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Drosophila T-Box Transcription Factor Midline Functions in the Notch-Delta Signaling Pathway to Regulate Sensory Organ Precursor Cell Fate and Cell Survival and Embryonic Development Profile of Midline, Senseless, and Achaete in the CNS and PNS

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Drosophila T-Box Transcription Factor Midline Functions in the Notch-Delta Signaling Pathway to Regulate Sensory Organ Precursor Cell Fate and Cell Survival
and
Embryonic Development Profile of Midline, Senseless, and Achaete in the CNS and PNS

By

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I would like to thank Dr. Leal for allowing me to perform research in her lab. She taught me the importance of research and, with a highly involved approach, continuous encouragement, and understanding, made sure that I was comfortable in the lab and understood the background of our research at all times. She has helped me prepare for a future career and is the definition of a true mentor. I would also like to thank Sudeshna Das, a graduate student who I worked very closely with in the lab and was really a second mentor to me. Sudeshna aided me in staining embryos, data collection, and taking confocal images. It is an honor to be listed as second author with Sudeshna on a paper that is currently under revision for publication.

Abstract:

The gene *mid* of *Drosophila* is a highly conserved gene that codes for a T-box transcription factor with similar functionality to its vertebrate homolog Tbx20. Mid and Tbx20 are important for their roles in heart and CNS development. Additionally, these transcription factors aid in proper eye development but this area of research is vastly understudied. This study uses the eye of *Drosophila* to report that *mid* and its paralog *H15* expression aid in the specification of sensory organ precursor (SOP) cell fates and cell survival in the pupal eye imaginal disc. Using RNAi interference to reduce *mid* expression resulted in the loss of interommatidial bristles as well as cell death due to the misspecification of SOP cells during pupal development. We completed genetic studies to place *mid* in the Notch-Delta genetic pathway because it is known to specify SOP cell fates and were able to determine that Mid functions downstream of Notch, upstream of the Enhancer of Split (E(Spl)) gene complex, and tentatively parallel with Suppressor of Hairless (Su(H)) in the pathway. Additionally, *mid* interactions with *extramacrochaete* (*emc*) and *Senseless* (*sens*) play a role in cell survival. These studies suggest that Mid functions within the Notch-Delta signaling pathway with a dual role of cell-fate specification and cell survival.

Another aspect of this research study was to evaluate the role of Mid in the developing central nervous system (CNS) and peripheral nervous system (PNS) of *Drosophila* embryos. Mid expression was compared to the expression of Sens and Achaete (Ac), SOP cell markers during various stages of embryonic development. Our results show a coordinated co-expression pattern of Sens and Ac with Mid. Sens is highly expressed in the PNS of stages prior to stage 12 and then fades. Ac is

expressed in the neurons of the CNS and PNS in early stages and continues after stage 12, which is when Mid expression begins. Ac is co-expressed with Mid beginning in stage 12. Further experiments will be performed using *mid*-RNAi embryos to evaluate if reducing *mid* expression affects the expression patterns of Sens and Ac.

This research has clinical applications to further the understanding of developmental and neurodegenerative diseases of the CNS, PNS, and eye. Additionally, Mid may have a link to the development of cancer, an area of research that will be studied in the Leal lab in the future.

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Introduction:

This research study seeks to improve the understanding of genetic interactions between the transcription factor gene, *midline,* and other candidate genes that regulate the development of the eye, central nervous system (CNS), and peripheral nervous system (PNS) of *Drosophila melanogaster,* the fruit fly.

Drosophila melanogaster

Drosophila melanogaster is a useful model organism for this study because it shares 70% similarity with the human genome. The similarity between the organisms' genomes is represented by evolutionarily conserved gene homologues within both genomes. While the genes between differing species may not be exactly the same, homologous genes share functionality in both species. Thus, understanding the function of genes in the fruit fly will help understand the function of the homologous genes in humans. In addition, the genome of the fruit fly offers a wide range of unique tools that make it a perfect candidate for genetic studies.

Drosophila melanogaster was the first species to have the entirety of its genome sequenced. The genome of the fruit fly is composed of only four chromosomes and has fewer repeating units than larger eukaryotes, which can be seen by comparing the genomes of humans and fruit flies. The human genome is composed of 23 pairs of chromosomes totaling about 3 billion base pairs. Of these, it was found that only 1-2% are genes coding for protein products, while the rest is comprised of non-coding DNA (U.S. Department of Energy Genome Programs, 2012). The smaller genome of *Drosophila* encodes approximately 13,600 genes. (Adams et al., 2000). A large number of genes are contained in a much smaller

amount of DNA in fruit flies, since there are fewer non-coding regions of DNA in fruit flies than in humans.

The generation time of some model organisms including mice is long. The time required to complete experiments in mice requires months. In *Drosophila,* however, eggs laid by a female will mature into a full adult stage in about 11 days after which the results of an experiment can be seen (Powell, 1997). This is especially useful in genetic studies when the function of a gene is unknown because it allows a quick assessment of the function of the gene. Additionally, short generation time allows for experiments to be repeated and validated over a much shorter period of time than what is required in other organisms.

Meiosis, the production of eggs and sperm, is a process of cellular division that helps produce genetic variance. During meiosis, a phenomenon referred to as crossing over occurs where portions of chromosomes exchange genetic material. Crossing over allows the combination of many differing sets of chromosomes in gametes, ensuring that the genotypes of the parental and offspring genotypes differ. In *Drosophila melanogaster*, however, crossing over does not occur in male specimens and occurs in moderation within females (Schug). This is useful in genetic studies because it ensures the inheritance of a specific gene, or lack thereof, from the male, allowing for the study of a specific gene via mutations when crossed with a female of a known genotype.

T-box Genes

The expression of genetic characteristics occurs through the processes of transcription and translation. Transcription is the conversion of DNA into form that

can leave the nucleus, called mRNA. Translation is the process of reading mRNA and building proteins; certain sections of DNA that encode for a specific trait are referred to as genes. During transcription and translation, these genes are expressed with the production of proteins being the end result.

The T-box transcription factor genes are highly conserved throughout the animal kingdom serving a variety of functions in development including "mesoderm formation, morphogenetic movements, cell adhesion, cell migration, tissue patterning, limb patterning, limb bud outgrowth, and organogenesis (Leal et al., 2009)." T-box genes are split into families that are further divided into subfamilies. In *Drosophila melanogaster*, there are eight identified T-box genes from four different subfamilies (Leal et al., 2009). Although these genes may have varied functions, all share a common region known as the T-box. The T-box is a conserved sequence involved in protein dimerization and DNA binding (Minguillon and Logan, 2003).

The general role of a transcription factor is to bind to another region of DNA and either promote or inhibit the transcription of other genes. Therefore, transcription factors play an integral role in proper development of different tissues. Specifically, T-box genes have been found to play an important role in proper heart and forelimb abnormalities in mammals. Mutations in T-box genes may result in different diseases, such as Holt-Oram syndrome, which is characterized by heart and forelimb abnormalities (Bruneau et al., 2001). *Tbx20* is a T-box gene that has been highly studied in vertebrates, primarily for its effect on heart development but also for its effect on the developing CNS and eye. *mid*, an equivalent gene in

invertebrates, shares a similar function with the *Tbx20* gene but is less researched in many aspects. Researching whether *mid* and *Tbx20* have parallel functionality in many different aspects of developing invertebrates and vertebrates, respectively, will lead to a deeper understanding of the genetic pathways involved in heart, eye, CNS, and PNS development.

Role of *midline* and *Tbx20* in Development: Clinical Implications

mid and its paralog, *H15*, are vital to the development of the dorsal vessel which is equivalent to the heart in vertebrates. Research showed that *mid* and *H15* interact in a functionally redundant manner to regulate heart development. The complete knockout or los of *mid* resulted in severe defects of the dorsal vessel, while the loss of *H15* had no effects on heart development. These studies show that *mid* has a stronger effect on dorsal vessel development than *H15* (Miskolczi-McCallum et al., 2004).

Stennard et al. reported a similar result in vertebrates and determined that *Tbx20* plays a key role in genetic pathways that lead to cardiac cell specification (Stennard et al., 2003). The researchers' experiments using mice showed that *Tbx20* is expressed in the myocardium and endocardium. Through physical interactions with cardiac specific transcription factors, such as Nkx2-5, GATA4, and GATA5, *Tbx20* activates the expression of cardiac genes responsible for heart cell specification (Stennard et al., 2003). Another research study by Takeuchi et al. reiterates that *Tbx20* is crucial for heart development. Specifically, *Tbx20* is important for proper valve formation and mutations in the *Tbx20* gene lead to congenital heart defects. Additionally, the research found that *Tbx20* is necessary for

the proper development of motoneurons in developing mouse and chick embryos. Motoneurons extend from the CNS and act as a relay system to the organs of the body, integrating signals from the brain with responses from organs. The researchers found that with a knockdown of *Tbx20* expression, there was a lack of differentiation into the multiple motoneuron subtypes and a lack of patterning was also detected. These defects occurred because *Tbx20* was found to interact with *Islet 2* (*Isl2*) and *Hb9* which regulate differentiation and patterning in developing motoneurons (Takeuchi et al., 2005).

mid was also found to be an important factor for motoneuron development in *Drosophila melanogaster* embryos. In normal embryos, axons develop outwardly from the CNS in a ladder-like scaffold and *mid* is highly expressed in these developing axons. However, in embryos where *mid* is knocked-down, this scaffolding pattern is disrupted. It is likely that *mid* is necessary for the secretion of guidance factors that lead the developing axon to its required destination (Liu et al., 2009).

As the mentioned research studies show, there is a distinct similarity between the functions of *mid* and *Tbx20* even though the mechanism for their effects may differ. The similarities between these genes can have clinical implications that will help toward developing therapies for the prevention of heart developmental defects in humans and other higher-level vertebrates. Understanding the function of these genes will also allow for predicting the chances of developing congenital heart diseases, CNS disorders, and specific diseases that affect motor function through the use of genetic screening. Genetic screening processes are currently used to detect

genetic disorders such as Huntington's disease, Alzheimer's disease, and some forms of breast cancer. Currently, there is no cure for these diseases. However, it is probable that treatment of these diseases could come from understanding the nature of the genetic variations that cause them (Risch and Merikangas, 1996). The future of medicine potentially lies in genetic screening and gene therapy. Thus, it is important to identify gene mutations that give rise to specific heart, eye, and CNS disorders. Before potential treatments are tested on humans, it will be beneficial to experiment on model organisms such as the fruit fly and mouse.

Role of *mid* in CNS Development

Buescher et al. state that "the *Drosophila melanogaster* ventral nerve cord derives from neural progenitor cells called neuroblasts (2006)." Within these neuroblasts, the expression of different genes gives rise to neurons that differ from one another. *midline* was found to contribute to the formation of these neuroblasts within the anterior of the ventral nerve cord and is a key factor in the development of the CNS (Buescher et al., 2006). Leal et al. found that *mid* (also known as *neuromancer 2* or *nmr2)* and *H15* (*also known as neuromancer 1* or *nmr1)* interact with *even-skipped* (*eve*), a gene that affects the fate of neurons in the developing organism to control the specification of these neurons in the CNS (2009). However, the specific role of *mid* in the development of the CNS is understudied. In order to place it in a regulatory network guiding CNS development, more research is required.

Role of *mid* in PNS Development

The peripheral nervous system (PNS) of *Drosophila melanogaster* is a network of neurons that innervate the organs connecting them to the CNS. The neurons of the PNS act to relay electrical signals from the CNS to the organs leading to a response from the organs. Neurectoderm cells of the fruit fly can either differentiate into epidermal cells or neurons (Simpson, 1990). The genetics of the developing PNS, however, is vastly understudied with many gaps remaining in the genetic model describing it. Witt et al. described a model describing the coordination between the *senseless* (*sens*) transcription factor gene and another gene *atonal* in the abdominal sensory organ precursors (Witt et al., 2010). While these genes are expressed in the abdominal region SOPs, this research thesis found that *mid* is also expressed in the developing neurons, suggesting that it could play a role in this genetic pathway. However, the role of *mid* in the developing neurons of the PNS is an area that is completely void of research and one goal of this research is to affirm that *mid* plays a role in the developmental pathway of the PNS.

Eye Development

The compound eye of *Drosophila* is composed many smaller units called ommatidia. Approximately 750 ommatidia combine to create the compound eye and each ommatidium is an arrangement of eight photoreceptor cells and 12 accessory cells; the complex of these cells that constitute each ommatidium create a hexagonal shape with a mechanosensory bristle projecting from each alternative vertex of the hexagon (Tomlinson, 1988). There are approximately 450 bristle in each *Drosophila* eye.

The Notch-Delta signaling pathway is a defined regulatory pathway involved in eye development. In the Notch-Delta signaling pathway, a sensory organ precursor (SOP) is selected from a field of cells depending on its expression of Delta. Delta and Notch are transmembrane proteins that act as ligand (Delta signal) and receptor (Notch) on neighboring cells. When Delta meets Notch on the outside of a cell, it activates an intracellular signaling pathway. SOPs are selected to become proneural cells because they express higher levels of Delta on their cell surface than neighboring cells. This high level of Delta on the surface of the cell selects it to become a proneural cell by inhibiting the surrounding cells, which become epidermal cells (Heitzler et al., 1996). The internal cell signaling pathways involve the inhibition and activation of many different genes to cause differentiation into either an epidermal or SOP proneural cell. One goal of this research was to place *mid* in the Notch-Delta signaling pathway that specifies the fate of SOP cells (Das et al., 2013).

Role of *midline* in Eye Development

The role of *mid* in eye development is highly understudied. The role of its vertebrate homolog, *Tbx20,* in retinal development has been documented in mice. Meins et al. found that *Tbx20* is expressed in the periphery of the neural retina and the optic cup in early staged fetuses (2000). In later stage fetuses, the presence of *Tbx20* was detected in more parts of the eye, including the sclera, optic nerve, cornea, and ganglion and neuroblastic layers of the neural retina (Meins et al., 2000; Kraus et al., 2001; Pocock et al., 2008). The studies mentioned focus on the vertebrate *Tbx20* gene, highlighting its function in eye development. However, there

is much similarity between the *Tbx20* and *mid* gene functions, as observed in the studies that explain the development of the CNS and heart. Therefore, this research addresses the function of *mid* on eye development.

Knockdown of *mid* results in apoptosis and fusion of ommatidial units as well as the loss of bristles. By knocking down other genes of interest that are believed to interact with *mid* in the *mid*-RNAi background, a rescue or further depletion in number of bristles affirms that the gene of interest interacts with *mid* to regulate eye development. Additionally, using suppressors and enhancers of the genes allows the placement of the genes interacting with *mid* either upstream or downstream of *mid* in the Notch-Delta Pathway. This is used to genetically place *mid* within the Notch-Delta pathway.

Materials and Methods:

UAS-Gal4 System and RNA Interference

Brand and Perrimon developed the UAS-GAL4 system, which relies on crossing two genetically different parental lines (1993). One of the parents will have the *B-galactosidase* gene that codes for GAL4, a transcription factor DNA binding protein. The other parent's genome contains an upstream activator sequence (UAS) that is present before the transcriptional start site of the gene of interest (*mid*). The UAS serves as the binding site for the GAL4 transcription factor. The transcription of *mid* in the progeny relies on the presence of both of these sequences (Brand and Perrimon, 1993).

In order to target the eye tissue for these experiments, the *glass multiple reporter* (*GMR*) driver line was utilized. Driver lines can be used to target specific tissues with the UAS-Gal4 system by "placing specific elements upstream of Gal4", known as promoters, that lead to the directed expression of genes in target tissues (Duffy, 2002). *GMR* is an eye-specific promoter region. Placing *mid* under the control of UAS-GMR-Gal4 allows for its targeted expression in the eye but not other tissues.

By combining the UAS-Gal4 system with another method, referred RNA interference (RNAi), the expression of *mid* can be reduced in order to detect its effect on eye development. RNAi depends on a cell's natural ability to degrade double-stranded RNA molecules (dsRNA). When *mid* mRNA is produced through transcription, it pairs with short, complementary RNA sequences placed into the UAS construct (UAS-*mid*-RNAi), and an antisense strand is formed. This antisense strand marks the dsRNA for degradation of both the inserted RNA molecules and the

endogenous mRNA transcribed from DNA. Destroying the products of a gene before they can be translated into protein is known as gene silencing (Hannon, 2002). When *mid* expression was silenced in the eye tissues, there was an approximate 50% loss of bristles in the eye as well as other tissue defects as compared to Oregon-R (OR) wild-type flies with no mutations.

In order to find genes that interact with *mid* to regulate eye bristle development, flies with certain genes removed from their genome, referred to as chromosomal deficiency lines, were crossed with *mid*-RNAi flies. The rescue or further loss of bristles in these progeny determines whether the removed gene suppresses or enhances the *mid*-RNAi phenotype, respectively (Fig. 1). This is also referred to as a genetic modifier screen. Genetics is an ongoing process in the Leal lab and I was able to assist a current graduate student, Sudeshna Das, in the generation of genetic crosses, identification of different genotypes, and collection of flies.

Bristle Counts

To quantify bristle counts for F_1 progeny, images were taken of each compound eye of ten adult female flies using a high-power Leica M165C dissection microscope. Flies were transfixed to a slide using clear nail polish and submerged in water to reduce the amount of refracted light in the photograph. A series of images which focused on different areas of the eye were collected over 10-15 focal planes. The images were compiled together to create a single, flattened montage with the entire eye in focus. Next, the image was imported using Image Pro Plus software where an annotation tool was used to distinguish the dorsal and ventral regions of

the eye and each eye bristle was manually tagged. The Image Pro Plus software counted each tag and provided a final total count of eye bristles as well as dorsal and ventral counts (Media Cybernetics, Inc., Bethesda, MD).

Scanning Electron Microscopy

Sudeshna Das, a graduate student in the Leal lab at The University of Southern Mississippi acquired scanning electron microscopy (SEM) images of one-day old adult fly eyes of the Oregon-R (wild-type) and UAS-*mid*-RNAi phenotypes. The compound eyes were gold sputter coated to a thickness of 10nm and high-resolution images were acquired on an FEI (FEI Company, Hollsboro, OR) Quanta 200 scanning electron microscope with an accelerating voltage of 20kv (The Department of High Performance Materials and Polymers, USM).

I assisted with the manual bristle counts of the SEM images. Images were divided into dorsal, ventral, anterior, and posterior segments and bristles were counted separately for each segment. These counts were used to support that a further loss of bristles were observed in the ventral region compared to the dorsal region of the eye field. Additionally, overlapping deficiency mapping in the specific areas where deficiencies were seen in the UAS-*mid*-RNAi phenotype was used when screening for gene candidates that interact with *mid* to regulate eye development (Das et al., 2013).

Embryo Collection and Preparation

In order to stain embryos with specific antibodies that identify the presence of proteins, they must first be collected and fixed. Embryos can be collected continuously over a period of 0-20 hours to produce batches of embryos that have

more stages common than others. Collection in the early hours of development will produce more early stage embryos, while collection at the end of 20 hours after being laid will result in more late stage embryos.

Two outer layers protect *Drosophila* embryos: the outer layer or the chorion and the inner layer, the vitelline membrane. To prepare embryos for staining, the outer layers must be removed. The chorion is removed by soaking the embryos in a 50% bleach solution for 3 minutes, followed by a rinse with distilled water. Next, 2mL of heptane and 2mL of 37% formaldehyde are added, fixing the tissues. Fixing tissues induces chemical crosslinks that stiffen and preserve the tissue. This mixture is removed and 6-10mL of methanol is added, followed by shaking for 1-2 minutes to remove the vitelline membrane. Embryos that sink should have both membranes removed and stored in methanol at -20°C for 3-4 months (Forstall, 2012).

Immunofluorescent Studies

Immunofluorescence uses antibodies that have been tagged with fluorescent molecules that can be excited at specific wavelengths of light with the use of lasers to detect the presence of proteins in tissues. Primary antibodies, because of their very specific affinities, are used to mark the protein of interest. For example, when looking for Mid presence in tissues, anti-Mid is used to interact with Mid proteins. Next, a secondary antibody bound to a fluorophore, a molecule with fluorescent properties, is added. The secondary antibody has a specific affinity for the primary antibody. In order to complete a double immunostaining experiment, where two different antibodies are used to detect different proteins in a single specimen, it is crucial that the antibodies be bred from different species. For example, anti-Mid is

bred in rabbit. As such, the secondary antibody is specific for the anti-Mid antibody and must be bred in a rabbit as well. To mark a second protein, such as Senseless or Achaete, another species must be used to produce the antibody. Anti-Sens and its secondary antibody are produced in guinea pigs. Anti-Ac and its secondary antibody are produced in mouse. Using two separate species prevents the possibility of cross reactivity.

Before embryos are incubated with antibodies, they are removed from methanol and rinsed three times with phosphate buffered saline (PTX), composed of 0.1% Triton-X and PBS, which is a set solution of NaH_2PO_4, Na_2HPO_4, and NaCl, for 10 minutes each time. Then, three washes with PBT, a mixture of 1% Bovine serum albumin and PTX, are performed for 10 minutes each. The primary antibody may be added after these washes occur. The wash cycle is repeated after the addition of primary and secondary antibodies. Embryos are preserved in PBS containing 50% glycerol for mounting purposes. Some antibodies require additional buffers to allow proper binding between proteins and antibodies. Embryos younger than Stage 10 required the addition of PEMF, a mixture of 100 mM Pipes at pH 6.9, 2mM EGTA, and 1mM $MgSO_4$, to allow for optimal binding of the antibody to Mid and Sens proteins in younger tissues, as detailed by Skeath and Carroll (1994).

The primary antibodies used in these experiments were used at the following dilutions: guinea pig anti-H15 (1:2000), rabbit anti-Mid (1:1000) (Leal et al., 2009), anti-Achaete (1:2) (Skeath and Carroll, 1991), and anti-Senseless (1:800) (Nolo et al., 2000). The monoclonal antibody Elav (1:10, O'Neill et al., 1994) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of

the National Institute of Child Health and Development at The University of Iowa. Alexafluor 488, 594, and 633 secondary antibodies with coordinating species specificity were use for immunofluorescent labeling (Molecular Probes).

Embryo Staging

Drosophila melanogaster embryos mature over a series of 24 hours and their development has been divided into distinct stages, depending on the development of the stomodeum, the position and length of the ventral nerve cord and the development of the cephalic cleft. In order to create a developmental profile, these different stages had to be collected, mounted, and viewed under a confocal microscope.

Stages of embryo development were studied prior to mounting using the website *flymove.uni-meunster.de*, which has detailed descriptions and photographs of each stage and how it can be uniquely identified from other stages (2013). After embryos were separated by stage, they were mounted on a slide with 1,4-Diazabicyclo[2.2.2]octane (DABCO) and a coverslip for preservation. The cover slips were sealed to the slide using clear nail polish. To create a full developmental profile, collection began with stage 8 or stage 9 embryos and continued through stage 17 embryos.

Confocal Microscopy

Confocal microscopy uses lasers to excite the fluorophores bound to secondary antibodies to visualize the expression of proteins in tissues. Different fluorophores are excited at different wavelengths, allowing them to be viewed

separately. Images can be taken of the emitted fluorescence and merged together to detect whether different proteins are co-localized in the same tissues.

Confocal images were captured by a Zeiss LSM510 META confocal microscope and analyzed using the accompanying Zeiss LSM Image Browser software (version 5). Immunofluorescent probes were excited at 488 nm (to detect Mid) and 594 nm (to detect Sens) of the absorption spectrum (Das et al., 2013).

Data Representation

Confocal images were assembled using Adobe Photoshop CS6 software (Adobe Systems, Inc.) and GraphPad Software, Inc. (La Jolla, CA) was used to represent data as bar graphs.

Results:

Using the GMR-Gal4 driver line and *UAS-mid-RNAi* transgenes, we reduced *mid* expression in the eye. The eyes of ten *UAS-mid-RNAi/CyO;GMR-Gal4/TM3* (*mid*-RNAi, Fig. 1B) one-day old adult female flies were examined using SEM and compared to the images of ten Oregon-R (WT, Fig. 1A) females. By counting the interommatidial bristles, we detected an approximate 50% decrease of bristles in the *mid*-RNAi mutants (Fig. 1H). Bristle counts of the different regions of the eye revealed a more significant loss of bristles in the ventral region of the eye compared to the dorsal region of the eye (Fig. 1C). Anterior and posterior regions were also counted and compared but a less significant difference was noted between these regions when compared to the loss in the ventral region compared to the dorsal region (Fig. 1D). Additionally, *mid*-RNAi mutants displayed a variety of other tissue defects including ommatidial fusion, bristle polarity defects (Fig. 1B'), and the presence of patches of discolored tissue suggestive of cell death (Fig. 1G).

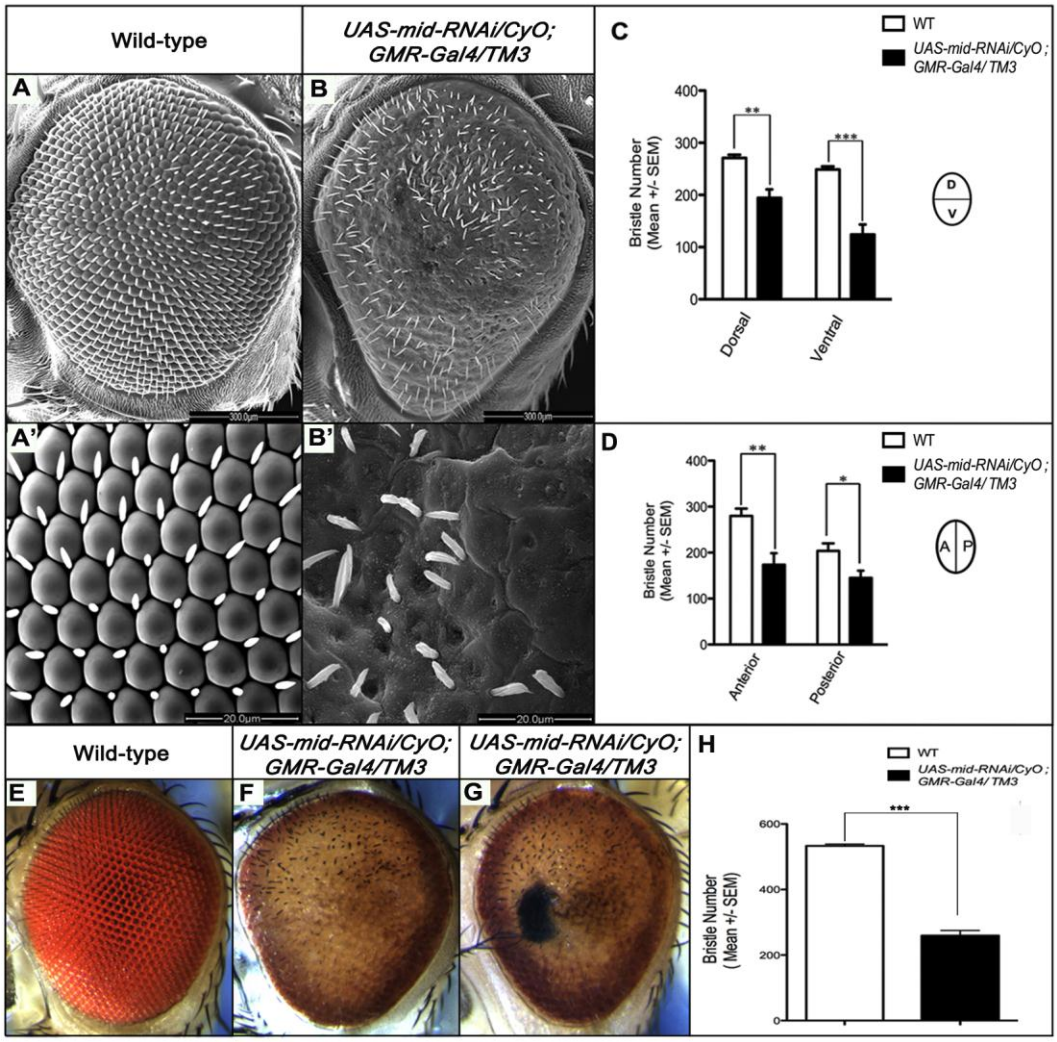


Figure 1: Light microscope and SEM images of wild-type and *mid*-RNAi eyes. A, A': SEM images of wild-type eye. B,B': SEM images of *mid*-RNAi eye. C: Graphical representation of bristle counts of wild-type and *mid*-RNAi eyes of dorsal and ventral regions. D: Graphical representation of bristle counts of wild-type and *mid*-RNAi eyes of anterior and posterior regions. E: Wild-type eye under light microscope. F: *mid*-RNAi eye under light microscope. G: *mid*-RNAi eye under light microscope presenting loss of bristles and melanized tissue. H: Graphical representation of bristle count totals of wild-type and *mid*-RNAi eyes.

To verify that Mid expression is occurring in proneuronal SOP cells in the eye, the expression pattern of Mid and H15 was examined in the eye imaginal discs of P1 and P2 pupal stages. Eye imaginal discs were stained with an antibody specific for Elav, a neural-specific, RNA binding protein important for proper neuron development of the eye and CNS (Yao et al., 1993). During the P1 stage, H15 and

H15 were co-expressed with Elav within photoreceptor neuron clusters (Fig. 4A-D). In the P2 stage, when many cells have selectively become SOP cells, Mid and H15 expression shifted with respect to Elav, now co-expressed in photoreceptors neurons and a population of SOP cells (Fig. 2G-H; Das et al., 2013).

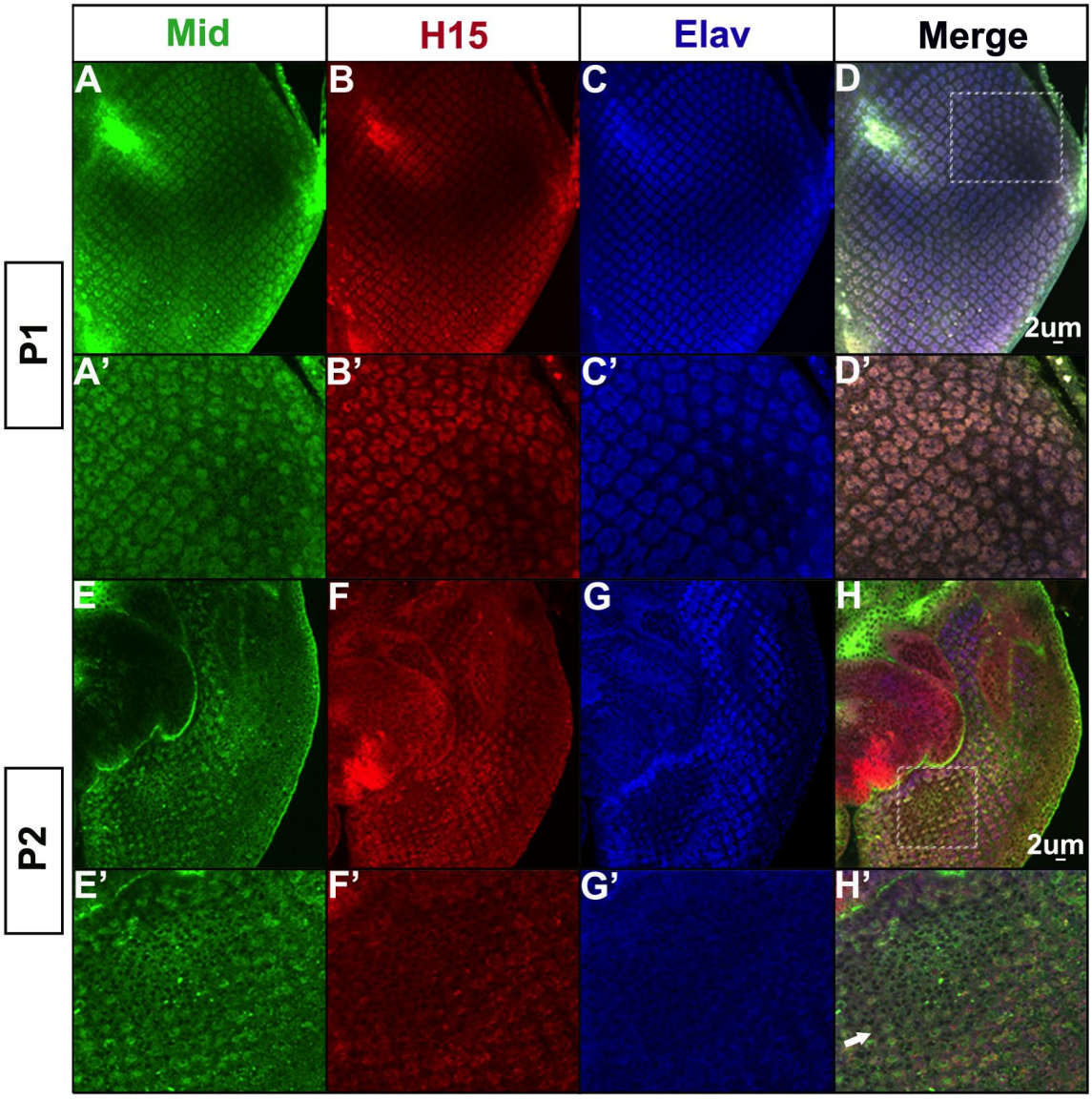


Figure 2: Immunostaining of eye imaginal discs with anti-Mid, anti-H15, and anti-Elav at P1 and P2 stages during pupal development. A-D: Mid, H15, and Elav expression during the P1 stage. A'-D': Zoomed in region of A-D. E-H: Mid, H15, and Elav expression during the P2 stage. E'-H': Zoomed in region of E-H.

To validate the expression of Mid in SOP cells, stage P2 pupae were labeled with anti-Achaete (Ac), which is an SOP cell marker. Many of the SOP cells highlighted by Ac expression also co-expressed Mid (Fig. 3A-C). When immunostained stage P2 pupae of the *mid*-RNAi genotype were compared to WT pupae, a reduction in the expression of Ac in SOP cells was observed suggesting that Mid positively regulates Ac expression in SOP cells (Fig. 3D-F). To validate this result, another protein present in SOP cells, Senseless (Sens), was labeled with antibodies. Mid and Sens were also co-expressed in SOP cells (Fig. 3G-I) of WT P2 pupae and Sens expression was reduced in the SOP cells of the *mid*-RNAi pupae (Fig. 3J-L). This suggests that Mid also positively regulates the expression of Sens in SOP cells.

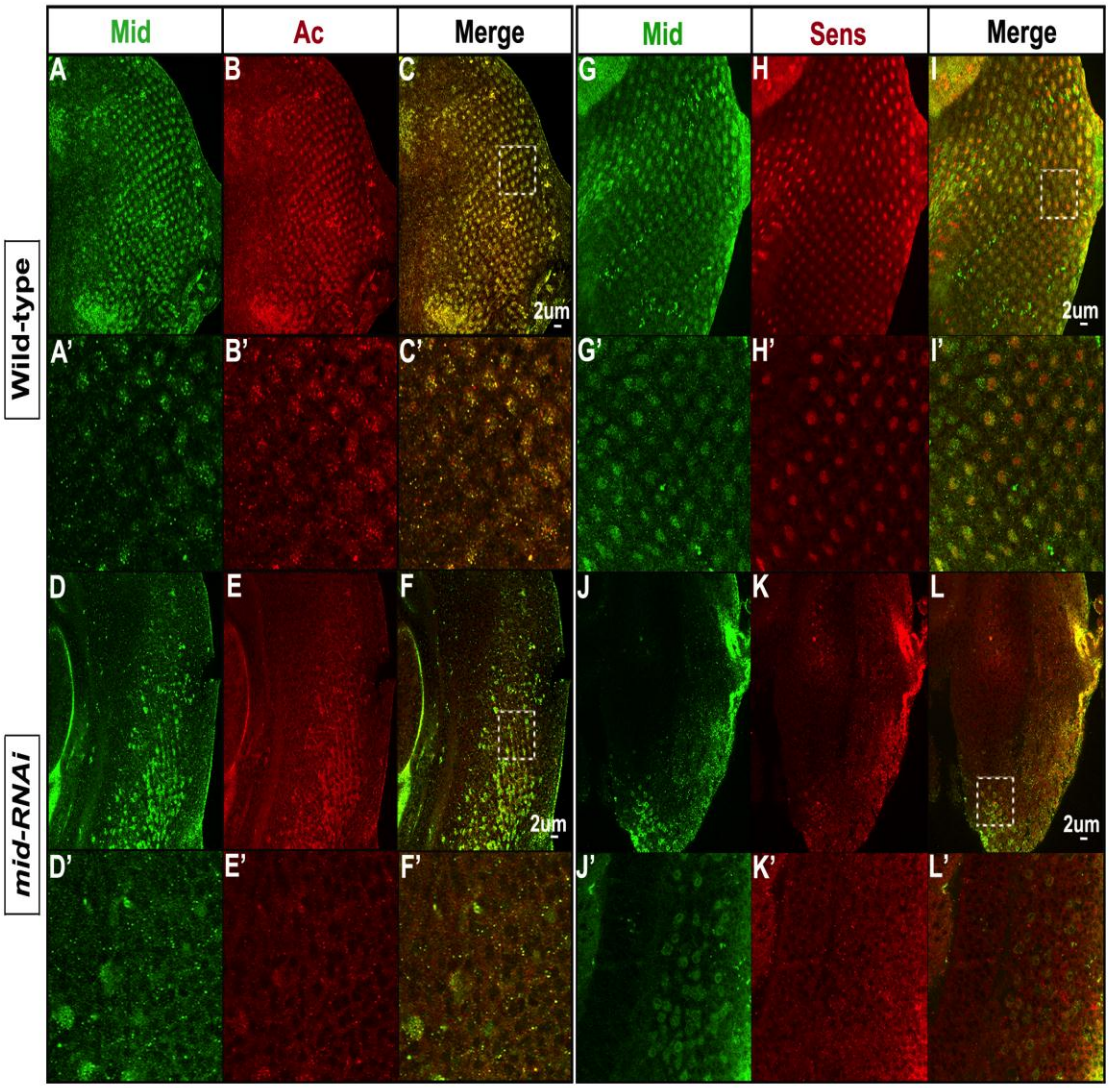
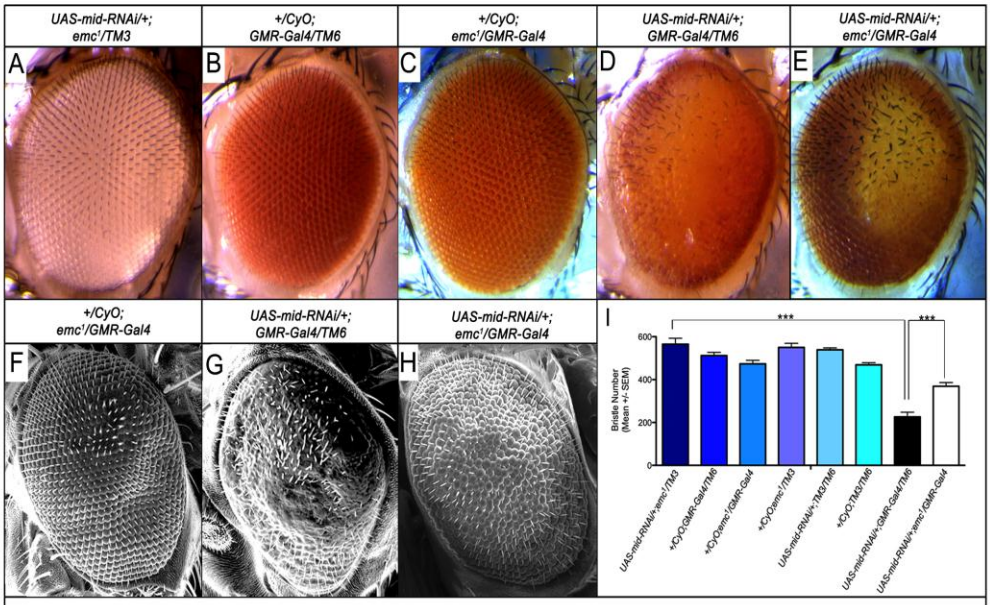


Figure 3: Immunostaining of eye imaginal discs of wild-type and *mid*-RNAi P2 staged pupae with anti-Mid, anti-Ac, and anti-Sens. A-C, A'-C': Mid and Ac expression in wild-type eye discs. D-F, D'-F': Mid and Ac expression in *mid*-RNAi in wild-type eye discs. G-I, G'-I': Mid and Sens expression in wild-type eye discs. J-L, J'-L': Mid and Sens expression in *mid*-RNAi eye discs.

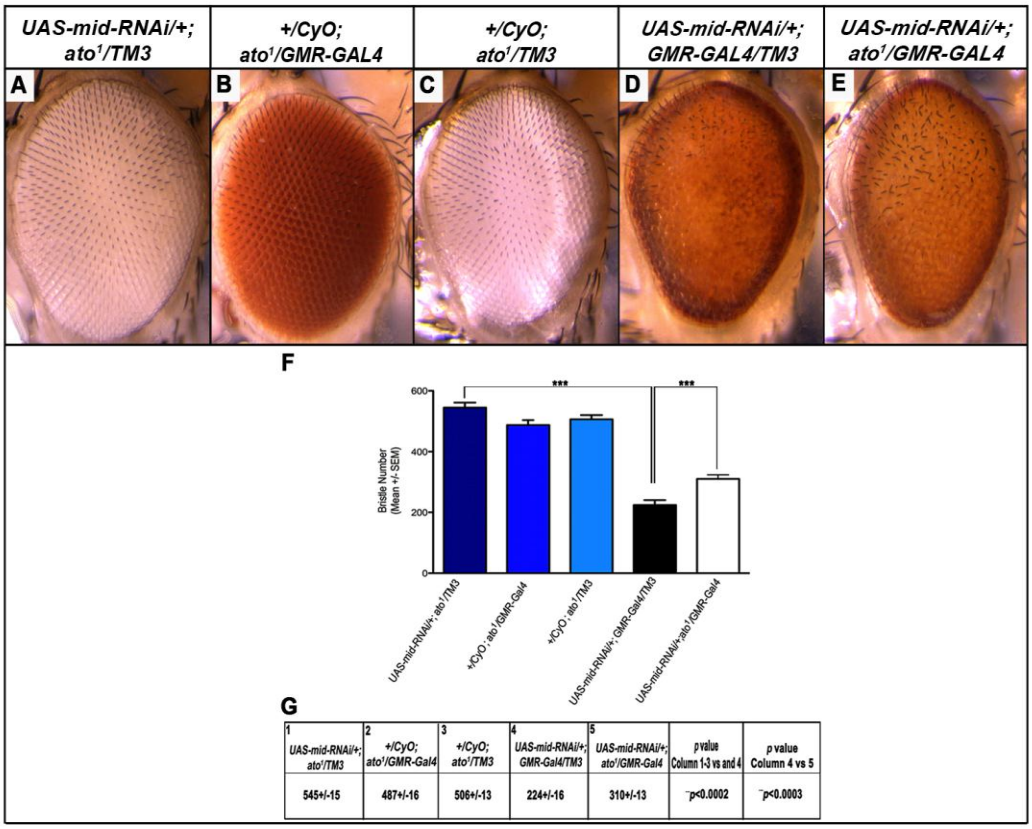


1 UAS-mid-RNAi/+; emc¹/TM3	2 +/CyO; GMR-Gal4/TM6	3 +/CyO; emc¹/GMR-Gal4	4 +/CyO; emc¹/TM3	5 UAS-mid-RNAi/+; TM3/TM6	6 +/CyO; TM3/TM6	7 UAS-mid-RNAi/+; GMR-Gal4/TM6	8 UAS-mid-RNAi/+; emc¹/GMR-Gal4	p value Column 1-6 vs 7	p value Column 7 vs 8
564+/-28	512+/-15	473+/-16	550+/-19	539+/-9	469+/-10	226+/-22	369+/-17	***p<0.0001	***p<0.0001

Figure 4: Light microscope and SEM images of eyes when removal of *emc* was placed in the *mid*-RNAi background. A-C: Light microscope images of the F1 progeny generated from the parental cross. A and B show no significant loss of bristles while C shows an approximate 6% decrease in bristles. D: *mid*-RNAi eye under light microscope. E: Light microscope image of eye with one functional copy of the *emc* gene removed. F: SEM image of +/*CyO;emc*^{1}*/GMR-Gal4* eye presenting ~6% loss of bristles. G: SEM image of *mid*-RNAi eye. H: SEM image of eye with one functional copy of the *emc* gene removed. I: Graphical representation of bristle counts of the listed genotypes. J: Representation of ANOVA Single Factor statistical analysis of bristle counts.

Preliminary data suggested that *mid* functions within the Notch-Delta signaling pathway to specific SOP cell fates. A chromosomal deficiency line ,*Df(3L)ED4196*, was observed to suppress the *mid*-RNAi phenotype, recovering bristles, when placed in the *mid*-RNAi background. Within this large stretch of DNA, 85 genes were surveyed and genes known to interact with the Notch-Delta pathway were chosen for further study. *emc* has a documented relationship with the Notch-Delta pathway for the regulation of the development of mechanosensory bristles and lies within the mentioned deficiency line (Chen et al., 1996). For these reasons,

emc was chosen as a gene for further study. The F1 progeny produced from the parental cross exhibited no significant loss of bristles (Fig. 4A-B) except for +/*CyO;emc*^{1}*/GMR-Gal4*, which presented an approximate 6% loss of bristles. When one copy of the *emc* gene was removed and placed in the *mid*-RNAi background, a significant recovery of bristles was observed as well as removal of tissue defects seen in the *mid*-RNAi phenotype. We were able to easily collect and separate the different genotypes using marker genes such as *curly of oster* (CyO), that when present results in a curly wing phenotype, and TM3, which causes a stubble bristle phenotype seen in the dorsal region. Finding and evaluating the flies with the missing *emc* gene allowed us to conclude that Mid and Emc interact with Mid playing a cell-survival role as an antagonist of Emc function.

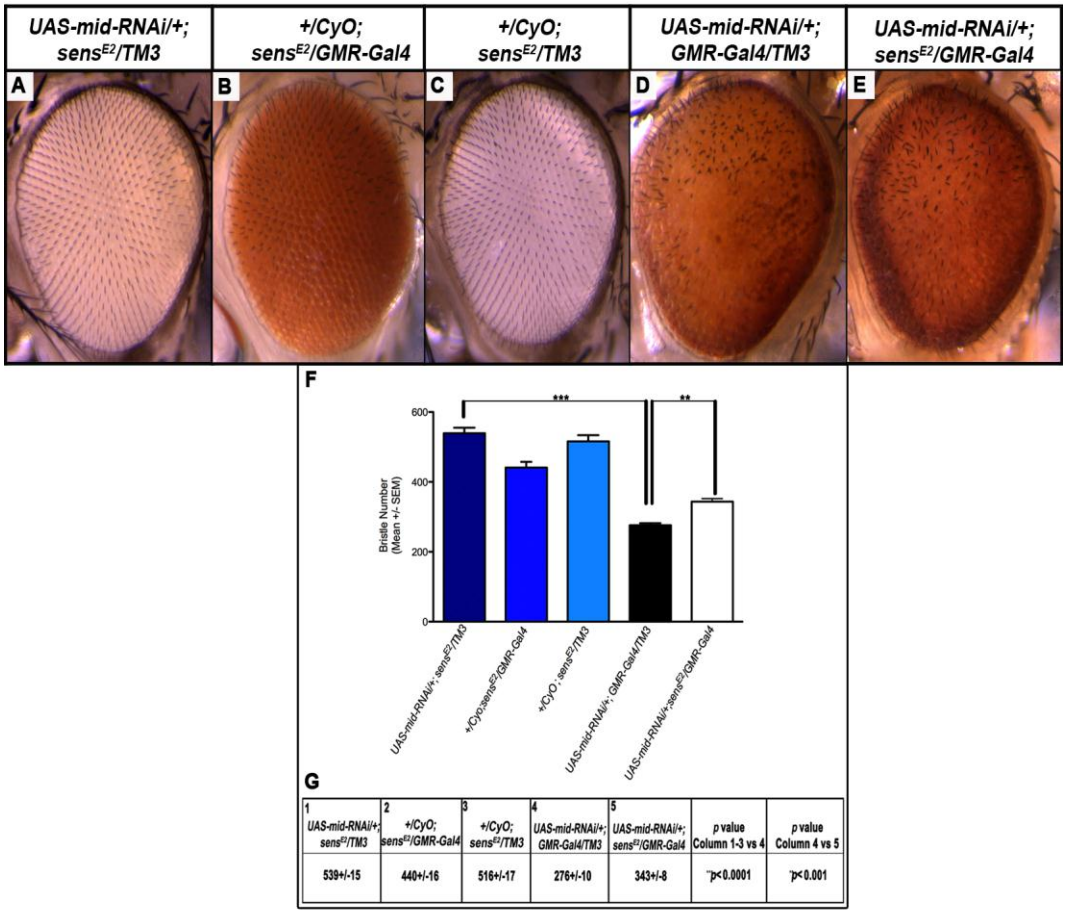


G

1 UAS-mid-RNAi/+; ato1/TM3	2 +/CyO; ato1/GMR-Gal4	3 +/CyO; ato1/TM3	4 UAS-mid-RNAi/+; GMR-Gal4/TM3	5 UAS-mid-RNAi/+; ato1/GMR-Gal4	p value Column 1-3 vs and 4	p value Column 4 vs 5
545+/-15	487+/-16	506+/-13	224+/-16	310+/-13	¯p<0.0002	¯p<0.0003

Figure 5: Light microscope images of eyes when *ato*^{1} was placed in the *mid*-RNAi background. A-C: Light microscope images of F1 progeny of crossed parental flies exhibiting no significant loss of bristles. D: Light microscope image of a *mid*-RNAi fly eye. E: Light microscope image of eye with one functional copy of *ato*^{1} gene removed shows suppression of *mid*-RNAi phenotype. F: Graphical representation of bristle counts of A-E. G: Representation of ANOVA Single Factor statistical analysis of bristle counts.

Locating *mid* within the Notch-Delta signaling pathway required further genetic studies by placing *mid*-RNAi flies within heterozygous mutant backgrounds of genes that known to function within the Notch-Delta signaling pathway. Of the surveyed genes, including *Su(H), E(spl)-m8, H, hairy (h), ato, da, ac, sc, and sens*, many exhibited normal bristle count numbers when compared to WT flies. Two genes, ato^{1} (Fig. 5) and $sens^{E2}$ (Fig. 6), were observed suppressing the *mid*-RNAi phenotype through a recovery of bristles. The other members of the Notch-Delta pathway that were tested displayed no recovery of bristles when compared to the *mid*-RNAi phenotype. Using these genetic studies and the immunolabeling showing the effects of reduction of Mid on Ac activity (Fig. 3), *mid* was placed upstream of *E(Spl)* parallel to *Su(H)* in the Notch-Delta pathway to specify SOP cell fates. Additionally, the expression of the genes downstream of *mid* in the Notch-Delta pathway rely on the expression of *mid* for their own expression.



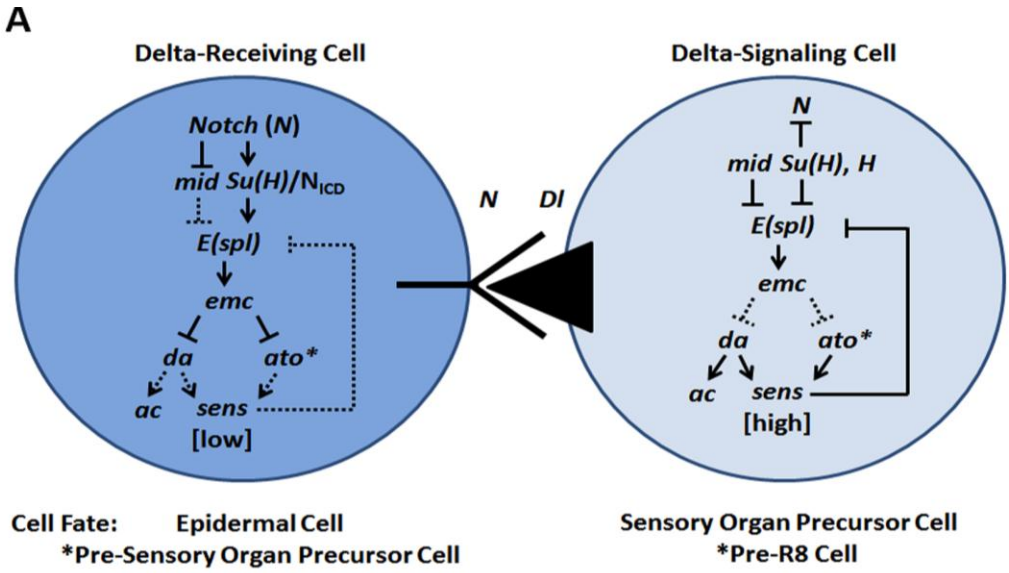
G

1 UAS-mid-RNAi/+; sens^{E2}/TM3	2 +/CyO; sens^{E2}/GMR-Gal4	3 +/CyO; sens^{E2}/TM3	4 UAS-mid-RNAi/+; GMR-Gal4/TM3	5 UAS-mid-RNAi/+; sens^{E2}/GMR-Gal4	p value Column 1-3 vs 4	p value Column 4 vs 5
539+/-15	440+/-16	516+/-17	276+/-10	343+/-8	*p<0.0001	*p< 0.001

Figure 6: Light microscope images of eyes when *sens*^{E2} was placed in the *mid*-RNAi background. A-C: Light microscope images of F1 progeny of crossed parental flies exhibiting no significant loss of bristles. D: Light microscope image of a *mid*-RNAi fly eye. E: Light microscope image of eye with one functional copy of *sens*^{E2} gene removed shows suppression of *mid*-RNAi phenotype. F: Graphical representation of bristle counts of A-E. G: Representation of ANOVA Single Factor statistical analysis of bristle counts.

Notch regulates apoptosis, or programmed cell death, of excess interommatidial precursor cells in the developing eye disc of WT flies (Wolff and Ready, 1991). We used this research to explain the patches of melanized tissue observed in some of the eyes of *mid*-RNAi flies. Due to the reduction of the expression of *mid*, a higher number of cells were incorrectly specified as epithelial cells. We predicted that these excess cells were undergoing apoptosis, resulting in the loss of pigmentation and bristles. To test this, flies that overexpressed *p35,* an

anti-apoptotic factor, were bred in the *mid*-RNAi background. The flies of genotype *UAS-p35/w^{1118}; UAS-mid-RNAi/+;GMR-Gal4/+* suppressed the *mid*-RNAi phenotype, recovering bristles and reducing the amount of discolored tissue. This supports the prediction that, to an extent, loss of bristles occurs due to apoptosis (Fig. 7).



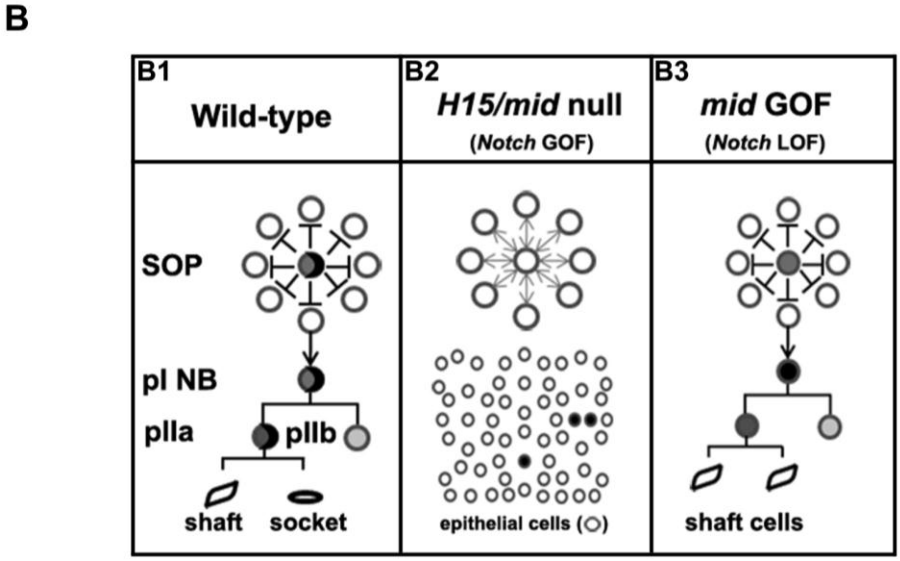


Figure 7: Diagram showing the Notch-Delta signaling pathway in neighboring cells, deciding cell fate as SOP cell or epidermal cell. A: Cell-to-cell interactions between Delta-Signaling cells and Delta-receiving cells, determining cell fate as SOP or epidermal. B1: Formation of differentiated cells in WT flies. B2: Formation of excess epithelial cells that undergo apoptosis in *mid*-RNAi flies. B3: Replacing *mid* results in the recovery of bristles.

Another goal of this research was to expand the understanding of the importance of *mid* in the developing CNS and PNS of *Drosophila.* To accomplish this, we created a developmental profile using double immunostaining techniques to highlight the expression of Mid and its co-expression with Ac and Sens in the stages of embryonic development of Oregon-R (WT) *Drosophila* embryos.

Sens was previously described in the results of our genetics studies as a protein that is present in SOP cells. By looking for co-expression of Mid and Sens, we can determine if Mid is present in proneural cells of the CNS and PNS as it was in the eye imaginal disc. Sens expression is turned on in SOP cells of the CNS and PNS in stages 9 through 11 of embryonic development (Fig. 8A-C'''). Witt et al. report that Sens is present in C1 SOP cells and its lineage during these stages (Fig. 8A-B''') and that the expression begins to fade in stage 12 embryos (2010). We support these results, showing very little Sens expression in the stage 12 embryo (Fig. 8D-D'''). Mid expression begins around stage 11 (Fig. 8B-B''') and is highly expressed in both the CNS and PNS after stage 11. In late stage 11 and stage 12 embryos, Sens and Mid are co-expressed in SOP cells but this may be an artifact of Sens providing false results. Further research is required to affirm the co-expression of Sens and Mid during these stages.

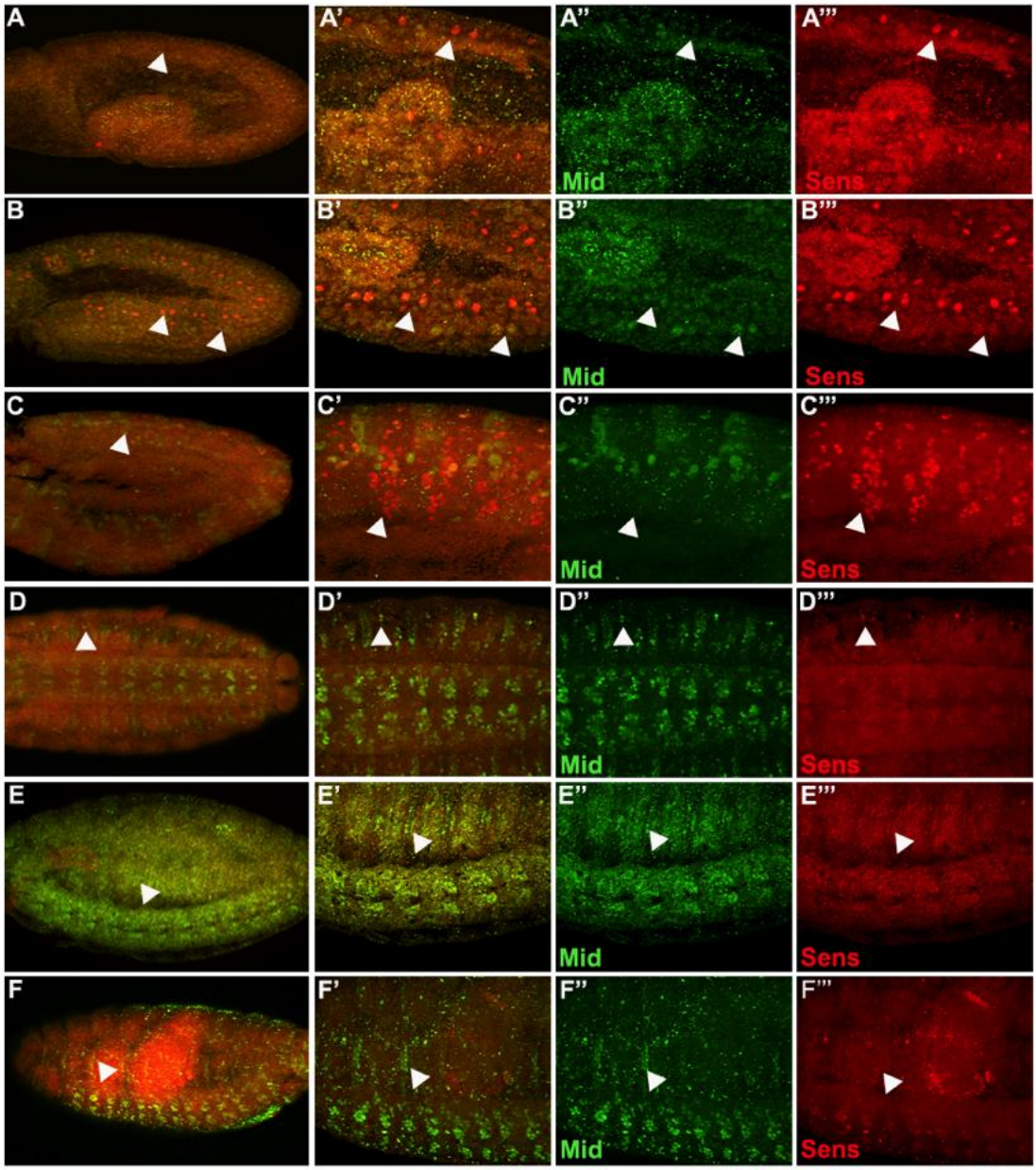


Figure 8: Expression of Mid and Sens in PNS and CNS during stages of embryonic development. A-A’’’: Mid and Sens expression in dorsal abdominal region of stage 9 embryo. B-B’’’: Mid and Sens expression in ventral abdominal region of early stage 11 embryo. C-C’’’: Mid and Sens expression in dorsal abdominal region of late stage 11 embryo. D-D’’’: Mid and Sens expression in ventral abdominal region of stage 12 embryo. E-E’’’: Mid and Sens expression in ventral abdominal region of stage 15 embryo. F-F’’’: Mid and Sens expression in ventral abdominal region of stage 17 embryo.

Mid expression continues through the later stages of embryonic development but appears to begin to fade after stage 15 (Fig. 8 E-E'''). Dissections of the VNC of a stage 15 embryo validates that Sens is not expressed in the CNS during later stages. Another useful area of future research will be dissection of the VNC of stage 12 embryos to detect if Sens and Mid are co-expressed in the CNS at earlier stages, which is hard to detect with whole mount embryos.

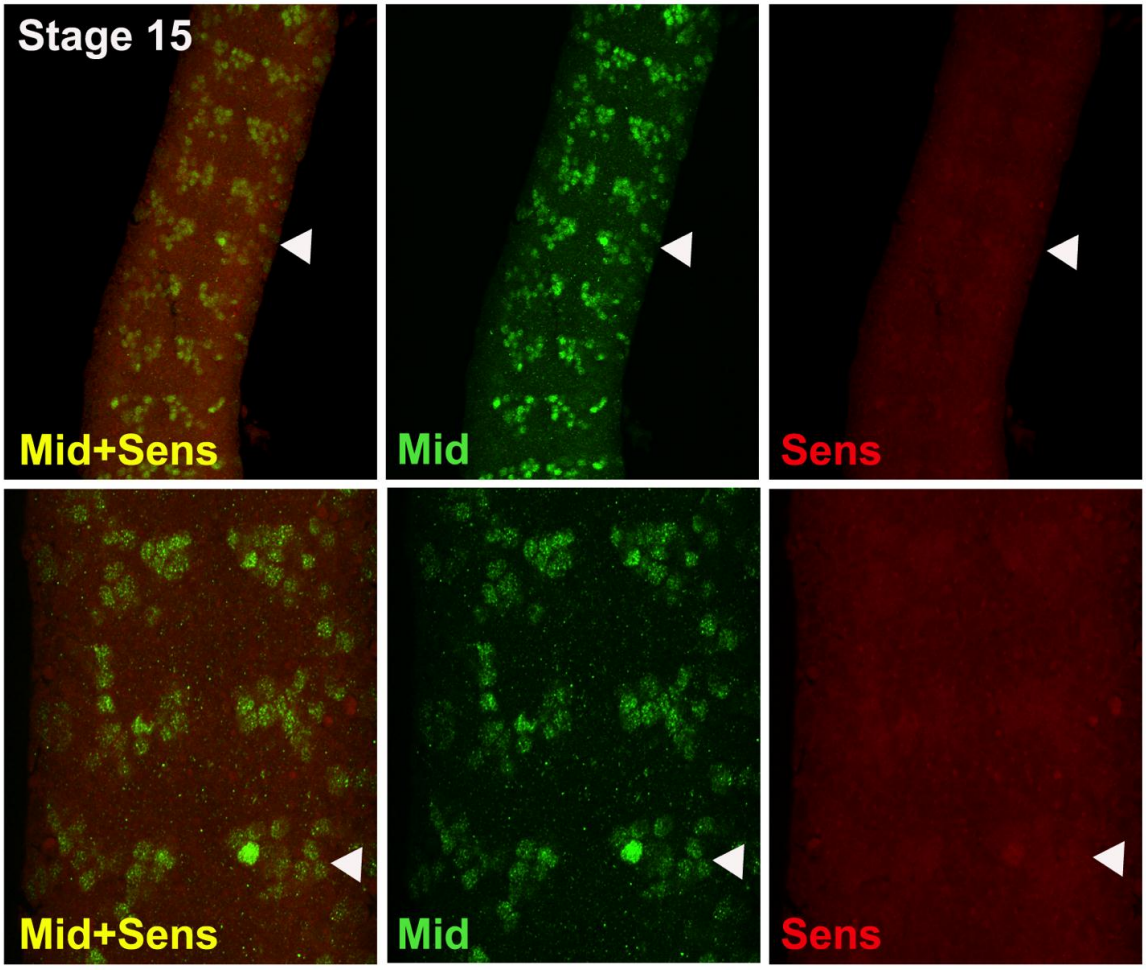


Figure 9: Expression of Mid and Sens in dissected Stage 15 Ventral Nerve Cord. The ventral nerve cord of an Oregon-R (wild-type) embryo that was double immunostained with anti-Mid and anti-Sens antibodies was dissected and viewed under a confocal microscope. At stage 15 Mid is highly expressed in the neurons of the CNS but Sens protein expression has ceased.

We also screened for another SOP cell marker, Ac, to detect the presence of Mid in SOP cells. As with Sens, Ac was present beginning in stage 8, when Mid is not yet expressed. Skeath et al. noted that Ac was expressed in three distinct rows in stage 8 embryos (Fig. 10A-B'''), marking SOP cells that develop into the neurons of the CNS and PNS at later stages (1994). Our data shows that there is co-expression of Mid and Ac in a few SOP cells beginning at stage 9 (Fig. 10B-B''') but this may be an artifact of Mid, since it has been shown that Mid expression does not begin until around stage 11 or 12. More confocal images will need to be gathered to rule out Mid expression at stage 9.

As seen in the confocal images presented of Mid and Sens expression (Fig. 8), Mid is highly expressed after stage 12. However, our data shows that Ac expression, unlike Sens, does not stop after late stage 11 or stage 12. Ac is observed co-expressing with Mid in the CNS and PNS neuronal cells continuing through stage 17. VNC dissection of a stage 15 embryo supports that Mid and Ac are co-expressed in the CNS neurons in these later stages. This may also be an artifact, allowing the anti-Ac antibody to bind to these cells, providing false results. No prior research could be found detailing the expression of Ac during the stages of embryonic development past stage 8, so, repetition of this study will be needed to affirm that Ac expression continues past the early stages and that this is not mislabeling.

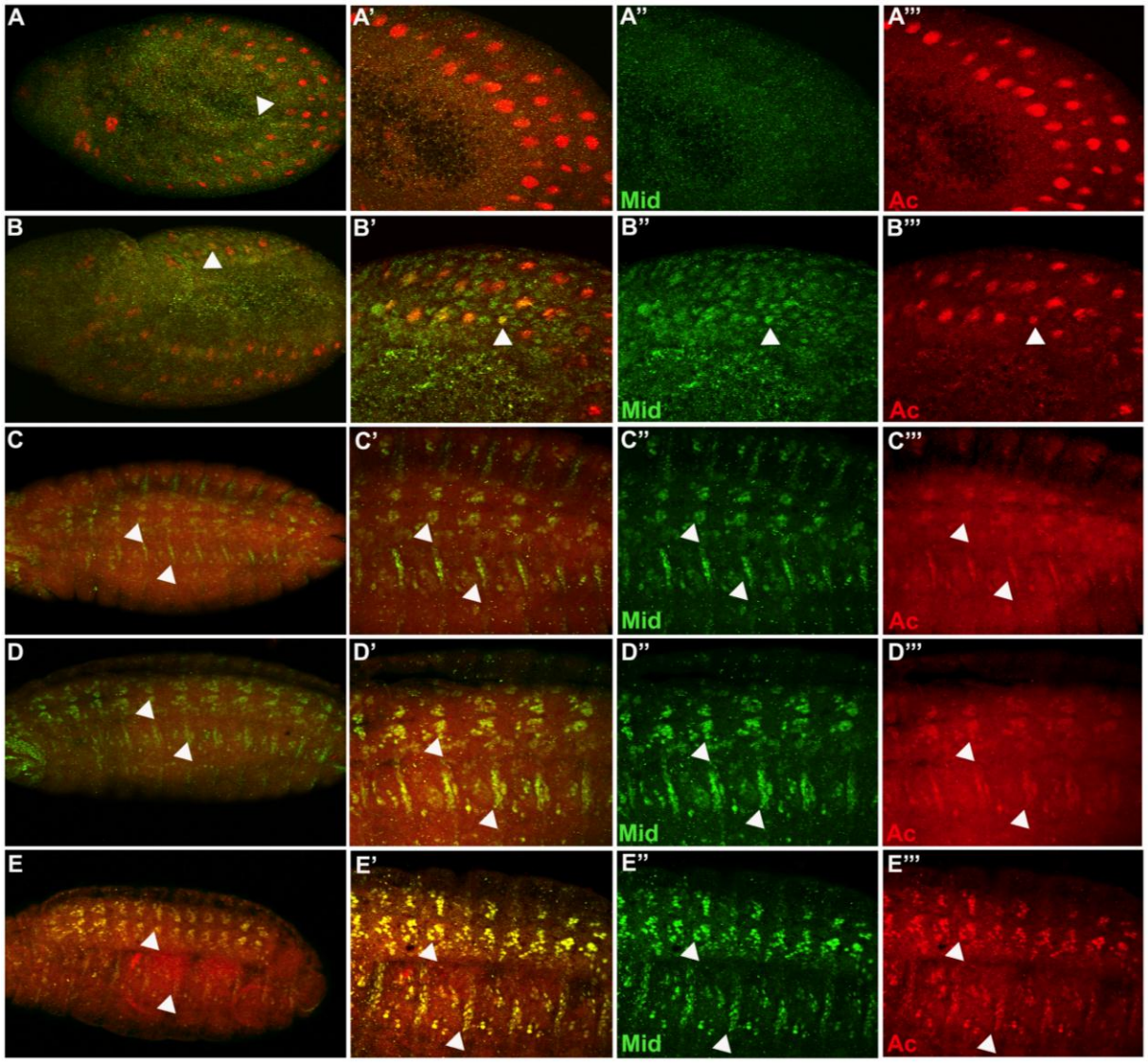


Figure 10: Expression of Mid and Ac in PNS and CNS during stages of embryonic development. A-A''': Mid and Ac expression in the thoracic region of a stage 8 embryo. B-B''': Mid and Ac expression in the dorsal abdominal region of a stage 9 embryo. C-C''': Mid and Ac expression in the ventral abdominal region of a stage 13 embryo. D-D''': Mid and Ac expression in the ventral abdominal region of a stage 15 embryo. E-E''': Mid and Ac expression in the ventral abdominal region of a stage 17 embryo.

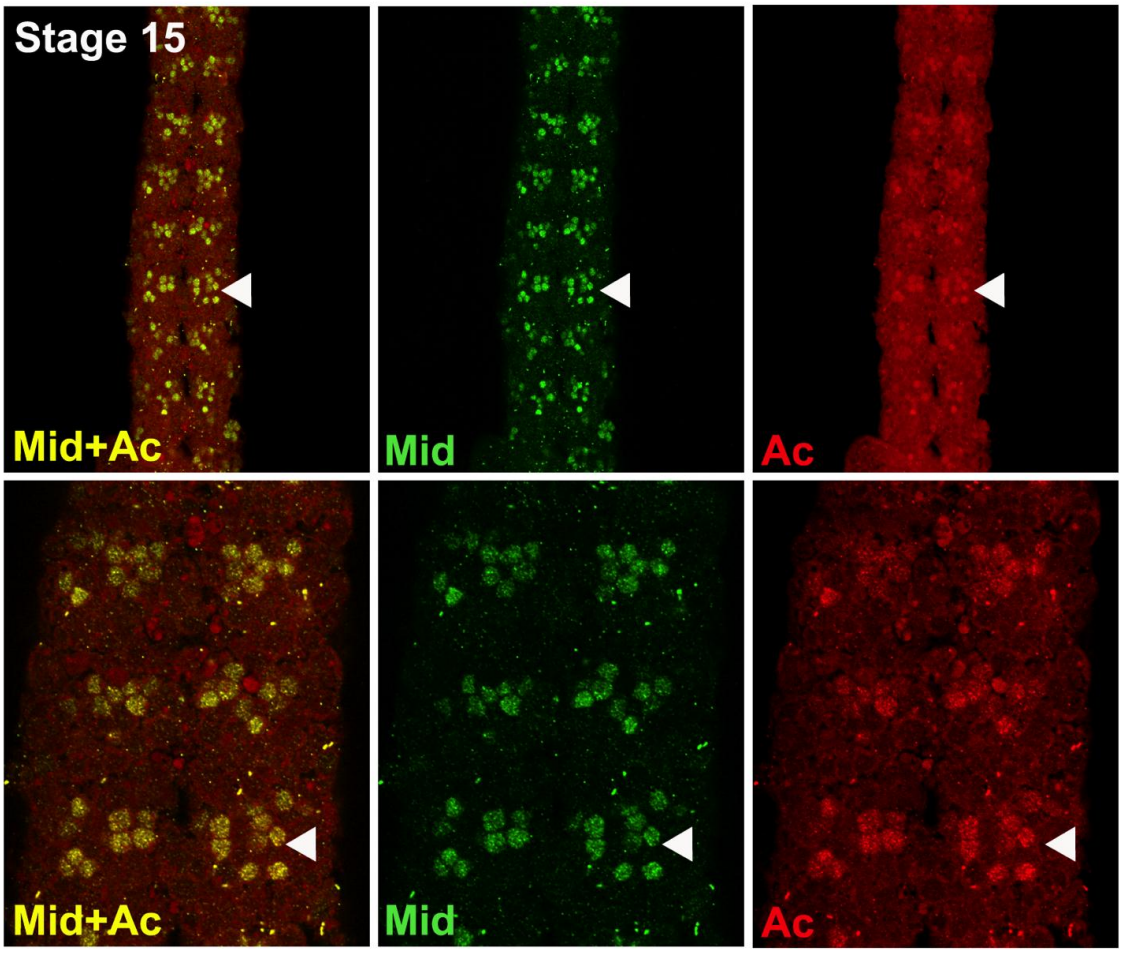


Figure 11: Expression of Mid and Ac in dissected stage 15 ventral nerve cord. The ventral nerve cord of an Oregon-R (wild-type) embryo that was double immunostained with anti-Mid and anti-Ac antibodies was dissected and viewed under a confocal microscope. At stage 15, Mid and Ac proteins are co-expressed in the neurons of the CNS.

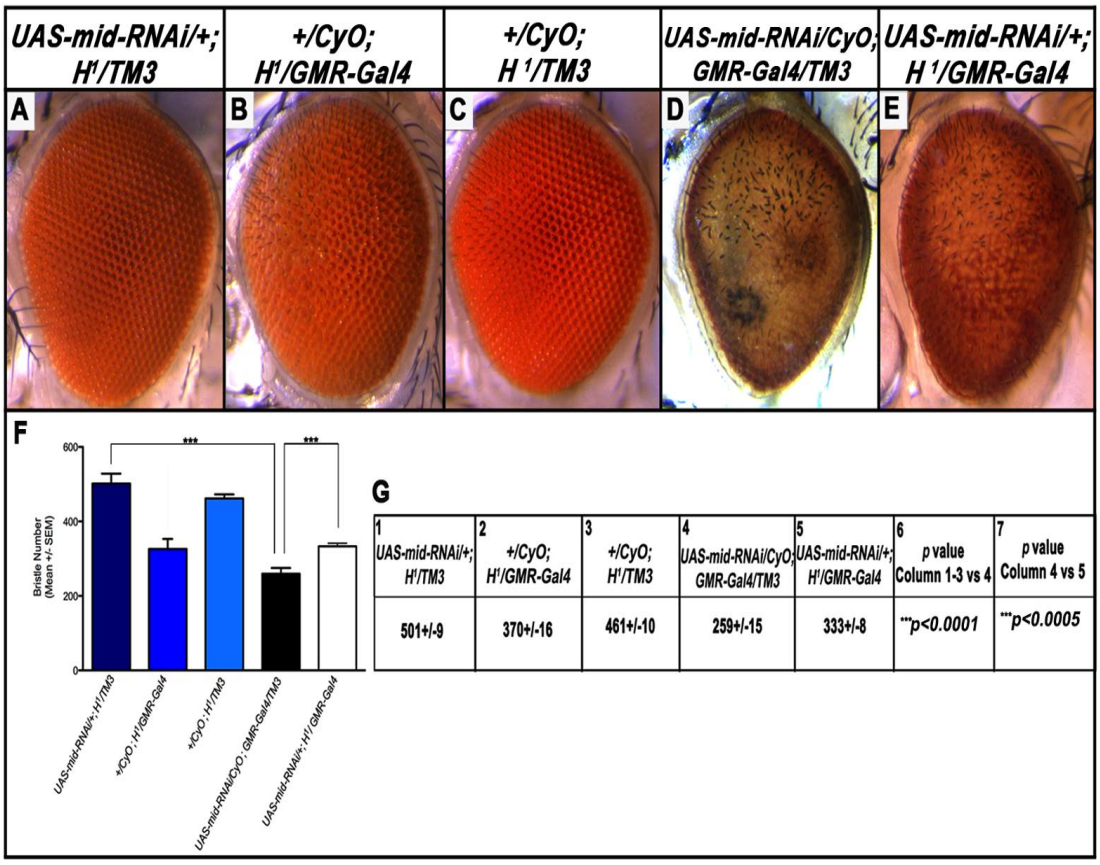
Discussion:

The Notch-Delta signaling pathway regulates SOP cell specification and is a highly organized intercellular and intracellular process. A single cell in a cluster of proneural cells is selected to become a proneural cell by expressing Delta, a surface protein, in higher levels than the surrounding cells. The Delta receptor protein Notch receives the signal from the Delta signaling cell and triggers a downstream, intracellular pathway regulating the expression of genes that leads to the formation of epithelial cells. Knock-down of *mid* using RNAi techniques and the *GMR-Gal4* driver line allowed us to find that eye development is highly affected without the presence of *mid* and hypothesized that *mid* was a member of the Notch-Delta pathway, acting to regulate eye development.

Using genetic modifier studies to place mutant alleles of the Notch-Delta pathway within the *mid*-RNAi background, we were able to place *mid* in the pathway downstream of *Notch*, upstream of *E(spl)-m8*, and in parallel for the signaling in both SOP cells and epidermal cells (Das et al., 2013). With a loss of mid, no cells have high levels of delta expressed and no cell is selected from a proneural cluster as an SOP cell. A lack of SOP cells to develop into shaft and socket cells results in a loss of bristles and an excess of epidermal cells that undergo apoptosis. This result puts Mid in both a cell survival and cell specification role.

An additional source of bristle loss, the inclusion of the *GMR-Gal4* driver line, must also be accounted for in this research as it could have slightly altered the amount of bristle loss in the progeny. Several combinations of mutant alleles with the driver line resulted in a 5-15% loss of bristles, while the most significant loss

occurred with the *Hairless*, H^1, mutant alleles. A loss of 25% occurred in these flies when compared to the WT phenotype (Fig. 12). Therefore, it was not possible to accurately analyze H^1 effects on the *mid*-RNAi phenotype. Besides H^1, the loss of bristles was much more extensive than the 5-15% that could have been due to the *GMR-Gal4* driver line, so, we are accurately able to identify that they do have effects on the *mid*-RNAi phenotype.



1 UAS-mid-RNAi/+; H1/TM3	2 +/CyO; H1/GMR-Gal4	3 +/CyO; H1/TM3	4 UAS-mid-RNAi/CyO; GMR-Gal4/TM3	5 UAS-mid-RNAi/+; H1/GMR-Gal4	6 p value Column 1-3 vs 4	7 p value Column 4 vs 5
501+/-9	370+/-16	461+/-10	259+/-15	333+/-8	***p<0.0001	***p<0.0005

Figure 12: Light microscope images of the effect of the *GMR-Gal4* driver line on bristle loss when combined with H^1 mutant allele. A and C: Light microscope images of F1 progeny displaying minimal bristle loss in comparison to WT phenotype. B: Light microscope images of F1 progeny displaying an ~25% loss of bristles. D: Light microscope image of *mid*-RNAi phenotype. E: Light microscope image of eye with removal of one functional copy of H^1. F: Graphical representation of bristle counts of the listed genotypes. G: Representation of ANOVA Single Factor statistical analysis of bristle counts.

For the future directions of this research, the Leal lab is interested in investigating why the ventral region of the *mid*-RNAi eyes exhibited greater bristle loss than the dorsal region. Additionally, this research does not eliminate the possibility of interactions between *mid* and other genes of the Notch-Delta pathway so further analyses of the interactions between these two may be undertaken.

The developmental profiles of the Oregon-R (WT) embryos revealed that expression of Mid, Sens, and Ac in the CNS and PNS occurs in a choreographed manner during embryonic development. Due to extremely limited research on PNS development, extensive research will be required to determine the developmental significance of Mid in the PNS.

Sens expression fades immediately when Mid expression begins. Whether Sens expression or activation is due to interactions with Mid or regulated by Mid in the CNS and PNS will require further research. From our immunostaining images (Figs. 8-11), it is clear that Mid is highly expressed in the neurons of the PNS and CNS, so determining whether Mid exhibits a cell survival and specification role in these areas, as it does in the eye, will further develop a more extensive understanding of a possible universal role of Mid in *Drosophila.*

Discovering that Ac expression was continuous from early to late stages of embryonic CNS development and its high levels of co-expression was an exciting finding from this research. No other genes have been found that are so highly co-expressed with *mid*, asserting that there may be a very close relationship with *mid* and *achaete* that is worth further research. Additionally, the Leal lab plans to repeat this experiment to verify that Ac expression was not just an artifact.

To continue this research, the Leal lab plans to repeat the double-immunostaining procedures on *mid*-RNAi embryos and evaluate its effects on the expression of Sens and Ac during the stages of embryonic development. If Ac expression was not an artifact, it can be expected that reducing Mid expression will also reduce Ac expression in the CNS and PNS. This will be an area of future research and will further distinguish a close relationship between *achaete* and *midline*.

Clinical Implications

This research may have future clinical implications as it reveals that *mid* plays a cell survival and cell specification role in the eye. The expression of Tbx20 has been documented in mice photoreceptor neuron development (Meins et al., 2000) and our research shows that Mid is also expressed in the photoreceptors of *Drosophila*. Since *Tbx20* heterozygotes display developmental disorders of the heart and CNS, *Tbx20* could also be involved in photoreceptor neurodegenerative diseases.

Fortini and Bonini report that *Drosophila* can serve as a model for understanding human neurodegenerative diseases by studying the basic protein functions of gene homologues, such as *mid* and *Tbx20* (2000). Also, since mutations of *mid* and *Tbx20* are known to cause developmental disorders of the heart and CNS in heterozygotes, it may be a useful area of research to look for neurological disorders, eye disorders, the rate of cataract development compared to homozygotes, etc. in heterozygotes. By placing Mid in the Notch-Delta pathway, we observed interactions between Mid and Notch. Alagille syndrome, which displays

symptoms of liver, eye, cardiac, and skeletal deformities has been linked to mutations in *JAGGED1* (*JAG1*), a ligand for the for Notch receptors (Jones et al., 2000). Overlap in symptoms of the heart and CNS with neurological disorders would provide further evidence that Tbx20 has a multifaceted role in development, including the regulation of Notch receptors leading to the development of Alagille syndrome. Future research to determine if *mid* also plays a cell survival role in the PNS could lead to a better understanding of degenerative diseases affecting motor function in humans by screening for motor function disorders in vertebrates.

Another student in the Leal lab is experimenting to determine if Mid regulates cell proliferation. Uncontrolled cell division leads to the development of cancer. From our research, the gene *emc*, which has a known relationship with the Notch-Delta pathway, was found to interact with *mid* to regulate eye development. The vertebrate homologue of Emc is the Inhibitor of DNA-binding protein (Id), which has been highly studied for its relationship to the development of various types of cancer. Maw et al. found that Id-4 expression is elevated in patients with ovarian cancer and may serve in the diagnosis of ovarian cancer (2009). Another research study reports that Id-1 regulation has a link to melanoma progression (Zigler et al., 2011). Since we found that Mid plays a cell survival role as an antagonist of Emc function, there may also be a relationship between these two genes concerning the development of cancer. Considering this relationship in *Drosophila* can lead to discoveries between interaction of Tbx20 and Id in vertebrates, potentially finding that Mid plays a role in the regulation of cell proliferation.

While research with *Drosophila* provides a basic understanding of protein function that does not fully match the complexity of protein function and expression in vertebrates, this basic understanding will lead to greater developments in the research of diseases in vertebrates. *Drosophila* also has a fast generation time, which can lead to faster developments in the understanding of diseases than research in mice and without the ethical constraints faced in human research. In the near future, genetic screening for mutations of Tbx20 genes could lead to the prevention and enhanced, personalized treatment of diseases.

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