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Amino Acid Functions in the Juxtamembrane Region of the Transmembrane Serrate Ligand, and Their Effects on Subcellular Localization of Serrate Protein.

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AMINO ACID FUNCTIONS IN THE *JUXTAMEMBRANE* REGION OF THE TRANSMEMBRANE *SERRATE* LIGAND, AND THEIR EFFECTS ON SUBCELLULAR LOCALIZATION OF SERRATE PROTEIN.

BY

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Honors Thesis Committee

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Susan M. Bush

Date: _____

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ABSTRACT

The Notch signaling pathway is a highly conserved cell-to-cell signaling system that is present in eukaryotic animals. This pathway plays a significant role during animal development. The Notch gene codes for a protein that functions as a receptor belonging to the single-pass transmembrane protein group. The Notch receptors can interact with ligands that are also single-pass transmembrane proteins of the *Delta/Serrate/Lag-2* (DSL) family of ligands. The regulation of the Notch signaling pathway can be controlled through different types of interactions with ligands such as *cis*-inhibition and *trans*-activation. The *trans*-activation interaction occurs when the receptor and ligand proteins, present in neighboring cells, interact. Ligand–receptor interactions can also take place within the cell and on the cell surface, and the *cis*-interactions can reduce or inhibit the ability of a cell to receive an activating signal from neighboring cells. In activation of Notch, ligands are trafficked through endocytosis and are often regulated by ubiquitin ligase. While it was found that *Serrate*, a ligand for Notch, is relevant for Notch activity, the importance of the specific localization of *Serrate* is much less well defined. We postulate that in order to successfully activate Notch, *Serrate* must be located on the cell surface, however, our data suggest that there may be other properties beyond *Serrate* localization on cell surface that dictate the activation of Notch. To test this, we analyzed the different locations of *Serrate* in cells expressing specific constructs, previously made in Fleming laboratory, with different amino acid lengths in the Juxtamembrane domain, a key component in the structure of *Serrate* for successful activation. The mechanisms of Notch activation by ligand endocytosis models such as the ligand-recycling model or classical endocytosis model can be the explanation for why *Serrate* is located on the cell surface while being unable to activate Notch.

INTRODUCTION

Cell-to-cell signaling mechanism: Notch signaling pathway

In living organisms, there are many ways that cells can communicate intercellularly. One well-studied method of cellular communication is cell-to-cell signaling. Cell signaling pathways are critical for cells to signal their surrounding neighbors to work together. This provides the overall mechanism for cells to perform important bodily processes that are necessary for survival (Reece and Neil, 2014). The specific cell signaling mechanism on which this study is focused is the *Notch* signaling pathway that plays an important role in organismal developmental changes. Notch is present in most multicellular eukaryotic organisms and when combined with other cellular factors it influences cell fate, differentiation, proliferation, and apoptotic events (Artavanis-Tsakonas, 1999). The *Notch* gene encodes a single-pass transmembrane receptor that is essential for successful cell signaling. It is evident that mutation or disruption of the *Notch* gene can cause numerous severe defects in the development of living organisms, including abnormal anterior-posterior polarity in vertebrate somites (Feller et al., 2008), because Notch signaling is essential for the regulation of polarity in eukaryotic cells. Notch signaling is also important for vertebrates to determine left-right asymmetry in embryonic development (Levin et al., 2005).

Mutations of the *Notch* gene can also lead to a number of different human diseases that may affect the development of the central nervous system (CNS), cardiovascular system, and endocrine system. Other *Notch* mutations have also been implicated in the formation and progression of cancers such as promotion of leukemia cell growth due to activation of anabolic pathways including ribosome and protein biosynthesis (Palomero at al., 2007). These mutations can cause abnormal developmental phenotypes that affect the liver, skeleton, heart, eye, face,

kidney, and vasculature. Since *Notch* receptor proteins and their ligands are expressed in cells of the adult nervous system, Notch signaling plays an important role in the CNS throughout life. Additionally, mutations in either Notch receptor (NOTCH1) or CBF1/RBP-Jkappa (CBF1) of mice have deficits in spatial learning and memory (Costa et al., 2003). Patients who have Alzheimer's disease and mild cognitive impairment demonstrated statistically significant worsening of cognition when several γ-secretase inhibitors were employed, affecting *Notch* signaling (De Strooper, 2014). This is expected as γ-secretase plays an important role in S3 cleavage of the transmembrane domain of the *Notch* receptors and is crucial for activating *Notch* signaling pathways. When the γ-secretases are inhibited, the *Notch* signaling pathway cannot be activated since the S3 cleavage cannot be carried out successfully.

Mutations in either the ligand (Jagged1; JAG1) or the receptor (NOTCH2) can lead to autosomal dominant, multi-system Alagille syndromes, a genetic disorder that affects the liver, heart, and other body parts due to abnormalities in the bile duct or impairment in blood flow. Similarly, mutations in the ligand (Delta-like-3; DLL3) can cause autosomal recessive spondylocostal dysostosis, which is a type of bone developmental disease and mutations of the NOTCH2 receptor gene are also correlated with the development of Hajdu-Cheney syndrome, a dominant disorder causing focal bone destruction, osteoporosis, craniofacial morphology and renal cysts (Penton et al., 2012).

The majority of the studies done on *Notch* signaling are applicable across species since the *Notch* pathway is highly conserved. One of the reasons why *Drosophila melanogaster* is used as a model organism for *Notch* signaling research stems from the advantage of genetic simplicity for the pathway compared to other organisms. One of the key differences between mammalian systems and *Drosophila* is that the *Drosophila* genome contains only one gene copy of

(NOTCH) that encodes for the Notch receptor, whereas the human genome contains four copies (NOTCH1 to 4; Kopan and Illigan 2009). Similarly, single copies of each ligand molecule, *Serrate* and *Delta*, are found in *Drosophila* while multiple copies of each ligand gene are typically found in mammals (Kumar et al., 2016). The Notch protein itself also differs between flies and mammals in that it has been demonstrated that mammalian Notch undergoes an early (S1) proteolytic cleavage mediated by a furin-like protease, which produces a cleaved yet linked two-piece receptor molecule prior to its placement on the cell membrane. In contrast, the majority of the Notch receptor protein found at the cell membrane in *Drosophila* consists of the single, uncleaved ~300 kDa full-length protein.

Despite the minor differences in the structural components of the Notch pathway between mammals and flies, *Drosophila* remains a popular genetic research species for a number of different reasons. Compared to many species it has a relatively short life cycle making it easy to grow and reproduce in a short amount of time, and it has a simple genome consisting of four pairs of chromosomes (Adams, 2000). Furthermore, model organisms such as *Drosophila*, can be used in a translational manner to study underlying mechanisms of Notch-related human diseases and to investigate the function of novel disease associated genes and variants (Salazar and Yamamoto, 2018). The majority of Notch research is still studied extensively on *Drosophila* mainly due to those reasons. My thesis will focus on Notch signaling in the *Drosophila* with the main emphasis on the *Serrate* ligand protein, specifically examining how the structural changes in the protein affect the subcellular localization of *Serrate* trafficking in cells.

Structure of the *Notch* receptor and ligands

As mentioned, the *Notch* gene codes for a protein that functions as a receptor belonging to the single-pass transmembrane protein group. The receptor can interact with ligands that are also single-pass transmembrane proteins, specifically the members of the Delta/Serrate/Lag-2 (DSL) family of ligands (Fehon et al., 1990). In order to have successful cell signaling, cells expressing the *Notch* receptors must be adjacent to the cells expressing the ligands. The *Notch* receptors have extracellular (NECD), intracellular (NICD) and transmembrane (TMD) domains (*Figure 1)*.

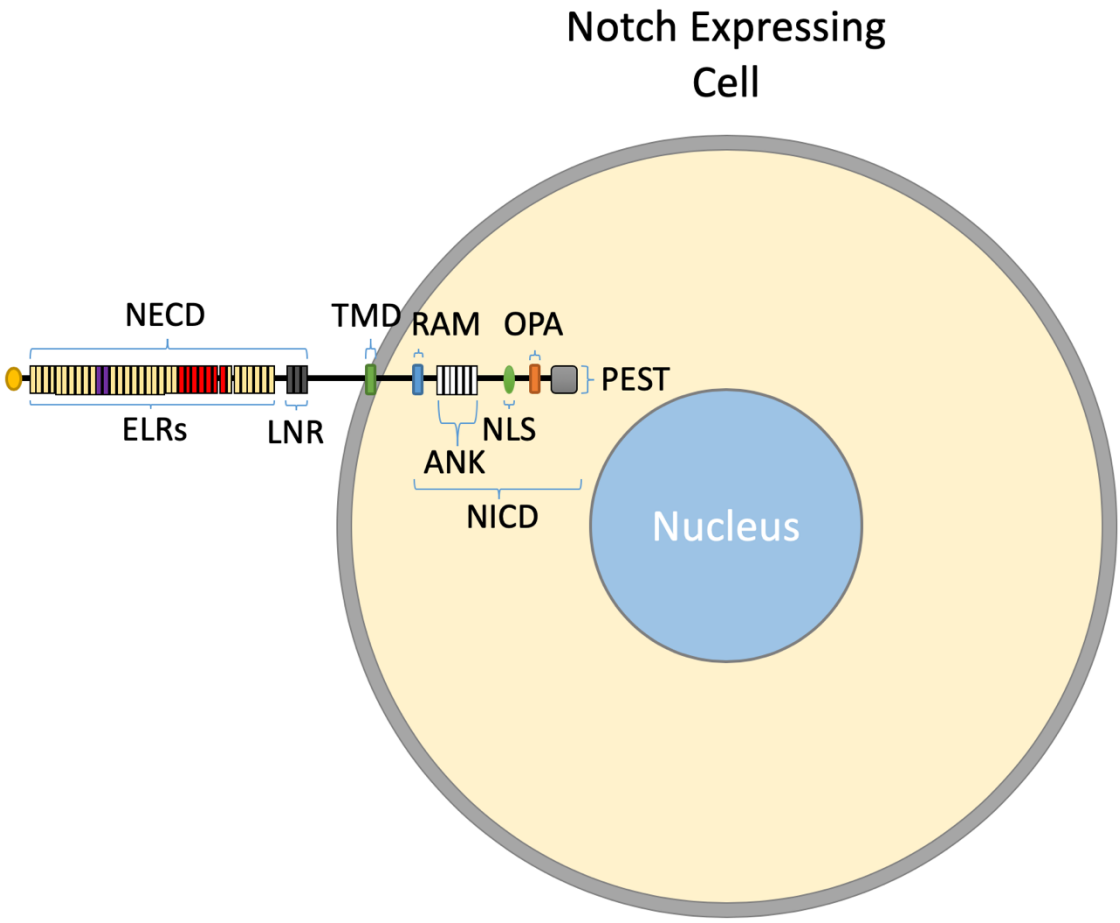


Figure 1. The *Notch* receptor expressing cell. This figure shows the Notch Extracellular Domain (NECD) consisting of 36 Epidermal growth factor-like repeats (ELRs) and 3 LIN-12/Notch repeats (LNRs). Also shown are the transmembrane domain (TMD), and the Notch intracellular domain (NICD) consisting of a RBP_{jk} association module (RAM), 7 ankyrin-like repeats (ANKs), a nuclear localization signal (NLS), and proline-glutamate-serine-threonine–rich domain (PEST). All the domains make up the membrane bound receptor.

The *Notch* extracellular domain (NECD) is composed of small cysteine-rich motifs called epidermal growth factor-like repeats (ELRs). In one of the most common *Notch* receptors, NOTCH1, there are 36 contiguous ELRs where each ELR is composed of around 40 amino acids

and its structure is defined by six conserved cysteine residues that form three conserved disulfide bonds (Rebay et al., 1991). It also contains a *lin12-Notch* region (LNR) that is also very cysteine-rich. The ELRs are the key component of the Notch receptors' structure and maintenance. The *Delta* and *Serrate* ligand interactions with Notch are dependent on a number of different ELRs on the NECD. In the case of a successful interaction, the ELRs 11 and 12 (*Figure 1*) are considered necessary for Notch to interact with the *Serrate* or *Delta* ligands. They are sufficient to induce cells expressing *Notch* and *Delta* to aggregate (Rebay et al., 1991).

The *Notch* intracellular domain (NICD) on the other hand, contains several conserved sequence motifs. The NICD includes a RBP_{jk} (recombination signal binding protein) association module (RAM), which has high attraction for binding to specific transcriptional factors in the nucleus, a block of six CDC10, or ankyrin (ANK) repeats, one or two nuclear localization signals (NLS) which allows the NICD to go to the nucleus, a homopolymer repeat of glutamines (OPA domain) which is a non-conserved sequence with little or no known functionality, and a proline glutamate serine threonine rich (PEST) domain that is significantly important for the degradation of the NICD (Struhl and Adachi, 1998).

The *Notch* transmembrane domain (TMD) is a 21 amino acid long segment allowing a single pass through the cell membrane. The TMD is essential for connecting through the cellular membrane and locating the protein in place securely. When the receptor is attached to a ligand on an adjacent cell at the cell membrane, the signaling pathway becomes activated. During this process, Notch undergoes cleavage where the extracellular domain (NECD) is removed first and then the intracellular domain (NICD) later (Figure 3; Deatherage et al., 2015). An important step in activating the *Notch* signaling pathway is S3 cleavage of its transmembrane domain by γ-

secretase. It has been suggested that receptor and ligand interactions on the membrane play an important role in modulating *Notch* cleavage by γ-secretase (Wolfe et al., 1999).

The *Notch* receptor cannot activate the signaling pathway by itself, hence the Notch ligands are responsible for activation or inhibition of the pathway by interacting with the Notch receptor. The ligands in the DSL family are characterized by three related structural motifs: An N-terminal Delta, Serrate, Lag-2 domain (DSL), specialized tandem EGF repeats called the Delta and OSM-11-like protein domain (DOS; Komatsu et al., 2008), and ELRs similar to the ones found in *Notch* receptors (both calcium binding and non-calcium binding; Kopan and Iligan, 2009). Another ligand worth mentioning is *Jagged* which is a vertebrate homolog of a *Serrate* ligand (*Serrate* is found in *Drosophila*). The region defined by ELRs 4, 5 and 6 are conserved among *Jagged* family ligands and is known as the Notch Inhibitory Region (NIR), this region is crucial since removal of any of the 3 ELRs of the NIR will result in the loss of most inhibitory functions (Fleming et al., 2013). The main difference between the *Jagged* and *Serrate* ligands is that *Jagged* contains 16 ELRs while *Serrate* contains 14 and they both have a cysteine-rich domain. The *Delta* ligand in mammals, on the other hand, only has 8 or 9 ELRs without the cysteine-rich domain. The *Jagged* and *Serrate* ligands are transmembrane proteins; hence, they also have a transmembrane and intracellular domain (*Figure 2).*

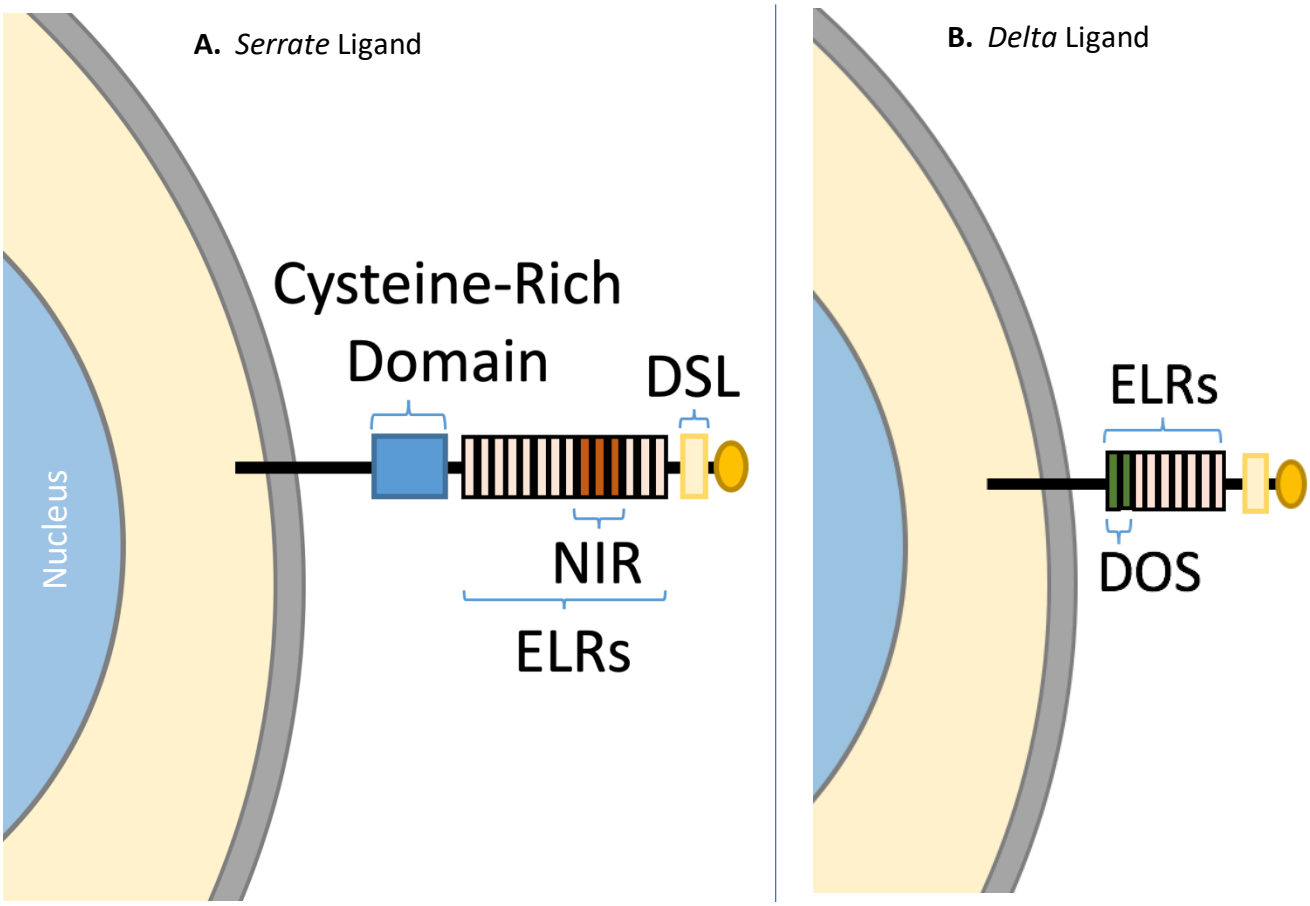


Figure 2: The DSL ligands expressing cells. **A.** This figure shows the structure of *Serrate* ligand consisting of a Delta/Serrate/Lag-2 (DSL) domain, 14 epidermal growth factor-like repeats (ELRs) similar to the ones on *Notch* receptors, Notch inhibitory region (NIR) located at the 4th, 5th and 6th ELRs, and a Cysteine Rich Domain. **B.** The structure of the *Delta* ligand is similar to *Serrate* consisting of a DSL domain and 9 ELRs with the OSM-11-like protein domain (DOS) located at the 8th and 9th ELRs.

Mechanism and regulation of Notch signaling

The regulation of the *Notch* signaling pathway can be controlled through different types of interactions with ligands such as *trans*-activation and *cis*-inhibition. The *trans*-activation

interaction occurs when the receptor and ligand proteins present in neighboring cells interact (*Figure 3*). Ligand–receptor interactions can also take place within the same cell, and these *cis*-interactions can reduce or inhibit the ability of a cell to receive an activating signal from neighboring cells. This process is called *cis*-inhibition of the receptor by the ligand (Álamo et al., 2011; *Figure 4*).

In the case of *trans*-activation of receptor by ligand, where the transmembrane proteins are on neighboring cells, their interaction creates a conformational change that causes S2 cleavage of the *Notch* receptor by a metalloprotease (*Figure 3A*). The NECD is endocytosed by the cells expressing the ligand after the ligation is recognized by the receptors as soon as the S2 cleavage is initiated. Before Notch activation, the γ-secretase is normally blocked by the presence of the NECD, hence, after the NECD is endocytosed the γ-secretase interacts with and cleaves the NICD at the S3 site. The S2 cleavage, therefore, leads to the S3 cleavage as seen from *Figure 3B*. Overall, it is suggested that that S2 cleavage is a ligand-regulated step in the proteolytic cascade leading to Notch activation (Mumm et al., 2000). Following the release of an active NICD fragment through S3 cleavage, the NICD travels to the cell nucleus where the NICD links with a DNA binding protein. This interaction assembles a transcription complex that binds and activates the downstream target genes leading to the activation of the *Notch* pathway (Figure 3C; Kopan and Illigan 2009).

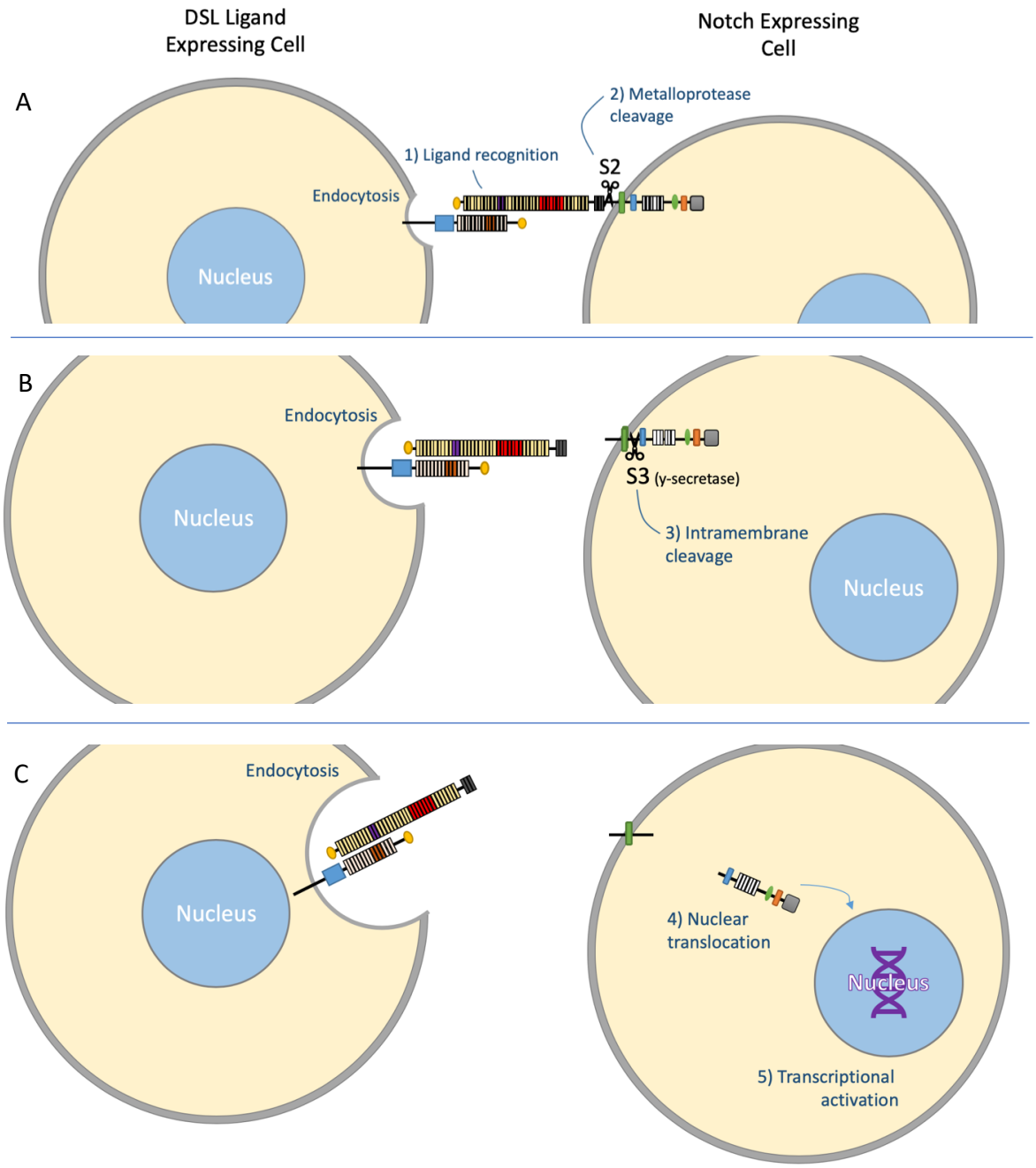


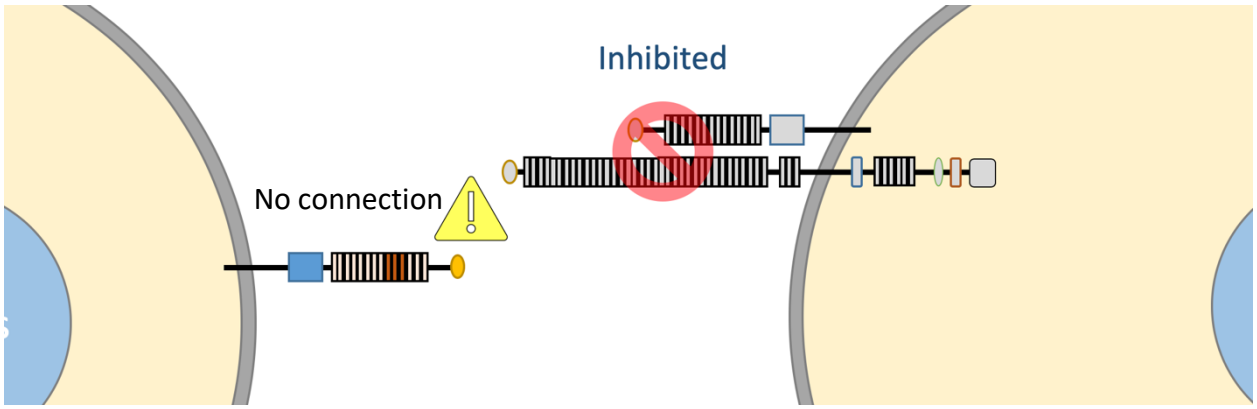
Figure 3. Regulation of *Notch* signaling pathways though *Trans*-activation ligand-receptor interactions. **A.** The *Notch* receptor recognizes and binds with the ligand (DSL) producing a conformational shift allowing for endocytosis and S2 cleavage of the NECD. **B.** S2 cleavage is then followed by S3 intramembrane cleavage of the NICD. **C.** Once released, the NICD is translocated to the nuclease to activate transcriptional activities.

Cis-inhibition happens when ligand and receptor are expressed within the same cell and ultimately reduces or inhibits the ability of a cell to receive an activating signal from neighboring cells. The exact location of where *cis*-inhibition is localized at the cellular level, however, is still not fully understood. There are a number of different suggested models for DSL ligand expressing cells to inhibit Notch. In the case of *Notch-Serrate* interactions, it is broadly suggested that *cis*-inhibition takes place at the cell surface during disruption of Notch signaling pathway (Glittenberg et al., 2006). This is supported since strong inhibition is observed for Serrate proteins such as Ser^{*LL} (Serrate protein construct with a change in the two leucines to alanines; Glittenberg et al., 2006), which are defective for endocytosis and accumulate Notch at the cell surface. Also, *cis*-inhibition is often enhanced by NeurΔRING (E3 ubiquitin ligase Neuralized without the RING domain), which has lost the capacity to ubiquitinate and can trap ligands at the cell surface and increase the opportunity for *cis*-inhibition to occur (Pavlopoulos et al., 2001; Yeh et al., 2001). This ultimately suggests that ubiquitination of Serrate is not required for the ligand to impart cis-inhibition.

While the model of *cis*-inhibition at the cell surface may hold true, different models suggest inhibitory interactions of ligands can also occur within a same-cell before reaching the surface in an intracellular fashion. These models suggest that ligand-receptor interactions occur cell-autonomously by forming homomeric or heteromeric complexes, which are not present on the cell surface. This suggests a possible association of ligand and receptor occurring in the endoreticulum or Golgi apparatus (Sakamoto 2002). As previously suggested in *trans*-activation (Figure 3), ligand and Notch receptors are located in neighboring cells exclusively, but this is not strictly true since the fate of a cell's functionality as a signal receiver or signal sender is determined by the relative levels of ligands and Notch receptors present. For instance, signal

receiving cells express more Notch than ligand but some of the Notch receptors are *cis*-inhibited by the ligand while sufficient number of Notch remains available to interact with ligands from neighboring cells, making the cell capable of receiving signals (Figure 5a). The reciprocal situation arises with signal sending cells (Bray 2016; Figure 5b). Hence, cells expressing both ligands and Notch autonomously associate ligand/receptor complexes within the cell. Notch participates in formation of the cell-autonomous complexes before protein processing, therefore, a transmembrane protein like Notch is cotranslationally transferred to the endoplasmic reticulum (ER) and modified in the ER and Golgi apparatus before being transported to the cell surface (Sakamoto 2002). It was also proposed that ligand binding displaces intramolecular interactions between EGF-repeats within Notch to promote a change in conformation necessary for the activating cleavage (Xu et al., 2005; Glittenberg et al., 2006).

A. Cell surface *cis*-inhibition



B. Intracellular heteromeric complex *cis*-inhibition

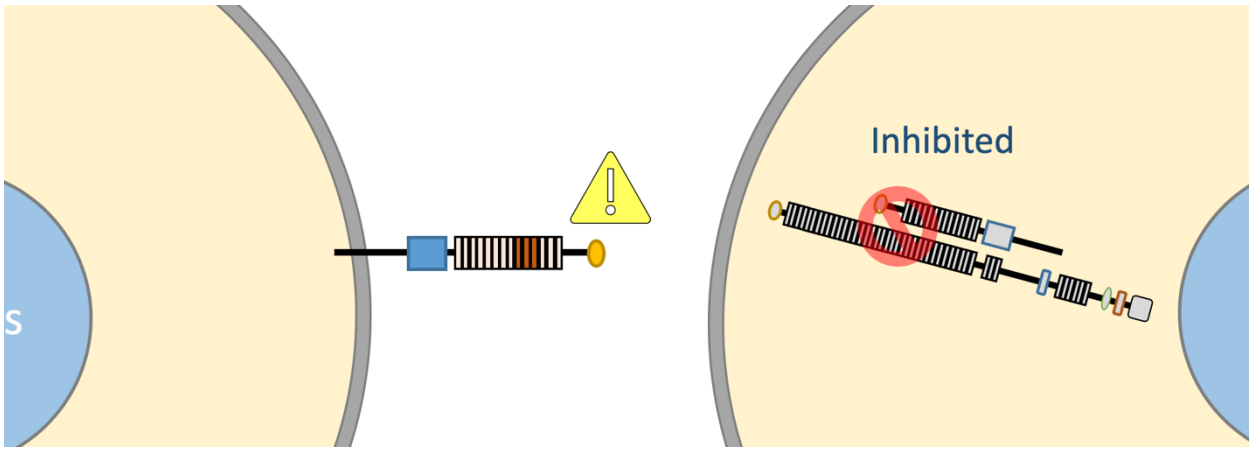


Figure 4. Hypothesized models of *cis*-inhibitions of Notch and ligand. This figure illustrates the possible ways of disrupting the Notch activation pathway through *cis*-inhibition. **A.** The cell surface *cis*-inhibition suggests the Notch receptor is inhibited by a ligand on the cell surface due to possible disturbance in ubiquitin ligases and could be caused by failure of ubiquitinating ligands from the cell surface. **B.** The intracellular *cis*-inhibition mechanism suggests that heteromeric complexes form between ligand and Notch intracellularly before the ligand, or the receptor make it to the cell surface independently. This implies that the association between ligand and Notch occurs in the endoplasmic reticulum or Golgi apparatus.

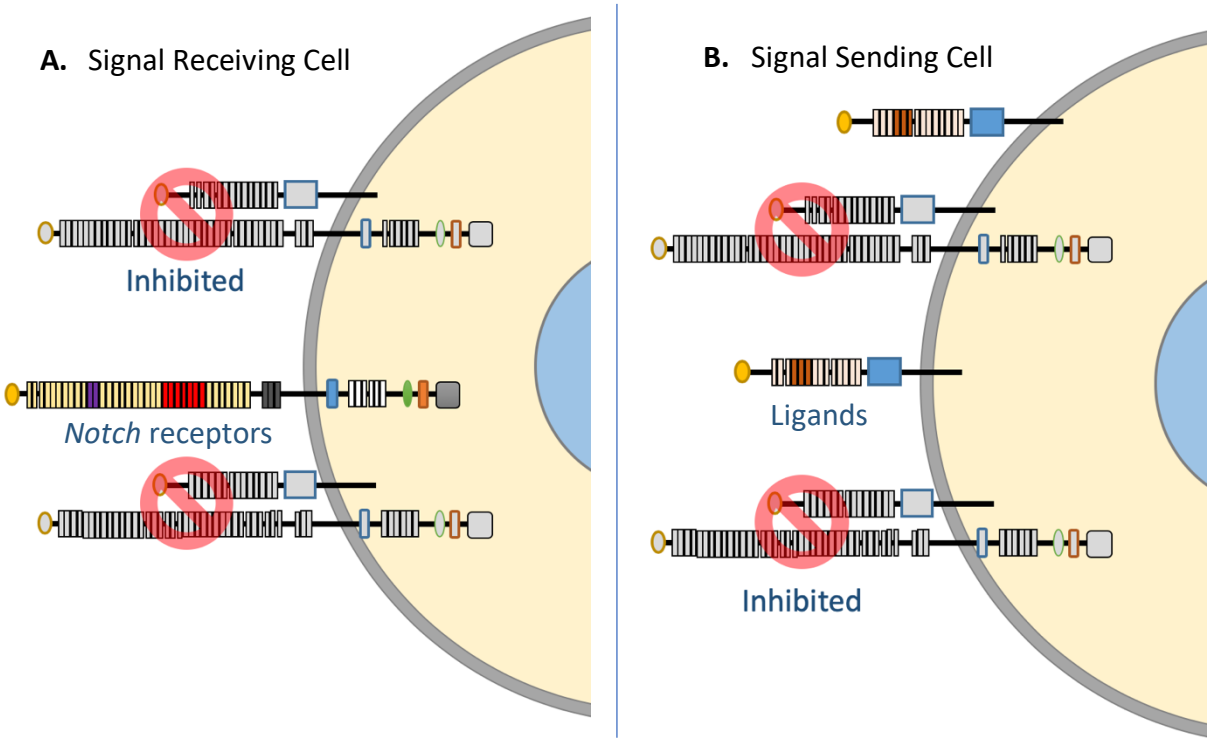


Figure 5. **Consequences of cell surface *cis*-inhibition on cell fate of Notch signaling.** This figure illustrates how the relative quantity of ligands and Notch determines whether cells send or receive signals due to *cis*-interactions between ligands and receptors. **A.** a signal receiving cell expressing more *Notch* than ligands. While some *Notch* receptors are *cis*-inhibited by adjacent ligands, sufficient *Notch* remains available to interact with ligands from neighboring cells, making the cell capable of receiving signals. **B.** a signal sending cell expressing more ligands than Notch. All *Notch* receptors are *cis*-inhibited by adjacent ligands but sufficient level of ligands remains available to interact with Notch from neighboring cells, making the cell capable of sending signals (Bray 2016).

In *Drosophila*, major regulations in the activity and availability of *Notch* receptors and ligands are done through endocytic trafficking, the process of internalization of membrane components, which can be modulated by the activity of different ubiquitin ligases (Kandachar and Roegiers 2012). The requirement of endocytosis in both signal-sending and signal-receiving cells was first demonstrated by the clonal analysis of temperature-sensitive *shibire* mutants (Seugnet et al., 1997). *Shibire* encodes the *Drosophila* homolog of Dynamin (van der Bliek and Meyerowitz 1991) a protein required to pinch off endocytic vesicles from the plasma membrane. Experiments showed that in order to activate the signal-receiving cell in Notch signaling, the endocytosis of the Notch ligand Delta in the signal-sending cell is essential (Parks et al., 2000).

The trafficking of the ligands through endocytosis is regulated often through ubiquitin ligase, as mentioned previously. There are two structurally unrelated RING-type E3 ubiquitin ligases, Neuralized and Mindbomb that promote Notch ligand endocytosis by monoubiquination; an enzymatic post-translational regulation in which a ubiquitin protein is added to the lysine residues on target proteins (Kopan and Iligan 2009). Neuralized and Mindbomb are known to bind to the same ASN-based tripeptide stretch (NNL) on *Serrate*. While it is still being explored, the process after endocytosis to produce more active cell surface ligand is poorly characterized to date. The current models of ligand modification include clustering of the ligand, posttranslational modifications to the ligand, and recycling of the ligand into specific membrane microdomains, which will be further discussed later (Le Borgne 2006). Consequently, if Neuralized and Mindbomb were limiting, some *Serrate* could remain un-ubiquitinated and would reside for longer periods on the cell surface. This would allow increased opportunity for *cis*-interactions, although this would only be true if *cis*-inhibition occurs on the cell surface instead of intracellularly (Glittenberg et al., 2006).

The mechanisms of Notch activation by ligand endocytosis can be described by two hypothesized models so far. The *ligand-recycling* model (Nichols et al., 2007) suggests that a ligand is internalized prior to its interaction with *Notch* receptor in a ubiquitination and Epsin-dependent manner (a conserved adaptor protein for Clathrin-mediated endocytosis) and then recycled back to the surface with a modification that renders the ligand signaling-competent (Wang and Struhl 2004). Since Epsin is oftentimes not required for bulk endocytosis of DSL proteins, it implies that targeting DSL proteins to an endocytic pathway may be required for those ligands to acquire signaling activity.

The *classical endocytosis* model, on the other hand (Le Borgne 2006), suggests that the endocytosis of the ligand bound to the *Notch* receptor induces a conformational change that allows access of the metalloproteases to the S2 cleavage site on the *Notch* receptor. While *Delta* is still endocytosed in a Dynamin and Epsin dependent manner, it does not require any other endocytic/recycling factors to promote signaling (Parks et al., 2000). The proposed two mechanisms of Notch activation by the ligand are important when analyzing DSL trafficking since endocytosis activity will demonstrate how ligands are usually regulated.

Activity of modified *Serrate* ligand's *Juxtamembrane* domain

Previous studies in the Fleming lab have identified an unexpected *Juxtamembrane* (JM) domain, located extracellularly adjacent to the transmembrane domain of the *Serrate* ligand. This JM segment of approximately 65 amino acids long is essential for the activation of the *Notch* receptor (Curlin 2015; Fleming et al., 2013). These studies have demonstrated that the function of *Serrate* varies with the length of the JM segment. A 65 amino acid segment near the transmembrane domain is essential for wild-type levels of Notch activation while a 32 amino

acid segment on the JM region demonstrates only a weak activation of the Notch pathway. The focus of this thesis is to explore how the length of amino acids in the JM region of the transmembrane *Serrate* ligand affects Notch activation and *Serrate*'s effects on subcellular localization in a cell using fluorescence staining methods. The trafficking of the *Serrate* ligand is of essential focus in this study since depending on the extent of activation in Notch signaling, the ligand may be trafficked or withheld in different regions of the cell. Such locations include membrane bound trafficking by Epsin or Dynamin transmembrane proteins. It is expected that *Serrate* may face cellularly localized changes depending upon whether it functions as a Notch activating or non-activing form due to modifications in its structure.

A fluorescence staining method is utilized in the detection of *Serrate* ligands in a cell for this study. The *Serrate* ligand is tagged by a fusion protein fluorescent tag, constructed in the protein through genetic engineering, which was placed directly into the coding region of the *Serrate* constructs previously made in the Fleming lab (elaborated more in later section). A red fluorescent protein tag, tandem dimer Tomato (tdTomato), containing RFP-type termini, was utilized in the detection of *Serrate* ligands by genetically adding to the *Serrate* construct that was used in this study (Shaner et al., 2004). The tdTomato tag was used because previously developed wild-type yellow-to-red fluorescent proteins have been known to be toxic and disruptive when inserted to proteins (Lai et al., 2015) and tdTomato proved to be an accessible dye for this study's purposes. Under a fluorescent microscope, this allows easy visualization of the distribution of *Serrate* ligands throughout the cell.

As mentioned, the Fleming lab has successfully constructed *Serrate* expression vectors with different numbers of amino acid lengths in the JM region. While it was also mentioned that during *trans*-activation, the *Notch* receptors are able to facilitate the cleavage release of the

NICD in signal receiving cells to activate Notch signaling pathway, the purpose of cleaving the ligands in signal sending cells is unknown. Nonetheless, the Notch ligands *Serrate* and *Delta* are known to be extracellularly cleaved near the transmembrane domain during Notch signaling pathway (LaVoie and Selkoe, 2003). However, it was hypothesized that the cleavage of *Serrate* functions as a down regulator of the Notch signal (Curlin, 2015). In other words, truncated forms of *Serrate* and *Delta* ligands without the Transmembrane region (TM; colored yellow in *Figure 6A*) and the Intracellular Domain (ICD; colored red in *Figure 6A*) are incapable of activating Notch and exhibit dominant-negative properties (Hukriede and Fleming, 1997). In order to disrupt the normal cleaving of *Serrate, a human* Discoidin Domain Receptor (DDR) that are tyrosine kinase proteins that control the interactions between collagen and ligands, were used; specifically, the TM of DDR2 leads to no such cleavage by a metalloprotease in ligands (Fu et al., 2013). Hence, some of the vectors that the Fleming lab constructed had cDNA encoding the transmembrane and JM regions of DDR2 replacing the comparable region of *Serrate* to eliminate metalloproteinase cleavage. Such a construct would be expected to activate but not inhibit the Notch signaling pathway (Curlin, 2015; *Figure 6B*). Such vectors are employed in this study to analyze the cellular localization of noncleaved *Serrate*s that are expected to exclusively activate Notch signaling with no *cis*-inhibition.

Other vectors that have been constructed in the Fleming lab and been used in this study include: the minigene (MG), a *Serrate* construct with JM region and ELRs from 7-14 removed that experiences the loss of ability to activate Notch signaling (*Figure 6C*); the minigene 65 (MG65), a *Serrate* MG construct as above with 65 amino acids of the JM region restored. This construct retains the ability to activate and inhibit Notch signaling similar to wild type Serrate forms *(Figure 6E*); Deletion of 4 to 6 minigene 65 (D46MG65), minigene 65 with ELRs 4 to 6

removed which is known as the NIR region (Fleming et al., 2013), removal of such regions results in the loss of inhibitory functions (*Figure 6G*); *Serrate* DDR2 full length ligand without the 65 amino acid JM region (*Ser*DDR2FL-65), full length (with all ELRs) *Serrate* construct with DDR2 domain inserted along with the removal of 65 amino acids in the JM region, this construct lacks the ability to activate the signaling pathway (*Figure 6D*); DDR2 Delta (DDR2DL), a Ser MG construct with DDR2 domain inserted along with extra JM region of *Delta* in replacement of *Serrate* JM region, this construct has a normal activation property (*Figure 6F*). All the constructs were generated from the wild-type *Serrate* cDNA sequence mentioned in Fleming et al., 1990. The initial DNA used in this experiment was the full-length *Serrate* (*Figure 6A*) *Bsp tom* DNA in *pUAST attB* vector used for transformation into *Drosophila melanogaster* (Biscof, et al. 2007).

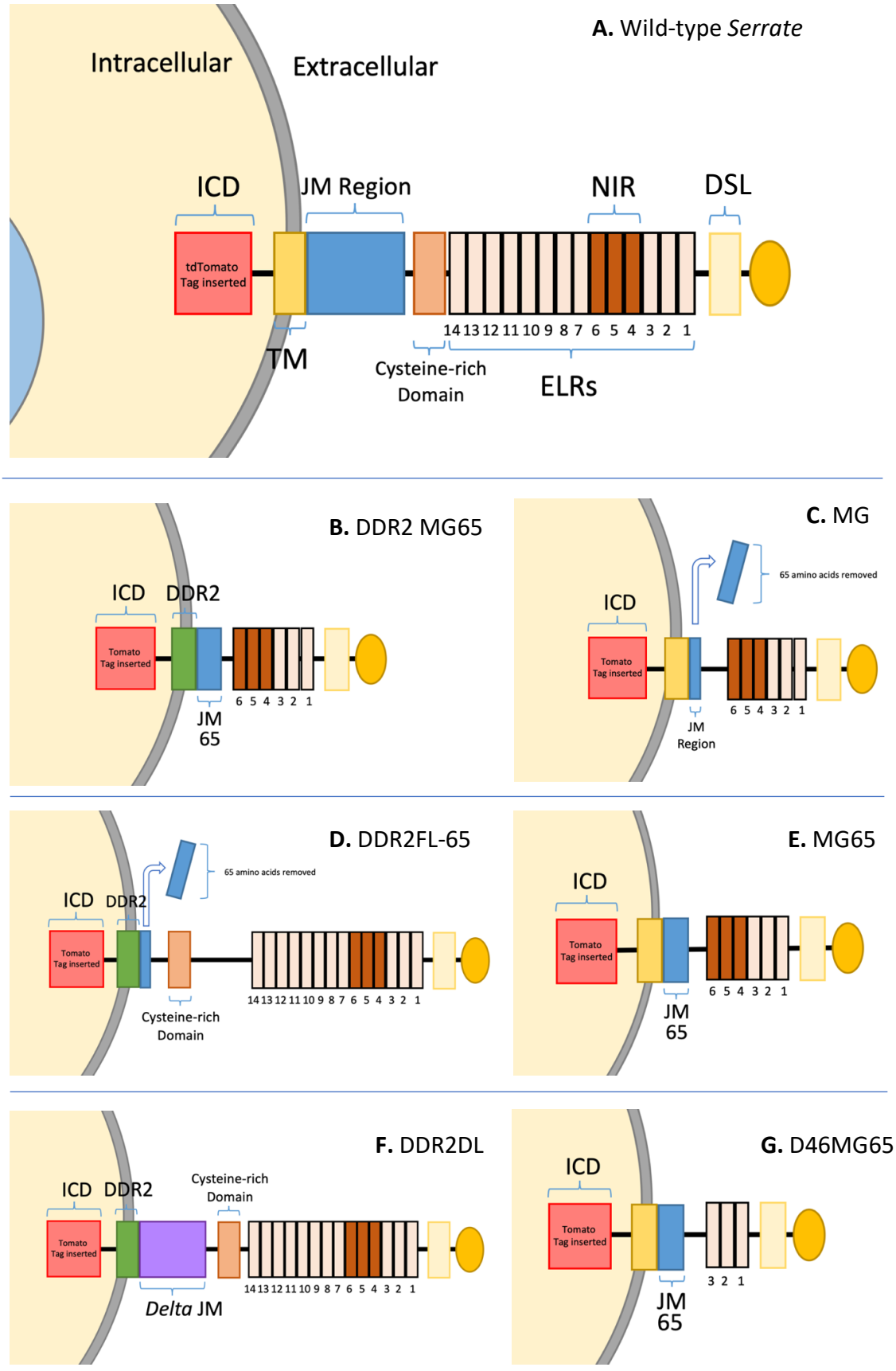

A. Wild-type Serrate
Intracellular
Extracellular
ICD
JM Region
NIR
DSL
tdTomato
Tag inserted
14 13 12 11 10 9 8 7 6 5 4 3 2 1
TM
Cysteine-rich
Domain
ELRs
B. DDR2 MG65
ICD
DDR2
Tomato
Tag inserted
6 5 4 3 2 1
JM
65
C. MG
65 amino acids removed
ICD
Tomato
Tag inserted
6 5 4 3 2 1
JM
Region
D. DDR2FL-65
65 amino acids removed
ICD
DDR2
Tomato
Tag inserted
14 13 12 11 10 9 8 7 6 5 4 3 2 1
Cysteine-rich
Domain
E. MG65
ICD
Tomato
Tag inserted
6 5 4 3 2 1
JM
65
F. DDR2DL
ICD
DDR2
Cysteine-rich
Domain
Tomato
Tag inserted
14 13 12 11 10 9 8 7 6 5 4 3 2 1
Delta JM
G. D46MG65
ICD
Tomato
Tag inserted
3 2 1
JM
65

Figure 6. Different constructs of *Serrate* ligands made in the Fleming laboratory. A. Wild-type *Serrate:* ligand with an extracellular domain (ECD) consisting of Delta/Serrate/Lag-2 (DSL) domain, 14 epidermal growth factor-like repeats (ELRs) similar to the ones on *Notch* receptors, Notch inhibitory region (NIR) located at the 4^{th}, 5^{th} and 6^{th} ELRs, a *Juxtamembrane* region (JM) and a Cysteine Rich Domain. Located on the cell membrane is the transmembrane region (TM) and in the intracellular region is the intracellular domain (ICD) with tdTomato tag inserted. B. DDR2 Minigene 65: TM Discoidin Domain Receptor 2 (DDR2) in replacement of the TM region to hinder the metalloproteases cleavage in *Serrate* minigene 65 construct (65 Amino acids 'AAs'; *Figure 6E*). C. *Serrate* minigene construct with only 14 amino acids in the JM region (65 amino acids removed) and ELRs 7-14 removed. D. DDR2 Full Length without 65 amino acids in JM region: wild type full length *Serrate* construct with DDR2 in replacement of the TM region and the JM region missing the essential 65 amino acid JM regions. E. Minigene 65: *Serrate* minigene (*Figure 6C*) with 65 amino acids in the JM region restored. F. DDR2 *Delta*: *Serrate* DDR2 not full length – only ELRs 1-6 like the minigene construct (*Figure 6D*) construct with DDR2 in replacement of the TM region and *Delta* JM region in replacement of the *Serrate* JM region. G. Deletion of 4-6 ELRs Minigene 65: *Serrate* minigene 65 (*Figure 6E*) construct with ELRs 4 to 6, known as the NIR region removed.

The red tdTomato tag was constructed in the known active, inactive or partially active (*Figure 6*) forms of different vector constructs of *Serrate* as mentioned above. Independent of the property of the specific constructs of the vector, the *Serrate* ligands were observed within the cells of the salivary gland of *Drosophila melanogaster* when expressed by the promoter of the

gene *patched (ptc).* The *Drosophila* salivary gland is a simple tubular organ derived from an adjoining epithelial primordium, which is established by the activities of the homeodomain-containing proteins (Haberman 2003). Due to its tubular organ development, imaginal rings that are larval tissues composed of progenitor cells are essential for the formation of salivary glands (Yang and Deng 2018). It is also evident that both *trans*-activation and *cis*-inhibition between the *Serrate* and *Notch* receptor control Notch activation in the imaginal ring (Yang and Deng 2018) which makes the salivary gland ideal for the localization of *Serrate* constructs within the cell. The size of the salivary gland (*Figure 7*) cells also makes it attractive for the subcellular localization of *Serrate* compared to cells of other organs which exhibit *Serrate* induced Notch signaling such as the wing imaginal discs (Dye et al., 2017).

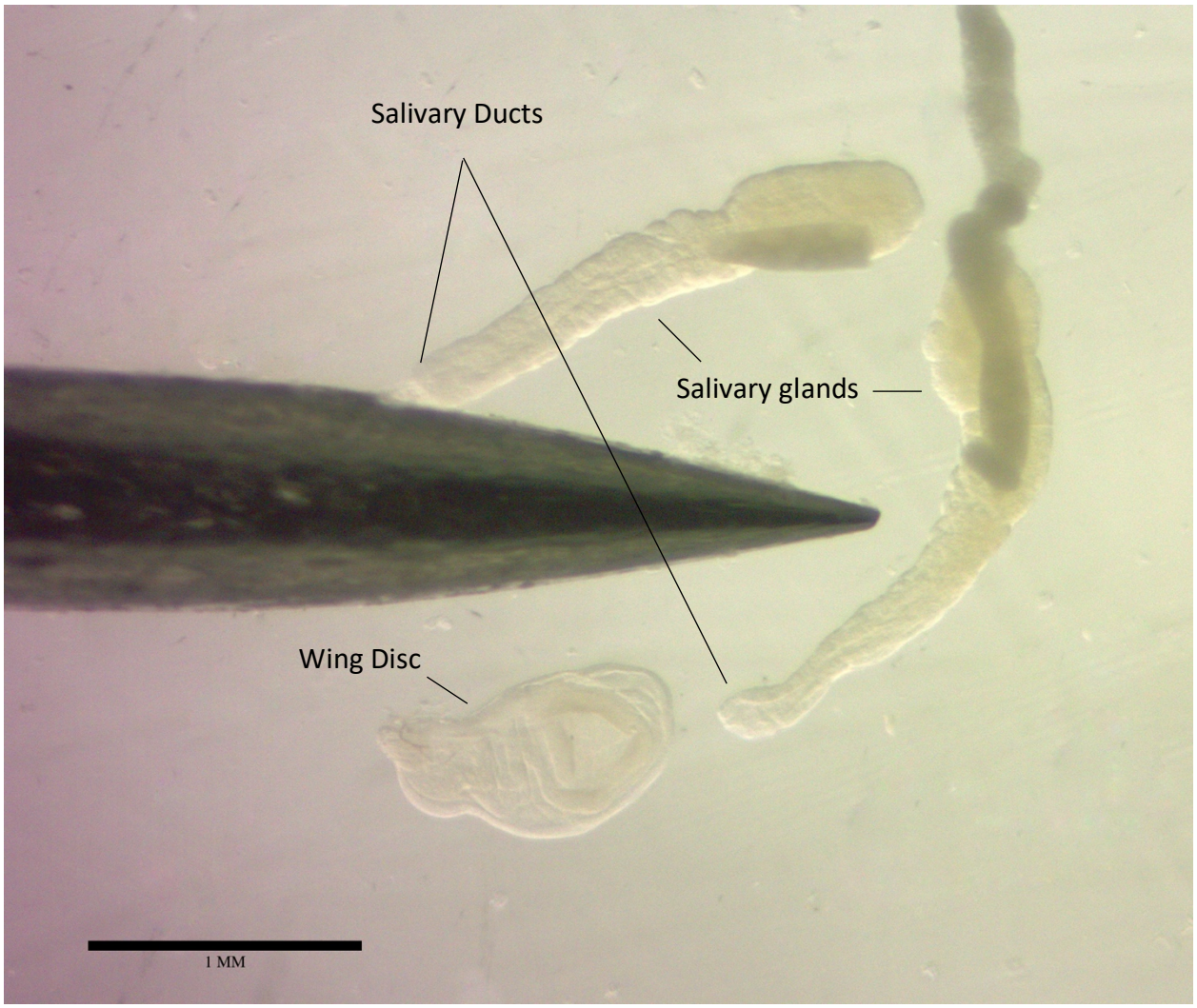


Figure 7. Comparison sizes of *D. melanogaster* salivary glands and wing disc. This figure shows the size differences of two salivary glands (each with approximately 100 cells) with a duct attached to each of the glands (~2mm) and a wing disc composed of approximately 50,000 cells (~1mm) next to a tip of a common pin.

MATERIALS AND METHODS

Drosophila Culture and Strains

For our genetic crosses, we utilized cornmeal, dextrose, yeast food media (Fleming et al., 2013; Hinz et al., 1994) as a food source to maintain the crosses. *D. melanogaster* were all grown in an incubator set to 18°C. Transgenic expression lines were produced by crossing UAS-Ser* lines (where * indicates different modified forms of the *Serrate* protein) with a *Gal4*-expressing *patched* (*ptc*) gene promoter (*ptcGal4*). The *patched* promoter expresses in a stripe crossing the dorsal and ventral compartments in the developing *Drosophila* wing disc and expresses strongly in the salivary gland (Hinz et al., 1994). The combination of the UAS-Ser* and *ptcGal4* constructs causes the expression of UAS-Ser* in the *ptc* promoter pattern.

Construction of *Serrate* DNA vector.

All the constructs (*Figure 6*) that were used in the study were generated from the wild-type Ser cDNA sequence (Fleming et al., 1990) and each of the constructs was placed in the pUAST attB transformation vector (Brand and Perrimon, 1993). The tdTomato tag (Shaner et al., 2004) is located in the intracellular domain of the *Serrate* coding region (Fleming et al., 2013).

Additionally, for all the constructs that were prepared for subcellular localization of *Serrate,* each of the samples were randomized and were observed blindly in order to contrast the extension of activation in correlation to the number of amino acids present for each of the constructs. In other words, although there were specific predictions that were made for each of the constructs previously, to minimize biases and fabricated assumptions, the samples were observed without any indication of what the actual constructs were.

Genetic crossing and generation of expression lines

The specific expression crosses performed for this study are summarized below:

1. ptcGal4/ptcGal4 x MG65 B72/B72 on II
2. ptcGal4/ptcGal4 x DDR231 B9/B9 II
3. ptcGal4/ptcGal4 x D46MG65 C31/C31 III
4. ptcGal4/ptcGal4 x DDR279 A9/A9 II
5. ptcGal4/ptcGal4 x SerDDR2FL-65 A5/A5 II
6. ptcGal4/ptcGal4 x DDR2Dl A9/A9 II
7. ptcGal4/ptcGal4 x Bsp Xho A3/A3 II (wild-type; constructed previously in Fleming lab)

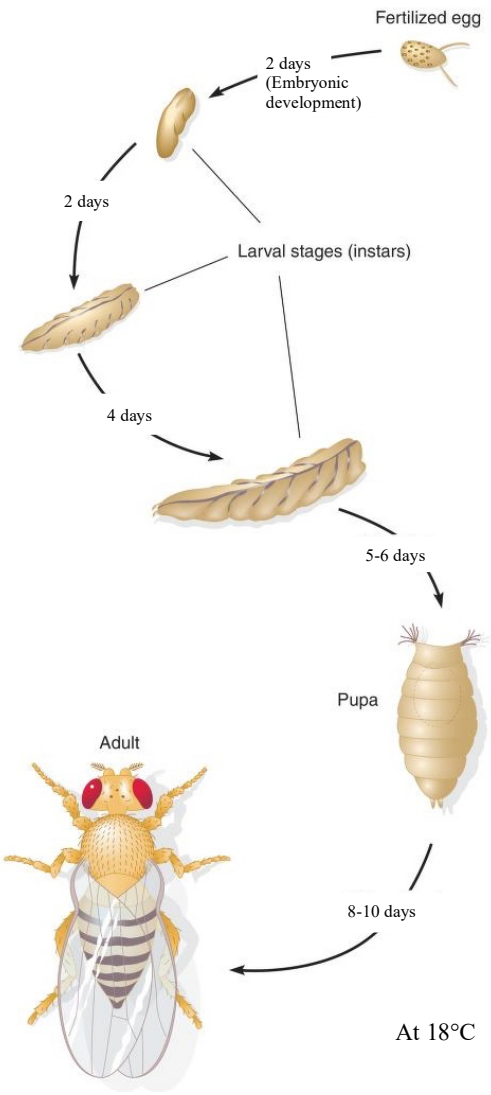
The DDR231 is a *Serrate* construct containing the DDR2 TM domain with an additional 31 amino acids inserted immediately adjacent to the TM and activates but not inhibit Notch signaling. The DDR279 is similar to DDR231 but with an additional 79 amino acids added instead of 31 amino acids. All other constructs are same as the construct explained previously in Introduction.

When the crossing was completed the constructs were grown in the food culture at 18°C until the larvae were ready for dissection to isolate the salivary glands. Each of the germ line batches were identified as *unknowns* in order to not disclose the actual crossing.

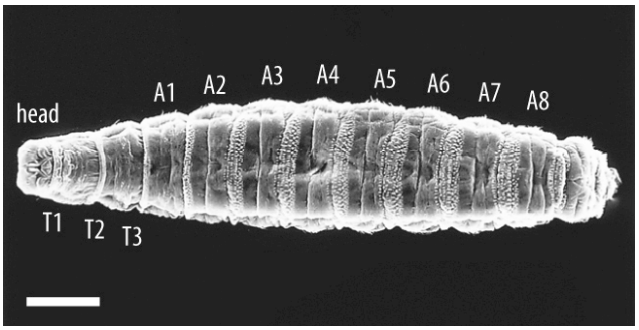
Preparation of specimen and clearing agents.

The egg of *Drosophila* hatches to a larva around 24 hours post-fertilization when incubated at 25°C. The larvae would undergo three molts taking about 3 days after which would be called a pupa (Russell 2010). However, when incubated at 18°C their growth slows down by 50% taking about 5 to 6 days until the larvae grows up to be pupa. The larvae have 3 thoracic

segments and 8 abdominal segments from the head during its third stage of larval development (Gilbert 2000). Larvae at the third stage of their development were carefully selected from each of the *unknown* batches and dissected around the A6 abdominal segment (*Figure 9*) of the larvae. The head portion of each larva was turned inside out to increase accessibility of the internal tissues to which the clearing agent is exposed. Clearing agent allows better observation of the salivary gland (located around the A3 abdominal segment of the larvae) in the prepared specimen under a fluorescent microscope.



(*Left*) *Figure 8.* Life cycle of *Drosophila melanogaster* from a fertilized egg to an adult at 18°C (Russell, 2010; *modified*). After fertilization, the egg would go three instar larval stages of molts during six days of period to become a pupa at 18°C which would develop up to an adult *Drosophila*.



(*Above*) *Figure 9.* Segmental morphology of a larval during the third instar larva stage of its development (~4 days post-fertilization. Staveley, 2019). The larval stage during dissection is processed.

In order to observe the specimen as clearly as possible different types of clearing agents were prepared to allow enhanced visualization of the salivary glands once isolated. Hence, *Clear*^{T} and *Clear*^{T2} (Kuwajima et al., 2013) clearing reagents were made. For *Clear*^{T}, 20%, 40%, 80% and 95% formamide solutions were made by adding formamide to deionized water (vol/vol). For *Clear*^{T2}, a 50% formamide/20% polyethylene glycol (PEG) solution was made by mixing formamide (as made for *Clear*^{T}) with 40% PEG/H2O (wt/vol) at a ratio of 1:1 (vol/vol). A 25% formamide/10% PEG solution was made by mixing 50% formamide plus 20% PEG/H2O (wt/vol) at a ratio of 1:1 (vol/vol). A 40% PEG solution was made by stirring powdered PEG

1450 MW (Sigma) in deionized water for 30 minutes and is stable at room temperature for several months (Kuwajima et al., 2013). Tissue incubation times for each of the diluted solution were 30 minutes for 20% formamide, 30 minutes for 40% formamide, 2 hours for 80% formamide, and 5,6 hours for 95% formamide in *Clear*^{T} for desired transparency, this methodology is referred to as a serial dilution method throughout this paper. For *Clear*^{T2}, 1 hour for 25% formamide/10% PEG and 5-6 hours for 50% formamide/20% PEG (Kuwajima et al., 2013).

Another clearing agent that was utilized in this study was glycerol. Different dilution series were made in order to gradually clear the salivary glands. This was done by preparing 20%, 40%, 80%, and 100% glycerol solutions by adding glycerol to deionized water. The isolated larval heads including the salivary glands were incubated in the glycerol solutions for 30 minutes in each 20% glycerol, 40% glycerol, 80% glycerol solutions sequentially and finally for 1 full day in 100% glycerol. To improve the clearing of the tissue and to inhibit endogenous peroxidases, 10mM Sodium Azide (NaAzide) was used in the preparation of 20% glycerol's diluting instead of deionized water. Each of the clearing agents was compared in terms of its clarity, clearing ability, and resolution of the *Serrate* fluorescence.

Each of the *unknown* salivary gland samples was observed under Nikon® Eclipse E600 with U-III Film Camera System to compare and contrast each of the clearing agents and the activity of each construct that was made. The image acquisition, analysis, and visualizations were all processed using the NIS-Elements d 4.40.00, Nikon's universal software platform. The characterization of membrane localization of *Serrate* is identified when *Serrate* fluorescence along the membrane. The intracellular *Serrate* localization is observed when *Serrate* fluorescence among the cytoplasm.

RESULTS

Glycerol is a rapid tissue clearing agent

In an attempt to find the best clearing agent for the preparation of the salivary gland to view the *Serrate* proteins under the fluorescent microscope, the resolution, and the accessibility to view each gland was compared using different clearing agents. The *Clear*^{T2} clearing reagent exhibited better clearing effects and defined resolution of the *Serrate* fluorescent lighting than *Clear*^{T} reagent. This was readily seen when the salivary glands were incubated in the diluted reagents series with gradual increases in clearing reagent concentration (Figure not shown since the tdtomato tag in Serrate did not fluorescent in *Clear*^{T}). Contrastingly, the *Clear*^{T2} reagent did not exhibit better clearing effects when compared to glycerol clearing. When the salivary gland with the Minigene-65 (MG65) and Nominal minigene (NomMG; 32 amino acids long with partial activation) construct expressing cells were incubated in glycerol for 24 hours, they exhibited brighter fluorescence and finer resolution of the *Serrate* fluorescence when compared to *Clear*^{T2} (*Figure 10*).

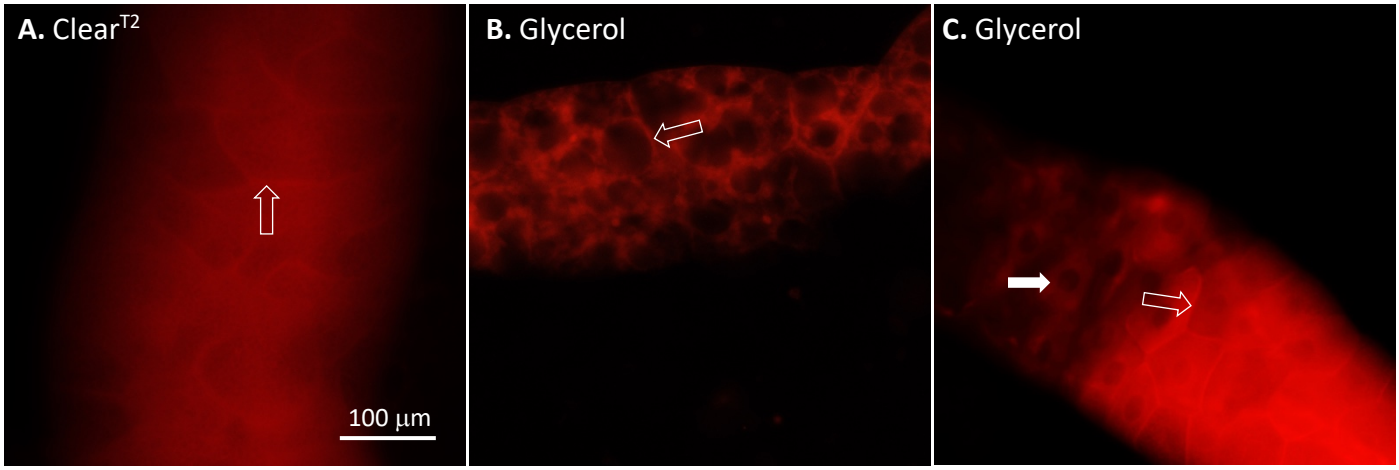


Figure 10. Fluorescent microscope view of salivary gland with *Serrate* protein expressing the MG65 and NomMG construct. The fluorescent *Serrate* proteins are displayed partially inside the cell (*solid arrow*) and mostly on the cell membrane (*open arrow*). Salivary gland incubated in: **A.** serial dilution of *Clear^{T2}* clearing agent with different incubation period (MG65) where *Serrate* is located mostly on the cell membrane (*open arrow*); **B.** Glycerol clearing agent for a day (NomMG) where *Serrate* is exclusively on the cell membrane (*open arrow*); **C.** Glycerol clearing agent for a day, showing a different region of a salivary gland compared to B (MG65).

MG65 construct expressing cells are known to activate *Notch* signaling and fluorescence microscopy reveals that cells display fluorescent Serrate mostly on the plasma membrane (Figure 10A). On the other hand, the NomMG construct expressing cells are known to exhibit partial activation of the Notch signaling pathway, yet display fluorescent *Serrate* on both the cell membrane and in the cytoplasm (*Figure 10B)*. When the salivary glands were incubated in 100% concentrated (no dilution) glycerol reagent for a day, the gland cells became distorted with water moving out of the cells rapidly (*Figure 10B*).

When the salivary glands were gradually incubated in serial dilutions of glycerol reagent the resolution and the accessibility to view each gland increased (*Figure 11*) without distortions as was seen in glands incubated in 100% concentrated glycerol for a day (compare *Figure 10B* with *Figure 11B* and *C*).

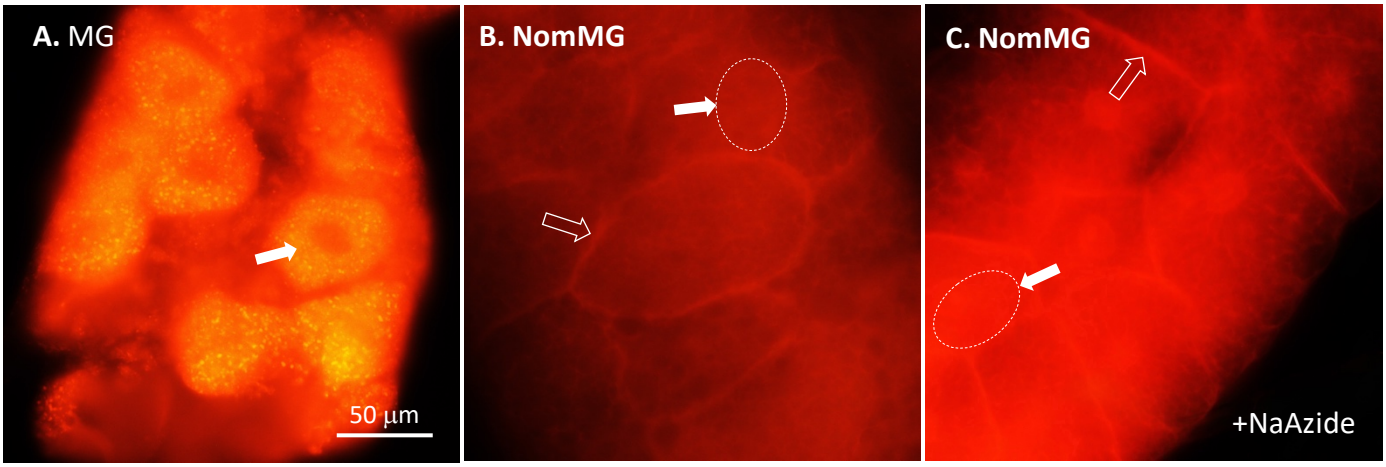


Figure 11. Fluorescent imaging of salivary glands incubated in serial dilution of glycerol clearing agents in different incubation periods. **A.** Minigene (MG) construct expressed salivary gland cells with *Serrate* proteins located inside the cell (*solid arrow*). **B.** Nominal MG (NomMG) construct expressed cell with *Serrate* proteins located on both the cell membrane (*open arrow*) and cytoplasm (*solid arrow* highlighting *the dashed line area*). **C.** Nominal MG construct same as B, Improved fluorescence of *Serrate* located on both membrane (*open arrow*) and cytoplasm (*solid arrow* highlighting *the dashed line area*), when Sodium Azide (NaAzide) was added only to the first dilution series (see Materials and Methods).

Different construct vectors display varied traffic patterns

The MG construct expressing cell does not have the ability to activate Notch and fails to show localization of *Serrate* protein at the membrane (*Figure 11A*), instead *Serrate* is mostly located inside the cell (cytoplasm). The cause of accumulated *Serrate* in the cytoplasm is unknown. These cells would not be expected to activate the Notch signaling pathways without *Serrate* located on the cell membrane for *trans*-activation. The NomMG construct expressing cells have the minimum number of amino acids to activate Notch, and it displayed fluorescent *Serrate* both on cell membrane and inside the cell (*Figure 11B*). Having *Serrate* located on the cell membrane allows the cells to activate Notch. The localization of *Serrate* in this construct is very similar to the same construct expressing cells incubated in glycerol for a day (*Figure 10C*) and gradually incubated samples did not exhibit distortion as seen in cells incubated in glycerol for a day. When the salivary gland samples were incubated in glycerol with NaAzide as a diluent, the fluorescent *Serrate* displayed brighter fluorescing effects (*Figure 11B and C*). These results show that incubation of the samples in serial dilution of glycerol exhibit a brighter and finer resolution of the cells under fluorescent microscope.

When the samples with different construct expressions (1-7) were incubated in serial concentrations of glycerol, they showed a clear resolution view of the gland cells with different localization of *Serrate* proteins (*Figure 12A*).

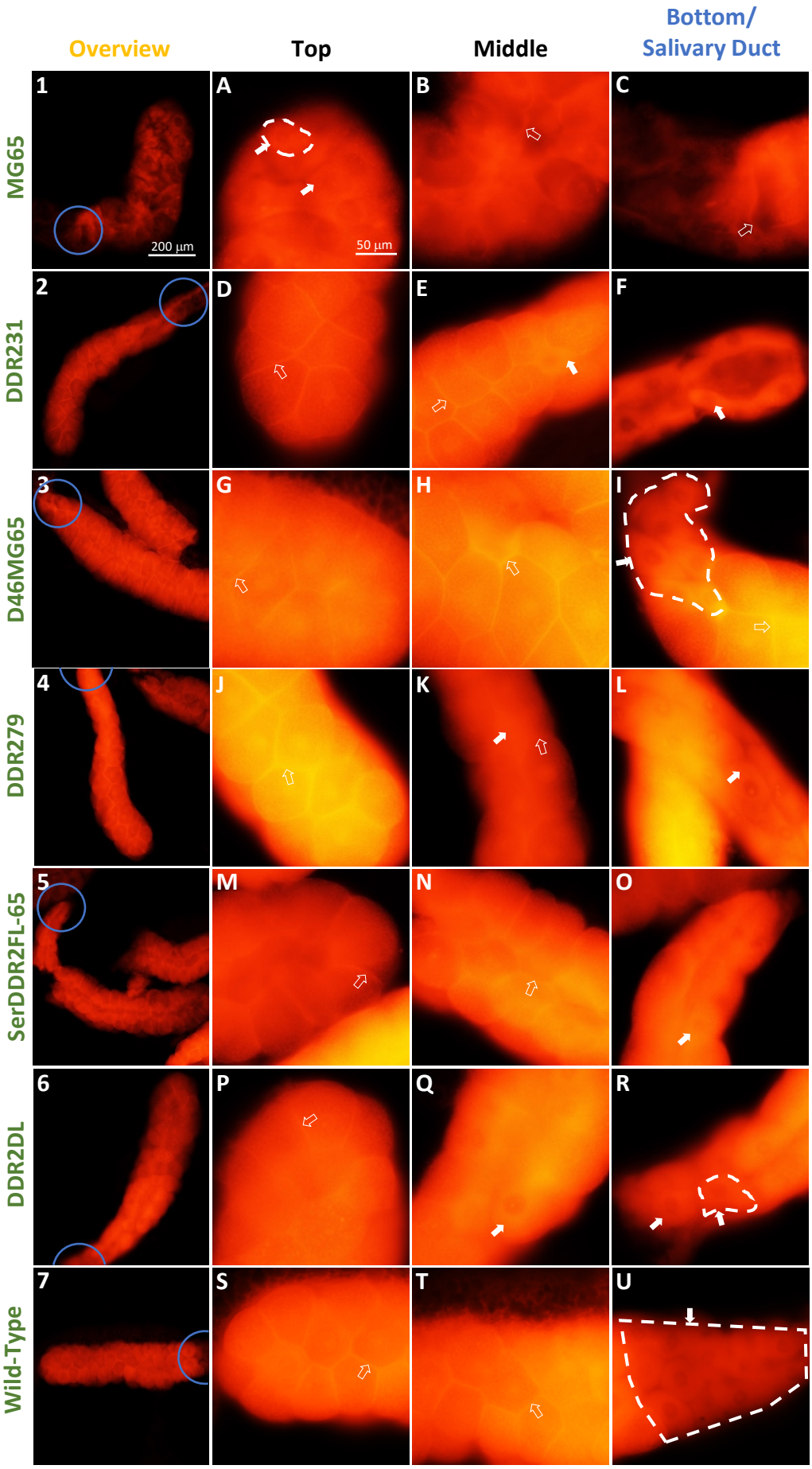

Overview
Top
Middle
Bottom/
Salivary Duct
MG65
DDR231
D46MG65
DDR279
SerDDR2FL-65
DDR2DL
Wild-Type
1
A
B
C
2
D
E
F
3
G
H
I
4
J
K
L
5
M
N
O
6
P
Q
R
7
S
T
U
200 µm
50 µm

Figure 12. Subcellular localization of *Serrate* in salivary gland cells expressing different constructs. Each row demonstrates the expression of different constructs (*green text*), depicting the cells expressing the constructs, from top to bottom, MG65 (1A-C), DDR231 (2D-F), D46MG65 (3G-I), DDR279 (4J-L), SerDDR2FL-65 (4J-L), DDR2D1 (6P-R), and Wildtype (7S-U). Each row demonstrates different viewing areas of the salivary gland: the first column (from left to right; *yellow text*) displays the overview of the whole gland while the second, third, and fourth columns display the top, middle, and bottom (salivary duct; *blue open circles*) areas of the overview of gland, respectively. The *open arrow* (⇨) illustrates *Serrate* located on the cell membrane while the *solid arrow* (➡) highlighting *the dashed line area* (◌) illustrates *Serrate* located inside the cell. The MG65 (A-C) construct expressing cells shows unexpectedly almost no localization of *Serrate* located on the cell membrane, hence it should be unable to activate *Notch*. The *dashed line area* highlighted by the *solid arrow* in A illustrates one of the cells where *Serrate* is located inside the cell in MG65. The DDR231 (D-F) construct containing the DDR2 is expected to have *Serrate* located mainly on the cell membrane, which is the case as seen in D and E, with the possibility of intracellular *cis*-inhibition of *Serrate*. The D46MG65 (G-I) construct is expected to have *Serrate* located almost exclusively on the cell membrane, which is the case (G and H) except for the salivary duct/bottom area (I). The *dashed line area* in I illustrate group of cells in the salivary duct area where *Serrate* is located inside the cell. The DDR279 (J-L) construct is the same case with DDR231 with *Serrate* located on the cell membrane but more abundantly in the cytoplasm. The SerDDR2FL-65 (J-L) construct is expected to have *Serrate* located inside the cell but this isn't necessarily the case since considerable quantity of Serrate is located on the cell membrane. The DDR2DL (P-R) construct is expected to have no *Serrate* localization since *Delta*

ligand was constructed and there is no restrictive area where Serrate should be located which is the case in images **P** and **R**. The *dashed line area* here highlighted in **R** illustrates one of the cells that has *Serrate* is diffusely located inside the cell. The Wild-type (**S-U**) construct should exhibit similar localization of *Serrate* as the MG65 construct (**A-C**), this is not the case. Regardless of the constructs, the gland duct area (bottom) exhibit more intracellular localization of the *Serrate,* illustrated by the *dashed line area* where group of cells have *Serrate* located inside the cell. Compared to the top and middle portion of the gland. All images are adjusted to 20% less bright than the images in *Figure 11 and 10* in post-production*,* due to their high fluorescence level.

There are different intensities of fluorescent *Serrate* in different areas of the cell when different constructs are expressed. Comparing the location of *Serrate* in a wild-type construct expressing cell (*Figure 12.7S-U)* to cells expressing other different constructs, the different regions of where *Serrate* is located can be seen. In the example of the wild-type construct, *Serrate* is located mainly on the cell membrane (*Figure 12.7 S and T; open arrow*) compared to the cytoplasm of the salivary gland cells. However, this does not hold true for the salivary duct cells, where *Serrate* is mainly located within the cell (*Figure 12.7 U; solid arrow with dashed line area*). The MG65 construct, (*Figure 12.1*) that has the minimum number of amino acids in the JM region capable of activating Notch, should have *Serrate* located on the membrane very much similar to the wild-type construct. However, this is not the case in the cells of the salivary gland or the salivary duct (*Figure 12.1 A, B; solid arrows*). Both DDR279 (*Figure 12.4*) and DDR231 (*Figure 12.2*) constructs are incapable of fully activating Notch with the DDR2 JM domain regions added. The, DDR279 is neither capable of activating nor inhibiting Notch. Both of these constructs have almost no effect on Notch when they are expressed. Unexpectedly, both

of the constructs have *Serrate* mainly concentrated on the cell membrane (*Figure 12.2 D, E and Figure 12.4 J, K; open arrows*). The DDR279 expressing cells exhibit much brighter fluorescent effect compared to DDR231. Following up, the SerDDR2FL-65 (*Figure 12.5*) construct should have much less definite localization of *Serrate* on the cell membrane but have *Serrate* mainly inside the cell due to its Notch non-activating ability with 65 amino acids removed from the JM region, and this is partially exhibited in the cells of salivary glands (*Figure 12.5 N; open arrow*). The D46MG65 (*Figure 12.3*) construct, which has lost the overall ability to inhibit *Serrate*, should have the most distinct localization of *Serrate* on the cell membrane out of all the constructs. However, this is not necessarily the case with few distinct areas show *Serrate* located on the cell membrane (*Figure 12.3 G-I; open arrow*) especially the middle range cells of the salivary gland (*Figure 12.3 H; open arrow*). Finally, the DDR2DL (*Figure 12.6*) construct with DDR2 TM and *Delta* JM region replacing the corresponding region of *Serrate* acts very similar to DDR231 where it cannot activate Notch. In this construct, *Serrate* is located less distinctly when compared to the other constructs where the protein is almost equally located both the membrane (*Figure 12.6 P; open arrow*) and inside the cell (*Figure 12.6 Q, R; solid arrow*).

DISCUSSION

A number of different adjustments had to be made for the specifics of our experimental setup to achieve the most definitive results. The constructs, that were prepared in the Fleming laboratory for observing the *Serrate* localizations, included heat shock (HS) promoters which are specific promoters that regulate the expression of the heat shock proteins (Sorger, 1991). The HS promoters are transcriptional activators of heat shock genes (Clos et al., 1990). The pUAST transformation vector, which was used for the constructions of different *Serrate* vectors for this

experiment, use the HS promoter since it useful for regulating transgene expression. The promoter easily responds to temperature where higher temperatures lead to higher levels of promoter expression. Hence, *Drosophila* cultures, with cells expressing such constructs, are incubated in a controlled, low temperature thermal environment of 18°C to keep the promoters less active in order to generate a low level of the expressed *Serrate* during embryogenesis when high levels of Serrate activity generate lethality. The modified temperature also makes the development stage of the larvae twice as long. One of the beneficial side effects of slow development in larvae is the bulkier body composition of larvae due to its constant consumption of a food source whilst having delayed growth. This is favorable since bulky larvae make the salivary gland accessible to isolate from the larvae during its third stage of development. The salivary gland is gradually cleared to be viewed under fluorescent microscope for a successful localization of *Serrate*.

Different clearing agents were prepared to allow clearing of the gland and support enhanced visualization once isolated. This is essential since biological specimens are intrinsically three dimensional, hence, they can have obscuring effects of light scatter imaging deep into a tissue volume which can be problematic for visualizing the details of labeled proteins and specific pathways leading to Notch activation (Tainaka et al., 2014). The clearing reagents that were prepared (Clear^{T}, Clear^{T2}, and glycerol) have the overall ability to clear or render tissue transparent, making fluorescent visualization marked for the *Serrate* activity easy to locate within the cells of salivary glands and salivary ducts to attain high microscopic resolution for the images (Richardson and Lichtman, 2016).

While Clear^{T} and Clear^{T2} were successful clearing agents for observing neuronal tissue (Kuwajima et al., 2013), which has denser tissue content compared to salivary glands, glycerol

was the best clearing agent for our purposes. Glycerol has a proficient ability to clarify tissues as noted by other clearing analyses (Richardson and Lichtman, 2016). However, glycerol solutions can be difficult to work with because of their high viscosity (Segur and Oberstar, 1951). Because of this, serial dilutions were prepared when clearing tissues. The serial glycerol method, incubating salivary glands in series of different diluted concentrations at specific incubation periods, had the most successful clearing effect overall. This was especially the case because incubating salivary glands in single incubation period at 100% glycerol made the cells distort significantly, making it particularly difficult to localize *Serrate* in a cell.

Sodium Azide (NaAzide) was chosen as the dilution solution for the glycerol due to its ability to inhibit oxidative phosphorylation via inhibition of cytochrome oxidase, the final enzyme in the mitochondrial electron transport chain, resulting in a rapid depletion of intracellular ATP (Tsubaki and Yoshikawa, 1993; Harvey et al., 1999). Having NaAzide as the initial dilutor of glycerol, not only maintained the osmotic activity of the cells in salivary gland, but also minimized the activity of oxidative phosphorylation taking place in the cells.

A series of complex genetic alterations usually controls the Notch ligand and receptor activities which ultimately signifies the fate of one cell that communicates with another. It is well established that cells utilizing the Notch signaling pathway to communicate with each other express both the receptor and a ligand simultaneously (Sprinzak et al., 2010). Hence, whether a cell becomes a signal sender, or a signal receiver can be a crucial part of its fate and cell decision making. The ratio of the expressed number of receptors and ligands in a cell can guide the developmental fate of the cell and signify the *cis* and *trans* interactions of ligand-receptor (Bray 2016). The models that describe Notch activation by ligand endocytosis and the importance of *cis* and *trans* interactions for the development of Notch signaling pathway from different cell

decisions has been the main subject of this study. In this study, we expressed *Serrate* constructs at very high levels using the *ptc* promoter, allowing us to directly compare the effects of each construct with its cellular localization. This in turn, helps us predict the mechanism of *Serrate* interaction to allow Notch signaling.

As previously implied for the Notch signaling pathway, the extent of success in activation is correlated to the localization of *Serrate* that is directed through the different constructs that is designed in Fleming Lab. The specific constructs that express the ability to activate Notch is expected to have *Serrate* located on the cell membrane allowing *trans*-activation. On the other hand, the specific constructs that do not activate Notch may very well have *Serrate cis*-inhibited intracellularly before it being localized on the cell surface or have *Serrate* inactivated through *cis*-inhibited. Through the data that we gathered, it is shown that *Serrate* constructs that lack the ability the activate Notch signaling are still able to express *Serrate* on the cell membrane surface. This finding is consistent with the model of a *ligand-recycling* mechanism (Nichols et al., 2007). In this model, a ligand is initially expressed on the cell membrane and later internalized for a modification that renders the ligand signaling-competent prior to its interaction with the *Notch* receptor (Wang and Struhl, 2004). This, however, does not rule out the possibility of the *classical endocytosis* model (Le Borgne, 2006) of Notch activation since the change in the structure of *Serrate* in these constructs could alter the range of activation for *Notch* (Whiteman et al., 2013). In other words, the cell surface *cis*-inhibition of *Serrate* can be induced in *classical endocytosis* model case where, despite the fact that *Serrate* is located on the cell surface, it still may not be able to activate Notch signaling due to an undiscovered restriction of the ligand from an alteration of its structural base.

The *ligand-recycling* model is well supported through results observed from SerDDR2FL-65 construct (*Figure 12.5*), a modified construct with the 65 amino acids removed from the JM region in the full-length construct, and a DDR2 region added to create non-cleaving effect on *Serrate*. This construct was expected to have *Serrate* located almost exclusively inside the cell, however this is not the case in our results (*Figure 12.5 H; open arrows*). The reason for having *Serrate* located on the cell surface with such construct can be explained through either the *ligand recycling* model, or the *classical endocytosis* model with the exception of having *Serrate* changed in its structural base hence imposing a constraint on Notch activation (Nichols et al., 2007; Le Borgne 2006)

On the other hand, the D46MG65 (*Figure 12.3*) construct has lost all inhibitory ability while retaining full ability to activate Notch, hence, was likely to have *Serrate* located almost exclusively on the cell membrane. Our data are consistent with this implication where *Serrate* is distinctly located on the cell membrane (*Figure 12.3 G* and *H; open arrow*) with the exception of the salivary duct/bottom area (*Figure 12.3 I*) where *Serrate* is inside the cell. The distinct distribution of *Serrate* location in the different areas of the gland (top and bottom areas of the gland show different localization of *Serrate*) is also observed across other construct expressing cells, including the Wild-type (*Figure 12.7*). The Wild-type construct expressing cells have *Serrate* located mostly in the cell membrane (*Figure 12.7 S* and *T*) of the salivary gland upper region but is contrasted in the salivary duct area cells where *Serrate* is located inside the cell (*Figure 12.7 U*). Since Wild-type exhibits such trends, other constructs can also be expected to exhibit similar trends as seen in the case of D46MG65. Having *Serrate* located on the cell membrane could initiate the *trans*-activation of Notch signaling without any *cis*-inhibition to hinder the pathway. This observation is consistent with the expected *classical endocytosis* model,

which suggests that the endocytosis of the ligand bound to the *Notch* receptor induces a conformational change that allows access of the metalloproteases to the S2 cleavage site on the *Notch* receptor (Le Borgne 2006), ultimately activating Notch signaling.

The DDR231 (*Figure 12.2*) construct expression contains the DDR2 region, with 31 amino acids added immediately adjacent to the membrane domain. This construct is known to have specific properties such as not fully activating Notch signaling due to the trimming of the JM region in *Serrate.* This means, since the ligand is unable to activate Notch, it would exhibit strong intracellular *cis*-inhibition where Serrate is located primarily inside the cell. This is not the case from our data (*Figure 12.2 D* and *E*) in which *Serrate* is clearly located on the cell membrane (*open arrow)*. Contrary to the D46MG65 construct, the DDR231 construct supports the *ligand recycling* model, where the ligand must be endocytosed from the cell surface to be *recycled* to allow modification of the ligand for successful Notch signaling.

Similar to the DDR231 construct, the DDR279 (*Figure 12.4*) construct has the DDR2 region to prohibit extracellular cleavage but conversely has 71 amino acids added immediately adjacent to the membrane domain instead of 31 amino acids, which should allow DDR279 to fully activate Notch. However, DDR279 has neither the ability to activate nor the ability to inhibit Notch. Our data demonstrate *Serrate* localized on the cell membrane, even more so than DDR231. The fluorescent effect of *Serrate* is especially brighter in DDR279 compared to DDR231, (Figure 12.4 and 12.2) suggesting that having longer amino acids in the JM region possibly allow the cells to have more *Serrate* located on the membrane. While it is interesting for a construct that fails to interact with Notch at all to still have *Serrate* located on the cell surface, it implies that localization of Serrate is not the only agent involved in Notch activity. Undiscovered properties of *Serrate,* therefore, may be the main operator for Notch activity.

The DDR2DL construct (*Figure 12.6*) with the DDR2 JM region replaced by *Delta* sequences instead of *Serrate* allowed us to contrast the activation effects of *Delta* for *Serrate* in Notch activation. Compared to the other constructs, the DDR2DL construct does not have any effect on Notch activity, whether that is negatively or positively. Hence, analyzing the result of this construct is crucial for understanding the mechanism of *Serrate* localization in regard to Notch activation. In this case, the construct did not have a distinct localization of the *Serrate* on the membrane, while there were some observable regions with membrane localization (*Figure 12.6 P; open arrow*). However, in the majority of the regions of the gland, the distribution of *Serrate* was indistinguishable between the cell surface and the cytoplasm with some incident of intracellular localization (*Figure 12.6 Q* and *R; solid arrow*).

Another possibility for the extended explanation for *Serrate'*s unexpected localization is segmented structure, or the missing domain of the *Serrate*. The red tdTomato tag, the main trigger for the fluorescent property of *Serrate*, is located on the intracellular domain (IC) adjacent to the transmembrane domain (TM) of *Serrate*. However, we do not have an absolute confirmation that any of the constructs that fail to interact with Notch are intact. Therefore, whereas the TM and IC domains with tdTomato tag might be intact, we cannot confidently state that the extracellular, Notch interacting domain remains associated. In other words, it is formally possible that the extracellular domain (ECD) is missing from the proteins, hence, it cannot interact with the *Notch* receptor at all, but we are still able to recognize *Serrate* because of the intact IC and TM domains.

The majority of the observed results displayed an unexpected behavior of *Serrate* localization compared to the known nature of confinement where *Serrate* is located mostly on the cell membrane for positive Notch activity. The unanticipated observation of *Serrate* being

located intracellularly for constructs which allow for full activation of Notch, as well as *Serrate* extracellular surface localization for constructs which do not activate Notch, both suggest that mechanisms of Notch activity are not simply dictated through protein location. Whether the *ligand recycling* model or the *classical endocytosis* model is the absolute mechanism of ligand processing for Notch activity is still under review. Determining stability of the native states of *Serrate* in correlation to specific constructs may be of a possible further study to investigate since specifics of *Serrate*'s structure may help us understand the three-dimensional shape of the protein to its involvement with Notch activation. The essential features of protein folding through different lengths of amino acids in the JM region of *Serrate* and analyzing the specific thermostability as well as kinetic properties of the protein may be a great place to start.

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