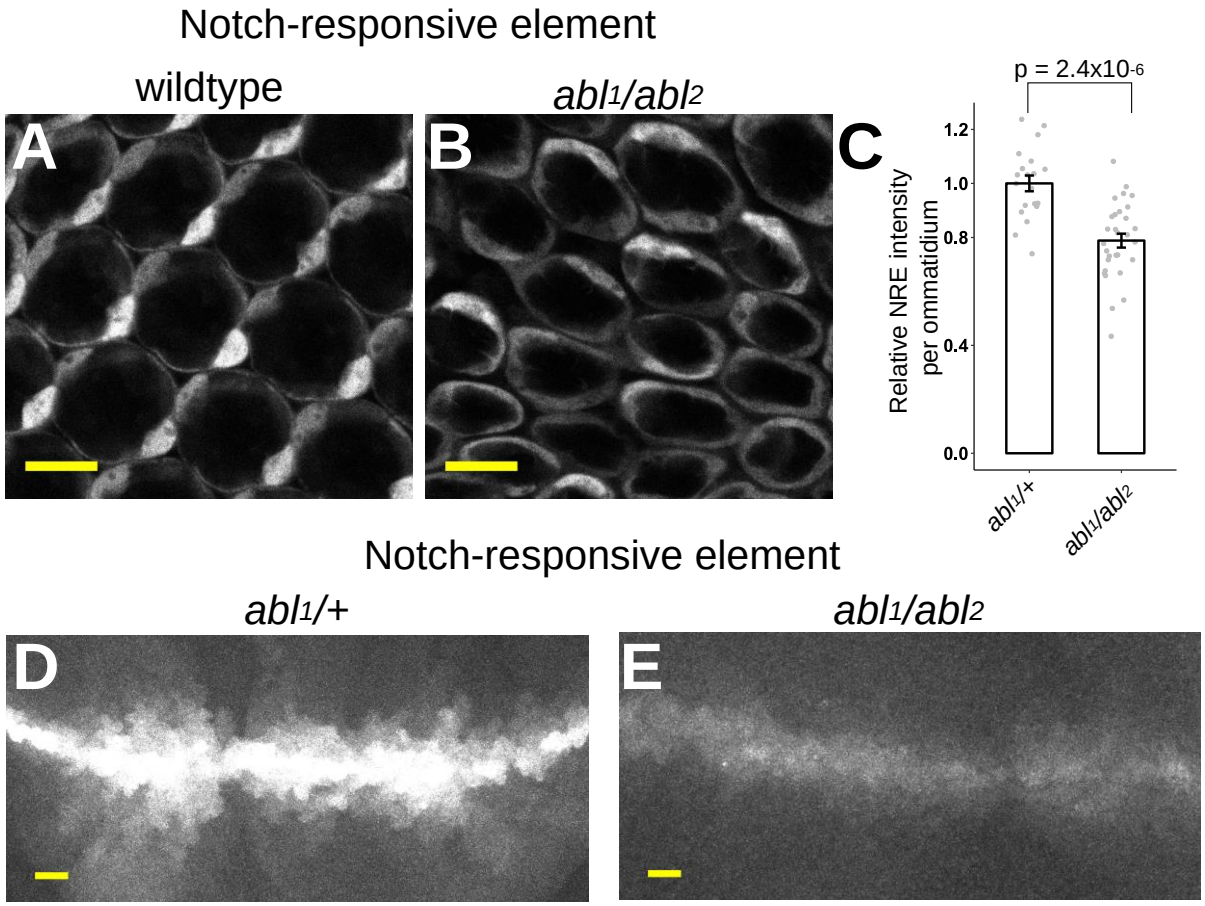


Figure 2.3 Abl promotes Notch signaling *in vivo*
(A-B) Panels A and B show confocal slices of 48h APF retinas depicting GFP intensity corresponding to NRE expression. Scale bar = 10 µm. (A) NRE expression in primary pigment cells from an $abl^{1}/+$ retina. (B) NRE expression in primary pigment cells from an abl^{1}/abl^{2} retina. (C) Quantification of GFP intensity per ommatidium. Each gray circle is a reading from a

Figure 2.3, continued: single ommatidium. Loss of Abl causes a 21% decline in NRE expression. (D) A maximal confocal projection showing NRE expression along the D-V boundary in an abl^{1}/+ wing imaginal disc. Scale bar = 10 μm. (E) A maximal confocal projection showing NRE expression along the D-V boundary in an abl^{1}/abl^{2} wing imaginal disc.

We then asked whether Abl regulates Notch signaling beyond the eye. Notch signaling is active along the D-V boundary of the wing imaginal disc (de Celis et al., 1996). Therefore, we asked whether loss of Abl also causes a loss in NRE expression in this context. NRE-GFP shows visibly reduced expression at the D-V boundary in abl^{1}/abl^{2} retinas at 48h APF (Figure 2.3 D, E). Therefore, Abl is also required for normal Notch signaling at the D-V boundary of the wing imaginal disc. However, we were unable to assess the effect of complete Abl loss on adult wings because *abl* mutant animals die during pupal development. Therefore, we used a protein knockdown strategy to observe the phenotype of Abl loss in adult wings.

We used the deGradFp system to knockdown an endogenously-tagged *abl-GFP* allele, provided by the MIMIC protein trap library (Caussinus and Affolter, 2016; Nagarkar-Jaiswal et al., 2015). The *engrailed-GAL4* element drives expression of deGradFP nanobody in the posterior compartment of the wing imaginal disc, driving ubiquitination and degradation of Abl-GFP protein. Knockdown using this system causes an approximately 15% loss of Abl-GFP in the posterior compartment of the wing imaginal disc when compared to the anterior compartment (Figure 2.5). We used heterozygosity of the null abl^{2} allele to further lower the amount of functional Abl in the experiment. We found that Abl knockdown causes an 81% penetrant ectopic veinlet formation phenotype (Figure 2.4 A, B). Complete posterior cross vein forking occurs at 28% penetrance. As a control, knockdown of *abl-GFP* in trans to wildtype *abl* causes no discernible wing phenotype.

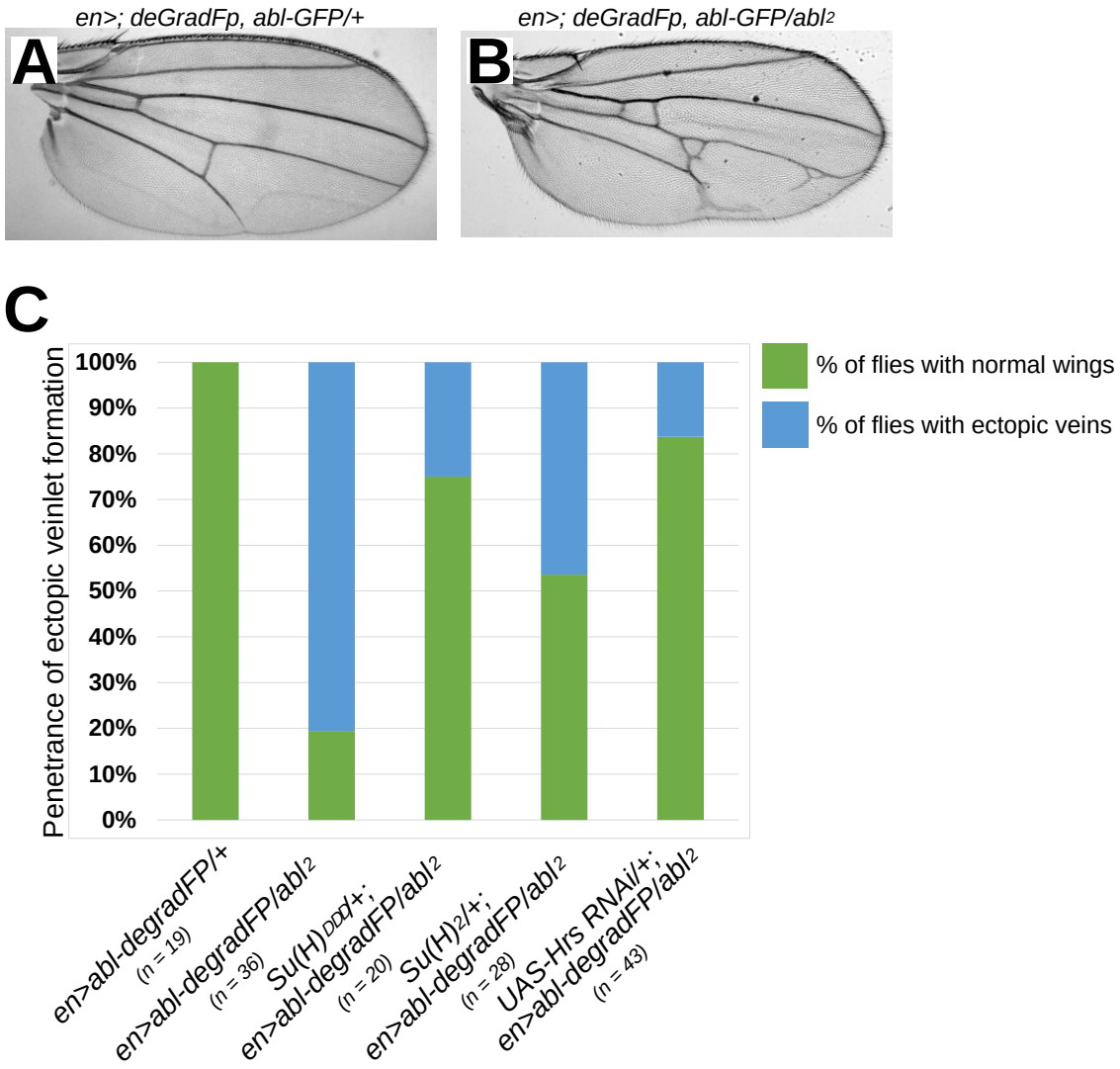


Figure 2.4 Abl regulates Notch signaling during wing vein development
(A-B) Adult wings from *en>; deGradFP, abl-GFP/+* (A) and *en>; deGradFP, abl-GFP/abl*^{2} (B) animals, respectively. Abl knockdown causes ectopic veinlet formation with 81% penetrance. (C) Quantification of ectopic veinlet penetrance under Abl knockdown. Green represents absence of ectopic veins. Blue represents presence of ectopic veins. *Su(H)* and Hrs knockdown suppress ectopic veinlet penetrance. $p < 0.05$ by chi-squared test for all comparisons to baseline penetrance.

The ectopic veinlet phenotype caused by Abl knockdown is consistent with a loss of Notch signaling. During pupal stages, Delta expression in the primordial veins drives Notch signaling in neighboring intervein tissue (Celis, 2003). Notch signaling represses vein thickening

and ectopic vein formation to produce the stereotypical vein pattern. To test whether the ectopic veins under Abl knockdown result from a decrease in Notch signaling, we asked whether Abl knockdown phenotype interacts with the canonical Notch transcriptional cofactor, Su(H). Two different *Su(H)* alleles, namely $Su(H)^{2}$ and $Su(H)^{\Delta 47}$, dominantly suppress the penetrance of ectopic veinlets to 25% and 46% respectively (Figure 2.4 C). Su(H) acts as a transcriptional repressor of Notch targets while not bound to NICD. Therefore, *Su(H)* heterozygosity suppresses phenotypes caused by Notch signaling decrease (Fuwa et al., 2006). This interaction suggests that Abl regulates wing veinlet formation by strengthening Notch signaling. Since Abl loss causes an accumulation of Notch in Hrs+ compartments, decreased Notch signaling may result from sequestration in these compartments. Therefore, we knocked down Hrs using RNAi in the background of Abl degradFp to test whether this would suppress the decrease in signaling. Indeed, Hrs knockdown suppresses the penetrance of ectopic veinlets to 16% (Figure 2.4 C). Therefore, the decrease in signaling under Abl loss likely results from sequestration in Hrs+ compartments.

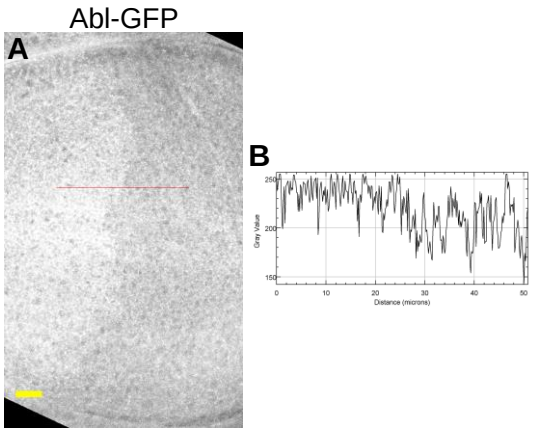


Figure 2.5 Knockdown efficiency of deGradFP in the wing imaginal disc
(A) Maximal projection of a wing imaginal disc from a *en>; deGradFP, abl-GFP/*abl^{2} animal. The posterior compartment shows a visible loss of GFP signal. Scale bar = 10 µm. A red indicates the signal profile in panel B. (B) A signal profile along the red line depicted in panel A.

Figure 2.5, continued: The red line spans the anterior/posterior compartment boundary. The deGradFp knockdown lowers GFP intensity by approximately 20% in the posterior compartment.

Abl regulates Notch trafficking and signaling in a kinase-dependent way

How does Abl strengthen Notch signaling? We first tested whether Abl kinase activity is required to prevent Notch accumulation. We transfected Notch into S2 cells, which express Abl endogenously (Zhang et al., 2010) but do not express Notch (Fehon et al., 1990), and treated the cells with 200 µM imatinib mesylate, an Abl kinase activity inhibitor (Hunter, 2007). In untreated cells, Notch localizes to the cell membrane, diffusely within the cytoplasm, and in distinct puncta (Figure 2.6 A). Under imatinib mesylate treatment, Notch concentrates into large cytoplasmic puncta, similar to the effect of Abl loss *in vivo* (Figure 2.6 B). These structures are Hrs+, similar to those that arise in *abl* mutants *in vivo*. Therefore, Abl kinase activity is required to prevent punctal Notch accumulation in S2 cells. We then tested the effect of decreased Abl kinase activity on Notch signaling. We performed a transcription assay using an NRE-luciferase reporter in S2 cells. Transfected Notch causes an immense induction of NRE activity in untreated cells (Figure 2.6 C). Treatment with 200µM imatinib mesylate lowers Notch activity to approximately 10% of baseline. The decrease in Notch signaling does not result from a general effect of imatinib mesylate on the reporter system, since treatment caused an increase of signal from the reporter alone. These results suggest that Abl kinase activity is required to maintain Notch signaling levels.

Position	Peptide sequence	Probability Score
1850	GNNGGYASDHT	3.1×10^{-12}
1860	TMVSEYEEADQ	0
2097	AREGSYEACKA	6.9×10^{-12}
2299	**NLPSPYDTSSM**	**8.9×10^{-11}**
2305	DTSSMYSNAMA	0
2328	**KQPPSYEDCIK**	**7.9×10^{-11}**
2354	IKLDNYAYSMG	1.1×10^{-11}
2356	LDNYAYSMGSP	4.6×10^{-13}
2415	GLSPPYSNQSP	5.0×10^{-11}
2435	LSPHAYLGSPS	3.0×10^{-11}
2627	**NQQAFYQYLTP**	**1.1×10^{-10}**
2629	QAFYQYLTPSS	1.0×10^{-11}
2652	**QTLDSYPTPSP**	**1.2×10^{-10}**
2687	AANNLYISGGH	2.9×10^{-12}
2702	GSEAIYIxxxx	NA

Table 1. Position-based probability scores of potential Abl target sites in the NICD
The left column contains the position for each tyrosine residue in NICD. The middle column contains10 surrounding amino acids for each tyrosine residue. The right column contains the position-based probability of that tyrosine being an Abl target, based on the position probability matrix in (Colicelli, 2010). The last site was not scored because it occurs at the end of Notch and thus does not contain 10 surrounding amino acids. The top four sites are shown in bold.

Does Abl phosphorylate Notch? The *Drosophila* NICD contains 15 tyrosine residues (Wharton et al., 1985b). We scored each tyrosine residue as potential Abl targets using a position probability matrix derived from reported target sites of human ABL proteins (Colicelli, 2010). Only two of the 15 residues reside in previously described NICD domains, and these residues are among the top four scoring residues in our position-based probability analysis (Table 1). The first residue is within the PPxY motif, which is required for Su(dx) recognition (Jennings et al., 2007), and the second residue is within the PEST motif, which limits NICD stability (Weng et al., 2004). Given that Abl regulates Notch trafficking, we tested whether Abl phosphorylates Notch at the PPxY tyrosine, which is recognized by Su(dx). We performed *in vitro* kinase assays in which recombinant murine Abl is incubated with GST-NICD, GST-NICD^{Y2328F}, or GST

alone. We calibrated the amount of Notch protein using western blots for the NICD (Figure 2.6 D'). Abl phosphorylates fragments of GST-NICD, including the full-length 105 kD species, but not GST alone (Figure 2.6 D). Strikingly, Abl has less activity on GST-NICD^{Y2328F} fragments, indicating that at least some Abl activity targets Y2328. These results show that Abl can phosphorylate Notch at the PPxY domain *in vitro.* Furthermore, this novel biochemical activity lends support to the model that Abl phosphorylates Notch *in vivo* to regulate signaling.

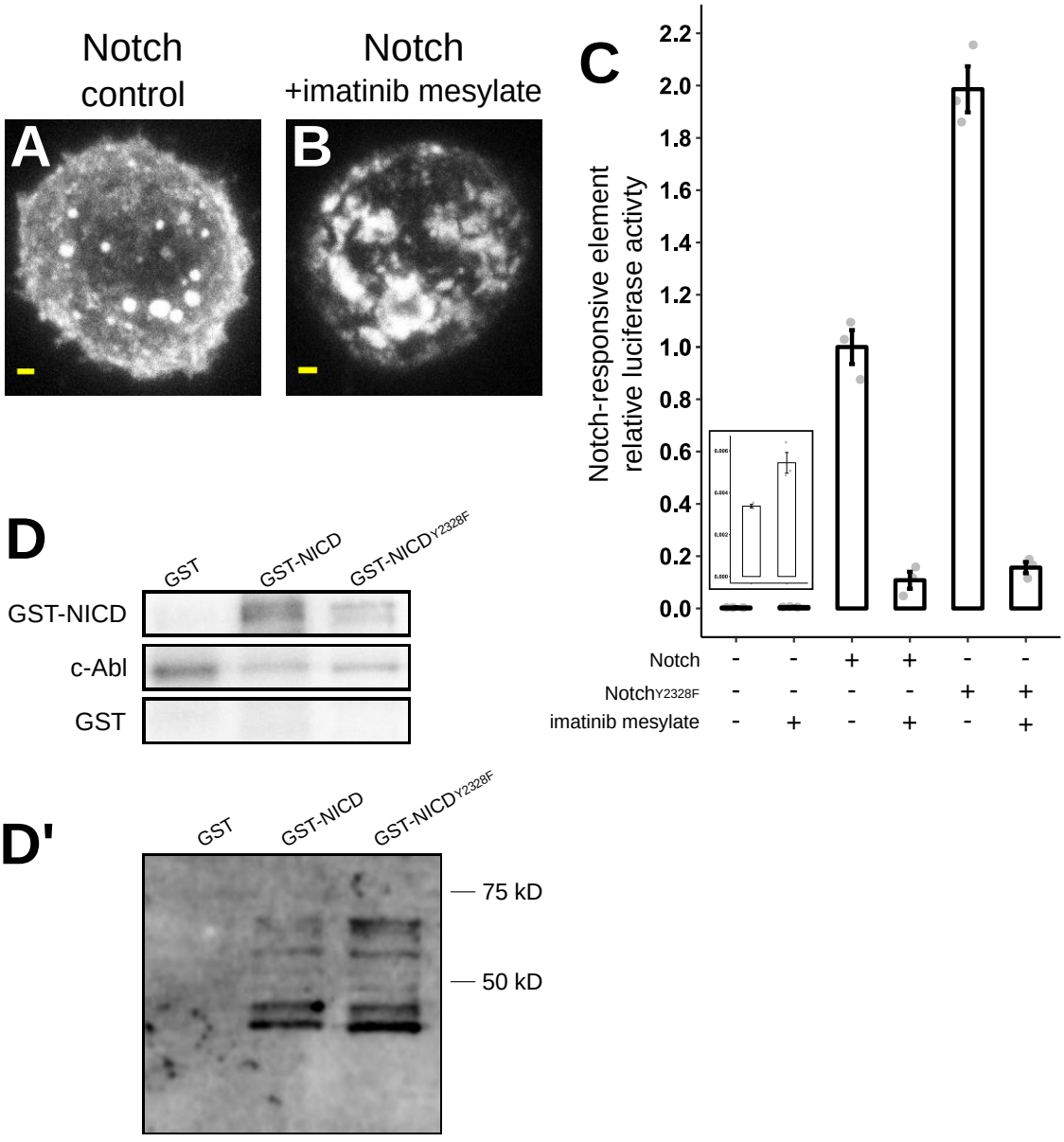


Figure 2.6 Abl kinase activity regulates Notch signaling
(A-B) Maximal projections of S2 cells expressing Notch. Scale bar = 5 µm. (A) An untreated cell exhibiting Notch at the cell membrane, diffusely in the cytoplasm, and concentrated into small puncta. (B) A cell treated with 200 µM imatinib mesylate for 24 hours. Notch concentrates into large cytoplasmic puncta. (C) Imatinib mesylate treatment lowers Notch activation of the *NRE*-luciferase reporter by tenfold. Imatinib mesylate treatment increases the activity of reporter alone (see inset plot for appropriate scale). Notch^{Y2328F} activates the *NRE*-luciferase reporter with 2.1-fold higher activity than Notch. Imatinib mesylate treatment lowers Notch^{Y2328F} activation down to 15% of baseline. (n = 3 biological replicates for each condition) (D) *In vitro* kinase assays show that recombinant murine Abl phosphorylates the NICD with some activity at

Figure 2.6, continued: Y2328. In lane 1, Abl phosphorylates itself but not GST alone. In lane 2, Abl phosphorylates itself and GST-NICD, including both full-length and co-purifying degradation products (not shown). In lane 3, Abl phosphorylates itself with slightly higher intensity as lane 2. However, Abl phosphorylates GST-NICD^{Y2328F} with less intensity than lane 2 (including co-purifying degradation products), indicating that at least some Abl activity targets Y2328. (D') A western blot of the membrane from panel B using anti-NICD antibody. The antibody specifically recognizes species between ~40-70 kD. The full-length GST-NICD species is not recognized after *in vitro* exposure to Abl activity. Slightly more GST-NICD^{Y2328F} was loaded into the kinase assay than GST-NICD.

Two points of evidence suggest that a PPxY motif within the NICD functions as an important site of Nedd4 family regulation. First, Nedd4 family ubiquitin ligases bind the PPxY motif as a recognition motif within their substrates (Ingham et al., 2004b). In fact, nuclear magnetic resonance reveals the structure of a Su(dx) WW domain bound to the Notch PPxY motif (Jennings et al., 2007). Second, the eponymous member, Nedd4 exhibits less binding and ubiquitination activity upon a PPxY to PPxF Notch mutant (Notch^{Y2328F}) (Sakata et al., 2004). Indeed, Notch^{Y2328F} robustly exhibits an approximately twofold increase in activation of the NRE reporter when compared to wildtype Notch (Figure 2.6 C). This shows that the tyrosine in Notch's PPxY motif is required to repress Notch signaling, in addition to its known requirement for recognition and ubiquitination by Su(dx) and Nedd4.

Why does Abl loss cause a decrease in signaling when mutating a relevant Abl target site causes an increase in signaling? One explanation is that, in addition to promoting Su(dx) activity, Abl is required for signaling downstream of impaired Su(dx) activity. To test this model, we treated S2 cells expressing Notch^{Y2328F} with imatinib mesylate. If Abl is not required for the increased activation, then the Y2328F mutation would be epistatic to Abl loss. However, we find that imatinib mesylate reduces the twofold Notch^{Y2328F} activation down to 15% of the wildtype Notch baseline (Figure 2.6 C). Therefore, Abl is also required for the increased activation caused

by impairment of MVB internalization. This epistasis may explain why Abl loss causes a decrease in overall signaling.

If Abl phosphorylates the Notch PPxY *in vivo*, then loss of Abl would impair the ubiquitin-ligase dependent activity of Su(dx) to internalize Notch into the MVB. However, the endocytic, ubiquitin-ligase independent activity of Su(dx) would remain intact. Therefore, overexpression of Su(dx) would predictably enhance Notch accumulation in the absence of Abl. Indeed, overexpression of Su(dx) using the pan-eye GMR driver significantly enhanced Notch accumulation in abl^2 clones (Figure 2.7). No noticeable difference in Notch puncta was apparent in wildtype tissue, although Su(dx) overexpression causes an excess of specified retinal cell types, consistent with a decrease in Notch signaling (Cagan and Ready, 1989) (Figure 2.8). Quantification confirmed these observations, with the cross-sectional area of Notch puncta measured as 2.7-fold larger in abl^2 clones under Su(dx) overexpression than in abl^2 clones without Su(dx) overexpression (Figure 2.7 C). Therefore, loss of Abl sensitizes cells to Su(dx) activity with respect to Notch accumulation. This is consistent with the model that Abl promotes the ubiquitin-dependent function of Su(dx) to internalize and degrade Notch within the MVB.

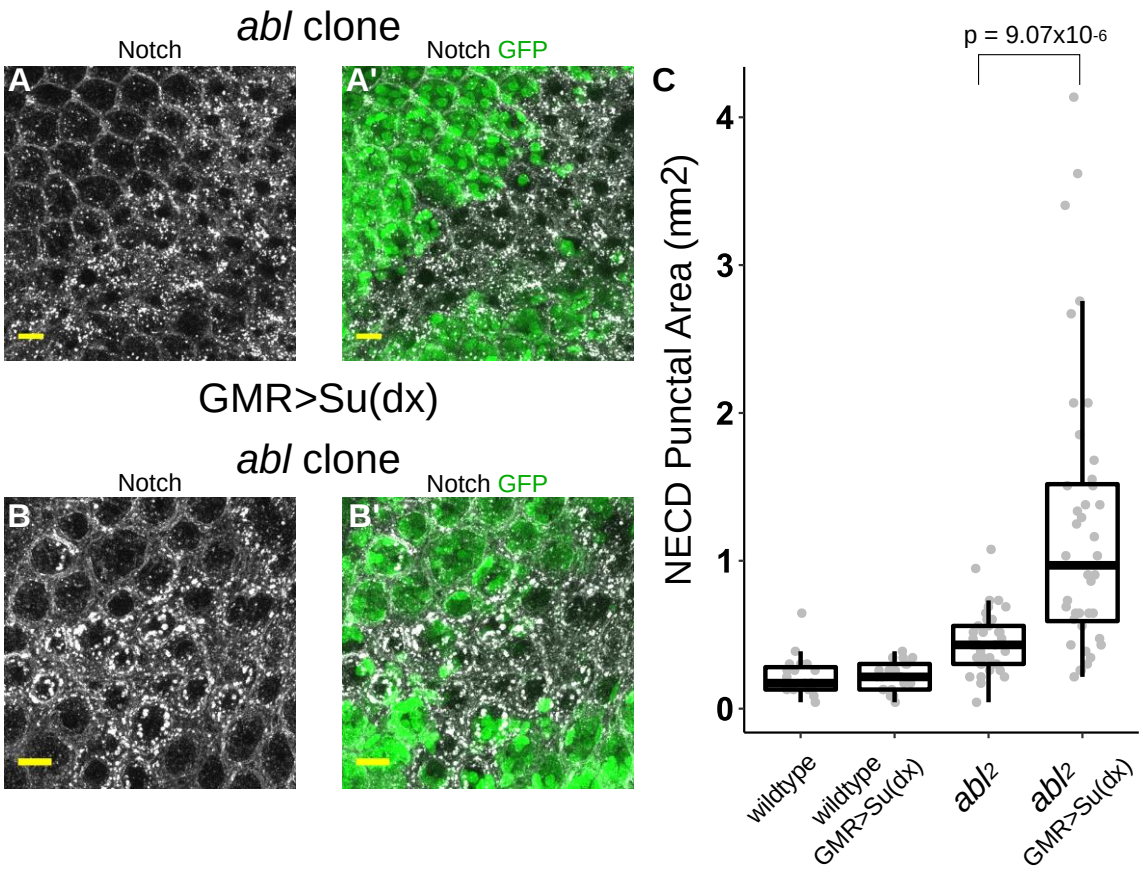


Figure 2.7. Su(dx) overexpression enhances Notch accumulation in *abl* clones
(A-B) Panels A and B show maximal confocal projections from 48h APF retinas containing an *abl*^{2} mutant clones. Scale bar = 10 µm Overexpression of Su(dx) enhances Notch punctation in *abl* clones while having no effect on punctation in wildtype tissue. (C) Quantification of the data in A and B, calculated as the maximal cross-sectional area of Notch puncta within each genetic category. Gray circles represent individual data points. Box plots indicate population quadrants. T-test p-values are indicated.

Given that Abl promotes both Notch signaling and Su(dx) activity upon Notch, it is unclear whether Abl overexpression predictably strengthens or represses Notch signaling. One possibility is that Abl overexpression represses Notch signaling by hyper-phosphorylating Notch and drives its internalization into the MVB via Su(dx) activity, overriding the requirement of Abl for normal Notch signaling levels. To address how Abl overexpression affects Notch activity *in vivo*, we performed an Abl overexpression assay in the wing. Flies that overexpress GFP-tagged Abl under control of *engrailed-Gal4 (en>)*, which drives expression in the posterior

compartment of the wing imaginal disc, exhibit wing notches at 34.6% penetrance (n=52) (Figure 2.9 A). Flies that express *engrailed-Gal4* alone exhibit a 0% penetrance (n=41). The Abl overexpression phenotype is completely suppressed by a point mutation, K417N, that disrupts Abl kinase activity (Henkemeyer et al., 1990). Specifically, *en>Abl^{K417N}-GFP* flies exhibit a 0% penetrance of wing notches (n=37). Wing discs dissected from *en>Abl-GFP* flies show the expected abrogation of Cut expression along the D-V boundary in the posterior compartment where Abl is overexpressed (Figure 2.9 B). Cut abrogation does not occur throughout the posterior compartment. Instead, smaller lesions of Cut absence are present within the compartment. This suggests that Notch signaling is spatially sensitized to Abl overexpression along the boundary. Strikingly, *en>Abl^{K417N}* discs exhibit normal Cut expression throughout the D-V boundary (Figure 2.9 C). These results suggest that Abl overexpression represses Notch signaling in a kinase-dependent way.

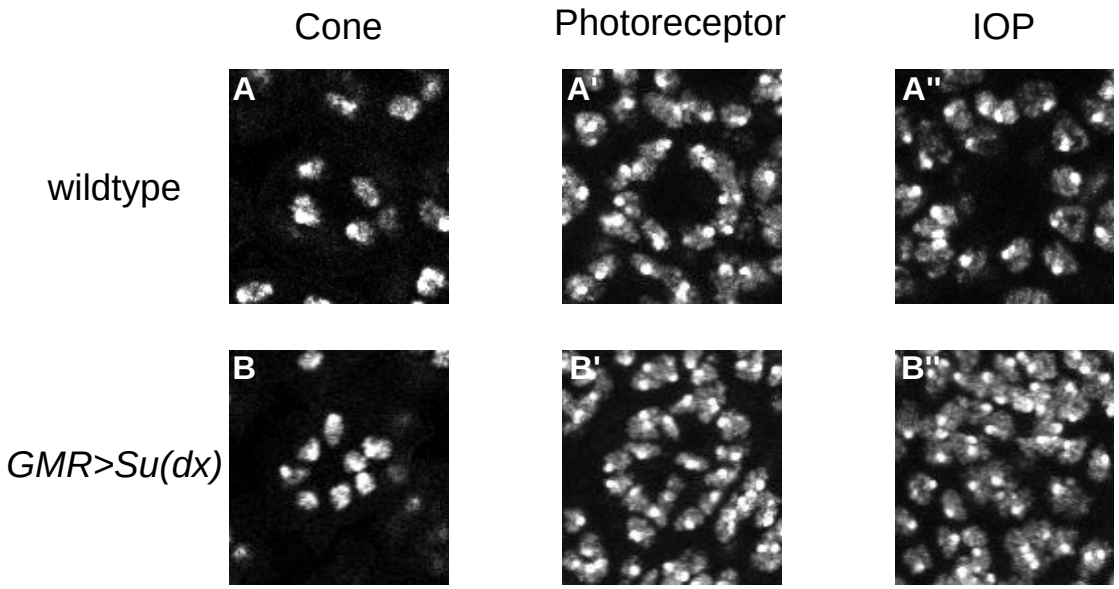


Figure 2.8 Su(dx) overexpression causes an increased number of cells among retinal cell types regulated by Notch signaling
All panels show confocal slices of 48h APF retinas stained with the nuclear marker DAPI. Cell types are identified based on stereotypical arrangement in the retinal epithelium. (A) wildtype ommatidia with cone cells, (A') photoreceptor cells, and (A'') IOPs. (B-B'') *GMR>Su(dx)* retinas exhibit an increased number of each cell type, consistent with decreased Notch signaling.

Discussion

Here we used cell biology, genetics, and biochemistry to identify Abl as a novel regulator of Notch signaling. We find that Abl regulates Notch trafficking in the endocytic pathway and that Abl promotes Notch signaling. Abl phosphorylates Notch *in vitro* at the PPxY motif, a known recognition motif for Nedd4-family ubiquitin ligase activity. In addition, mutating the PPxY tyrosine to phenylalanine confers higher signaling activity to Notch. Abl overexpression can in fact repress Notch signaling, consistent with an activity at the PPxY motif that promotes Su(dx) activity. Therefore, we propose a model in which Abl targets the PPxY to prevent Notch accumulation in Hrs+ endosomes, with the caveat that Notch activation also requires Abl activity.

Our results are consistent with a biochemical model in which Abl potentiates the ubiquitin-ligase activity of Su(dx) upon Notch. A recent analysis instructs this model (Shimizu et al., 2014). Su(dx) has two distinct biochemical activities. The first is to promote internalization into sterol-enriched endosomes. This activity is independent of ubiquitin ligase activity, since catalytically deficient forms of Su(dx) are sufficient to drive Notch endocytosis and cause ligand-independent activation. The second activity, which is ubiquitination-dependent, promotes internalization into the MVB to degrade Notch and silence signaling. Since Abl targets a tyrosine that is required for Notch ubiquitination, it is likely that Abl loss specifically impairs MVB internalization, the ubiquitin-ligase-dependent Su(dx) function. The endocytic Su(dx) function likely remains intact. Therefore, Su(dx) overexpression enhances Notch accumulation in *abl* clones as expected. Su(dx) drives more Notch into endocytosis, however Notch is further accumulated because the MVB-internalization activity remains impaired under Abl loss.

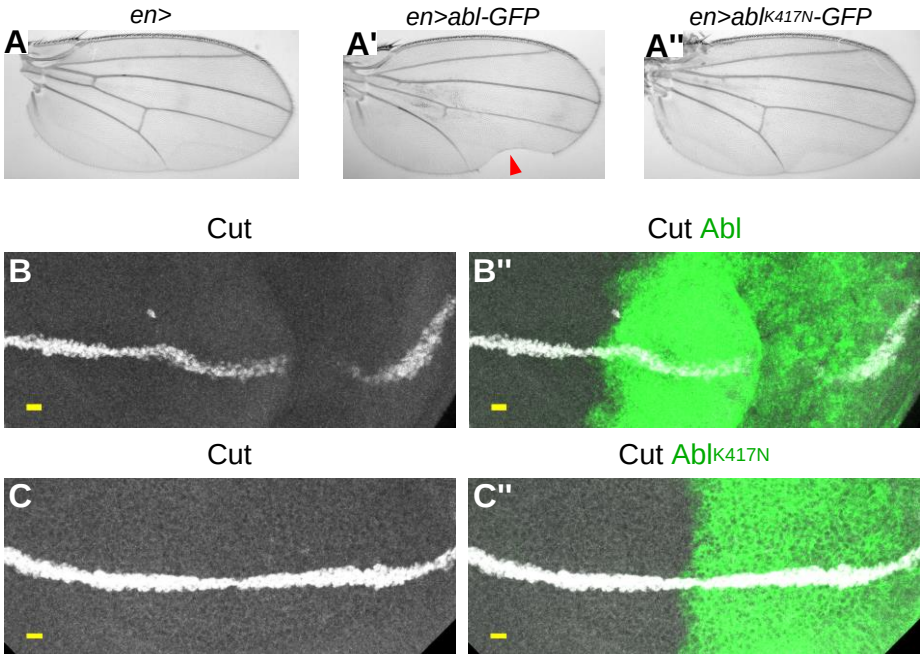


Figure 2.9 Excess Abl represses Notch activity in a kinase-dependent way
(A) Adult wings from *en>* (A′), *en>abl-GFP* (A″), and *en>abl^{K417N}-GFP* (A‴) animals, respectively. Abl overexpression causes wing notching at 34.6% penetrance (red triangle). Wing notching is completely suppressed under kinase-dead Abl overexpression. (B-C) Panels B and C show maximum projections of third instar wing imaginal discs. Scale bar = 10 µm. Cut is stained using anti-Cut antibody. GFP signal indicates the domain of Abl overexpression. (B) Abl overexpression abrogates Cut expression along the D-V boundary. (C) Kinase-dead Abl^{K417N} overexpression has no effect on Cut expression.

Why does Abl loss cause a decrease in Notch signaling? Indeed, the endocytic activity of Su(dx) was previously shown to drive increased signaling when MVB internalization is impaired (Shimizu et al., 2014). Therefore, one might predict an increase of overall signaling under Abl loss, since Su(dx)'s endocytic activity remains intact. One explanation is that Abl is also required to potentiate signaling. Therefore, under this model, Su(dx) merely sequesters Notch at the limiting membrane of the MVB in the absence of Abl, since vesicle internalization is impaired.

One mechanistic possibility is that Abl also phosphorylates the C-terminal PEST sequence to increase NICD stability, given that the PEST sequence was among the highest scoring putative Abl targets (Table 1). Interestingly, the activity of Abl in promoting Su(dx) activity apparently overrides the signal-promoting role under Abl overexpression. As a result, Abl overexpression causes a loss signaling at the D-V boundary in the wing. This suggests that Abl can hyper-phosphorylate Notch and drive enough Su(dx) activity to lower the amount of available Notch for activation.

The finding that Abl, kinase activity can regulate Notch trafficking and signaling is important for understanding the molecular mechanism of leukemias and tumors driven by Abl activity (Greuber et al., 2013). Abl acts as an oncogene in various malignancies. For example, the BCR-Abl fusion protein, a result of the Philadelphia chromosomal translocation, drives chronic myeloid leukemia (CML). However, the specific Abl kinase targets in oncogenesis remain elusive and poorly characterized. This work raises the possibility that Notch is a direct target of Abl in oncogenesis. The human Notch homolog NOTCH3 contains a PPxY domain, further enabling this possibility. A previous analysis suggests an antagonistic relationship between BCR-Abl and Notch in CML (Aljedai et al., 2015). Inhibiting BCR-Abl increases Notch signaling, and increasing Notch dosage inhibits growth in CML cell lines. Further studies will be needed to identify whether Abl directly targets Notch in human malignancies.

One open question relates to the basal requirement for Notch signaling. Why is Notch sometimes expressed in tissues lacking ligand exposure, and at undetectable levels of transcriptional signaling? Some studies have characterized non-canonical, transcription-independent roles for Notch (Andersen et al., 2012). However, it remains possible that Notch signaling functions in a basal regime, perhaps to promote cellular homeostasis. Abl may act to

stabilize a basal, ligand-independent signaling regime, in addition to the canonical signaling contexts we analyzed here. Homeostatic roles for Notch signaling, along with the mechanisms that would stabilize this low signaling regime, merit further investigation.

To summarize, we found a novel role for Abl in regulating Notch trafficking and signaling. Our results support a model in which Abl phosphorylates Notch to promote ubiquitination by Su(dx), which leads to degradation in the MVB. Abl promotes Notch activity by preventing Notch accumulation in the endocytic pathway. This work provides further understanding of how Notch signaling is actively regulated at a molecular level, with implications for both developmental regulation and human disease.

Materials and Methods

Fly genetics

All strains were obtained directly from the Bloomington stock center (BSC) or derived from BSC lines unless otherwise noted. To generate *abl*^{2} retinal clones, *abl*^{2} *FRT80B/TM6, Tb* males were crossed to *ey-Flp; ubi-GFP FRT80B* females. To generate *abl*^{2} retinal clones that overexpress Su(dx), *GMR>Su(dx)/Cyo, actin-GFP; abl*^{2} *FRT80B/TM6, Tb* males were crossed to *ey-Flp; ubi-GFP FRT80B* females. To generate *abl*^{2} retinal clones with marked Rab7, *abl*^{2} *FRT80B, rab7-myc-YFP/TM6, Tb* males were crossed to *ey-Flp; ubi-GFP FRT80B* females. To generate whole mutant *abl* retinas, *abl*^{1}*/TM6, Tb* males were crossed to *abl*^{2} *FRT80B/TM6, Tb* females. To knock down Abl in the posterior wing compartment, *engrailed>/Cyo, actin-GFP; abl-Mimic, UAS-degradFp/TM6, Tb;* males were crossed to *abl*^{2} *FRT80B/TM6, Tb* females. To overexpress Abl and Abl^{K417N} in the posterior wing compartment, *UAS-Abl-GFP or UAS-Abl*^{K417N}*-GFP* (courtesy of Greg Bashaw) males were crossed to *engrailed>* females.

Immunostaining and antibodies

Wing imaginal discs and 48 hours after puparium formation (APF) pupal retinas were dissected in S2 cell media and fixed for 10 minutes in 4% paraformaldehyde in PBS with 0.1% Triton X-100 (PFA+PBT). For 72-hour APF retinas, whole heads were fixed for 20 minutes in PFA+PBT. Then retinas were dissected and fixed for 10 minutes in PFA+PBT. After fixation, all samples were washed three times in PBT, blocked for 30 minutes in 1% normal goat serum in PBT, and incubated overnight at 4C with primary antibodies in PBT. After primary incubation, samples were washed three times in PBT, incubated with secondary antibodies in PBT for 2 hours at room temperature, washed three times in PBT, and mounted in n-propyl gallate mounting medium. Samples were imaged on Zeiss LSM 800 and 880 confocal microscopes. GFP and mCherry were imaged using the endogenous fluorescent signal. Antibodies: mouse anti-NECD (1:100, Developmental Studies Hybridoma Bank (DHSB)), mouse anti-Cut (1:100, DHSB), guinea pig anti-Eps15 (1:1000, provided by Hugo Bellen), guinea pig anti-Hrs (1:1000, Hugo Bellen), rabbit anti-Myc (1:1000, Rick Fehon).

Data analysis and quantification

NECD punctal area were quantified by measuring the maximal cross-sectional area in the X-Y plane for each punctum. The measurement was made using the magic wand tool in ImageJ with a threshold set to 40. Relative NRE intensity for each ommatidium was measured by measuring the total intensity of GFP signal in an elliptical selection encompassing the ommatidium. Values were then normalized to the average value in the control group. Penetrance of wing vein duplication phenotypes were counted manually under a light microscope.

S2 cell staining

S2 cells were transfected with 150 ng of pMT-Notch using dimethyldioctadecylammonium bromide (DDAB). They were induced with 0.7 mM $CuSO_4$ for 24 hours. Imatinib mesylate solution in water was added to 250 µM in the experimental group. Cells were settled on poly-L lysine coated slides for 1 hour. Cells were then fixed for 10 minutes in PFA+PBT. After fixation, they were washed three times in PBT, incubated for one hour at room temperature with mouse anti-NECD antibody (1:100) in PBT. After primary incubation, samples were washed three times in PBT, incubated with secondary antibodies in PBT for 2 hours at room temperature, washed three times in PBT, and mounted in n-propyl gallate mounting medium. Cells were imaged on a Zeiss LSM 880 confocal microscope.

Transcription assays

2.25 million S2 cells were transfected with 150 ng of each listed gene construct. They were also transfected with 2 ng of *NRE-luciferase* reporter (courtesy of Sarah Bray), and 40 ng of *actin-Renilla* control. Blank pMT vector was used to normalize DNA amount across transfections. Each biological replicate represents a separate transfection. Cells were induced and treated with imatanib mesylate as previously described. Cells were lysed in 100mM Potassium Phosphate, 0.5% NP-40, 1 mM DTT, pH7.8 for 1 hour on ice. Lysates were loaded in tripclicate into an Autolumat Plus LB 953 luminometer. Firefly and Renilla luciferases were activated in separate reactions using luciferase buffer (10mM Mg Acetate, 100mM Tris Acetate, 1mM EDTA, 4.5mM ATP, and 77uM D-luciferin, pH 7.8) and renilla buffer (25mM sodium pyrophosphate, 10mM Na Acetate, 15mM EDTA, 500mM Na2SO4 500mM NaCl, 4mM coelenterazine, pH 5.0), respectively. The ratio of Firefly RLU to Renilla RLU was averaged across technical replicates, and activation values were normalized to Notch activity.

Molecular cloning of pGEX-NICD, pGEX-NICD^{Y2328F}, and pMT-Notch^{Y2328F}

pMT-Notch^{Y2328F} was cloned by subcloning the XhoI-XbaI NICD fragment from pMT-Notch into pBluescript. The Y2328F point mutation was made using Quickchange PCR of this intermediate product. Then the mutated XhoI-XbaI fragment was cloned back into pMT-Notch to create pMT-Notch^{Y2328F}. Then XhoI-NotI NICD fragments from the pBluescript vectors were subcloned into SalI-NotI digested pGEX-4T-2 plasmid to create the bacterial expression constructs.

In vitro kinase assay

GST-fusion proteins were purified from BL21 *E. coli* cells. The kinase assays were performed using commercial murine c-Abl (NEB). The amounts of GST-fusion protein to add to the reaction were calibrated using western blots of the purification products with mouse anti-NICD (1:1000) antibody. The relative intensity of full-length bands was used to make equal dilutions of the GST fusion products. Approximately 1µg of GST-fusion protein, kinase buffer (50mM Tris-HCl, 10mM MgCl2, 1mM EGTA, 2mM DTT, .01% Brij 35, pH 7.5, 200 µM cold ATP), 1µl gamma-^{32}P ATP and 1 µL NEB Abl were incubated for 30 minutes at 30°C. Samples were run on a 10% polyacrylamide gel and transferred to a PVDF membrane using standard methods. The membranes were exposed on a Storm phosphoimager. Western blot using mouse anti-NICD antibody (1:1000) of the same membranes was then performed using standard methods.

Chapter 3: Discussion and future directions

Development requires robust deployment of Notch signaling to control cellular processes that include proliferation, cell fate specification, and programmed cell death. How ligand-dependent and ligand-independent Notch signaling is regulated is a long-standing question. In this dissertation, I contribute to the understanding of how Abelson kinase (Abl) regulates Notch trafficking and signaling. I used genetic, cell biological, and biochemical methods to show that Abl promotes Notch signaling in a kinase-dependent way. I showed that Abl can phosphorylate Notch *in vitro*. Furthermore, I specifically mapped some of its activity to the PPxY motif, which is critical for Suppressor of Deltex (Su(dx)) substrate recognition (Jennings et al., 2007). Mutating this tyrosine causes an increase in signaling, unlike the loss of signaling exhibited by Abl loss in general. Therefore, the results support a model in which Abl both promotes Notch internalization into the multivesicular body (MVB) by promoting Su(dx) activity and also permits signaling by an unknown mechanism. In this chapter, I highlight three open issues in Notch biology. I also highlight potential models for how Abl can promote signaling in light of these issues. First, Abl may phosphorylate other targets within Notch that promote signaling. Second, Abl may change the chemistry of endosomal compartments to promote signaling. Third, Abl may promote the requisite cholesterol-enrichment of the basal endocytic signaling network. I conclude the chapter with a broader discussion on the biological differences between ligand-dependent and ligand-independent Notch signaling, which remain elusive.

Post-translational modification of NICD

The finding that Abl can phosphorylate the Notch intracellular domain (NICD) supplements an increasing body of work describing post-translational modifications of NICD and their effect on signaling (Fortini, 2009; Lee et al., 2015). Given that Abl regulates Notch

trafficking and signaling in a kinase-dependent way, important aspects of Notch regulation will be learned by further analyzing this interaction.

A recent analysis shows that Notch is tyrosine phosphorylated in *Drosophila* embryos, and that tyrosine-phosphorylated Notch associates with Disabled and Trio, Abl kinase synergists (Kannan et al., 2018). Furthermore, three tyrosine residues in the Disabled-binding region are required for proper axon bundle formation in the embryonic central nervous system. However, details about which tyrosine kinases target the Notch intracellular domain (NICD) and how they regulate signaling remain elusive. Our *in vitro* experiments show that Abl phosphorylates other tyrosine residues in addition to Y2328, since tyrosine kinase activity is still detectable on $NICD^{Y2328F}$. Therefore, Abl may target tyrosine residues in the Disabled-binding domain within the NICD to coordinate axon bundle formation. Furthermore, transcription assay experiments suggest that Abl kinase activity is required for Notch activation, even in the $Notch^{Y2328F}$ PPxY mutant. Therefore, Abl may allow signaling via other less-understood tyrosine targets on NICD. I propose a mutational analysis of Notch to probe the function of other tyrosine residues. NICD contains 15 tyrosine residues. Therefore, one could feasibly mutate each one to phenylalanine and perform transcription assays to screen for signaling differences. Tyrosine residues that are required for basal activation would produce a loss of signaling when mutated. In addition, their mutation would be epistatic to Y2328F, since Notch would not be activated despite losing Su(dx) activity. Phosphorylation of these residues would perhaps recruit factors that promote Notch cleavage or stability of the NICD. Positive hits in the screen would be tested for Abl kinase activity *in vitro*, as was done with $NICD^{Y2328F}$.

NICD can also be modified by ubiquitination (Moretti and Brou, 2013), as exemplified by the role of Su(dx) in regulating Notch trafficking. The ubiquitin polypeptide is covalently

linked to lysine residues in the substrate. Since ubiquitin itself contains an internal lysine, polyubiquitin chains can be formed. Therefore, NICD may be ubiquitinated at multiple lysine residues, with variable chain lengths at each residue. Itch, a murine Nedd4 family ubiquitin ligase and Su(dx) homolog, drives Notch ubiquitination in cultured cells (Qiu et al., 2000). However, the specific pattern of ubiquitination is not known. To further understand specific ubiquitin states of NICD, I propose a mutational analysis to probe both the pattern of ubiquitination and the relevant lysine residues. Ubiquitination patterns can be probed using ubiquitin mutants that only contain a single exposed lysine. These mutants are restricted to monoubiquitination, since they can link with the NICD substrate but are unable to extend ubiquitin chains. If a given site is monoubiquitinated, then the signal between wildtype and chain-restricted ubiquitin will be the same. In the converse, a polyubiquitinated site loses signal when exposed to chain-restricted ubiquitin. The assay can be performed in S2 cell culture, where combinations of Notch mutants and tagged ubiquitin mutants are exposed to Su(dx) overexpression. The amount of tagged-ubiquitin incorporation on the NICD acts as a readout of Su(dx) activity. As a precursor to the screen, a traditional mutational analysis would map which lysine residues are targeted by Su(dx). Lysine to arginine mutants that cause a loss of ubiquitin incorporation in NICD identify the important site to test for mono- or polyubiquitination. The availability of modern gene fragment synthesis allows for the feasible cloning of a Notch expression construct in which all lysine residues in the NICD are mutated. Individual residues can be reverted to lysine and exposed to ubiquitin and Su(dx). Individual lysine residues can be probed for mono- or polyubiquitination by Su(dx) in this manner. Ultimately, this analysis may reveal the ubiquitin “code” that marks Notch for internalization into the MVB. The S2 cell system advantageously offers transcription assays in addition to tractable biochemistry. Mutating

critical Su(dx)-target lysine residues in Notch would predictably increase signal output from *NRE-luciferase*, similar to Notch^{Y2328F}. Ultimately, gain of function mutants can be verified *in vivo* using either CRISPR/Cas9 editing of the endogenous *Notch* gene or transgenic overexpression.

What promotes Notch activation from endosomal compartments?

How is Notch activated in endosomal compartments in the absence of ligand? S2 cleavage depends on conformational change such that the cleavage site is exposed to Kuzbanian (Hori et al., 2013). The Notch negative regulatory region (NRR) normally protects the cleavage site from Kuzbanian activity. Ligand recognition causes a conformational change that exposes the site for S2 cleavage. However, the biochemical events of ligand-independent signaling remain poorly understood. A recent review proposes three potential models of how the cleavage site is exposed: (1) lysozymes within endosomal compartments completely proteolyze the NECD (2) the chemistry within the compartment sufficiently denatures the NRR, and (3) the chemistry within the compartment disassociates the NECD heterodimer (Steinbuck and Winandy, 2018). These models are still speculative. However, there is evidence that compartment chemistry is important for Notch activation. For example, loss of the vacuolar ATPase complex, which acidifies compartments by pumping in protons, causes endosomal Notch accumulation and a loss Notch signaling (Vaccari et al., 2010). Therefore, compartment acidification is important for Notch activation.

Genetic screening can be used to identify novel factors that regulate Notch signaling from endosomal compartments. The most comprehensive genetic screen to date for Notch regulators used a combined cell culture and genetic approach (Saj et al., 2010). S2 cells were transfected with a full-length Notch fused with a VP16 activator domain. Activation of a Notch-responsive

element, *NRE-luciferase* served as a readout while a genomic RNAi library was co-transfected. RNAi hits were confirmed *in vivo* by expressing RNAi in the background of NRE reporters in the wing and eye. There are conflicting perspectives on the degree to which Notch signaling, without ligand transfection, in S2 cells is ligand-dependent. While S2 cells do not express Delta, quantitative reverse transcriptase PCR shows substantial Serrate expression in S2 cells. However, Serrate knockdown produces a negligible difference in activity, suggesting that activation is largely ligand-independent in S2 cells. While the screen identified various membrane transporters as Notch interactors, no lysozymes or proteases were identified (Saj et al., 2010). Therefore, it is more likely that compartment chemistry drives signaling within endosomal compartments, rather than intraluminal proteases. The importance of compartment chemistry for Notch signaling may have implications for non-canonical signaling roles. Speculatively, cells could use Notch to detect environmental acidity, which would perhaps trigger a ligand-independent signaling event at the cell surface. To test this, a sudden drop in media pH would predictably trigger NRE induction in S2 cells. Similarly, culturing wing discs from *NRE-GFP* animals in a lower pH media would induce increased activation of the reporter.

Given that Abl loss causes endosomal accumulation and loss of signaling, it is possible that Abl regulates the acidity of endosomal compartments. This can be tested with Lysotracker dye, which accumulates in acidic compartments (Chazotte, 2011). Mosaic mid-pupal retinas that contain abl^{2} clones can be exposed to Lysotracker, and the relative intensity of Lysotracker in mutant tissue can be compared to that of wildtype tissue. A loss of Lysotracker intensity in *abl* clones would motivate the hypothesis that Abl in part maintains compartment acidity to facilitate Notch signaling. This would reconcile the finding that the Abl-target mutant, $Notch^{Y2328F}$, exhibits increased signaling while a general loss of Abl activity causes decreased signaling.

Sterol-rich membrane trafficking and Notch signaling

The results described in Chapter 2 suggest that Abl partly promotes the ubiquitin-dependent activity of Su(dx). The ability of Su(dx) to drive Notch endocytosis, a ubiquitin-ligase independent activity, remains intact. A previous analysis showed that Su(dx) drives Notch flux into a sterol-rich compartment network marked by Glycophosphatidylinositol (GPI)-anchored proteins (Shimizu et al., 2014). This is in contrast to Deltex, which drives flux into GPI-negative compartments. Furthermore, cholesterol depletion causes a loss of Notch signaling in S2 cells, while having no effect on Deltex-induced or ligand-induced signaling. The authors conclude that Notch signaling in S2 cells acts through a cholesterol-dependent route. How cholesterol promotes Notch signaling remains unknown.

Given that Abl promotes Notch signaling, there are two possibilities for how Abl regulates cholesterol-dependent trafficking. The first is that Abl acts independently of cholesterol. Under this model, Notch would merely accumulate in GPI-rich compartments under Abl loss, since the Su(dx) activity that trafficks Notch into GPI-rich compartments remains intact. The second possibility is that Abl promotes the cholesterol enrichment of the trafficking network itself. Under this model, Notch would accumulate in GPI-negative compartments under Abl loss. In the absence of Abl, Su(dx) fails to internalize Notch into the MVB, but additionally the compartments are insufficiently cholesterol-rich to promote signaling. To test these models, GPI-GFP can be expressed in *abl*^{2} mosaic retinas. If Notch accumulates in GPI-rich compartments, then the first model is supported. Conversely, if Notch accumulates in GPI-negative compartments then the second model is permitted. To test the second model, S2 cells treated with imatinib mesylate can also be overloaded with cholesterol, which diverts Notch into GPI-rich compartments (Shimizu et al., 2014). If cholesterol overloading suppresses the loss of

basal signaling caused by imatinib mesylate, it would support the model that Abl maintains cholesterol-enrichment to allow for signaling in the basal network.

One curiosity of the connection between basal Notch trafficking and cholesterol is how it relates to systemic cholesterol at the organism level. A recent publication suggests that dietary cholesterol levels can modulate Notch signaling during tissue homeostasis in the *Drosophila* midgut (Obniski et al., 2018). Approximately 100 million Americans have elevated cholesterol. In addition, systemic cholesterol is epidemiologically and cell biologically correlated with cancer risk (Kuzu et al., 2016). Therefore, Notch signaling may partly mediate the connection between cholesterol and cancer.

A molecular memory of signaling mechanism?

Thus far, no genetic or molecular difference between ligand-dependent or ligand-independent signaling has been identified. The phenotypes produced by ligand-independent mechanisms mimic the traditionally studied ligand-sensitive phenotypes. Therefore, the prevalent view is that all NICD molecules regulate the same Notch targets, as defined by the spatiotemporal context, downstream of S3 cleavage. In other words, there is no relevant memory of the signaling mechanism that released NICD. However, this view has not been formally tested, and may prove oversimplified.

The ubiquitin tags conferred by Deltex and Su(dx) create an obvious biochemical memory of signaling route. NICD molecules derived from Deltex induction presumably carry the Deltex ubiquitination pattern. It has not been tested whether different transcriptional complexes are formed, and different targets are activated, as a result of these biochemical differences. The core NICD-Su(H)-Mastermind complex can recruit various chromatin modifying complexes, including histone acetyltransferases (Hori et al., 2013). Therefore, ligand-independently derived

NICD may form distinct complexes and activate different targets. To test this, I propose an *in vivo* experiment in which GFP (as a control), Delta, and Deltex are overexpressed in the imaginal wing disc using the UAS/Gal4 system. Both Delta and Deltex overexpression cause ectopic Notch activation in the wing. A Gal4 driver with broad wing expression, such as *apterous-gal4*, would suffice. Delta overexpression would represent a ligand-dependent response, and Deltex overexpression would represent a ligand-independent response. Genome-wide transcriptomics using high-throughput RNA sequencing of wing discs from each group could identify differential targets between the two groups. The fold change of every gene (compared to control) can be plotted along the axes of the two groups, and genes that fall away from the identity line would exhibit differential transcriptional response. In addition, the sensitive and specific NICD monoclonal antibody allows for chromatin immunoprecipitation and sequencing to identify the genomic location of NICD in the two groups of wing discs. Once again, loci are plotted along the axes of the two groups to identify loci with differential NICD enrichment. The null hypothesis of this approach, and the conventional wisdom, is that Delta and Deltex overexpression will affect the same loci, perhaps at different magnitudes given that they induce signaling with completely different mechanisms. However, a fundamental difference between ligand-dependent and ligand-independent mechanisms would produce different target loci, both in activation and NICD localization.

The finding that NICD carries a memory of ligand-dependent or ligand-independent routes would have significant implications for biology and medicine. For biology, it would add yet another layer of diversification and complexity to the Notch network. Trafficking would not only control signal magnitude, but also produce different transcriptional outputs. The relative contributions of ligand-dependent and ligand-independent signaling would allow for many more

output states than the simplistic model. For medicine, a memory of signaling route would imply that drugs produce different transcriptional effects depending on whether they target ligand-dependent or ligand-independent mechanisms. Drugs that target Deltex or Su(dx) would produce different effects than drugs that target Delta, such as ligand-specific antibodies. This would potentially allow for more targeted Notch therapies as opposed to outright inhibition of the entire pathway. The transcriptional difference between various Notch activation mechanisms demands further inquiry.

Appendix I: *abl* mutant photoreceptors enter the lamina during terminal differentiation

Results and Discussion

During development, the process of differentiation ensures that each cell type both expresses the genes necessary for its proper morphogenesis and function and represses genetic programs that govern competing fates. The traditional model of this process, proposed by Waddington (Waddington, 1957), describes a series of choice points whereby a cell's fate is increasingly restricted towards its terminal fate after each decision. This model suggests that terminal fates are permanent, and that, in modern terms, cells are epigenetically buffered against acquiring new fates. However, the ability of fully differentiated cells to become pluripotent, even without genetic manipulation, suggests that terminal cell fate is malleable and actively regulated (Hou et al., 2013). The original goal of my dissertation research was to address how photoreceptor cell fate is stabilized following specification and the extent to which cells can deviate from this fate when maintenance is abrogated.

The Rebay lab had previously established that the Abelson kinase (Abl) is required to stabilize photoreceptor terminal differentiation, but the cellular details of this phenomenon were not known. Briefly, *abl* mutant photoreceptors initially express neuronal and photoreceptor markers, suggesting that Abl is not required for the specification of photoreceptor fate. Mutant tissue then progressively loses expression of the neuronal fate marker Elav and the photoreceptor marker Chaoptin (Xiong et al., 2013). An increase in the number of Sine oculis positive pigment cells was noted at later pupal stages, leading to speculation of possible transdifferentiation (Xiong et al., 2013). My project sought to formally describe what happens to *abl* mutant photoreceptors.

Various models can account for the loss of neuronal marker expression in *abl* retinas. Experimental findings ruled out several possibilities. Mutant photoreceptors do not show

elevated markers of cell death, or irregular nuclear morphology, ruling out a cell death model (Xiong et al., 2013). Similarly, retinal precursor markers are not elevated, arguing against reversion to precursor fate (Xiong et al., 2013). I did, however, find that the interommatidial pigment cell (IOP) population, marked by the IOP-specific *LL54-Gal4* driver, is expanded in *abl* mutant clones at a late pupal stage in which neuronal marker expression is largely absent (Figure AI.1). Mutant ommatidia contain approximately 5 more IOPs per ommatidium. This concomitant expansion of pigment cells and loss of photoreceptors supported a trans-differentiation model, in which *abl* photoreceptors trans-differentiate into IOPs. To formally address the trans-differentiation model, I used a lineage trace system to track the *abl* mutant photoreceptor population after neuronal marker expression is lost.

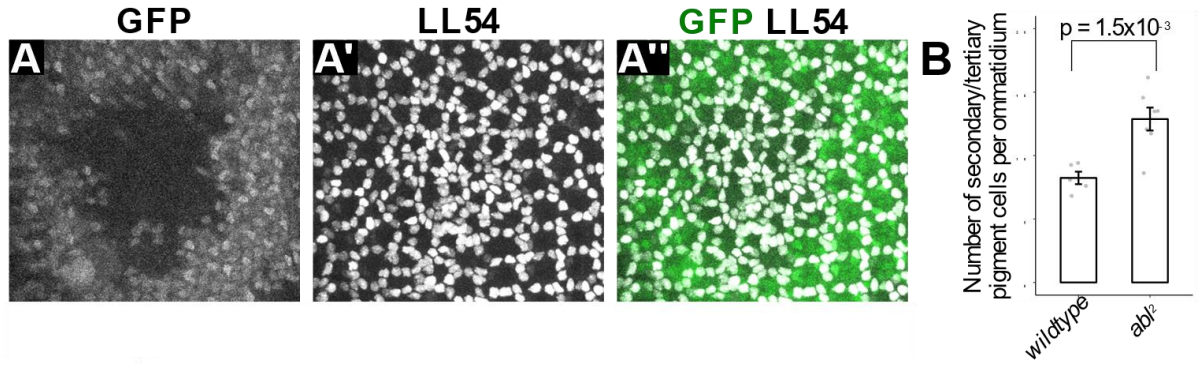


Figure AI.1 IOP cell number is increased in *abl* clones
(A) Panel A depicts a maximal projection from a 72h APF retina containing abl^2 clones. The number of IOP nuclei, marked by *LL54>mCherry* (A'), is increased within *abl* clones. Lack of GFP marks the *abl* clone. (B) Quantification of IOP cell number per ommatidium. Each gray circle represents a clone for which the number of IOP cells is divided by the number of ommatidia by area. Loss of Abl causes an increase of approximately 5 cells per ommatidium. T-test p-value indicated.

To trace the fate of *abl* photoreceptors, I combined the photoreceptor-specific *chaoptin (chp)-gal4* with the G-TRACE lineage trace system (GTRACE). The G-TRACE system relies on the FLP-mediated excision of a STOP codon to permanently drive nuclear GFP in GAL4-expressing cells (Evans et al., 2009). The G-TRACE cassette also contains a UAS-RFP

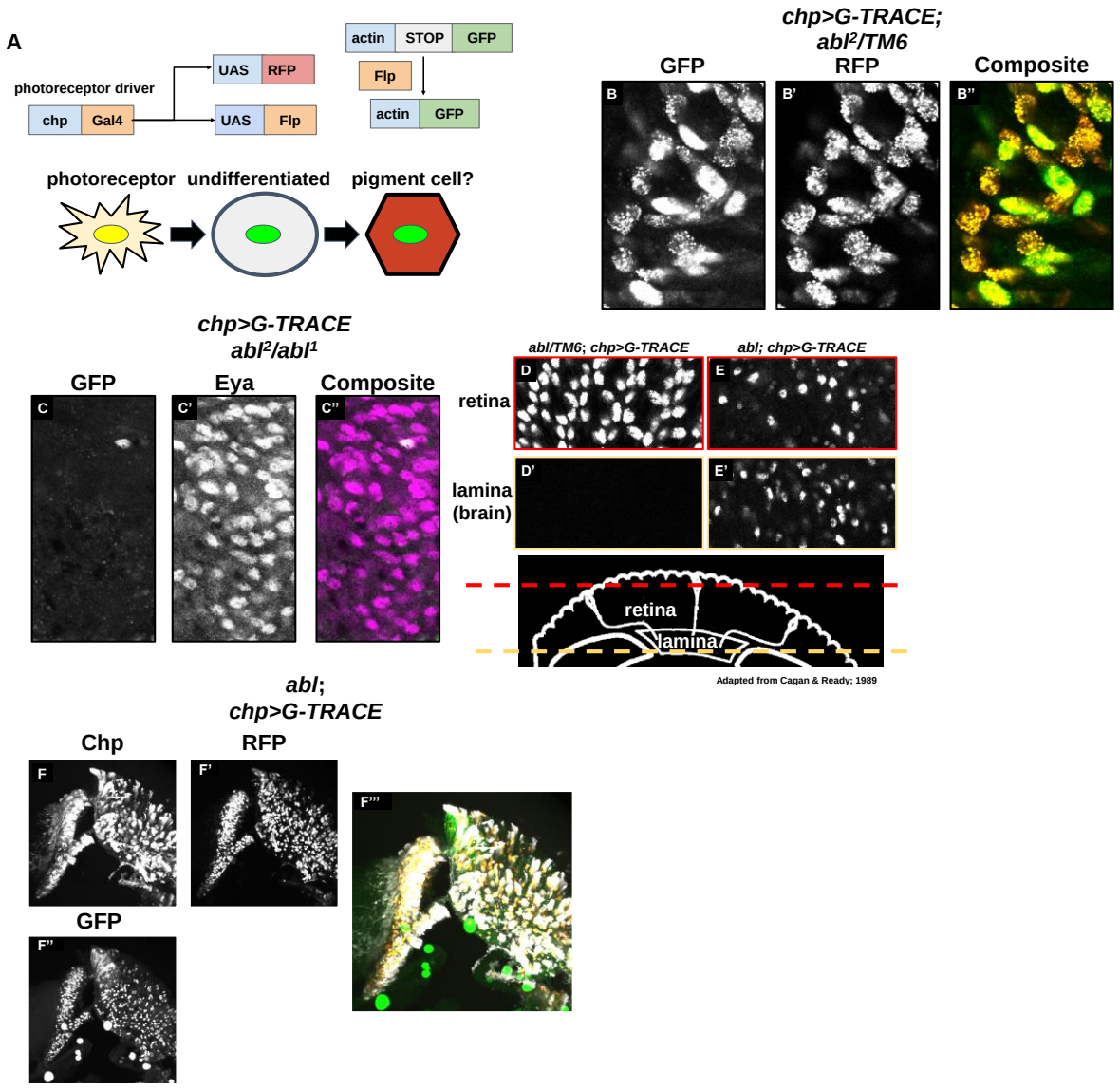


Figure AI.2 *abl* mutant photoreceptors enter the lamina during terminal differentiation
(A) The genetic lineage trace strategy to indelibly mark photoreceptors with GFP expression. (B-B'') A single confocal slice from an adult retina showing a test of the *chp>G-TRACE* system. GFP and RFP expression are activated in photoreceptors. (C-C'') A single confocal slice from an adult *abl* mutant retina with *chp>G-TRACE*. The expanded pigment cell population, marked by nuclear Eya expression, does not express GFP. (D -D') Single confocal slices show that lineage-traced photoreceptors remain the retina in control animals. (E-E') *abl* mutant, lineage-traced photoreceptors enter the lamina of the brain. (F-F''') Maximum projection of an adult *abl* mutant retina with *chp>G-TRACE*. The lamina brain layer is also visible on the left. Mutant photoreceptors express RFP while present in the lamina.

transgene, which marks current expression of *chp* (Figure AI.2 A). When combined with *chp-*

GAL4, G-TRACE predictably activates GFP and RFP expression in photoreceptors in control retinas (Figure AI.2 B). In abl^{1}/abl^{2} retinas, the expanded population of IOPs, marked by Eyes absent, does not express GFP (Figure AI.2 C). This result argues strongly against the original trans-differentiation model.

However, over the course of these experiments, I made the surprising observation that the *abl* mutant photoreceptor lineage is recovered in the lamina, the brain layer most proximal to the retina (Figure AI.2 E). Cells in the lamina express both RFP and GFP, suggesting that chaoptin expression, and thus photoreceptor fate, is maintained despite the cells occupying an aberrant niche (Figure AI.2 F).

Whether localization to the lamina reflects an active cellular migration or a more passive, structural abnormality of the retina remains an open question that is being investigated by another student in the lab, Xiao Sun. It should be noted that the marked nuclei appear very distinctly packed in the lamina (Figure AI.2 F), and are not seen in the adjacent medulla of the optic lobe. The distinct localization perhaps favors a migratory model, in which groups of photoreceptor cells maintain some of the cell-cell connections established in the retina as they invade the lamina. Alternatively, a structural or anatomical aberration of the retinal epithelium, the basement membrane that separates it from the lamina, or of the lamina itself might permit collapse of certain retinal cells into the lamina. Therefore, passive structural models are still viable, although less likely.

Materials and Methods

Fly genetics

All strains were obtained directly from the Bloomington stock center (BSC) or derived from BSC lines unless otherwise noted. To generate *abl*^{2} retinal clones, *abl*^{2} *FRT80B/TM6, Tb* males were crossed to *ey-Flp; ubi-GFP FRT80B* females. To generate *abl*^{2} retinal clones with marked IOPs, *LL54>mCherry; abl*^{2} *FRT80B/TM6, Tb* males were crossed to *ey-Flp; ubi-GFP FRT80B* females. To generate *abl* mutant retinas with *chp>G-TRACE, chaoptin-gal4, abl*^{2}*/TM6B* females were crossed to *G-TRACE; abl*^{1}*/TM6B* males.

Immunostaining and antibodies

Retinas were dissected in S2 cell media and fixed for 10 minutes in 4% paraformaldehyde in PBS with 0.1% Triton X-100 (PFA+PBT). For 72-hour APF and adult retinas, whole heads were fixed for 20 minutes in PFA+PBT. Then retinas were dissected and fixed for 10 minutes in PFA+PBT. After fixation, all samples were washed three times in PBT, blocked for 30 minutes in 1% normal goat serum in PBT, and incubated overnight at 4C with primary antibodies in PBT. After primary incubation, samples were washed three times in PBT, incubated with secondary antibodies in PBT for 2 hours at room temperature, washed three times in PBT, and mounted in n-propyl gallate mounting medium. Samples were imaged on Zeiss LSM 800 and 880 confocal microscopes. GFP, RFP, and mCherry were imaged using the endogenous fluorescent signal. Antibodies: mouse anti-Elav (1:50, Developmental Studies Hybridoma Bank (DHSB)), and mouse anti-Chaoptin (1:20, DHSB).

Data analysis and quantification

IOP cell numbers were quantified by first measuring the area of an ommatidia in the given confocal field. Clonal areas were then manually traced in ImageJ, and the number of LL54+ nuclei within the clone were counted using the point selection tool. Each clone

quantification produced a number of LL54+ nuclei normalized to the ommatidial area to produce a per-ommatidium count.

Appendix II: Genetic interaction between Abl and EGF repressor Argos

Results and Discussion

Given that Abl loss causes Notch accumulation (Chapter 2) and *Notch* dominantly suppresses the loss of photoreceptors from the retina in *abl* mutants (Xiong et al., 2013), I hypothesized that an increase of Notch signaling under Abl loss destabilizes photoreceptor differentiation. I therefore predicted that EGF, a known Notch antagonist during retinal cell specification (Freeman, 1996; Doroquez and Rebay, 2006), acts antagonistically to Notch in stabilizing photoreceptor terminal differentiation.

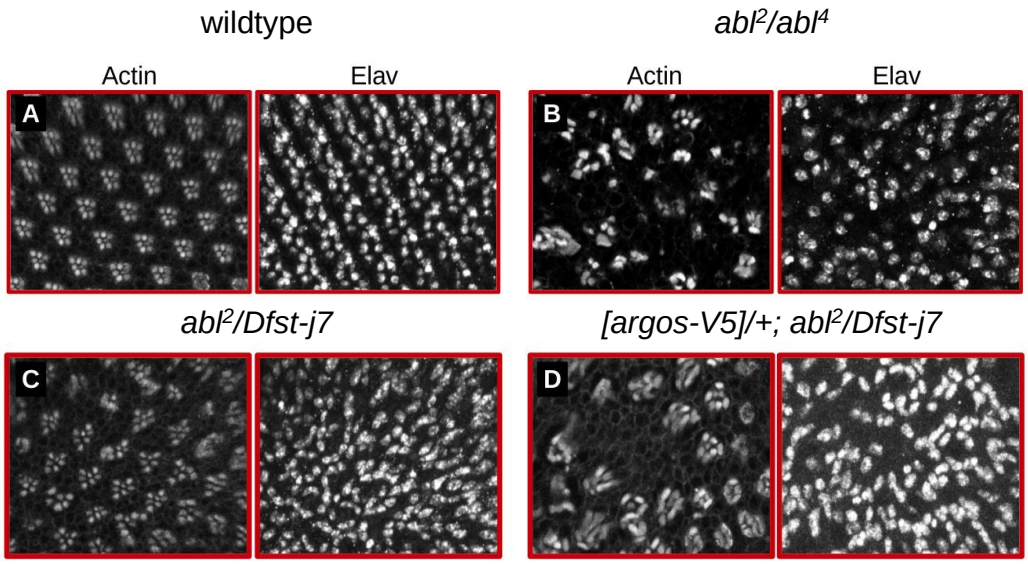


Figure AII.1 Loss of EGF inhibitor Argos dominantly suppresses the loss of photoreceptor fate in adult *abl* mutant retinas.
For each genotype, Actin marks rhabdomeres, and Elav marks photoreceptor nuclei. (A) Control adult eye has normal number of rhabdomeres and photoreceptor nuclei. (B) abl^2/abl^4 eye has severe loss of rhabdomeres and photoreceptor fate. (C) $abl^2/Dfst\text{-}j7$ eye has a suppressed phenotype. *Dfst-j7* is missing both *argos* and *abl*. (D) $[argos+]/+;\ abl^2/Dfst\text{-}j7$ enhances towards the *abl* mutant phenotype. Genetic rescue therefore identifies *argos* as the suppressor within *Dfst-j7*.

A preliminary analysis (Figure AII.1) suggested that EGF may indeed stabilize photoreceptor terminal differentiation. abl^2/abl^4 retinas show a severe loss of photoreceptors by adulthood (Figure AII.1 A, B). I used Elav to mark photoreceptor nuclei and phalloidin to mark

the actin-rich rhabdomeres at the center of each ommatidium. This phenotype is significantly suppressed in retinas that are also heterozygous for *argos* (*aos*), a potent inhibitor of EGF signaling (Schweitzer et al., 1995) (Figure AII.1 C). Because *aos* and *abl* are closely linked, I performed the experiment using abl^2 in trans to a small deficiency that lacks both genes, *Df(3L)st-j7* (Henkemeyer et al., 1987). To verify that *argos* is the key suppressor in this deficiency, I rescued the genetic dosage of *argos* using a functional genomic rescue transgene (Jemma Webber, unpublished), and found enhancement towards the abl^2/abl^4 phenotype (Figure AII.1 D). This result suggests that increasing EGF signaling by attenuating its repressor dosage stabilizes photoreceptor terminal differentiation in *abl* retinas.

Materials and Methods

Fly genetics

All strains were obtained directly from the Bloomington stock center (BSC) or derived from BSC lines unless otherwise noted. To generate *abl* mutant retinas, abl^2*/TM6B* females were crossed to abl^4*/TM6B or Dfst-j7/TM6B* males. To rescue *argos*, *[argos-V5];* abl^2*/TM6B* females were crossed to *Dfst-j7/TM6B* males.

Immunostaining and antibodies

Retinas were dissected in S2 cell media and fixed for 10 minutes in 4% paraformaldehyde in PBS with 0.1% Triton X-100 (PFA+PBT). Whole heads were fixed for 20 minutes in PFA+PBT. Then retinas were dissected and fixed for 10 minutes in PFA+PBT. After fixation, all samples were washed three times in PBT, blocked for 30 minutes in 1% normal goat serum in PBT, and incubated overnight at 4C with primary antibodies in PBT. After primary incubation, samples were washed three times in PBT, incubated with secondary antibodies and

phalloidin (1:1000) in PBT for 2 hours at room temperature, washed three times in PBT, and mounted in n-propyl gallate mounting medium. Samples were imaged on Zeiss LSM 800 and 880 confocal microscopes. GFP, RFP, and mCherry were imaged using the endogenous fluorescent signal. Antibody: mouse anti-Elav (1:50, Developmental Studies Hybridoma Bank (DHSB)).

Appendix III: Implementation of image segmentation and developmental time conversion

Three Python scripts, “segmentation.py”, “feeder.py”, and “time_converter.py” are attached to this dissertation. Details of how to run the files and relevant command line options are described in comments within the scripts. These scripts are useful for image segmentation and converting between spatial coordinates and developmental time in the 3rd instar *Drosophila* eye disc.

The first script, “segmentation.py”, implements segmentation of objects within microscopic images stored in the LSM format. The algorithm for segmentation is summarized as follows:

1. For each slice in the LSM file:
    1.1 Segment the “red” channel (Channel 1) using the following algorithm:
        1.1.1 Delineate foreground vs background using an intensity threshold (the default is the Otsu threshold (Otsu, 1979) if the threshold parameter is not provided).
        1.1.2 Convert the foreground mask into a distance map of the distance between a given pixel to the nearest background pixel.
        1.1.3 Apply a watershed algorithm (Vincent and Soille, 1991) to the distance map using local maxima of the distance map as the initial watershed “markers”. The minimum distance between markers is set to an arbitrary default of 4 pixels if the minimum distance parameter is not provided.
    1.2 The resulting segments are then outputted both in image and text format. Images are outputted such that every segment is given a random color, which facilitates visual analysis. The text is outputted in JSON format and contains summary statistics for each segment, including, for example, the average pixel intensity of each channel within that segment. This JSON format is compatible for import into

the *FlyEye Silhouette* software package (Pelaez et al., 2015; Bernasek et al., 2018), which allows manual labeling of segments.

The next script, “feeder.py”, is merely a Unix-style filter program that converts the JSON output from “segmentation.py” into a tab-delimited columnar format.

Finally, the “time_converter.py” script imports the columnar segment description from “feeder.py” and appends a new column representing developmental time in hours, assuming that the leftmost (lowest x-coordinate) segment labeled “r8” represents the anterior-most R8 nucleus in a 3rd instar imaginal eye disc. Pixel coordinates are mapped to developmental time according to previously described methods (Pelaez et al., 2015).

Appendix IV: CRISPR/Cas9 strategy for creating an endogenous *Notch*^{Y2328F} allele

Given that *Notch*^{Y2328F} confers increased Notch signaling in a cell culture assay (Chapter 2), we sought to generate an endogenous *Drosophila NotchY2328F* allele for *in vivo* analysis. I used an established CRISPR/Cas9 strategy (Bier et al., 2018):

1. Inserted oligos into the pU6-gRNA vector that correspond to the following CRISPR site at the *Notch* locus near the desired mutation: CCGAACACGGGCGCCAAGCAGCC
2. Created the PPSF-rescue construct using a standard Gibson cloning strategy. A report from the NEBuilder program detailing the construct sequence, primers used, and Gibson assembly protocol is show in Figure AIV.2. The rescue construct includes sequences from the *Notch* locus that were subcloned into the pHD-Scarless repair vector. The construct contains the desired point mutation along with three synonymous mutations within the CRISPR target site to prevent retargeting.
3. The resulting repair inserts a *dsRed* gene, which is useful for screening, into an intron near the desired mutation. The *dsRed* can be excised with expression of PiggyBac transposase.

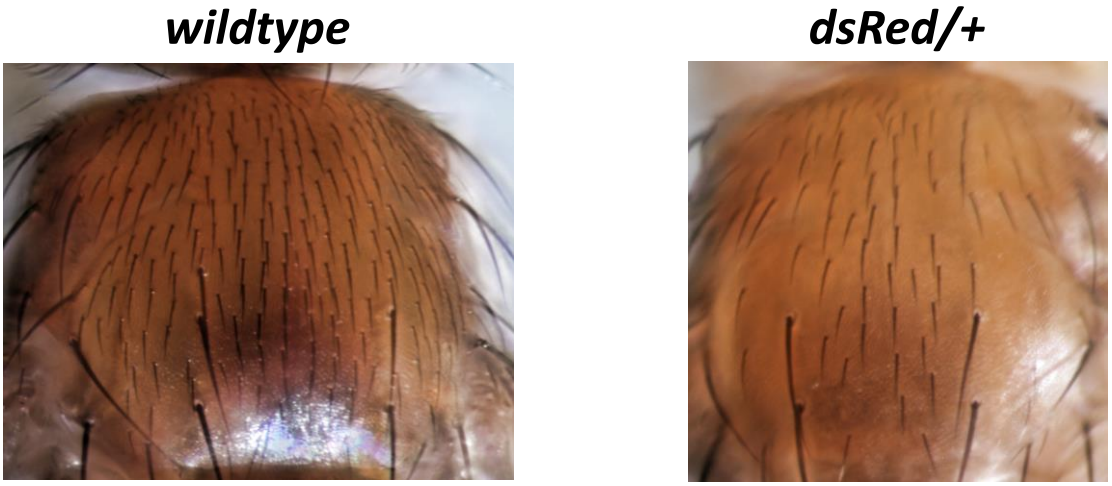


Figure AIV.1 The putative *Notch*^{Y2328F} mutants (*dsRed*+) exhibit a decrease in small thoracic bristle density

A 50:50 mix of pU6-gRNA and PPSF-rescue was injected into embryos from the *vasa-Cas9* line. A single male from approximately 50 fertile crosses produced dsRed+ females. These heterozygous females were infertile, but exhibited a decreased density of small bristles on the thorax (Figure AIV.1) reminiscent of Notch gain of function phenotypes (Rebay et al., 1993; Maier et al., 2013).

Figure AIV.2 Notch^{Y2328F} CRISPR cloning strategy

NEBuilder Assembly Tool v1.12.17

Timestamp: 28/1/2018 @ 19:21

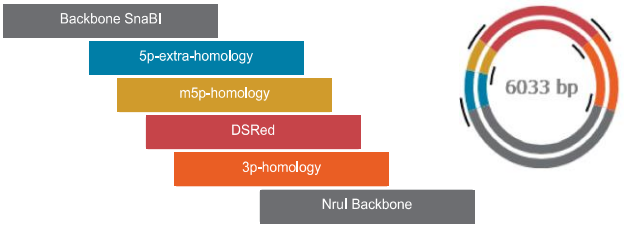
User Selected Settings

Product Version	E5520 - NEBuilder High-Fidelity DNA Assembly Cloning Kit
No. of Fragments	2-3 fragments (including vector)
Construct Length	less than 10 Kb
Min. Overlap	25 bp
Allow linear assemblies	No
PCR Polymerase	Generic
PCR Primer Conc.	200 nM
Min. Primer Length	18 nt

Fragment Arrangement

Vector Digestion

Vector backbone digested with SnaBI and NruI



Required Primers

Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta *	6-Frame
Backbone	agtatttattatgcatttagaatacTGGGCAGCGGCGGTTAAC	5p-extra-homology	Fwd	63.3°C	**61.7°C**	view
m5p-homology	cttgtctttttttgaacttgccttgcGTCGCAAACTGCCCGTGG	5p-extra-homology	Rev	61.7°C	**61.7°C**	view
m5p-homology	gtggaagtgggattgggtggttcacTTAACCCTAGAAAGATAATCATATTGTGACGTAC	DSRed	Fwd	55.5°C	**55.5°C**	view
3p-homology	taatatgagtgcaTTAACCCTAGAAAGATAGTCTGCGTAAAATTG	DSRed	Rev	56.2°C	**55.5°C**	view
DSRed	tttctagggttaaTGCACTCATATTACAATTGCAGAATGCGC	3p-homology	Fwd	60.1°C	**60.1°C**	view
Backbone	aattaaccaattctgaacattatcgCGGCGATGAGCCGGACATTG	3p-homology	Rev	63.2°C	**60.1°C**	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

Figure AIV.2, continued:

Figure AIV.2, continued:

AACAGCAGCAGCAGCAACAGCAACAGCAGCAACATCAGCAGCAGCAACAGCAGCAGCAA
CAGCAGCAGCAGCAACAGCAGCAGCAACTCGGAGGCCTGGAGTTCGGTTCAGCGGGCTTGGA
CCTGAATGGATTTTGTGGATCTCCGGGTAAGTGGTCACTCATGATGGACTCTATGGACTAGC
TAACTAGCTAACTAATCATTCTACCATCCCAACTTGCAGACTCATTTCACTCGGGTCAAATG
AATCCGCCCTCGATACAAAGTTCAATGTCCGGCTCATCGCCGCGATAATGTTCAGAATTGGT
TAATTGGTTGTAACACTGACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCC
GCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAATATGAGTA
TTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCT
CACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTA
CATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTC
CAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGG
CAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGT
CACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCA
TGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACC
GCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAA
TGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCGATGGCAACAACGTTGC
GCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATG
GAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGC
TGATAAATCCGGAGCCGGTGAGCGTGGTTCTCGCGGTATCATCGCAGCGCTGGGGCCAGATG
GTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGA
AATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAAAAGCAGAGCATTAC
GCTGACTTGACGGGACGGCGCAAGCTCATGACCAAAATCCCTTAACGTGAGTTACGCGCGCG
TCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTT
TCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGC
CGGATCAAGAGCTACCAAC

HiFi DNA Assembly® Protocol

Optimal Quantities

NEB recommends a total of 0.03–0.2 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector and 0.2–0.5 pmoles of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols.

50 ng of 500 bp dsDNA is about 0.15 pmols.

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly*	4-6 Fragment Assembly**	Positive Control†
Recommended DNA Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmols* X µl	0.2–0.5 pmols* X µl	10 µl
Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

* Optimized cloning efficiency is 50–100 ng of vectors with 2 fold excess of inserts. Use 5 times more inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.
** To achieve optimal assembly efficiency, it is recommended to design ≥ 20 bp overlap regions between each fragment with equimolarity (suggested: 0.05 pmol each).
† Control reagents are provided for 5 experiments.
‡ If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional Assembly Master Mix.

References

Adams, F.H., and Oliver, C.P. (1945). Hereditary deformities in man: due to arrested development. J. Hered. *36*, 3–7.

Aljedai, A., Buckle, A.-M., Hiwarkar, P., and Syed, F. (2015). Potential role of Notch signalling in CD34+ chronic myeloid leukaemia cells: cross-talk between Notch and BCR-ABL. PloS One *10*, e0123016.

Andersen, P., Uosaki, H., Shenje, L.T., and Kwon, C. (2012). Non-canonical Notch signaling: emerging role and mechanism. Trends Cell Biol. *22*, 257–265.

Arikawa, K., Hicks, J.L., and Williams, D.S. (1990). Identification of actin filaments in the rhabdomeral microvilli of Drosophila photoreceptors. J. Cell Biol. *110*, 1993–1998.

Artavanis-Tsakonas, S., and Simpson, P. (1991). Choosing a cell fate: a view from the Notch locus. Trends Genet. *7*, 403–408.

Aster, J.C., Pear, W.S., and Blacklow, S.C. (2017). The Varied Roles of Notch in Cancer. Annu. Rev. Pathol. *12*, 245–275.

Bailey, A.M., and Posakony, J.W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. Genes Dev. *9*, 2609–2622.

Baker, N.E., Yu, S., and Han, D. (1996). Evolution of proneural atonal expression during distinct regulatory phases in the developing Drosophila eye. Curr. Biol. *6*, 1290–1302.

Bao, S. (2014). Notch Controls Cell Adhesion in the Drosophila Eye. PLOS Genet. *10*, e1004087.

Barolo, S., Stone, T., Bang, A.G., and Posakony, J.W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. Genes Dev. *16*, 1964–1976.

Benmerah, A., Lamaze, C., Bègue, B., Schmid, S.L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998). AP-2/Eps15 Interaction Is Required for Receptor-mediated Endocytosis. J. Cell Biol. *140*, 1055–1062.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. J. Cell Sci. *112*, 1303–1311.

Bernasek, S.M., Lachance, J.-F.B., Peláez, N., Bakker, R., Navarro, H.T., Amaral, L.A.N., Bagheri, N., Rebay, I., and Carthew, R.W. (2018). Ratio-based sensing of two transcription factors regulates the transit to differentiation. BioRxiv 430744.

Bier, E., Harrison, M.M., O'Connor-Giles, K.M., and Wildonger, J. (2018). Advances in Engineering the Fly Genome with the CRISPR-Cas System. Genetics *208*, 1–18.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. *7*, 678–689.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R.A., and Israël, A. (2000). A Novel Proteolytic Cleavage Involved in Notch Signaling: The Role of the Disintegrin-Metalloprotease TACE. Mol. Cell *5*, 207–216.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., van Deurs, B., and Pfeffer, S.R. (2000). Rab7: A Key to Lysosome Biogenesis. Mol. Biol. Cell *11*, 467–480.

Cabrera, C.V. (1990). Lateral inhibition and cell fate during neurogenesis in Drosophila: the interactions between scute, Notch and Delta. Development *109*, 733–742.

Cagan, R.L., and Ready, D.F. (1989). Notch is required for successive cell decisions in the developing Drosophila retina. Genes Dev. *3*, 1099–1112.

Carthew, R.W. (2007). Pattern formation in the Drosophila eye. Curr. Opin. Genet. Dev. *17*, 309–313.

Caussinus, E., and Affolter, M. (2016). deGradFP: A System to Knockdown GFP-Tagged Proteins. Methods Mol. Biol. Clifton NJ *1478*, 177–187.

Celis, J.F. de (2003). Pattern formation in the Drosophila wing: The development of the veins. BioEssays *25*, 443–451.

Celis, J.F. de, Bray, S., and Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing. Development *124*, 1919–1928.

de Celis, J.F., Garcia-Bellido, A., and Bray, S.J. (1996). Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. Dev. Camb. Engl. *122*, 359–369.

Chazotte, B. (2011). Labeling lysosomes in live cells with LysoTracker. Cold Spring Harb. Protoc. *2011*, pdb–prot5571.

Chillakuri, C.R., Sheppard, D., Ilagan, M.X.G., Holt, L.R., Abbott, F., Liang, S., Kopan, R., Handford, P.A., and Lea, S.M. (2013). Structural analysis uncovers lipid-binding properties of Notch ligands. Cell Rep. *5*, 861–867.

Colicelli, J. (2010). ABL tyrosine kinases: evolution of function, regulation, and specificity. Sci. Signal. *3*, re6.

Comer, A.R., Ahern-Djamali, S.M., Juang, J.-L., Jackson, P.D., and Hoffmann, F.M. (1998). Phosphorylation of Enabled by the DrosophilaAbelson Tyrosine Kinase Regulates the In Vivo Function and Protein-Protein Interactions of Enabled. Mol. Cell. Biol. *18*, 152–160.

de-la-Concha, A., Dietrich, U., Weigel, D., and Campos-Ortega, J.A. (1988). Functional Interactions of Neurogenic Genes of Drosophila Melanogaster. Genetics *118*, 499–508.

Cornell, M., Evans, D. a. P., Mann, R., Fostier, M., Flasza, M., Monthatong, M., Artavanis-Tsakonas, S., and Baron, M. (1999). The Drosophila melanogaster Suppressor of deltex Gene, a Regulator of the Notch Receptor Signaling Pathway, Is an E3 Class Ubiquitin Ligase. Genetics *152*, 567–576.

Coumailleau, F., Fürthauer, M., Knoblich, J.A., and González-Gaitán, M. (2009). Directional Delta and Notch trafficking in Sara endosomes during asymmetric cell division. Nature *458*, 1051–1055.

Delidakis, C., Preiss, A., Hartley, D.A., and Artavanis-Tsakonas, S. (1991). Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of Drosophila melanogaster. Genetics *129*, 803–823.

Dexter, J.S. (1914). The Analysis of a Case of Continuous Variation in Drosophila by a Study of Its Linkage Relations. Am. Nat. *48*, 712–758.

Diederich, R.J., Matsuno, K., Hing, H., and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development *120*, 473–481.

Doroquez, D.B., and Rebay, I. (2006). Signal Integration During Development: Mechanisms of EGFR and Notch Pathway Function and Cross-Talk. Crit. Rev. Biochem. Mol. Biol. *41*, 339–385.

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D., and Sklar, J. (1991). TAN-1, the human homolog of the Drosophila Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell *66*, 649–661.

Evans, C.J., Olson, J.M., Ngo, K.T., Kim, E., Lee, N.E., Kuoy, E., Patananan, A.N., Sitz, D., Tran, P., Do, M.-T., et al. (2009). G-TRACE: rapid Gal4-based cell lineage analysis in *Drosophila*. Nat. Methods *6*, 603–605.

Fazioli, F., Minichiello, L., Matoskova, B., Wong, W.T., and Di Fiore, P.P. (1993). eps15, a novel tyrosine kinase substrate, exhibits transforming activity. Mol. Cell. Biol. *13*, 5814–5828.

Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A., and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. Cell *61*, 523–534.

Fehon, R.G., Johansen, K., Rebay, I., and Artavanis-Tsakonas, S. (1991). Complex cellular and subcellular regulation of notch expression during embryonic and imaginal development of Drosophila: implications for notch function. J. Cell Biol. *113*, 657–669.

Fleming, R.J. (1998). Structural conservation of Notch receptors and ligands. Semin. Cell Dev. Biol. *9*, 599–607.

Fortini, M.E. (2009). Notch signaling: the core pathway and its posttranslational regulation. Dev. Cell *16*, 633–647.

Fortini, M.E., Rebay, I., Caron, L.A., and Artavanis-Tsakonas, S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. Nature *365*, 555–557.

Fostier, M., Evans, D.A.P., Artavanis-Tsakonas, S., and Baron, M. (1998). Genetic Characterization of the Drosophila melanogaster Suppressor of deltex Gene: A Regulator of Notch Signaling. Genetics *150*, 1477–1485.

Freeman, M. (1996). Reiterative Use of the EGF Receptor Triggers Differentiation of All Cell Types in the Drosophila Eye. Cell *87*, 651–660.

Furie, B., and Furie, B.C. (1988). The molecular basis of blood coagulation. Cell *53*, 505–518.

Fuwa, T.J., Hori, K., Sasamura, T., Higgs, J., Baron, M., and Matsuno, K. (2006). The first deltex null mutant indicates tissue-specific deltex-dependent Notch signaling in Drosophila. Mol. Genet. Genomics MGG *275*, 251–263.

Garcia-Bellido, A., and Merriam, J.R. (1971). Parameters of the wing imaginal disc development ofDrosophila melanogaster. Dev. Biol. *24*, 61–87.

Garg, V., Muth, A.N., Ransom, J.F., Schluterman, M.K., Barnes, R., King, I.N., Grossfeld, P.D., and Srivastava, D. (2005). Mutations in *NOTCH1* cause aortic valve disease. Nature *437*, 270–274.

Gause, M., Eissenberg, J.C., Macrae, A.F., Dorsett, M., Misulovin, Z., and Dorsett, D. (2006). Nipped-A, the Tra1/TRRAP subunit of the Drosophila SAGA and Tip60 complexes, has multiple roles in Notch signaling during wing development. Mol. Cell. Biol. *26*, 2347–2359.

Gertler, F.B., Bennett, R.L., Clark, M.J., and Hoffmann, F.M. (1989). Drosophila abl tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with disabled. Cell *58*, 103–113.

Gertler, F.B., Comer, A.R., Juang, J.L., Ahern, S.M., Clark, M.J., Liebl, E.C., and Hoffmann, F.M. (1995). enabled, a dosage-sensitive suppressor of mutations in the Drosophila Abl tyrosine kinase, encodes an Abl substrate with SH3 domain-binding properties. Genes Dev. *9*, 521–533.

Gilbert, S.F. (2013). Developmental Biology, Tenth Edition (Sunderland, MA, USA: Sinauer Associates, Inc.).

Giniger, E. (1998). A role for Abl in Notch signaling. Neuron *20*, 667–681.

Giniger, E., Jan, L.Y., and Jan, Y.N. (1993). Specifying the path of the intersegmental nerve of the Drosophila embryo: a role for Delta and Notch. Development *117*, 431–440.

Gordon, W.R., Vardar-Ulu, D., Histen, G., Sanchez-Irizarry, C., Aster, J.C., and Blacklow, S.C. (2007). Structural basis for autoinhibition of Notch. Nat. Struct. Mol. Biol. *14*, 295–300.

Gordon, W.R., Arnett, K.L., and Blacklow, S.C. (2008). The molecular logic of Notch signaling – a structural and biochemical perspective. J. Cell Sci. *121*, 3109–3119.

Gorman, M.J., and Girton, J.R. (1992). A genetic analysis of deltex and its interaction with the Notch locus in Drosophila melanogaster. Genetics *131*, 99–112.

Greenwald, I. (2012). Notch and the Awesome Power of Genetics. Genetics *191*, 655–669.

Greenwald, I.S., Sternberg, P.W., and Robert Horvitz, H. (1983). The lin-12 locus specifies cell fates in caenorhabditis elegans. Cell *34*, 435–444.

Greuber, E.K., Smith-Pearson, P., Wang, J., and Pendergast, A.M. (2013). Role of ABL Family Kinases in Cancer: from Leukemia to Solid Tumors. Nat. Rev. Cancer *13*, 559–571.

Grevengoed, E.E., Loureiro, J.J., Jesse, T.L., and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in Drosophila. J. Cell Biol. *155*, 1185–1198.

Grevengoed, E.E., Fox, D.T., Gates, J., and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. J. Cell Biol. *163*, 1267–1279.

Gruenberg, J., and Stenmark, H. (2004). The biogenesis of multivesicular endosomes. Nat. Rev. Mol. Cell Biol. *5*, 317–323.

Haigler, H.T., McKanna, J.A., and Cohen, S. (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. J. Cell Biol. *81*, 382–395.

Hantschel, O., Nagar, B., Guettler, S., Kretzschmar, J., Dorey, K., Kuriyan, J., and Superti-Furga, G. (2003). A myristoyl/phosphotyrosine switch regulates c-Abl. Cell *112*, 845–857.

Hartenstein, V., and Posakony, J.W. (1989). Development of adult sensilla on the wing and notum of Drosophila melanogaster. Development *107*, 389–405.

Haynie, J.L., and Bryant, P.J. (1986). Development of the eye-antenna imaginal disc and morphogenesis of the adult head in Drosophila melanogaster. J. Exp. Zool. *237*, 293–308.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. Cell *64*, 1083–1092.

Henkemeyer, M., West, S.R., Gertler, F.B., and Hoffmann, F.M. (1990). A novel tyrosine kinase-independent function of Drosophila abl correlates with proper subcellular localization. Cell *63*, 949–960.

Henkemeyer, M.J., Gertler, F.B., Goodman, W., and Hoffmann, F.M. (1987). The Drosophila abelson proto-oncogene homolog: Identification of mutant alleles that have pleiotropic effects late in development. Cell *51*, 821–828.

Henne, W.M., Buchkovich, N.J., and Emr, S.D. (2011). The ESCRT Pathway. Dev. Cell *21*, 77–91.

Hershko, A., and Ciechanover, A. (1998). The Ubiquitin System. Annu. Rev. Biochem. *67*, 425–479.

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

Hoffmann, F.M., Fresco, L.D., Hoffman-Falk, H., and Shilo, B.-Z. (1983). Nucleotide sequences of the drosophila src and abl homologs: conservation and variability in the src family oncogenes. Cell *35*, 393–401.

Hoppe, P.E., and Greenspan, R.J. (1986). Local function of the notch gene for embryonic ectodermal pathway choice in drosophila. Cell *46*, 773–783.

Hoppe, P.E., and Greenspan, R.J. (1990). The Notch locus of Drosophila is required in epidermal cells for epidermal development. Development *109*, 875–885.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., and Matsuno, K. (2004). Drosophila Deltex mediates Suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development *131*, 5527–5537.

Hori, K., Sen, A., Kirchhausen, T., and Artavanis-Tsakonas, S. (2011). Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. J Cell Biol *195*, 1005–1015.

Hori, K., Sen, A., and Artavanis-Tsakonas, S. (2013). Notch signaling at a glance. J. Cell Sci. *126*, 2135–2140.

Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., Liu, K., et al. (2013). Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science *341*, 651–654.

Housden, B.E., Li, J., and Bray, S.J. (2014). Visualizing Notch signaling in vivo in Drosophila tissues. Methods Mol. Biol. Clifton NJ *1187*, 101–113.

Hunter, T. (2007). Treatment for chronic myelogenous leukemia: the long road to imatinib. J. Clin. Invest. *117*, 2036–2043.

Ingham, R.J., Gish, G., and Pawson, T. (2004a). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene *23*, 1972–1984.

Ingham, R.J., Gish, G., and Pawson, T. (2004b). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene *23*, 1972–1984.

Jarman, A.P., Grell, E.H., Ackerman, L., Jan, L.Y., and Jan, Y.N. (1994). atonal is the proneural gene for Drosophila photoreceptors. Nature *369*, 398–400.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., and Israel, A. (1995). Signalling downstream of activated mammalian Notch. Nature *377*, 355–358.

Jennings, M.D., Blankley, R.T., Baron, M., Golovanov, A.P., and Avis, J.M. (2007). Specificity and autoregulation of Notch binding by tandem WW domains in suppressor of Deltex. J. Biol. Chem. *282*, 29032–29042.

Kannan, R., Cox, E., Wang, L., Kuzina, I., Gu, Q., and Giniger, E. (2018). Tyrosine phosphorylation and proteolytic cleavage of Notch are required for non-canonical Notch/Abl signaling in Drosophila axon guidance. Dev. Camb. Engl. *145*.

Kelley, M.R., Kidd, S., Deutsch, W.A., and Young, M.W. (1987). Mutations altering the structure of epidermal growth factor-like coding sequences at the Drosophila Notch locus. Cell *51*, 539–548.

Knust, E. (2007). Photoreceptor morphogenesis and retinal degeneration: lessons from Drosophila. Curr. Opin. Neurobiol. *17*, 541–547.

Kopczynski, C.C., Alton, A.K., Fechtel, K., Kooh, P.J., and Muskavitch, M.A. (1988). Delta, a Drosophila neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. Genes Dev. *2*, 1723–1735.

Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., et al. (2000). Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. *14*, 1343–1352.

Kumar, J.P. (2011). My What Big Eyes You Have: How the Drosophila Retina Grows. Dev. Neurobiol. *71*, 1133–1152.

Kurada, P., and White, K. (1998). Ras promotes cell survival in Drosophila by downregulating hid expression. Cell *95*, 319–329.

Kurooka, H., Kuroda, K., and Honjo, T. (1998). Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region. Nucleic Acids Res. *26*, 5448–5455.

Kuzu, O.F., Noory, M.A., and Robertson, G.P. (2016). The Role of Cholesterol in Cancer. Cancer Res. *76*, 2063–2070.

Le Gall, M., De Mattei, C., and Giniger, E. (2008). Molecular separation of two signaling pathways for the receptor, Notch. Dev. Biol. *313*, 556–567.

Lecourtois, M., and Schweisguth, F. (1998). Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. Curr. Biol. CB *8*, 771–774.

Lee, H.-J., Kim, M.-Y., and Park, H.-S. (2015). Phosphorylation-dependent regulation of Notch1 signaling: the fulcrum of Notch1 signaling. BMB Rep. *48*, 431–437.

Lehmann, R., Jiménez, F., Dietrich, U., and Campos-Ortega, J.A. (1983). On the phenotype and development of mutants of early neurogenesis inDrosophila melanogaster. Wilhelm Rouxs Arch. Dev. Biol. *192*, 62–74.

Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trask, B.J., Kuo, W.L., Cochran, J., et al. (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat. Genet. *16*, 243–251.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M.W. (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 Dev. *7*, 1949–1965.

Longley, R.L., and Ready, D.F. (1995). Integrins and the Development of Three-Dimensional Structure in the Drosophila Compound Eye. Dev. Biol. *171*, 415–433.

Luca, V.C., Jude, K.M., Pierce, N.W., Nachury, M.V., Fischer, S., and Garcia, K.C. (2015). Structural basis for Notch1 engagement of Delta-like 4. Science *347*, 847–853.

Luca, V.C., Kim, B.C., Ge, C., Kakuda, S., Wu, D., Roein-Peikar, M., Haltiwanger, R.S., Zhu, C., Ha, T., and Garcia, K.C. (2017). Notch-Jagged complex structure implicates a catch bond in tuning ligand sensitivity. Science *355*, 1320–1324.

Lyman, D., and Young, M.W. (1993). Further evidence for function of the Drosophila Notch protein as a transmembrane receptor. Proc. Natl. Acad. Sci. U. S. A. *90*, 10395–10399.

Maier, D., Praxenthaler, H., Schulz, A., and Preiss, A. (2013). Gain of Function Notch Phenotypes Associated with Ectopic Expression of the Su(H) C-Terminal Domain Illustrate Separability of Notch and Hairless-Mediated Activities. PLOS ONE *8*, e81578.

Maitra, S., Kulikauskas, R.M., Gavilan, H., and Fehon, R.G. (2006). The Tumor Suppressors Merlin and Expanded Function Cooperatively to Modulate Receptor Endocytosis and Signaling. Curr. Biol. *16*, 702–709.

Mašek, J., and Andersson, E.R. (2017). The developmental biology of genetic Notch disorders. Dev. Camb. Engl. *144*, 1743–1763.

Matsuno, K., Diederich, R.J., Go, M.J., Blaumueller, C.M., and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development *121*, 2633–2644.

Matsuno, K., Ito, M., Hori, K., Miyashita, F., Suzuki, S., Kishi, N., Artavanis-Tsakonas, S., and Okano, H. (2002). Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. Development *129*, 1049–1059.

Mazaleyrat, S.L., Fostier, M., Wilkin, M.B., Aslam, H., Evans, D.A.P., Cornell, M., and Baron, M. (2003). Down-regulation of notch target gene expression by suppressor of deltex. Dev. Biol. *255*, 363–372.

McDaniell, R., Warthen, D.M., Sanchez-Lara, P.A., Pai, A., Krantz, I.D., Piccoli, D.A., and Spinner, N.B. (2006). NOTCH2 Mutations Cause Alagille Syndrome, a Heterogeneous Disorder of the Notch Signaling Pathway. Am. J. Hum. Genet. *79*, 169–173.

McWhirter, J.R., Galasso, D.L., and Wang, J.Y. (1993). A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol. Cell. Biol. *13*, 7587–7595.

Meester, J.A.N., Southgate, L., Stittrich, A.-B., Venselaar, H., Beekmans, S.J.A., den Hollander, N., Bijlsma, E.K., Helderman-van den Enden, A., Verheij, J.B.G.M., Glusman, G., et al. (2015). Heterozygous Loss-of-Function Mutations in DLL4 Cause Adams-Oliver Syndrome. Am. J. Hum. Genet. *97*, 475–482.

Mellman, I. (1996). Endocytosis and Molecular Sorting. Annu. Rev. Cell Dev. Biol. *12*, 575–625.

Micchelli, C.A., Rulifson, E.J., and Blair, S.S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. Development *124*, 1485–1495.

Miller, D.T., and Cagan, R.L. (1998). Local induction of patterning and programmed cell death in the developing Drosophila retina. Development *125*, 2327–2335.

Morata, G., and Lawrence, P.A. (1979). Development of the eye-antenna imaginal disc of Drosophila. Dev. Biol. *70*, 355–371.

Morel, V., Lecourtois, M., Massiani, O., Maier, D., Preiss, A., and Schweisguth, F. (2001). Transcriptional repression by Suppressor of Hairless involves the binding of a Hairless-dCtBP complex in Drosophila. Curr. Biol. *11*, 789–792.

Moretti, J., and Brou, C. (2013). Ubiquitinations in the Notch Signaling Pathway. Int. J. Mol. Sci. *14*, 6359–6381.

Morgan, T.H. (1917). The Theory of the Gene. Am. Nat. *51*, 513–544.

Nagarkar-Jaiswal, S., Lee, P.-T., Campbell, M.E., Chen, K., Anguiano-Zarate, S., Gutierrez, M.C., Busby, T., Lin, W.-W., He, Y., Schulze, K.L., et al. (2015). A library of MiMICs allows tagging of genes and reversible, spatial and temporal knockdown of proteins in Drosophila. ELife *4*.

Nam, Y., Weng, A.P., Aster, J.C., and Blacklow, S.C. (2003). Structural requirements for assembly of the CSL.intracellular Notch1.Mastermind-like 1 transcriptional activation complex. J. Biol. Chem. *278*, 21232–21239.

Nüsslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle inDrosophila melanogaster. Wilhelm Rouxs Arch. Dev. Biol. *193*, 267–282.

Obniski, R., Sieber, M., and Spradling, A.C. (2018). Dietary Lipids Modulate Notch Signaling and Influence Adult Intestinal Development and Metabolism in Drosophila. Dev. Cell *47*, 98-111.e5.

Otsu, N. (1979). A threshold selection method from gray-level histograms. IEEE Trans. Syst. Man Cybern. *9*, 62–66.

Palmer, W.H., and Deng, W.-M. (2015). Ligand-Independent Mechanisms of Notch Activity. Trends Cell Biol. *25*, 697–707.

Parks, A.L., Turner, F.R., and Muskavitch, M.A. (1995). Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. Mech. Dev. *50*, 201–216.

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

Pearse, B.M.F., and Robinson, M.S. (1990). Clathrin, Adaptors, and Sorting. Annu. Rev. Cell Biol. *6*, 151–171.

Pelaez, N., Gavalda-Miralles, A., Wang, B., Navarro, H.T., Gudjonson, H., Rebay, I., Dinner, A.R., Katsaggelos, A.K., Amaral, L.A.N., and Carthew, R.W. (2015). Dynamics and heterogeneity of a fate determinant during transition towards cell differentiation. ELife *4*.

Poulson, D.F. (1940). The effects of certain X-chromosome deficiencies on the embryonic development of Drosophila melanogaster. J. Exp. Zool. *83*, 271–325.

Prekeris, R., Yang, B., Oorschot, V., Klumperman, J., and Scheller, R.H. (1999). Differential Roles of Syntaxin 7 and Syntaxin 8 in Endosomal Trafficking. Mol. Biol. Cell *10*, 3891–3908.

Qiu, L., Joazeiro, C., Fang, N., Wang, H.Y., Elly, C., Altman, Y., Fang, D., Hunter, T., and Liu, Y.C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. J. Biol. Chem. *275*, 35734–35737.

Rafel, N., and Milan, M. (2008). Notch signalling coordinates tissue growth and wing fate specification in Drosophila. Development *135*, 3995–4001.

Raiborg, C., Bache, K.G., Gillooly, D.J., Madshus, I.H., Stang, E., and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat. Cell Biol. *4*, 394–398.

Ready, D.F., Hanson, T.E., and Benzer, S. (1976). Development of the Drosophila retina, a neurocrystalline lattice. Dev. Biol. *53*, 217–240.

Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and serrate: Implications for notch as a multifunctional receptor. Cell *67*, 687–699.

Rebay, I., Fehon, R.G., and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell *74*, 319–329.

Saj, A., Arziman, Z., Stempfle, D., van Belle, W., Sauder, U., Horn, T., Dürrenberger, M., Paro, R., Boutros, M., and Merdes, G. (2010). A Combined Ex Vivo and In Vivo RNAi Screen for Notch Regulators in Drosophila Reveals an Extensive Notch Interaction Network. Dev. Cell *18*, 862–876.

Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K., and Hayashi, S. (2004). Drosophila Nedd4 Regulates Endocytosis of Notch and Suppresses Its Ligand-Independent Activation. Curr. Biol. *14*, 2228–2236.

Schweitzer, R., Howes, R., Smith, R., Shilo, B.-Z., and Freeman, M. (1995). Inhibition of Drosophila EGF receptor activation by the secreted protein Argos. Nature *376*, 699–702.

Seugnet, L., Simpson, P., and Haenlin, M. (1997). Requirement for dynamin during Notch signaling in Drosophila neurogenesis. Dev. Biol. *192*, 585–598.

Shimizu, H., Woodcock, S.A., Wilkin, M.B., Trubenová, B., Monk, N.A.M., and Baron, M. (2014). Compensatory Flux Changes within an Endocytic Trafficking Network Maintain Thermal Robustness of Notch Signaling. Cell *157*, 1160–1174.

Shimizu, K., Chiba, S., Kumano, K., Hosoya, N., Takahashi, T., Kanda, Y., Hamada, Y., Yazaki, Y., and Hirai, H. (1999). Mouse Jagged1 Physically Interacts with Notch2 and Other Notch Receptors ASSESSMENT BY QUANTITATIVE METHODS. J. Biol. Chem. *274*, 32961–32969.

Shoup, J.R. (1966). THE DEVELOPMENT OF PIGMENT GRANULES IN THE EYES OF WILD TYPE AND MUTANT DROSOPHILA MELANOGASTER. J. Cell Biol. *29*, 223–249.

Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of Drosophila. Dev. Camb. Engl. *109*, 509–519.

Simpson, F., Bright, N.A., West, M.A., Newman, L.S., Darnell, R.B., and Robinson, M.S. (1996). A novel adaptor-related protein complex. J. Cell Biol. *133*, 749–760.

Smoller, D., Friedel, C., Schmid, A., Bettler, D., Lam, L., and Yedvobnick, B. (1990). The Drosophila neurogenic locus mastermind encodes a nuclear protein unusually rich in amino acid homopolymers. Genes Dev. *4*, 1688–1700.

Steinbuck, M.P., and Winandy, S. (2018). A Review of Notch Processing With New Insights Into Ligand-Independent Notch Signaling in T-Cells. Front. Immunol. *9*.

Stevens, T.H., and Forgac, M. (1997). STRUCTURE, FUNCTION AND REGULATION OF THE VACUOLAR (H+)-ATPase. Annu. Rev. Cell Dev. Biol. *13*, 779–808.

Stittrich, A.-B., Lehman, A., Bodian, D.L., Ashworth, J., Zong, Z., Li, H., Lam, P., Khromykh, A., Iyer, R.K., Vockley, J.G., et al. (2014). Mutations in NOTCH1 Cause Adams-Oliver Syndrome. Am. J. Hum. Genet. *95*, 275–284.

Strooper, B.D., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., et al. (1999). A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. Nature *398*, 518–522.

Struhl, G., and Adachi, A. (1998). Nuclear Access and Action of Notch In Vivo. Cell *93*, 649–660.

Struhl, G., Fitzgerald, K., and Greenwald, I. (1993). Intrinsic activity of the lin-12 and Notch intracellular domains in vivo. Cell *74*, 331–345.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T., and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). Curr. Biol. CB *5*, 1416–1423.

Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M., and Kirchhausen, T. (1996). Eps15 Is a Component of Clathrin-coated Pits and Vesicles and Is Located at the Rim of Coated Pits. J. Biol. Chem. *271*, 28727–28730.

Trump, B.F., Goldblatt, P.J., and Stowell, R.E. (1965). Studies of necrosis in vitro of mouse hepatic parenchymal cells. Ultrastructural and cytochemical alterations of cytosomes, cytosegresomes, multivesicular bodies, and microbodies and their relation to the lysosome concept. Lab. Investig. J. Tech. Methods Pathol. *14*, 1946–1968.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D., and Kitajewski, J. (1996). Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Dev. Camb. Engl. *122*, 2251–2259.

Vaccari, T., Lu, H., Kanwar, R., Fortini, M.E., and Bilder, D. (2008). Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. J Cell Biol *180*, 755–762.

Vaccari, T., Duchi, S., Cortese, K., Tacchetti, C., and Bilder, D. (2010). The vacuolar ATPase is required for physiological as well as pathological activation of the Notch receptor. Development *137*, 1825–1832.

Vincent, L., and Soille, P. (1991). Watersheds in digital spaces: an efficient algorithm based on immersion simulations. IEEE Trans. Pattern Anal. Mach. Intell. *13*, 583–598.

Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C.B., and Bucci, C. (1997). Role of the Small GTPase RAB7 in the Late Endocytic Pathway. J. Biol. Chem. *272*, 4391–4397.

Waddington, C.H. (1957). The strategy of the genes. A discussion of some aspects of theoretical biology. With an appendix by H. Kacser. Strategy Genes Discuss. Some Asp. Theor. Biol. Append. H Kacser.

Weinmaster, G., Roberts, V.J., and Lemke, G. (1992). Notch2: a second mammalian Notch gene. Dev. Camb. Engl. *116*, 931–941.

Weng, A.P., Ferrando, A.A., Lee, W., Morris, J.P., Silverman, L.B., Sanchez-Irizarry, C., Blacklow, S.C., Look, A.T., and Aster, J.C. (2004). Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. Science *306*, 269–271.

Wharton, K.A., Johansen, K.M., Xu, T., and Artavanis-Tsakonas, S. (1985a). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell *43*, 567–581.

Wharton, K.A., Johansen, K.M., Xu, T., and Artavanis-Tsakonas, S. (1985b). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell *43*, 567–581.

Wieschaus, E., and Nüsslein-Volhard, C. (2016). The Heidelberg Screen for Pattern Mutants of Drosophila: A Personal Account. Annu. Rev. Cell Dev. Biol. *32*, 1–46.

Wilkin, M., Tongngok, P., Gensch, N., Clemence, S., Motoki, M., Yamada, K., Hori, K., Taniguchi-Kanai, M., Franklin, E., Matsuno, K., et al. (2008). Drosophila HOPS and AP-3 Complex Genes Are Required for a Deltex-Regulated Activation of Notch in the Endosomal Trafficking Pathway. Dev. Cell *15*, 762–772.

Wilkin, M.B., Carbery, A.-M., Fostier, M., Aslam, H., Mazaleyrat, S.L., Higgs, J., Myat, A., Evans, D.A.P., Cornell, M., and Baron, M. (2004). Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. Curr. Biol. CB *14*, 2237–2244.

Wilson, J.J., and Kovall, R.A. (2006). Crystal Structure of the CSL-Notch-Mastermind Ternary Complex Bound to DNA. Cell *124*, 985–996.

Windler, S.L., and Bilder, D. (2010). Endocytic Internalization Routes Required for Delta/Notch Signaling. Curr. Biol. *20*, 538–543.

Winkelman, J.D., Bilancia, C.G., Peifer, M., and Kovar, D.R. (2014). Ena/VASP Enabled is a highly processive actin polymerase tailored to self-assemble parallel-bundled F-actin networks with Fascin. Proc. Natl. Acad. Sci. U. S. A. *111*, 4121–4126.

Wolff, T., and Ready, D.F. (1991a). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. Development *113*, 841–850.

Wolff, T., and Ready, D.F. (1991b). Cell death in normal and rough eye mutants of Drosophila. Dev. Camb. Engl. *113*, 825–839.

Wu, L., Aster, J.C., Blacklow, S.C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J.D. (2000). MAML1, a human homologue of *Drosophila* Mastermind, is a transcriptional co-activator for NOTCH receptors. Nat. Genet. *26*, 484–489.

Xiong, W., and Rebay, I. (2011). The Abelson tyrosine kinase is required for Drosophila photoreceptor morphogenesis and retinal epithelial patterning. Dev. Dyn. *240*, 1745–1755.

Xiong, W., Morillo, S.A., and Rebay, I. (2013). The Abelson tyrosine kinase regulates Notch endocytosis and signaling to maintain neuronal cell fate in Drosophila photoreceptors. Dev. Camb. Engl. *140*, 176–184.

Xu, T., and Artavanis-Tsakonas, S. (1990a). deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. Genetics *126*, 665–677.

Xu, T., and Artavanis-Tsakonas, S. (1990b). deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. Genetics *126*, 665–677.

Xu, T., Rebay, I., Fleming, R.J., Scottgale, T.N., and Artavanis-Tsakonas, S. (1990). The Notch locus and the genetic circuitry involved in early Drosophila neurogenesis. Genes Dev. *4*, 464–475.

Yamada, K., Fuwa, T.J., Ayukawa, T., Tanaka, T., Nakamura, A., Wilkin, M.B., Baron, M., and Matsuno, K. (2011). Roles of Drosophila Deltex in Notch receptor endocytic trafficking and activation. Genes Cells *16*, 261–272.

Yamamoto, S., Charng, W.-L., and Bellen, H.J. (2010). Endocytosis and intracellular trafficking of Notch and its ligands. Curr. Top. Dev. Biol. *92*, 165–200.

Yochem, J., and Greenwald, I. (1989). glp-1 and lin-12, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. Cell *58*, 553–563.

Yochem, J., Weston, K., and Greenwald, I. (1988). The Caenorhabditis elegans lin-12 gene encodes a transmembrane protein with overall similarity to Drosophila Notch. Nature *335*, 547–550.

Yu, S.-Y., Yoo, S.J., Yang, L., Zapata, C., Srinivasan, A., Hay, B.A., and Baker, N.E. (2002). A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye. Dev. Camb. Engl. *129*, 3269–3278.

Zacharioudaki, E., and Bray, S.J. (2014a). Tools and methods for studying Notch signaling in Drosophila melanogaster. Methods San Diego Calif *68*, 173–182.

Zacharioudaki, E., and Bray, S.J. (2014b). Tools and methods for studying Notch signaling in Drosophila melanogaster. Methods San Diego Calif *68*, 173–182.

Zhang, Y., Malone, J.H., Powell, S.K., Periwal, V., Spana, E., MacAlpine, D.M., and Oliver, B. (2010). Expression in Aneuploid Drosophila S2 Cells. PLoS Biol. *8*.