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Transcriptional Regulation of Notch target E(spl)mγ by Tramtrack69 and Putzig in Drosophila melanogaster

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Transcriptional Regulation of Notch target *E(spl)mγ* by *Tramtrack69* and *Putziq* in *Drosophila melanogaster*

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Abstract

The Notch pathway plays an important role during development by regulating whether a cell takes on a neuronal or non-neuronal cell fate in the peripheral nervous system of *Drosophila melanogaster*. When Notch signaling is present in a cell, the cell is prevented from becoming a sensory organ precursor because transcription of proneural genes is blocked by Notch downstream targets such as the *Enhancer of Split [E(spl)]* complex. Given the importance of neuronal vs. non-neuronal cell fate determination during development, tight regulation of the individual *E(spl)* genes, such as *E(spl)mγ,* must occur to ensure proper differentiation. Focusing on the transcriptional regulation of *E(spl)mγ*, it is possible that this gene is regulated by *Tramtrack69 (Ttk69)*, a transcriptional repressor known to associate with Notch downstream targets, and by *Putzig (Pzg)*, a transcriptional activator that interacts with both Notch downstream targets and TTK69.

A two-pronged approach was used to determine whether *E(spl)mγ* is regulated by *Ttk69* and *Pzg*. Changes in the expression patterns of *E(spl)mγ* were analyzed when ectopic TTK69 was driven in the imaginal eye discs of *D. melanogaster* larvae. I hoped to examine changes in *E(spl)mγ* expression patterns in the presence of ectopic Pzg , but no results were acquired. Using S2 transfections, the changes in expression levels of *E(spl)mγ* in relation to varying treatments of TTK69, Pzg, and MAM (a transcriptional activator of *E(spl)mγ)* were analyzed. The results of the ectopic TTK69 analysis indicate TTK69 specifically suppresses expression of *E(spl)mγ* in vivo. The results of the S2 cell transfection indicate Putzig requires the presence of Intracellular Notch (ICN) and MAM to upregulate expression of *E(spl)mγ.* TTK69 is capable of blocking this upregulation in the presence of MAM, Pzg, and ICN. Overall, the results provide evidence that both TTK69 and Pzg regulate expression levels of *E(spl)mγ*.

Introduction

The Notch Pathway

Multiple cell signaling pathways must work together to allow for proper cellular processes such as proliferation, differentiation, and apoptosis in developing and adult organisms. The Notch pathway, originally identified in *Drosophila melanogaster*, is one signaling pathway that influences every stage of development from specializing cells to halting cell growth and terminating cell life. The pathway is evolutionarily conserved throughout metazoans, from sea urchins to humans. Given its importance it is not surprising that malfunctions in Notch are associated with many human diseases.

The Notch pathway is most commonly known to control differentiation since the pathway determines whether adjacent cells will initiate or halt cell specialization. Notch signaling specifically controls how cells respond to both intrinsic and extrinsic developmental cues that lead the cell into a specified developmental pathway. This allows for the formation of precursor cells while also defining boarders between cells (for a review see Artavanis-Tsakonas et al. 1999; Bray 2006). In order to ensure that both proper cell boundaries form and proper cell differentiation occurs, the Notch pathway can laterally inhibit cells surrounding a differentiated cell from adopting the same fate (Castro et al. 2005). By allowing communication between adjacent cells, the Notch pathway is able to form distinct sets of cells, each having unique cell fates in the developing organism (Castro et al. 2005).

The Notch pathway regulates the differentiation processes in the developing central nervous system during embryogenesis while also regulating the development of peripheral nervous system associated sensory organ precursors (SOPs) in many developing organisms including *Drosophila*. By laterally inhibiting the proneural fate, the Notch pathway isolates a

SOP cell by preventing surrounding cells from adopting a SOP cell fate. This form of differentiation allows for specific tissue patterning during early development, such as in the imaginal discs of *D. melanogaster* (Vässin et al. 1987). One example of specific tissue patterning can be observed in the development of the *Drosophila* eye. The *Drosophila* eye consists of 800 ommatidia, each consisting of eight photoreceptor neurons (R1-R8) surrounded by a collection of non-neuronal support cells that begin to form as the larva approaches the third instar stage (Siddall et al. 2009; Cooper and Bray 1999). The movement of the morphogenetic furrow progresses anteriorly across the developing eye disc and initiates cell differentiation and pattern formation (Siddall et al. 2009). Once the R8 cell is recruited by the morphogenetic furrow, the cells around the R8 cell differentiate into R2/R5 cells, R3/R4 cells, and R1/R6 cells based on the Epidermal Growth Factor Receptor (EGFR) signaling followed finally by the differentiation of the R7 cell (Siddall et al. 2009). The specification of the different cell types is the result of lateral inhibition. Notch is one of the main signaling pathways involved in this differentiation, and is specifically involved in differentiating the R3/R4 cells. When a bias toward R3 develops in one cell, the expression of Delta (Dl) becomes upregulated in R3 to activate Notch in the adjacent cell forcing the cell into an R4 fate (Fanto and Mlodzik 1999). Because of the role Notch plays in regulating eye development, the imaginal discs of *Drosophila melanogaster* can be used to study the expression patterns created by Notch and aid in determining the function of specific genes within the Notch pathway.

Significance of Understanding the Notch Pathway

Previous research has linked the Notch pathway to a number of human diseases including cancer and neurologic degeneration. A number of studies indicate that Notch plays a key role in regulating tumor formation. If any of the many steps in the Notch signaling cascade are disrupted, tumor formation can occur. For instance, when the Notch1 receptor is altered, it results in a subset type of T-cell acute lymphoblastic leukemia (Sjölund et al. 2005). In addition, Notch has also been associated with breast cancer since Notch4 was identified as the target of the mouse mammary tumor virus, resulting in the formation of tumors in the mouse mammary glands (Sjölund et al. 2005). Renal cell carcinoma, prostate cancer, multiple myeloma, and Hodgkin's disease have all been linked to increased levels of Notch signaling. In addition, CADASIL, a late onset neurological disease has been linked to mutations in the *Notch3* gene. The changes in the Notch3 receptor that occur lead the person to experience prominent white matter abnormalities that eventually lead to strokes and dementia (Joutel and Tournier-Lasserve 1998). Alagille syndrome has also been linked to Notch. This developmental disorder causes liver disease in children because of a mutation in the *Jagged1* gene. The mutation that occurs leads to the formation of a truncated protein, and may potentially result in the impairment of proper cell specification (Joutel and Tournier-Lasserve 1998). With the continuing evidence that Notch plays a role in human disease, it will be necessary to develop strategies to control the signaling cascade in order to create treatments for diseases caused by Notch mutations. In order to control the Notch cascade, we must first fully understand how the cascade functions under specific circumstances.

Molecular Basis for Cell to Cell Communication Through the Notch Pathway

The highly conserved Notch pathway consists of a 300-KD single pass transmembrane domain that interacts with the extracellular domain of the ligands of adjacent cells, thus allowing Notch signaling to occur in cells that are in direct contact (Artavanis-Tsakonas et al. 1999). The canonical Notch pathway begins when either Delta (Dl) or Serrate (Ser) ligands sent from one cell bind to the Notch receptor on the adjacent cell (Castro et al. 2005). This binding allows for proteolytic cleavage to occur, creating intracellular Notch, also known as ICN (Wurmbach et al. 1999). ICN will then bind to *Suppressor of Hairless* [*Su(H)*] and initiate the transcription of specific target genes (Castro et al. 2005; Wurmbach et al. 1999). *Su(H)* most commonly activates the *Enhancer of Split* [*E(spl)*] complex located downstream of Notch in non-SOP cells and acts as a direct transcriptional repressor of the *E(spl)* genes in SOP cells (Castro et al. 2005; de Celis et al. 1996). *E(spl)* is a complex locus which includes seven genes that encode the following basic-helix-loop-helix proteins that act as transcriptional repressors: m8, m7, m5, m3, mβ, mγ, and mδ (de Celis et al. 1996; Maeder et al. 2009). This complex is collectively required for the inhibition of the neural precursor cell fate in the *Drosophila* peripheral nervous system. Over-expression of the *E(spl)* genes prevents proper development thus resulting in numerous morphological changes, such as a lack of vein development in the *Drosophila* wing (de Celis et al. 1997). The *E(spl)* genes are initially inhibited in proneural clusters by *Su(H)* that binds to the S DNA binding sites in the absence of Notch and puts the *E(spl)* genes in a default repression state by recruiting co-repressors such as *Hairless (H)* and *Groucho (Gro)* (Figure 1A) (Cave et al. 2011). When a cell is signaled to become a non-neuronal cell, ICN will allow for the expression of the *E(spl)* genes by binding to *Su(H)* and preventing the recruitment of Hairless and Gro. As seen in Figure 1B, each *E(spl)* gene associates with a specific enhancer that allows

binding to proneural proteins such as Achaete (Ac) and Scute (Sc) that eventually will lead to the formation of the epidermal cell fate by repressing Ac/Sc expression (Artavanis-Tsakonas et al. 1999). The function of individual *E(spl)* genes has yet to be determined given that these bHLH genes are functionally redundant and can substitute for each other (Schrons et al. 1992). Thus far, it appears all *E(spl)* genes are activated by Notch, yet the individual genes show distinct expression pattern in embryos and in imaginal discs (Maeder et al. 2009). Because there are only a limited number of expression patterns that could be created when Su(H) and Notch regulate the expression of the *E(spl)* genes, it is likely that other transcriptional regulators, such as Mastermind, Tramtrack69, and Putzig, may be recruited to regulate the expression of the *E(spl)* genes in the Notch pathway (Cooper et al. 2000).

Mastermind: An Enhancer in the Notch Pathway

The *Mastermind (MAM)* gene is a member of the original group of zygotic neurogenic loci identified in *Drosophila melanogaster* during development, and this gene leads to the formation of the evolutionarily conserved MAM protein that has homologues in *C. elegans*, mice, and humans. Each homologue of MAM acts as a nuclear protein that binds directly to chromatin and acts on downstream targets of Notch (McElhinny et al. 2008; Petcherski and Kimble 2000). When MAM loss-of-function analysis was performed in *Drosophila*, the number of neuronal cells increased significantly, while the number of epithelial cells decreased significantly, indicating that MAM plays an important role in regulating cell differentiation and development (McElhinny et al. 2008). In addition, numerous genetic screens have continually identified MAM as a modifier in the Notch pathway (McElhinny et al. 2008). This transcriptional regulator is believed to be an activator in the Notch pathway because MAM is

rich in glutamine and proline, components commonly seen in transcriptional activators (Petcherski and Kimble 2000).

Previous research indicates that MAM acts as a co-activator bound to Su(H) and ICN in *Drosophila* (Cave and Caudy. 2008). This complex is also conserved in mammals in which the MAML protein forms a complex with ICN and CSL, one of the mammalian targets of the Notch pathway (McElhinny et al. 2008). If the MAML protein is mutated, a decrease in overall transcriptional activity occurs and MAML becomes unable to bind with ICN, thus preventing the activation of the Notch signal (McElhinny et al. 2008). The trifecta is commonly known as the Notch Transcription Complex. The interactions that occur among the components of the Notch Transcription Complex allow for the activation of tissue specific transcriptional activators that are bound to the target genes (Cave 2011). In this way, the MAM complex aids in selectively activating transcription. The upregulation of the target gene expression occurs as the Notch Transcription Complex recruits additional co-activators and chromatin remodeling enzymes to enhance the transcription of target genes (Cave 2011). Current research is now focused on identifying the additional co-activators and chromatin remodeling enzymes that couple with MAM in the Notch Transcription Complex to regulate the *E(spl)* complex and other downstream targets.

Tramtrack: A Potential Repressor in the Notch Pathway

One potential repressor of the *E(spl)* genes is *Tramtrack (Ttk).* TTK protein was originally identified as a transcriptional repressor of the pair-rule genes, which play an important role in determining the body plan for *Drosophila* during development. It is known to also repress neuronal identity, thereby cementing non-neuronal fates in various contexts (Murawsky

et al. 2001). Given that both the *E(spl)* genes and *Ttk* aid in determining non-neuronal cell fate during differentiation it is possible that these two genes interact to regulate the differentiation process.

Previous research by Guo et al. (1995) showed that loss-of-function of *Ttk* causes non-neuronal sheath cells to convert into neurons, thus creating neuron duplications. Guo et al. (1995) also showed that over-expression of *Ttk* causes the opposite effect, converting neurons into sheath cells. While Guo's data support the role of *Ttk* in the peripheral nervous system, additional research indicates this gene also plays important roles in the formation of the larval and adult eye in *D. melanogaster* (Xiong and Montell 1993). Both TTK69 and TTK88, two alternate forms of *Ttk*, block neuronal fate determination in the eye discs of third instar larvae of *D. melanogaster* (Siddall et al. 2009) and can prevent ectopic photoreceptors from forming during adult *D. melanogaster* eye development (Badenhorst et al. 2002). In addition, it appears TTK69 may repress the expression of Lz, a Runt family transcriptional regulator that regulates cell fate determination, thereby preventing cells that are competent to take on the R7 fate from doing so, and thus regulating the determination of neuronal and non-neuronal cells (Sidall et al. 2009). Given the data supporting non-neuronal expression patterns when TTK is present, it appears that generally TTK plays a role in preventing neuronal fate, thus indicating there is a possibility this gene works in combination with Notch signaling and the *E(spl)* genes to regulate neuronal specification.

On the molecular level *Tramtrack* encodes two zinc finger DNA-binding proteins known as TTK69 and TTK88, each created through alternate splicing (Murawsky et al. 2001; Xiong and Montell 1993). Both isoforms share a common N-terminal portion that contains a Broad Complex Tramtrack Bric-a-Brac/Pox Virus and Zinc Finger (BTB/POZ) domain, but each

isoform has different C-terminal zinc finger domains (Murawsky et al. 2001; Siddall et al. 2009). The difference in the C-terminal zinc finger domain results in TTK69 and TTK88 having specific DNA binding specificities (Murwasky et al. 2001). It has been shown genetically that TTK69 is downstream of Notch acting as a transcriptional repressor to prevent neuronal specification (Okabe et al. 2001), while also acting as a positive regulator in order to maintain differentiated photoreceptor neurons during late stages of *D. melanogaster* development (Lai and Li 1999). Therefore TTK69 is able to both negatively and positively regulate cell differentiation and maintenance at different stages of photoreceptor development, indicating this gene may play numerous important roles in regulating proper development. It is possible that TTK69 may be able to enhance the expression of the *E(spl)* genes during one developmental time point, while repressing the expression of the *E(spl)* genes at other developmental time points. This ultimately may allow TTK69 to aid in controlling neuronal specification during eye development.

Two TTK69 binding sequences are conserved within the upstream regulatory sequence of *E(spl)mγ* in 12 *Drosophila* species indicating a role for TTK69 in regulating at least E*(spl)mγ* expression (Maeder et al. 2007;Maeder et al. 2009). Because TTK69 is involved in the same functions as the *E(spl)* genes, it is possible TTK69 could be regulating specific *E(spl)* genes, such as *mγ*, to create the unique *E(spl)* expression patterns. Previous research by Hildebrand (2010) explored the effects of TTK69 on *E(spl)mγ* expression in S2 cells through luciferase assays. The results indicate that TTK69 repressed the levels of Notch induced *E(spl)mγ* expression in S2 cells, even in the absence of the most conserved TTK69 binding site within the *E(spl)mγ* regulatory region, while the BTB domain was required for the repression of *E(spl)mγ* (Hildebrand 2010). Overall, these results indicate that TTK69 is capable of repressing the

expression of *E(spl)mγ* in vitro, but whether the repression also occurs in vivo remains undetermined.

While it is understood that TTK69 acts a repressor of transcription during development, the mechanism by which TTK69 represses is not understood. Thus far, a number of transcriptional co-repressors have been identified as direct interactors with TTK69 in order to remodel chromatin structure and block transcription. Research performed by Murawsky et al. (2001) indicates that TTK69 interacts with the dMi-2 subunit of the *Drosophila* NuRD chromatin remodeling complex. While there were a number of sites where TTK69 and dMi-2 bound, there were still other sites on TTK69 where dMi-2 did not bind, indicating that there are still other mechanisms that allow TTK69 to act as a repressor (Murawsky et al. 2001). More recent research indicates that TTK69 binds to MEP1, another subunit of the NuRD remodeling complex (Reddy et al. 2010). Interestingly, the researchers also found that TTK69 is able to bind chromatin in the absence of the NuRD complex, but that NuRD requires TTK69 to bind to the chromatin (Reddy et al. 2010). Because the presence of the chromatin remodelers is dependent on the presence of TTK69, it is probable that TTK69 is capable of recruiting a number of different chromatin remodeler complexes such as the Nucleosome Remodeling Factor (NURF).

***Putzig*: A potential enhancer in the Notch pathway**

Another possible regulator of *E(spl)mγ* is *Putzig (Pzg).* Previous mass spectrometry data indicate that Pzg and TTK69 proteins interact (Minsteris et al. 2009). To further support this, co-immunoprecipitation of TTK69 and Pzg proteins indicates a direct interaction between the proteins of the two genes (Figure 2. Unpublished data provided by Kugler and Nagel [2012]). In addition to this strong association with TTK69, Pzg has also been associated with Notch target

genes in a number of chromatin remodeling complexes (Kugler and Nagel 2010; Kugler et al. 2011). Because of these previous findings, we hypothesized that Pzg may be playing a role in regulating *E(spl)mγ* expression.

Pzg is part of a large multi-protein complex that includes the TATA-box-binding-protein-related factor 2 (TRF2) and the DNA-replication related element binding factor (DREF) (Kugler and Nagel 2010; Kugler et al. 2011). It appears Pzg works as a positive regulator of the cell cycle and is required for Notch target gene activation, hinting that Pzg may act as a chromatin activator (Kugler and Nagel 2010; Kugler et al. 2011). In addition to Pzg, the TRF2-DREF complex contains three members of the Nucleosome Remodeling Factor (NURF): imitation switch (ISWI), Nurf55, and Nurf38. The NURF complex has been known to cause chromatin activation and repression by triggering nucleosome sliding that changes the properties of the chromatin. The ISWI is a member of the SWI2/SNF ATPase family. This subunit provides the energy necessary for the nucleosome remodeling. Nurf 38 encodes inorganic pyrophosphatase that aids in forming growing nucleotide chains in addition to aiding in DNA repair, while Nurf 55 allows protein-protein interactions. While all of these units of NURF are in the TRF2-DREF complex, one subunit of NURF is not. This subunit, known as Nurf 301, is specific to the NURF complex. Nurf 301 contains transcriptional factor protein motifs in addition to other chromatin modifying protein (Kugler and Nagel 2010). Based on the N-terminal region of Nurf 301, it appears this protein may work as a DNA-binding protein by providing a platform for the recruitment of additional transcription factors (Kugler and Nagel 2010). Pzg associates with the NURF complex rather than the DREF complex when activating Notch target genes (Kugler and Nagel 2010). The research performed by Kugler and Nagel (2010) indicates that Pzg co-immunoprecipitates with the NURF complex. In mutants of the NURF complex and the subunit

Nurf 301, Pzg was not detectable at the Notch target genes (Kugler and Nagel 2010). Therefore, NURF is required to allow Pzg to mediate the expression of Notch target genes (Kugler and Nagel 2010).

Therefore, given that Pzg regulates the expression of Notch target genes through the use of the NURF chromatin remodeling complex, and that TTK69 most likely regulates transcription of the Notch downstream target genes by remodeling the chromatin or recruiting chromatin remodelers, it is possible that TTK69 and Pzg may be working together to regulate the transcription of Notch target genes. Mass spectrometry data indicate that TTK69 and Pzg do interact (Mintseris et al. 2009). To further support the idea that TTK69 and Pzg interact, co-immunoprecipitation of Pzg and TTK69 indicates that Pzg and TTK69 do interact in *Drosophila* embryos (Figure 2) (Unpublished data provided by Kugler and Nagel [2012]). It will now be important to determine where and when Pzg and TTK69 interact, in addition to how the proteins are interacting. Determining how Putzig and TTK69 interact will aid in determining the repression mechanism TTK69 uses. In addition, if Pzg does activate transcription of Notch downstream targets, it may also be interacting with other transcriptional regulators of Notch. It is possible that Pzg may be one of the chromatin remodeling enzymes that interacts with MAM to upregulate the expression of Notch target genes.

Means of Investigation

This thesis work took a two pronged approach to study both the genetic interactions between TTK69 and *E(spl)mγ* and Pzg and *E(spl)mγ*, while also examining the influence of TTK69 and Pzg on the expression levels of *E(spl)mγ* in S2 *D. melanogaster* tissue culture cells. In order to determine how TTK69 regulates the expression of *E(spl)mγ*, an ectopic expression

analysis was used to examine the expression patterns of the two genes in the imaginal eye discs of 3rd instar larvae using immunohistochemistry. In order to drive the ectopic expression of TTK69 in the eye discs where *E(spl)mγ* is highly expressed, the UAS-GAL4 system was used (Figure 3). The *Drosophila sevenless* promoter was used to drive the expression of TTK69 specifically in the eye disc during larval development. The same genetic tools were used to examine the effect of Pzg on *E(spl)mγ* in the *D. melanogaster* eyes discs. In order to examine how TTK69 and Pzg expression affects the expression of *E(spl)mγ* in S2 cells, luciferase assays were performed. Using various combinations of *E(spl)mγ*, ICN, TTK69, PzG, and MAM vectors, the expression levels of *E(spl)mγ* were analyzed.

Materials & Methods

***D. melanogaster* Stocks**

;P{GAL4-Hsp70.sev}/CyO was obtained from the Bloomington Drosophila Stock Center at Indiana University. *;;mγ0.6lacZ/TM36Bsb* was a gift from Dr. Sarah Bray. *;UASHATtk69* was a gift from Dr. Z.C. Lai. *;UAS-dsPzg* was a gift from Dr. Kugler. All stocks were cultured at 18°C with JAZZ Food medium and yeast. The crosses were kept in a room that remained at 25°C through the time the third instar larvae were dissected.

For the ectopic expression analysis of TTK69, virgin *;P{GAL4-Hsp70.sev}/CyO* females were crossed with *;;mγ0.6lacZ/TM36BSb* males in Cross A_1, and non-curly wing, non-stubble bristles progeny were selected.. In Cross B_1, virgin *;UASHATtk69/;UASHATtk69* females were crossed with *;;mγ0.6lacZ/TM36BSb* males and progeny with non-stubble bristle characteristics were selected. The A_2 cross consisted of crossing *;SevGal4/+ ;mγ0.6lacZ/+* virgin females with *;UASHATtk69/+ ;mγ0.6lacZ/+* males. The B_2 cross consisted of crossing *;SevGal4/+;mγ0.6lacZ/+* males with *;UASHATtk69/+* virgin females. The final progeny of this cross were *;SevGal4/;UASHATtk69 ;mγ0.6lacZ/+* (Figure 4).

For the ectopic expression analysis of Pzg, virgin *;P{GAL4-Hsp70.sev}/CyO* females were crossed with *;;mγ0.6lacZ/TM36BSb* males in Cross A_1, and non-curly wing, non-stubble bristles progeny were selected. In Cross B_1, virgin *;UAS-dsPzg/;UAS-dsPzg* females were crossed with *;;mγ0.6lacZ/TM36BSb* males, and non-stubble bristle phenotypes were selected. In the A_2 cross, *;SevGal4/+ ;mγ0.6lacZ/+* virgin females were crossed with *;UAS-dsPzg/+ ;mγ0.6lacZ/+* males. For the B_2 cross, *;UAS-dsPzg/+ ;mγ6lacZ/+* virgin females were crossed with *;SevGal4/+ ;mγ0.6lacZ/+* males. The final progeny of both the A_2 and B_2 crosses were *;SevGal4/;UAS-dsPzg ;mγ0.6lacz/+* (Figure 5). For both the TTK69 and the Pzg ectopic

expression analyses, all of the flies were stored in the same room maintained at 25 °C throughout the collection period and after the crosses were set up.

Imaginal Disc Fixation and Immuno-staining

For the imaginal disc dissection, wandering 3rd instar larvae were collected from the F_2 generation of the F_1 crosses. The larvae, kept on cold 1X PBS, were dissected using fine tip # 5 forceps and a dissecting scope, making sure the cuticle of the larvae had been flipped upwards and removed to ensure the exposure of the imaginal discs. The dissected tissues were placed into wells in the 96-well plate filled with fresh, cold PBS for no more than 30 minutes before the tissue was fixed. The tissue was then fixed [using CHS protocols (2007)] in 3 parts Brower's Fix buffer (2.268g PIPES, 40mL mQH_2O, 150µl $MgSO_4$, 75µl 1M EGTA, 750µl nonidet P-40 brought to a total volume of 50mL and at a pH of 6.9) and one part 8% formaldehyde in the 96-well plate for 2 hours at 4°C. The discs were then washed three times with 1X PBS containing 0.3% Triton (PBT) followed by a one hour wash in PBT on a shaker plate at 4°C. The discs were then blocked in PBT containing 5% normal goat serum (NGS) for at least 3 hours on a shaker plate at 4°C. After the blocking period, the discs were placed into the primary antibodies and blocking solution and incubated overnight on the shaker plate at 4°C. Roche Mouse Anti-HA (12CA5) in a 1:1000 dilution, Cappel Rabbit Anti-β-Galactosidase (55976) in a 1:2000 dilution, and UC Berkeley Rat Anti-Elav-7E8A10 in a 1:50 dilution were used as the primary antibodies. For the second set of dissections, Roche Rat Anti-HA was used in a 1:1000 dilution, Cappel Rabbit Anti-β-Galactosidase in a 1:2000, and Mouse Anti-Cut (2B10S) in a 1:50 dilution were used as primary antibodies. The next morning the discs were washed three times in 1X PBS, and then washed for one hour on the shaker plate at 4°C in 1X PBS. After the wash, the

discs were incubated in blocking solution containing the secondary antibodies for 1.5 hours at 4°C, making sure to wrap the tray in tinfoil to prevent fading of the fluorescent antibodies. For most dissections, Invitrogen Alexa Fluor 546 anti-mouse, Invitrogen Alexa Fluor 488 anti-rabbit, and Invitrogen Alexa Fluor 646 anti-rat for the Elav antibody staining were used. For some staining, the Alexa Fluor 546 anti-rabbit and Alexa Fluor 488 anti-mouse were used instead. Finally, the discs were washed for one hour in 1X PBS three times. The discs were then washed one more time in 1X PBS for one hour on the shaker plate at 4°C still wrapped in tinfoil. Once the wash was complete, the discs were dissected in 80% glycerol, isolating the eye discs, and mounting the discs in Vecta Shield with DAPI, and stored at -20°C in the dark until examined under the confocal microscope. Given there is no phenotypic way to determine the genotype of the larvae, discs that were dissected which did not express TTK69 were used to determine the wild-type expression pattern of *E(spl)mγ* and were used as experimental controls.

Plasmid Purification

The expression constructs for ICN, TTK69, and MAM had been previously isolated. In order to collect the D3 (a TTK double-stranded mutant vector with the second conserved *E(spl)mγ* binding site deleted) and Pzg constructs, the constructs were transformed in competent *E. coli* using the Qiagen EZ Competent Cells procedure. The transformed cells were grown in LB media at 37°C on a shaker plate and the constructs were then collected using Qiagen midi prep kits. The concentrations of the isolated DNA were measured in a spectrophotometer, set at an absorbance of 260nm.

Transfection, Cell Lysis, and Harvesting of S2 Cells

S2 cell transfection experiments were performed with pGL3mγ, a previously constructed pGL3 vector that contains the entire 1.2kb regulatory region of *E(spl)mγ* cloned upstream of the luciferase gene and pMT expression vectors containing ICN (Rebay et al. 1993), MAM (Cave and Caudy 2008) TTK69 (Badenhorst et al. 2002), or PZG (Kugler and Nagel 2007) expression vectors were also previously constructed. The parent pMT vector for all of the above vectors is pRmHa, which contains a metallothionein promoter inducible by $CuSO_4$ (Bunch et al. 1988). Empty pRmHa was used to equalize DNA concentrations for all transfections.

Throughout the transfection procedure, the S2 cells were kept at 25°C in Schneider's S2 media with 12.5% fetal bovine serum and 1X pen-strep fungizone. The cells were passaged weekly. Lipofectin™ (Invitrogen) was used to transfect the *Drosophila* S2 cells in 24-well plastic tissue culture plates. For each transfection performed, 0.5ml of complete media and 0.25 ml S2 cells from a stock culture were added at 25°C. The cells were left to sit for 30 minutes to allow for the adherence of the cells to the surface of the plate. For each transfection performed, 0.125ml incomplete media (not including the fetal bovine serum or the pen-strep fungizone), and 2.5 μg of DNA were combined in a polystyrene tube. In a different polystyrene tube, 0.125ml incomplete media and 12.5μl lipofectin™ were added for each transfection. Then, 0.125ml of diluted lipofectin™ was added to each tube containing the media and the DNA. Once the tubes were mixed, they were left to sit at room temperature for approximately 20 minutes.

The serum covering the cells that had adhered to the 24-well plate was removed by washing three times with incomplete media. Once rinsed, 0.25ml of the DNA/lipofectin mixture was added to each well. The plate was incubated at 25°C for 6 hours. Then, the DNA/lipofectin mix was removed and 0.75ml of complete media was added to each well. The cells were left to

rest overnight, before expression was induced by adding 0.75µl of $CuSO_4$ to each well approximately 24 hours after the initial transfection. The cells were left overnight at 25°C and were then harvested between 15 and 20 hours later.

The Promega Dual Luciferase Assay Passive Lysis Buffer (PLB) and protocol were used to lyse the S2 cells. 100µl of 1XPLB should be made for each transfected well. 0.5µl of 1X PBS (pH 7.4) was used to wash the cells after removing the media. Next, 100µl of 1XPLB were placed in the wells, and the plate was placed on the shaker platform at room temperature for 15 minutes. The lysate was then collected in microcentifuge tubes, and stored at -80°C until the Dual Luciferase Assay was performed.

Dual Luciferase Assay

The Dual-Luciferase Assay® was performed using the automated dual injection system of the Veritas Luminometer. LARII and Stop and Glo Reagents (Promega) were prepared as suggested in the Promega manual. For the injections, 100µl LARII and 20µl PLB Lysate were mixed first, and the Firefly Luciferase was read for 8 seconds. Then 100µl of Stop and Glo was added, and the luminosity of the Renilla Luciferase was read for 8 seconds. In order to accurately compare the levels of expression of the pGL3mγ expression vector, the Firefly Luciferase readings were standardized by dividing the Firefly Luciferase reading for a particular treatment by the Renilla Luciferase reading. Once standardized, the average level of expression and the average fold induction for each treatment were calculated.

Statistical Analysis

In order to analyze the results of the luciferase assay, SSPS was used to perform an ANOVA with Tukey HSD post hoc test on the data to determine whether the differences in expression levels were significant. In addition, mean expression levels, standard deviation, and standard error were calculated.

Results

Ectopic Tramtrack69 alters the expression of E(spl)mγ in 3rd instar imaginal eye discs

Ectopic expression analysis was used to determine whether TTK69 is capable of altering the wild-type expression pattern of *E(spl)mγ* in wandering 3rd instar larvae . Using the genetic crosses outlined in Figure 4, TTK69 tagged with HA (hemoagglutinin) epitope was ectopically expressed in the developing photoreceptors of larval eye discs. The eye discs were dissected and stained to determine whether the expression pattern of *E(spl)mγ* changed in the presence of ectopic TTK69. Green fluorescent Alexa Flour 488 was used to stain the expression of *E(spl)mγ* while red fluorescent Alexa Flour 546 was used to stain TTK69 expression. Under wild-type conditions, *E(spl)mγ* was expressed in numerous developing photoreceptors in the eye discs (Figure 6A and Figure 7A/7B). When TTK69 expression was driven in the eye discs, the expression pattern of *E(spl)mγ* changed. The photoreceptors that expressed TTK69 no longer expressed *E(spl)mγ*. While some *E(spl)mγ* expression is still present in the eye disc, the expression of *E(spl)mγ* in the developing photoreceptors never overlapped with the expression of TTK69 (Figure 6B). Therefore, the data suggest that TTK69 is sufficient to repress the expression of *E(spl)mγ* in the developing photoreceptors.

While the above data support the idea that TTK69 represses the expression of *E(spl)mγ*, we needed to ensure that ectopic TTK69 was specifically repressing *E(spl)mγ* and not repressing transcription generally within the eye discs. To do so, the cone cell marker *Cut* was used. *Cut* is expressed in cone cells which do not express *E(spl)mγ* but do express *sevenless.* If *Sev*-driven TTK69 was causing general repression of transcription, we would expect to see a change in the expression pattern of *Cut* in addition to a change in the expression pattern of *E(spl)mγ*. Figure 7

shows that while the expression of *E(spl)mγ* was repressed in the presence of ectopic TTK69, the expression of *Cut* still matched the wild-type expression pattern for the gene (Siddall et al. 2009). Because the *Cut* antibody did not stain the control discs, we cannot determine with certainty that TTK69 did not affect *Cut* expression. Even so, as compared to previously published data it appears *Cut* expression matched wild-type levels, thereby indicating that the ectopic TTK69 specifically suppresses the expression of *E(spl)mγ.*

Ectopic expression of Putzig in 3rd instar imaginal eye discs

Given that Pzg couples with the NURF complex to enhance the expression of downstream targets of Notch, we proposed that Pzg may be regulating the expression of specific genes within the *E(spl)* complex, such as *E(spl)mγ*. Because Pzg appears to act as an activator of transcription, the ectopic expression of Pzg would result in an increased expression of *E(spl)mγ,* thus indicating that Pzg regulates *E(spl)mγ* expression in vivo. Unfortunately, data could not be collected for this ectopic expression analysis. While the cross A_1 occurred successfully, the flies in cross B_1 containing the *;UAS-dsPzg* element struggled to produce viable offspring. Although development of the progeny occurred up to the pupal stage, adult flies rarely emerged from the pupal cases.

The lack of progeny for the B_1 cross may have resulted based on a partial lethality in the genotype or because of environment. Looking first at the partial lethality of the genotype, it is likely that the *;UAS-dsPzg* element sufficiently hinders developmental stages, even when not activated by the Gal4 system, thus resulting in a decrease in the reproductive success of the stocks. This hypothesis is supported by the fact that the parent *;UAS-dsPzg* homozygous stock struggled to produce viable progeny that could be collected for the B_1 cross. Clearly the health of

the stock is reduced when the *;UAS-dsPzg* element is present. The environment may have also played a role in the lack of successful reproduction. During the collection period, the food used for the crosses continually dried out, indicating a lack of moisture in the atmosphere. Given the lack of access to a regulated incubator, it is probable that the moisture fluctuation in the environment may have influenced the reproductive success of the ectopic *Pzg* flies. Future research should aim to complete this ectopic *Pzg* analysis because it will be important to understand whether Pzg protein interacts with the regulatory region of *E(spl)mγ* in vivo.

Putzig activates expression of E(spl)mγ in the presence of ICN and Mastermind

Given that Pzg has been shown to associate with the NURF complex in order to activate Notch target genes and that direct interaction between TTK69 and Pzg has been shown through mass spectrometry and co-immunoprecipitation, it is possible that Pzg and TTK69 work in combination to regulate the overall expression of specific *E(spl)* genes. In addition, because Pzg acts as a positive regulator of transcription, it is also possible that Pzg may interact with MAM in order to upregulate the expression of the *E(spl)* genes.

Looking first at whether Pzg activates the levels of expression of *E(spl)mγ*, we explored the expression levels of *E(spl)mγ* in relation to Pzg, MAM, and TTK69 expression vectors (Figure 8). When the *E(spl)mγLuciferase* vector was treated with the *pMTPZG* and *pMTICN* vectors, there was no significant increase in the levels of *E(spl)mγ* expression (P = 0.636). When the *pMTMAM* vector was added in place of the *pMTPzg* vector, the levels of *E(spl)mγluc* expression increased significantly, having a 54.8 fold induction (P=0.001). We then added the *pMTPZG* vector in combination with *pMTMAM*, *pMTICN*, and *E(spl)mγluc* vectors and an 82.8 fold induction was seen as compared to the *E(spl)mγluc* control. This increase in the *E(spl)mγluc*

expression level as compared to the control was found to be highly significant (P=0.001). Interestingly, the difference in the *mγ* expression level between [*E(spl)mγluc* + pMT*ICN* + pmT*MAM*] and [*E(spl)mγluc* +pMT*ICN* +pMT*MAM* +pMT*Pzg*] was also found to be highly statistically significant (P=0.001). Because Pzg was unable to cause a significant increase the *E(spl)mγ* expression in the absence of MAM, but caused a significant increase in *E(spl)mγ* expression levels when MAM was present, it appears the activation of *E(spl)mγ* by Pzg requires MAM.

Tramtrack69 blocks Putziq and Mastermind from activating expression of E(spl)mγ

Recent studies have shown that Pzg interacts directly with transcriptional repressor, TTK69, based on the previously described co-IP (Kugler and Nagel, unpublished data [2012]) and through mass spectrometry (Mintseris et al. 2009). Since previous results showed that TTK69 inhibits *E(spl)mγ* induction in S2 cells (Hildebrand 2010) and our results suggest that the same inhibition occurs in developing flies (Figure 6 and Figure7), we hypothesized that TTK69 would affect the Pzg-MAM-ICN induction of *E(spl)mγ*. As seen previously, when *pMTPZG* and *pMTMAM* were present at the same time, the expression levels of *E(spl)mγ* increased significantly. In the presence of *pMTICN*, *pMTMAM*, and *pMTTTK69*, there was only a 28.1 fold induction from the *E(spl)mγluc* control (P=0.001). This fold increase was significantly lower than the fold increases that were seen when [*E(spl)mγluc*, *pMTICN*, *pMTMAM]* and [*E(spl)mγluc*, *pMTICN*, *pMTMAM*, *pMTPZG*] were tested (P=0.001,P=0.001 respectively). We then tested to see whether *pMTPZG* would be able to activate the expression of *E(spl)mγluc* in the presence of *pMTTTK69* and pMT*MAM*. There was no significant increase in the expression

of *E(spl)mγluc* when [*E(spl)mγluc*, *pMTICN*, *pMTMAM*, *pMTTTK69*, *pMTPZG]* was tested as compared to when Pzg was not present in this combination (P=0.162). Over all, the presence of *pMTPZG* and *pMTMAM* did not enhance expression of *E(spl)mγluc* as previously observed when *pMTTTK69* was absent from the treatment. It therefore appears TTK69 is capable of blocking both Pzg and MAM activation of *E(spl)mγ*.

Discussion

In order to determine whether TTK69 represses the expression of *E(spl)mγ* in vivo, genetic crosses using *Drosophila melanogaster* were set up to create flies that ectopically express TTK69 in the imaginal eye discs of 3rd instar larvae, a location in which *E(spl)mγ* is normally highly expressed. This analysis revealed that ectopic TTK69 represses the expression of *E(spl)mγ*. In addition, the expression of *Cut*, a transcriptional control gene, did not change when ectopic TTK69 was present while *E(spl)mγ* was downregulated. These results indicate that TTK69 repression is specific to *E(spl)mγ.* In order to determine whether Pzg could activate the expression of *E(spl)mγ*, a luciferase assay was performed. There was a significant increase in the expression level of *E(spl)mγ* when ICN , MAM, and Pzg were present as compared to when only ICN and MAM were present. Therefore, Pzg activates the expression of *E(spl)mγ.* In addition, because Pzg was unable to increase expression *E(spl)mγ* in the absence of MAM, it is evident MAM is necessary for Pzg to act as an activator of *E(spl)mγ* expression. Also, because there was a significant decrease in the level of *E(spl)mγ* expression when TTK69 was added to the ICN, MAM, Pzg treatment, it is probable that TTK69 blocks MAM and Pzg from fully upregulating the expression of *E(spl)mγ.* Together, these findings hint at mechanisms by which TTK69 and Pzg regulate the overall expression of *E(spl)mγ* during development.

Tramtrack69 represses the expression of E(spl)mγ to regulate proper neuronal development in D. melanogaster

The results of the ectopic TTK69 analysis indicate that TTK69 specifically represses the expression of *E(spl)mγ* in the 3rd instar imaginal eye discs. These findings support the hypothesis that TTK69 acts as a repressor of transcription during development. Because ectopic TTK69

was able to noticeably down-regulate the expression of *E(spl)mγ,* it is likely that under normal developmental conditions TTK69 and *E(spl)mγ* interact to regulate proper neuronal differentiation during development. As de Celis et al. (1997) indicated, if the *E(spl)* genes are over-expressed during development, proper cell differentiation will not occur. Given that the *E(spl)* genes appear to be dose dependent, it is likely numerous transcription factors regulate the overall level of expression of these genes. Therefore, TTK69 most likely prevents the over-expression of *E(spl)mγ*, thus maintaining the dose dependent expression of *E(spl)mγ* and thereby allowing for proper regulation of neuronal development. Overall, it is possible that TTK69 may be partially responsible for creating the unique expression pattern observed for *E(spl)mγ* as compared to the other *E(spl) g*enes.

Future research should aim to determine whether TTK69 solely represses *E(spl)mγ* or if TTK69 regulates additional genes within the *E(spl)* complex. Once the *E(spl)* genes regulated by TTK69 are identified, it will be important to determine whether TTK69 regulates transcription in all developing photoreceptors or whether regulation by TTK69 is specific to certain types of photoreceptor precursor cells. Finding that TTK69 is specific to one type of photoreceptor precursor cell may aid in determining the unique function of *E(spl)mγ* and other E*(spl)* genes during *D. melanogaster* development.

Putzig Synergises with Mastermind to further activate gene expression of E(spl)mγ

Results from the luciferase assay indicate that Pzg is only capable of enhancing the transcription of *E(spl)mγ* when MAM and ICN are present. Current research involving MAM has attempted to identify additional co-activators and chromatin remodelers that work in

combination with MAM to regulate gene expression (Cave 2011). The findings in this thesis indicate that Pzg is most likely one of the chromatin remodeling complexes that MAM recruits. Based on this assumption, Pzg is most likely recruited to the Notch Transcription Complex to remodel the chromatin to allow for additional transcription. The remodeling of the chromatin will increase the expression of the *E(spl)* genes more so than when MAM is the sole activator (Figure 9). Future research should focus on where Pzg binds in the Notch Transcription Complex and whether Pzg and MAM are interacting directly or through other regulatory factors. In addition, because Pzg has been shown to regulate Notch target gene expression levels through the use of the NURF complex, it would be interesting to determine whether the NURF complex is recruited to the Notch Transcription Complex on the *E(spl)mγ* gene. Finally, luciferase assays using different *E(spl)* control vectors could be done in order to determine whether MAM and Pzg recruitment is specific to *E(spl)mγ* or used to activate the expression of other/all *E(spl)* genes. Since loss-of-function analysis cannot be performed on the *E(spl)* genes given the redundancy among *E(spl)* genes, these results will aid in determining the individual roles of the *E(spl)* genes in the Notch pathway.

Tramtrack69 prevents Mastermind and Putziq from activating transcription of E(spl)mγ

The results of the luciferase assay indicate that the activation of *E(spl)mγ* by MAM and Pzg could not occur in the presence of TTK69. Previous research indicates that the TTK69 binding site was not required for TTK69 repression of *E(spl)mγ* (Hildebrand 2010). Hildebrand (2010) attributed the ability of TTK69 to repress *E(spl)mγ* expression in the absence of the putative TTK69 binding site to the fact that TTK69 may bind to other co-factors or regulatory

proteins in the complex. Based on this hypothesis, it is probable that TTK69 is binding to either MAM or Pzg and preventing both from fully activating the transcription of *E(spl)mγ* (Figure 10). While it is possible that TTK69 binds to MAM and prevents the recruitment of Pzg, this model would not fit with the co-immunoprecipitation results that indicate Pzg and TTK69 interact in the same complex (unpublished data by Kugler and Nagel [2012]). Therefore, it is more likely the MAM/Pzg complex is prevented from activating expression. Future research should confirm definitively whether TTK69 binds to MAM or Pzg and in what way TTK69 is preventing this complex from activating expression of *E(spl)mγ*. In addition, previous research by Reddy et al. (2010) indicates that TTK69 appears to recruit the NuRD complex to prevent the opening of chromatin, thus preventing gene expression from occurring. For this reason, future research should determine whether the NuRD complex is recruited in the presence of TTK69. If both the NuRD complex is recruited by TTK69 and the NURF complex is recruited by Pzg, it is possible that both of these chromatin remodeling complexes are working against one another to regulate the expression of *E(spl)mγ*. If this is the case, it could explain the slight increase in the fold induction found in the results when TTK69, MAM, and Pzg were all present. Obviously, much more research needs to occur before any of these hypotheses can be given support.

Conclusions

The Notch pathway, which plays an important role in cell differentiation and development, is an important research topic because alterations in this pathway result in numerous human diseases such as CADASIL, a neurological disease, and Alagille Syndrome, one of the leading causes of childhood liver disease (Joutel and Tournier-Lasserve 1998). Because the *E(spl)* complex is a direct downstream target of Notch, it is important to determine the overall function of this complex in addition to determining the unique function of each individual *E(spl)* genes so that we may understand how to prevent diseases caused by Notch mutations. One approach to determining the unique functions of the *E(spl)* genes is to examine the transcriptional regulators that work in combination with specific *E(spl)* genes. Understanding how the individual *E(spl)* genes are regulated will ultimately aid in determining the function of the overall *E(spl)* complex, while also aiding in the understanding of how neuronal verse non-neuronal cell fate determination occurs.

The results of the research presented in this thesis supply information that can provide a basis for understanding the unique function of *E(spl)mγ* as it relates to the transcriptional repressor, TTK69 and the transcriptional activator, Pzg. The results of the ectopic TTK69 expression pattern analysis provide evidence that TTK69 specifically represses the expression of *E(spl)mγ* in vivo. The results of the luciferase assay indicated that MAM is required for the recruitment of Pzg in order to increase the levels of transcriptional expression of *E(spl)mγ* in S2 cells. It appears that TTK69 interacts with MAM and Pzg to prevent high levels of activation of *E(spl)mγ*, aiding in the overall regulation of *E(spl)mγ* expression levels. While the ultimate outcome of this TTK69 transcriptional regulation of *E(spl)mγ* has yet to be determined, it is

possible TTK69 regulation ultimately helps to determine whether a cell differentiates into a neuronal or non-neuronal cell.

Future research should aim to determine whether TTK69 regulates *E(spl)mγ* expression through chromatin remodeling or through a different mechanism. In addition, it will be important to determine where in the Notch Transcription Complex TTK69 binds to regulate *E(spl)mγ* expression. Along the same lines, it will also be important to understand if Pzg binds directly to MAM and to understand whether Pzg activates *E(spl)mγ* expression through chromatin remodeling or through some other mechanism. Completing the ectopic expression analysis for Pzg will be necessary to ensure that Pzg acts as a transcriptional activator in vivo. To further explore the ectopic expression analysis for TTK69, future staining experiments should solidify the results that TTK69 specifically represses *E(spl)mγ* and does not alter the expression pattern of non-TTK69 regulated genes such as *Cut*.

The research performed in this thesis was severely limited in time and space because of the current construction on New London Hall. For example, the luciferase assay was only performed once in duplicate due to time restrictions based on evening access to the laboratory, therefore providing few data sets in the statistical analysis. In addition, calculations for the amount of each vector in the luciferase assay were slightly off, which may have influenced the overall difference in expression of *E(spl)mγ* based on each treatment. Replication of this experiment in at least triplicate will need to occur to ensure that the preliminary data collected were valid. In regard to the ectopic expression analyses, under normal conditions, the flies would be kept in a 25°C incubator to constantly regulate temperature, light, and humidity. Because an incubator was not accessible in Olin during the construction period, the flies were

kept in a 25°C room, though it is likely the temperature, humidity, and light cycles varied, potentially influencing the amount of ectopic gene expression that occurred.

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Immuno-staining protocol adapted from: CSH Protocols;2007;doi:10.1101/pdb.prot4794 and prot796.

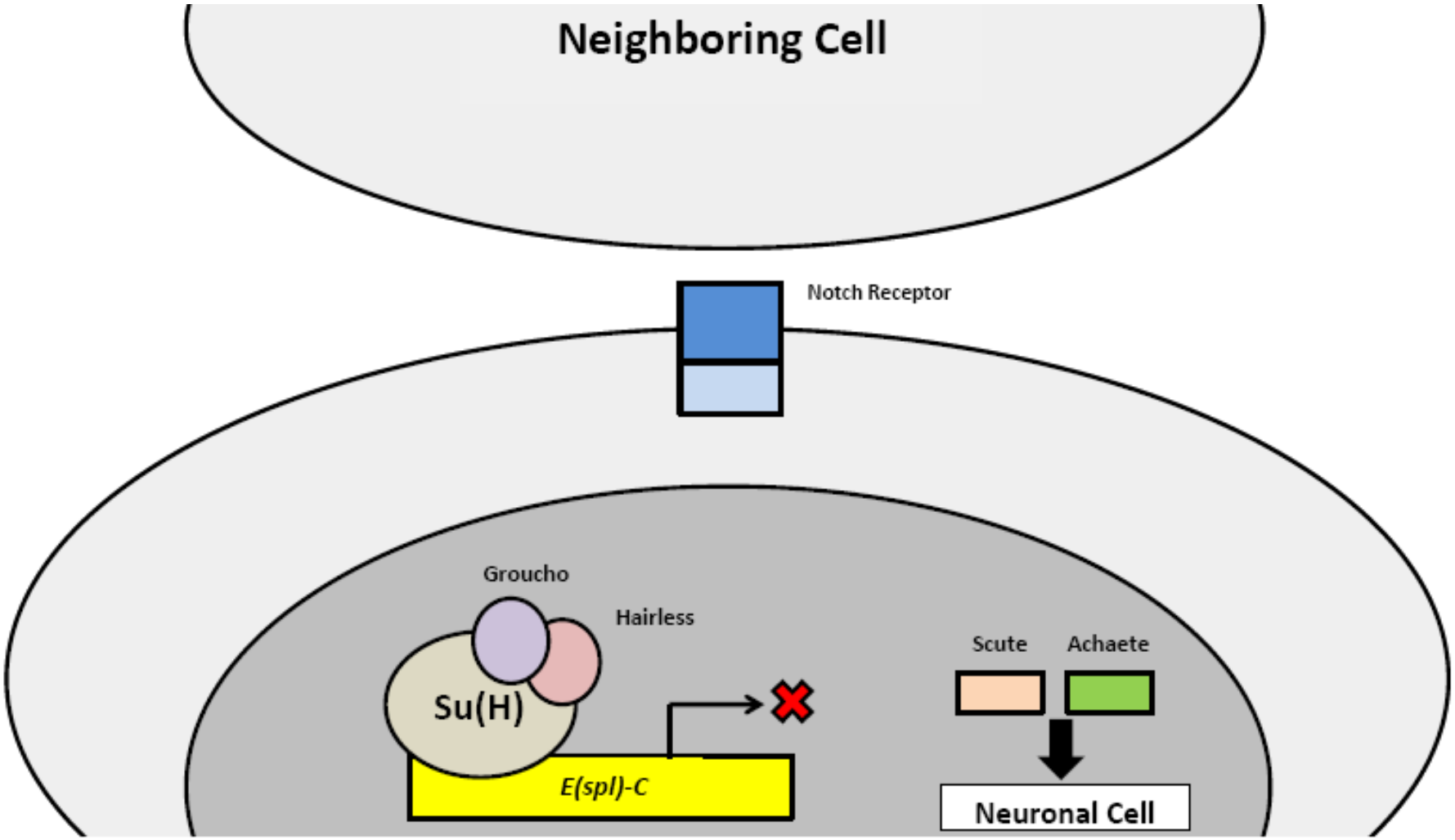

Figure 1A. Canonical Notch Signaling Pathway When Notch is Not Present. When Notch receptors are not activated, Su(H) binds transcriptional repressors, Groucho and Hairless, in the nucleus, thereby preventing the expression of *E(spl)* genes. The proneural genes Scute and Achaete are therefore transcribed, allowing the cell to take on a neuronal fate. This figure was modeled after the description of the Notch default setting in Cave et al. 2011.

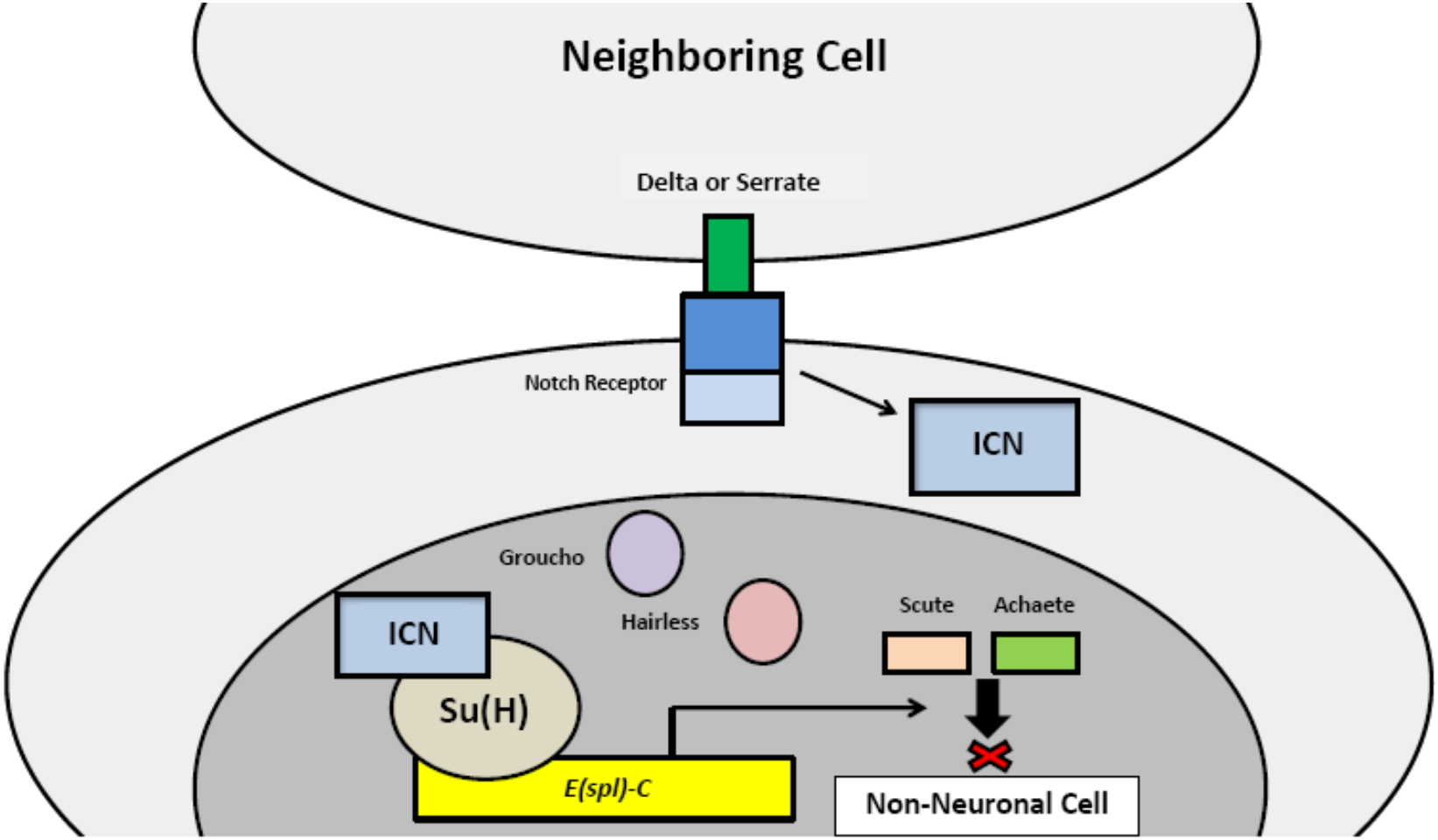

Figure 1B. The Canonical Notch Pathway: When the Notch receptor on a cell is activated by a Delta or Serrate ligand from an adjacent cell, the Notch receptor is cleaved into Intracellular Notch (ICN), which then binds to Su(H) in the nucleus, preventing the transcriptional repressors Groucho and Hairless from binding. The *E(spl)* genes are therefore expressed, which then blocks the expression of proneural genes such as Scute and Achaete. The cell therefore does not become proneural. The mechanism was modeled after the description of the canonical Notch pathway described in Artavanis-Tsakonas et al. 1999.

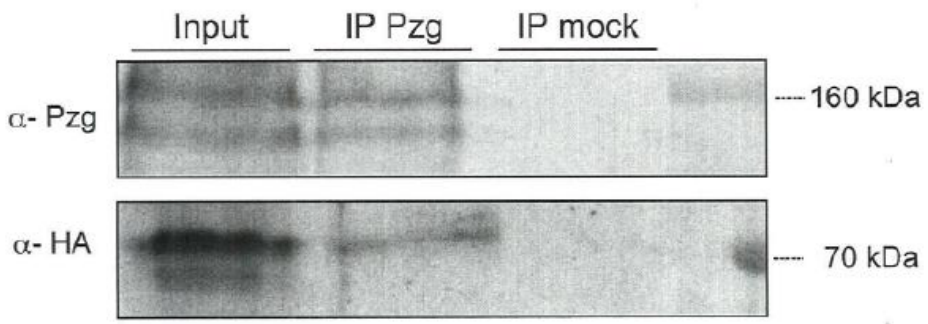

Figure 2. Co-Immunoprecipitation of *D. melanogaster* embryonic TTKHA and Pzg. The proteins were immunoprecipitated from *da-GAL4::UAS-TTKHA* embryos using anti-Pzg antibodies. Anti-Pzg was used to detect Pzg and anti-HA was used to detect TTK. Results indicate TTK and Pzg do interact in embryonic *D. melanogaster*. This image is courtesy of unpublished data by Kugler and Nagel (2012).

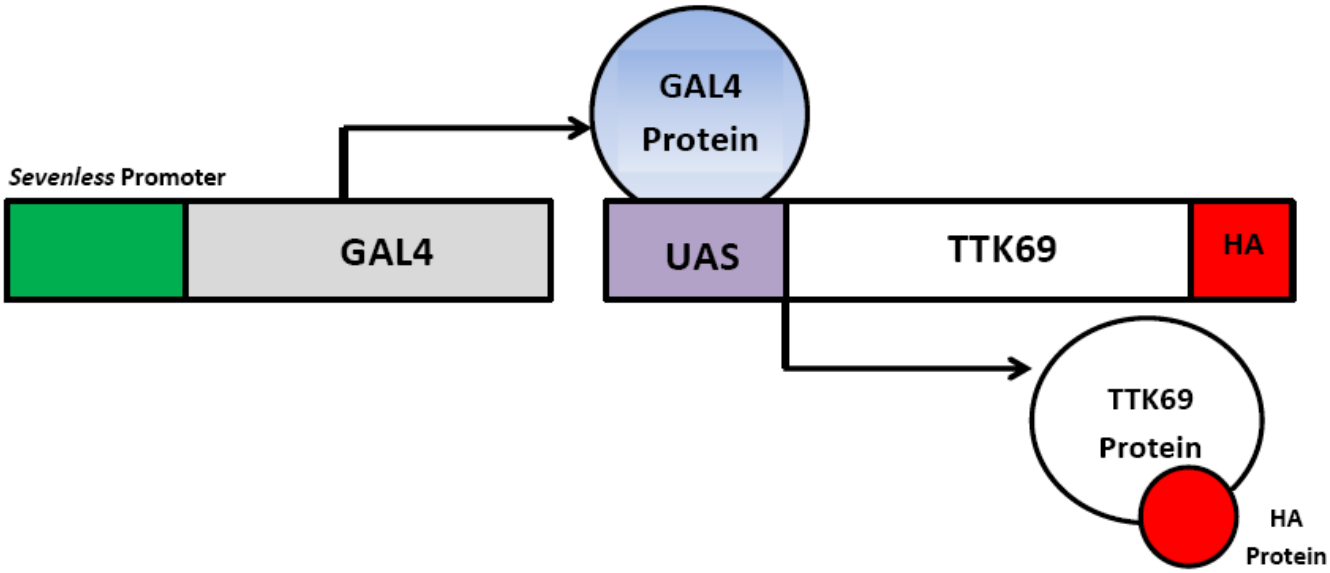

Figure 3. The UAS-GAL4 system used to overexpress TTK69 in the imaginal eye discs of *D. melanogaster*. The *Sevenless* promoter was used to drive the expression of GAL4 solely in the developing eye discs. The GAL4 protein binds to the UAS site driving the expression of TTK69 tagged with HA in the imaginal eye discs.

Cross A				Cross B		
Parental Cross A_1 and B_1	*;P{GAL4-Hsp70.sev}2/CyO* ♀	×	*;;myo.6lacZ/TM36BSb* ♂	*;;myo.6lacZ/TM36BSb* ♂	×	*;UASHATtk69/;UASHATtk69* ♀
Selected F_1	*;SevGal4/+ ;myo.6lacZ/+*	Non-*CyO*, Non-*Sb* ♀ and ♂		*;UASHATtk69/+ ;myo.6lacZ*	Non-*Sb* ♀ and ♂	
Final Crosses (A_2 and B_2)						
;SevGal4/+ ;myo.6lacZ/+ ♀	×	*;UASHATtk69/+ ;myo.6lacZ* ♂		*;UASHATtk69/+ ;myo.6lacZ* ♀	×	*;SevGal4/+ ;myo.6lacZ/+* ♂
F_2 Selected Progeny						
;SevGal4 / ;UASHATtk69 *;myo.6lacZ / +*						

Figure 4. Genetic Crosses for the ectopic expression of TTK69 in relation to E(spl)mγ expression in imaginal eye discs.

Cross A				Cross B		
Parental Cross A_1 and B_1	*;P{GAL4-Hsp70.sev}2/CyO* ♀	×	*;;myo.6lacZ/TM36BSb* ♂	*;;myo.6lacZ/TM36BSb* ♂	×	*;UAS-dsPzg/;UAS-dsPzg* ♀
Selected F_1	*;SevGal4/+ ;myo.6lacZ/+*		Non-*CyO*, Non-*Sb* ♀ and ♂	*;UAS-dsPzg9/+ ;myo.6lacZ*		Non-*Sb* ♀ and ♂
Final Crosses (A_2 and B_2)						
;SevGal4/+ ;myo.6lacZ/+ ♀		×	*;UAS-dsPzg/+ ;myo.6lacZ* ♂	*;UAS-dsPzg/+ ;myo.6lacZ* ♀	×	*;SevGal4/+ ;myo.6lacZ/+* ♂
F_2 Selected Progeny						
;SevGal4 / ;UAS-dsPzg				*;myo.6lacZ / +*		

Figure 5. Genetic Crosses for the ectopic expression of Putzig in relation to E(spl)mγ expression in imaginal eye discs.

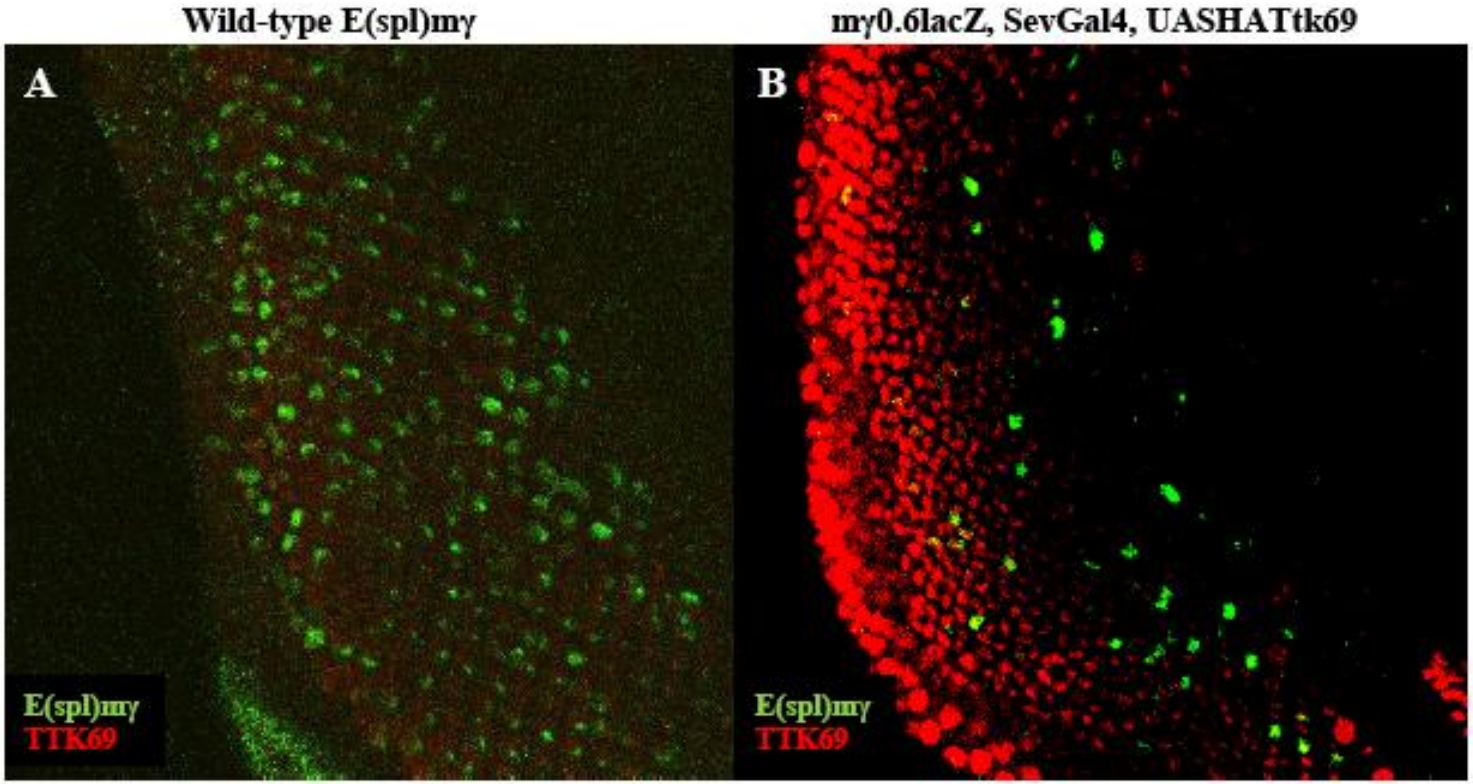

Figure 6. Ectopic TTK69 represses the expression of E(spl)mγ in 3rd instar imaginal eye discs. (A) Confocal image of the wild-type E(spl)mγ expression pattern represented in green. E(spl)mγ is present in most developing photoreceptors. **(B)** Confocal image of E(spl)mγ expression pattern in the presence of ectopic TTK69 (represented in red) in developing photoreceptors. The expression of E(spl)mγ in developing photoreceptors is much reduced in the presence of ectopic TTK69.

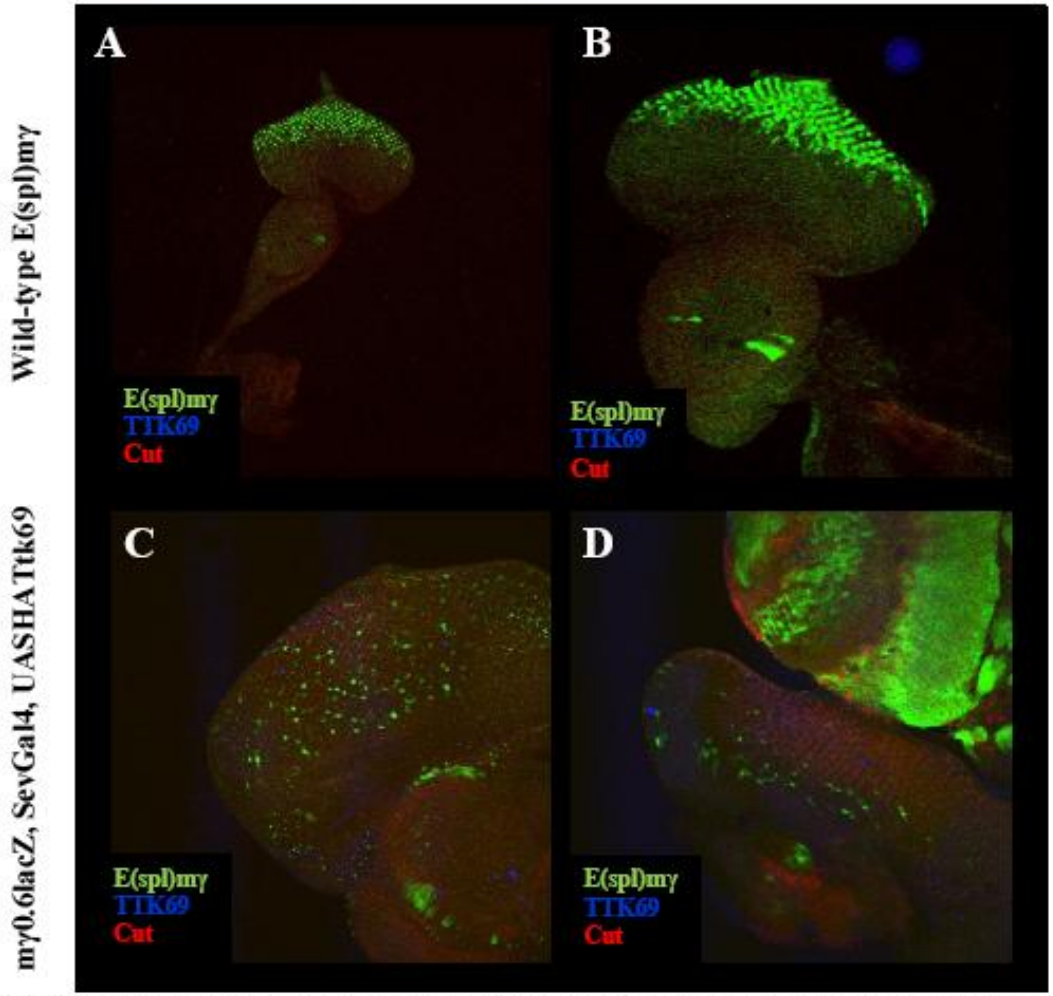

Figure 7. Ectopic expression of TTK69 specifically suppresses expression of E(spl)mγ in developing photoreceptor cells. (A-B) Confocal image of wild-type expression of E(spl)mγ (green) in developing photoreceptors of 3rd instar larvae. Cut should be present but did not stain in control images. **(C-D)** Confocal image of E(spl)mγ expression (green) and Cut (red) expression in the presence of ectopic TTK69. Cut expression is very high as compared to E(spl)mγ expression. TTK69 suppresses E(spl)mγ expression in developing photoreceptors but does not suppresses Cut expression, indicating *Sev*-driven TTK69 specifically represses E(spl)mγ expression.

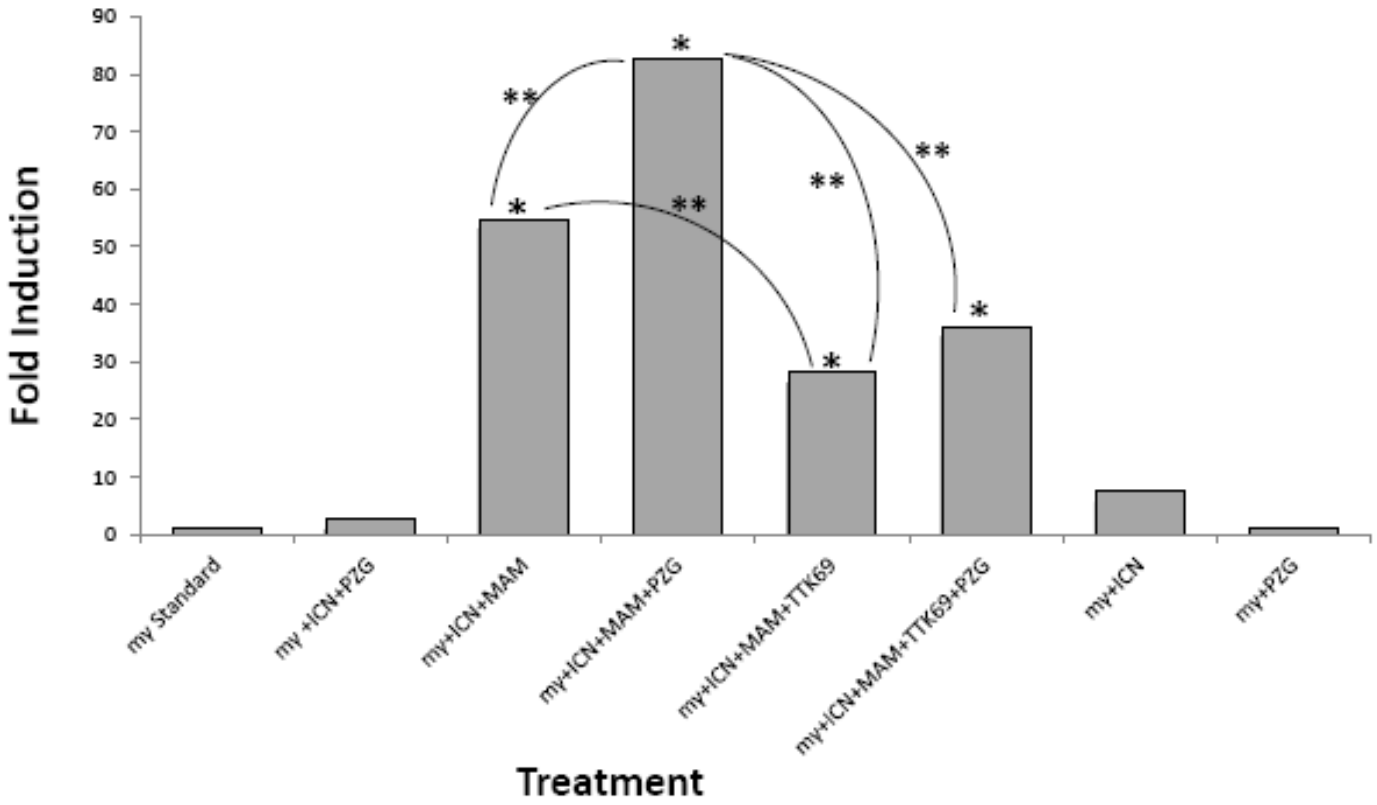

Figure 8. Effects of ICN, MAM, TTK69, and PZG on levels of E(spl)mγ expression. Treatments marked with * indicate a significant increase in the level of expression of pGL3mγ as compared to the standard ($p<0.05$). Brackets marked with ** indicate a significant difference in levels of expression of pGL3mγ between the two treatments in the bracket ($p<0.05$). Error bars are not indicated because there were only two replicates per treatment.

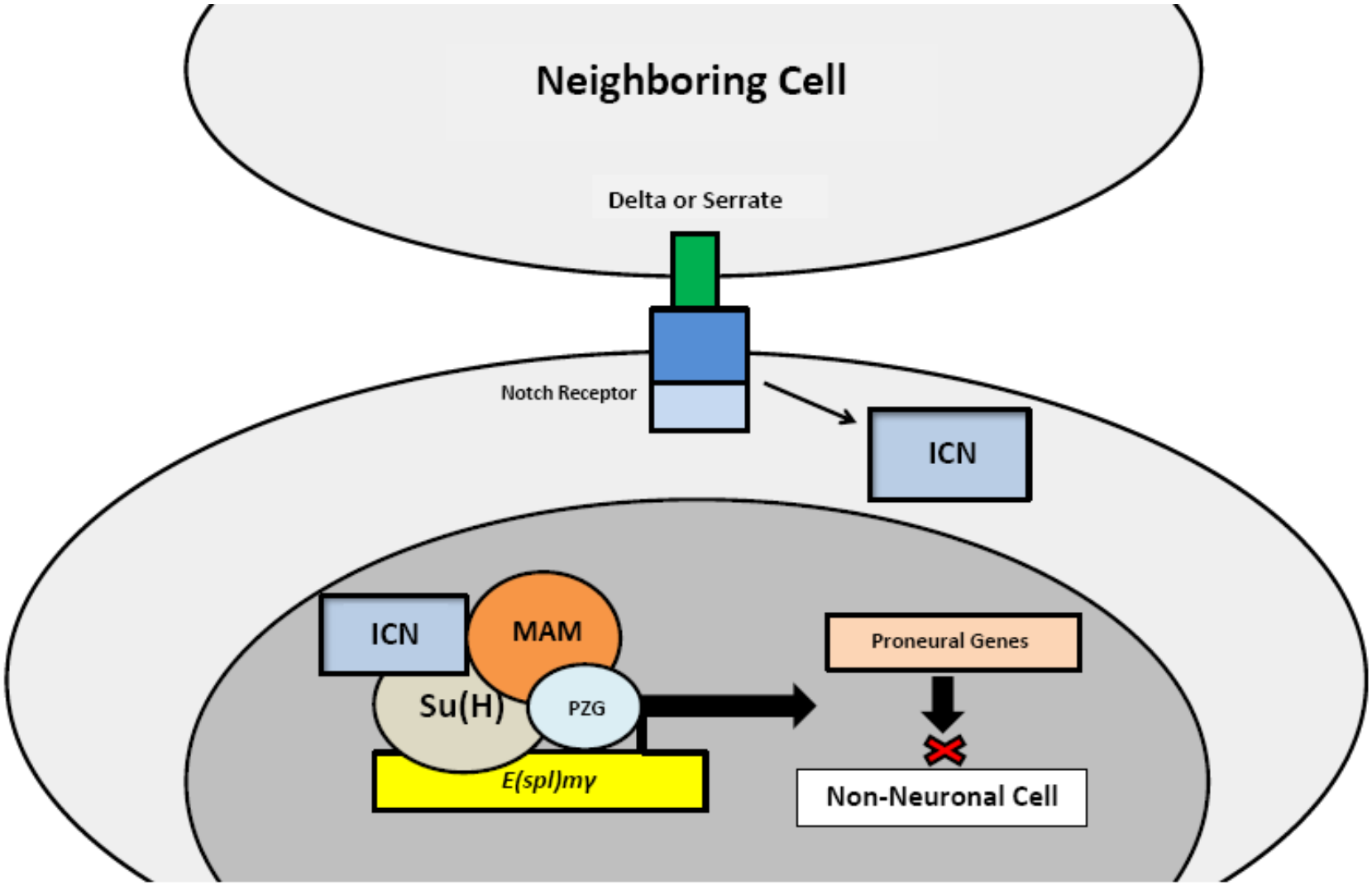

Figure 9. Potential Mechanism: Mastermind recruits Putzig to the enhance the levels of E(spl)mγ transcription. When MAM recruits Pzg to the Notch Transcription Complex, the transcriptional expression of *E(spl)mγ* increases. This increase in expression may further block expression of proneural genes, thus further preventing the formation of neuronal cells.

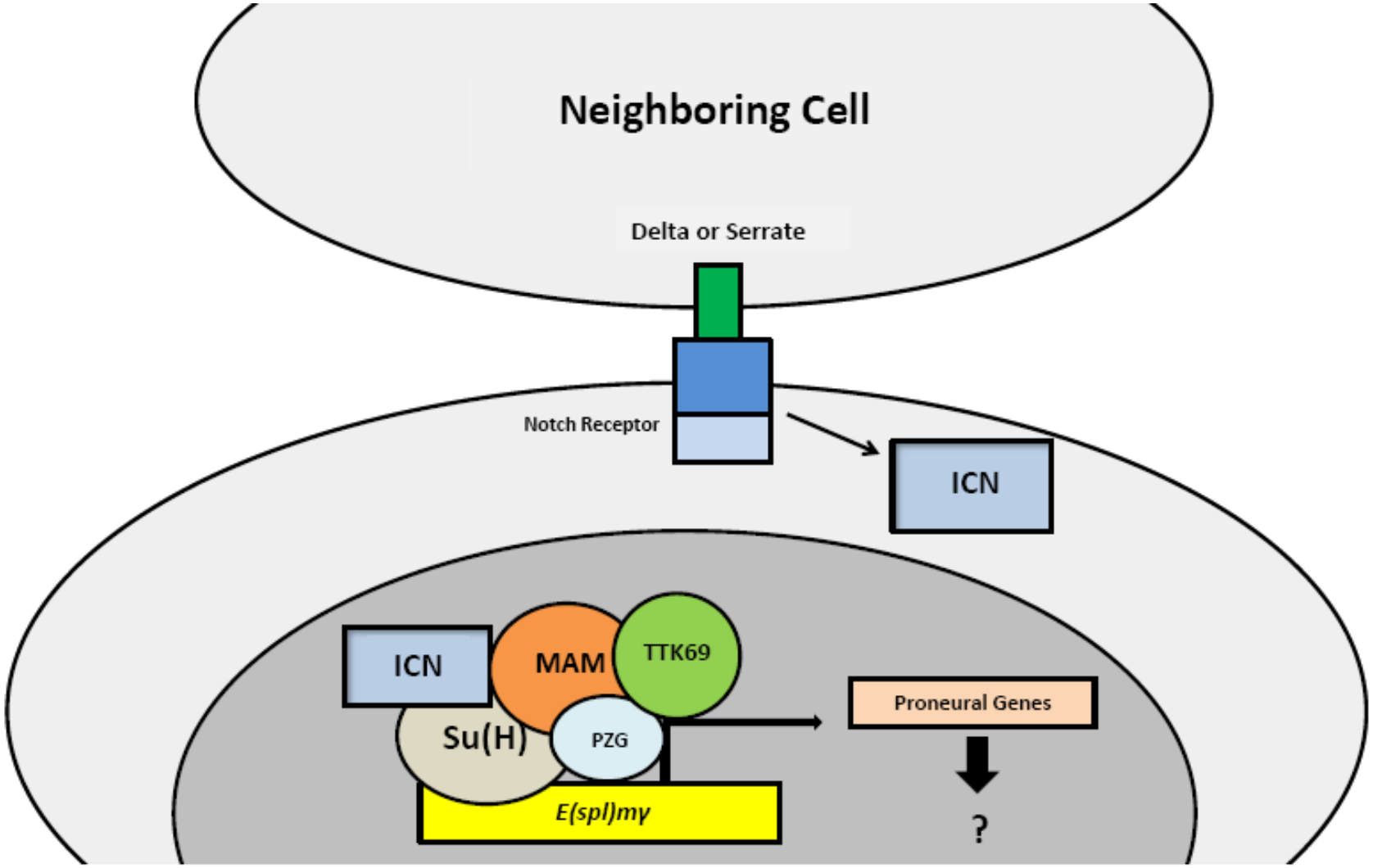

Figure 10. Potential Mechanism: *Tramtrack69* prevents activation of expression of *E(spl)mγ* by blocking MAM/Pzg activity. Based on the results collected in combination with previous research, it is probable TTK69 binds MAM and/or PZG to prevent increased activation of *E(spl)mγ* gene expression. This mechanism would allow for tighter regulation of *E(spl)mγ* gene expression in the Notch pathway.