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Investigating roles for RNA turnover processes in cell signaling through Drosophila melanogaster genetic mosaics

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Investigating roles for RNA turnover processes in cell signaling through *Drosophila melanogaster* genetic mosaics

by

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Abstract

The process of cell signaling is vital in organisms for proper development as it determines the fate and function of cells. This process is highly regulated by myriad interactions between signaling pathway components and gene expression mechanisms. RNA turnover is a type of RNA processing that degrades RNA. It plays an essential role in homeostasis and environmental changes; however, its influence on signaling pathways is currently unknown. In the present study, the effect of RNA turnover processes on cell signaling was analyzed using the genetic mosaics in *Drosophila melanogaster*. Fly mosaics were created by breeding RNAi lines targeting cellular ribonucleases under control of the UAS promoter with a fly line expressing GAL4 in the notum region of imaginal wing discs. Cell signaling phenotypes were analyzed in 3rd instar larva utilizing immunohistochemistry. It was observed that knockdown of RNA turnover genes resulted in substantial changes in signaling pathway proteins as well as evidence of hyperproliferation, apoptosis, and reduced proliferation. Morphologically, it was observed that the knockdown of RNA turnover genes resulted in thinner and shorter sensory notum and bristles in *Drosophila* adults. This indicates that RNA turnover influences developmental signaling pathways and may be an important mechanism in gene regulatory networks. Exercising control of developmental signaling pathways could be applied in therapeutics for conditions such as cancer and diabetes as well as in the development of regenerative medicine.

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List of Abbreviations

1. RNAi – RNA Interference
2. mRNA – messenger RNA
3. dsRNA – double stranded RNA
4. siRNA – short interfering RNAs
5. RISC – RNA-induced silencing complex
6. Dcp-1 – Death Caspase 1
7. Pcm – Pacman
8. Rat1 – Ribonucleic Acid Trafficking 1
9. Rrp6 – Ribosomal RNA Processing
10. UAS – Upstream Activation Sequence
11. GFP – Green Fluorescence Protein
12. SOP – Sensory Organ Precursor
13. PFA – Paraformaldehyde
14. PBS – Phosphate buffered saline
15. DAPI – 4’,6-diamidino-2-phenylindole
16. dDcp1 – Drosophila decapping Protein 1
17. dDcp2 – Drosophila decapping Protein 2
18. Ldbr – Lariat Debranching Enzyme
19. rRNA – Ribosomal RNA
20. snRNA – Small nuclear RNA
21. snoRNA – Small nucleolar RNA

Chapter 1: Literature Review

Drosophila melanogaster

Drosophila melanogaster, or the common fruit fly is an organism that has served as an exemplary genetic model in biological research (Greenspan, 2004). *Drosophila melanogaster* was first used as a model organism by William Ernest Castle (1867-1962) at Harvard University. However, the species potential as a model organism was discovered through work conducted by Thomas Hunt Morgan (1866-1945) in the early 1900s at Columbia University. This led to great discoveries such as sex-linked genes and the effect of radiation on genes (Stephenson & Metcalfe, 2013). Morgan's contribution to science led him to be awarded the 1933 Nobel Prize in Physiology or Medicine.

Further research was conducted that greatly advanced the field of genetics. This *Drosophila* research greatly contributed to the fields of genetics, developmental biology, cell biology and population genetics and later to neuroscience and neurodevelopment (Stephenson & Metcalfe, 2013). This research solidified the genetic possibilities of utilizing *Drosophila* as a genetic model and it became widely utilized by others in the scientific world. Although, it has been used for over a century, *Drosophila melanogaster* still remains as one of the most valuable model organisms and is still widely utilized in neuroscience, developmental and cellular biology research. Currently, it is being extensively used in research to understand the molecular basis of almost every disease (Stephenson & Metcalfe, 2013).

Drosophila melanogaster serves as a successful model organism for multiple factors. Firstly, the genetics are simple, and it contains less genetic redundancy than humans (Hughes et al., 2012), which allows for simpler genetic analysis. The species is less difficult and expensive to manipulate and maintain compared to mammalian model organisms and thus can be

genetically modified and propagated at low cost. *Drosophila* breed at high rates with females being able to lay up to a 100 eggs per days for up to 20 days (Jennings, 2011). Although, the rate of development depends on temperature and environmental conditions, *Drosophila* can usually develop from an egg to a mature adult in about 10 days at 25°C (Jennings, 2011). The life cycle is displayed in Figure 1.

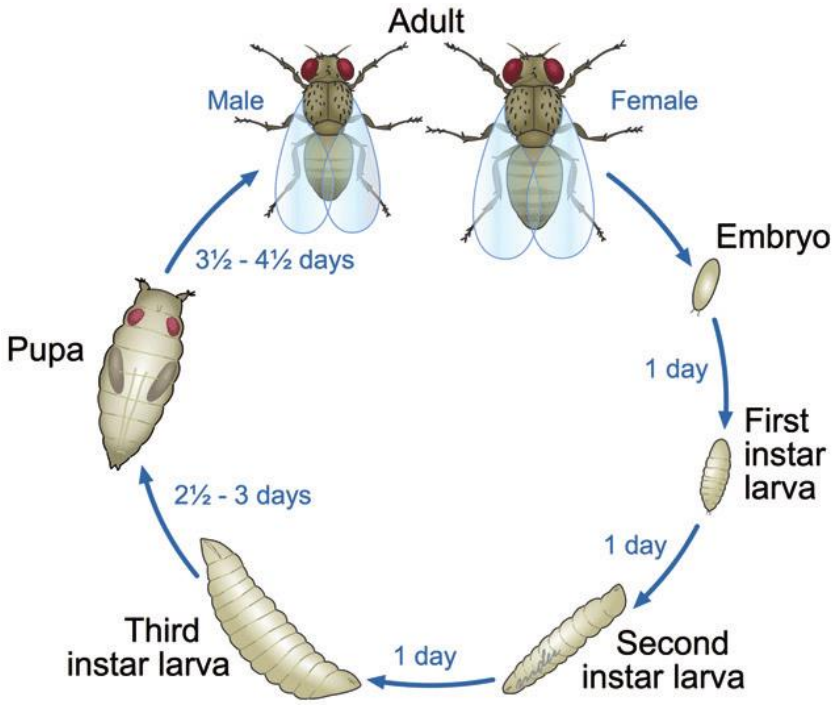

Figure 1: **Life cycle of *Drosophila melanogaster.*** (Ong et al., 2014)

Drosophila can also be easily maintained in the laboratory. The flies can be maintained in plastic vials with fly food made with cornmeal, agar, yeast, molasses and other components (Jennings, 2011). Thus, large stocks of flies can be maintained in a relatively small space. As the flies will constantly breed, new lines can be produced and as long as the population has food, it will be self-sufficient and keep growing. The flies can be anesthetized using carbon dioxide for easy handling (Jennings, 2011). This is utilized for creating crosses and for dissection and morphological analysis. Due to the widespread use of *Drosophila* use as a model genetic

organism, there are large-scale organizations that maintain fly stocks that can be ordered and utilized at individual institutions and laboratories. Thus, *Drosophila* species continue to serve as one of the most commonly used and sophisticated model organisms in biological research including developmental biology research.

RNA Turnover Processes

RNA transcripts are subject to many regulatory processes such as during splicing and adenylation. RNA Turnover is a type of regulatory mechanism related to the degradation of mRNA (messenger RNA), where its turnover plays an essential role in regulating mRNA expression stability and quality (Shyu, 2018). In mammalian cells, all the major modes of mRNA decay are initiated by deadenylation and there are two major pathways. The 3' pathway is predominant compared to the 5' pathway with both being regulated by exonucleases (Meyer et al., 2004).

There are a variety of factors that affect the involvement of mRNA turnover in the decay process. The rate of deadenylation appears to have to greatest influence on the rate of decay (Meyer et al., 2004). Poly(A) binding proteins also have an effect on the regulation of the deadenylation and 11 different proteins are known to be involved in mRNA decay (Shyu & Chen, 2010). Thus, it can be observed that mRNA turnover is not a simple pathway and consists of a complex set of processes independent of transcription (Shyu & Chen, 2010). However, the effect of mRNA turnover processes on cell signaling and specification is not yet fully understood.

Cell fate specification

The cells in the embryo are influenced by their 'environment', which is the surrounding tissues and interactions within the environment that determine the fate of the cell (Gilbert, 2000).

The specialization of cells is called differentiation. However, this process is preceded by the commitment of the cell to a certain developmental fate (Gilbert, 2000). This developmental commitment can be divided into two stages, specification and determination. During this process, the cell may remain physiologically the same, but its eventual function has been determined.

Signaling pathways play a vital role in cell specification by activating transcriptional programs in response to external signals (Perrimon et al., 2012). Although, there is a limited number of pathways, a variety of extracellular signals produce various products by varying the amplitude, duration, interaction, and integration of transcription factors (Perrimon et al., 2012). Responses to signaling pathways are complex and involve a myriad of regulatory processes, including cell proliferation and apoptosis. A variety of pathways can interact in the formation of adult structure precursor regions, such as the interaction of Fringe, Notch and Delta pathways in the specification of the wing margin in *Drosophila* wings (Perrimon et al., 2012). The effects of these various pathways can be observed by creating model genetic mosaics.

Genetic mosaics are model techniques that are used to observe cell development. An important prerequisite for using genetic mosaics is they need to be induced at the embryonic or larval stage when cells are still undifferentiated or undeveloped. Thus, in *Drosophila* we induce the genetic mosaic techniques in the larval stages. These techniques were utilized on the imaginal disc whose formation begins during embryogenesis. The anatomy of the larvae is displayed in Figure 2. The adult epidermis develops from precursor cells. The abdominal segments develop from histoblasts, whereas the rest of the epidermis is formed from imaginal discs (Bate, 1991). They undergo growth and differentiation during the larval and pupal development stages, which results in the development of adult structures such as the thorax,

wings, and sensory bristles (Jones, 2013). Imaginal discs play an essential role in development, whereby genetic phenotypes in the discs can be analyzed and provide important information about the effect modified genes on development. These effects can be observed at both the molecular and the morphological level in adult flies.

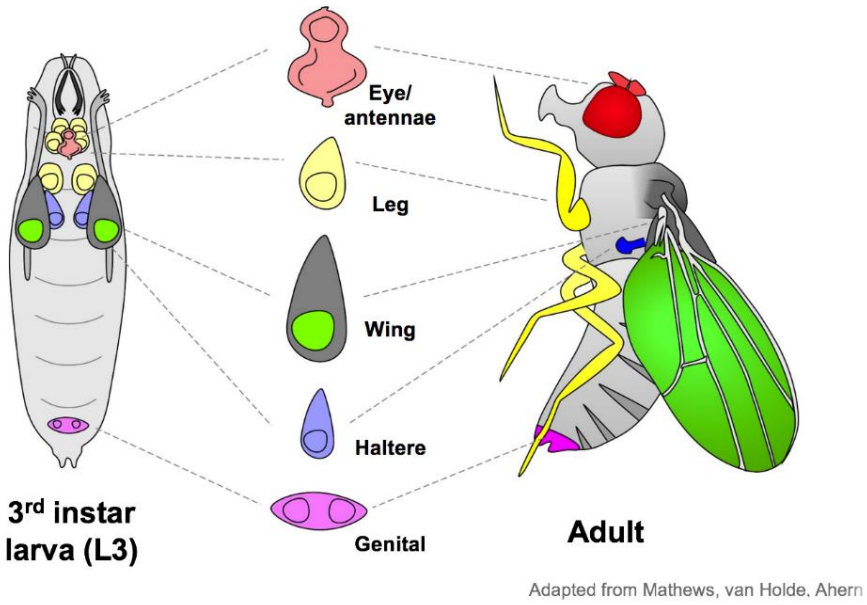

Figure 2. **Anatomy of discs in *Drosophila melanogaster* larvae** (Harris, 2020)

Wing discs serve as sensory organ precursor (SOP) cells to the sensory bristles present on the back, and thus analyzing the morphology in adult specimens could indicate the effect on the sensory organ precursor cells (Zitserman & Roegiers, 2011). The bristles on the back consist of the notum, which are smaller bristles and various larger bristles called macrochaetes (Figure 3). To observe the molecular and morphological interactions, a variety of molecular biology techniques such as RNAi and fly systems were used.

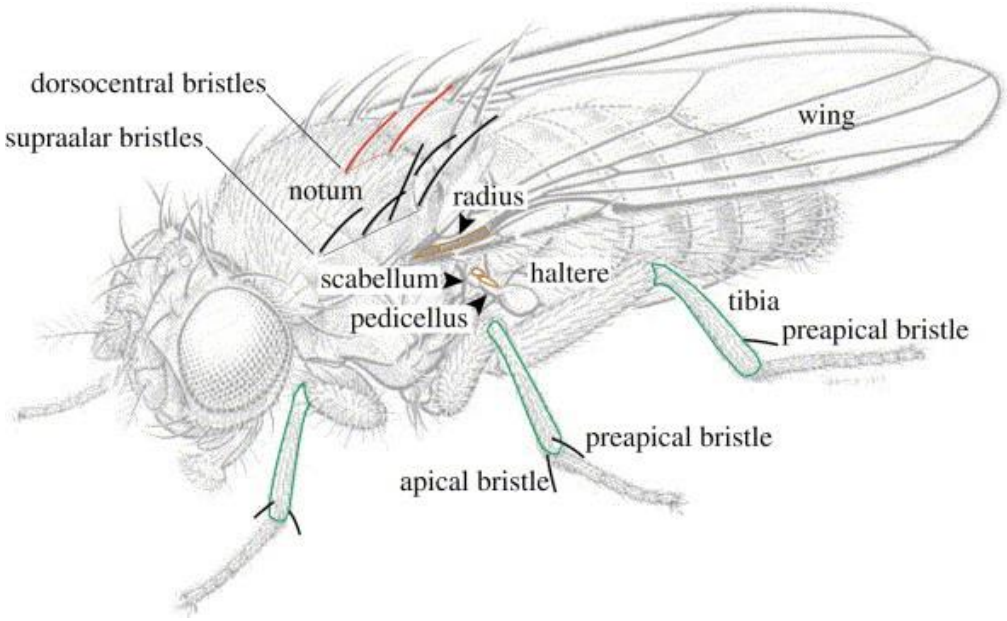

Figure 3. **Diagram illustrating the position of sensory organs in *Drosophila melanogaster***

RNA Interference

RNAi (RNA Interference) is a genetic technique utilized to silence specific genes (Rozowski, 2002). It utilizes the process of targeted degradation of the mRNA (messenger RNA) by a specific double stranded RNA (dsRNA). First, the dsRNA is processed by the cell into short interfering RNAs (siRNAs) by RNAse enzymes. Second, the siRNA forms an RNA-induced silencing complex (RISC), which then unwinds during RISC assembly. The single-stranded RNA then binds to the mRNA target and silences the gene expression. In *Drosophila*, multiple RNAi lines have been developed and the effects of genes can be studied by silencing the gene and analyzing its effect on the phenotype. Here, several RNAi lines were utilized to silence specific genes related to development to analyze their effect on expression and protein formation. The list of RNAi lines utilized to knockdown RNA turnover enzymes in this study is listed in Table 1.

Table 1: **RNAi fly lines utilized.**

Stock Number	Stock Name	Gene	Genotype
38315	Dcp-1	Death caspase 1	y^1 sc* v^1 sev^{21}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS01779}attP2
67919	Dis3	Dis3 3'-5' exoribonuclease	y^1 sc* v^1 sev^{21}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS05761}attP40
34690	Pcm	Pacman cytoplasmic exoribonuclease	y^1 sc* v^1 sev^{21}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS01169} attP2
57176	Rat1	Rat1 5'-3' nuclear exoribonuclease	y^1 sc* v^1 sev^{21}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMC04558}attP40
34809	Rrp6	Rrp6 exoribonuclease	y^1 sc* v^1 sev^{21}; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS00118}attP2/TM3, Sb^1

Death caspase 1 is a caspase that is a group of enzymes functioning in programmed cell death. It was observed that the expression of Dcp-1 was sufficient to induce cell apoptosis by causing cellular damage that would trigger cell death (Song et al., 1997). Dis3 or Tazman is a highly conserved exoribonuclease that degrades RNA in the 3’ to 5’ direction and functions in the processing of both nuclear and cytoplasmic RNA species (Towler et al., 2015). It has been observed that ribonucleases can target specific RNAs and thus, affect specific cellular pathways but their overall biological function is not well understood.

Pacman or xrn1 is a highly conserved 5’-3’ cytoplasmic exoribonuclease (Krzyszton et al., 2020). It plays a role in gene regulatory events such as control of mRNA stability, RNA Interference and regulation via miRNAs (Jones et al., 2013). Rat1 (Ribonucleic Acid Trafficking 1) or Xrn2 is a 5’ to 3’ nuclear exoribonuclease essential to cellular RNA turnover (Nagarajan et al., 2013). Rat1 degrades mRNA precursors in the nucleus (Nagarajan et al., 2013) and plays a role in the termination of RNA Polymerase II transcription to stop the formation of long mRNAs that may be harmful to the cell (Nagarajan et al., 2013). Rrp6 (Ribosomal RNA Processing) is an exoribonuclease. It is involved in mRNA biogenesis (Hessle et al., 2012). It also plays role in

RNA processing, quality control of gene expression, regulation of enhancer RNAs and the degradation of unstable transcripts synthesized at DNA double-strand breaks. (Eberle et al. 2015). As RNAi silences specific genes, it is silenced using the UAS/GAL4 (Upstream Activation Sequence) system until gene expression is required.

UAS/GAL4 System

The UAS/GAL4 system utilizes the UAS enhancer element's effect on the Gal4 transcription factor (Blair, 2003). Gal4 is a protein that is usually controlled by a driver gene and the UAS controls the expression of the target gene. Thus, the target gene is expressed in Gal4 expressing genes. This technique can be used to create cell mosaics by utilizing a one strain with the UAS and target gene as well as a marker such as GFP (Green Fluorescence Protein) and a second having the Gal4 gene. When the two lines are crossed and the UAS/GAL4 system is initiated the target gene is expressed resulting in a genetic mosaic. If the target gene is RNAi, the resulting genetic mosaic will have areas of the tissue with the target gene silenced allowing the phenotype of the gene to be analyzed alongside the normally expressed gene.

The RNAi fly lines are crossed with the EqGAL4 line that results in the UAS/RNAi genes being present in the new crossed offspring. This will result in expression of the GFP and the RNAi. This is depicted in Figure 4. The expression of the RNAi will cause silencing of specific genes resulting in the creation of genetic mosaics. Immunohistochemistry is utilized to understand the effect on development.

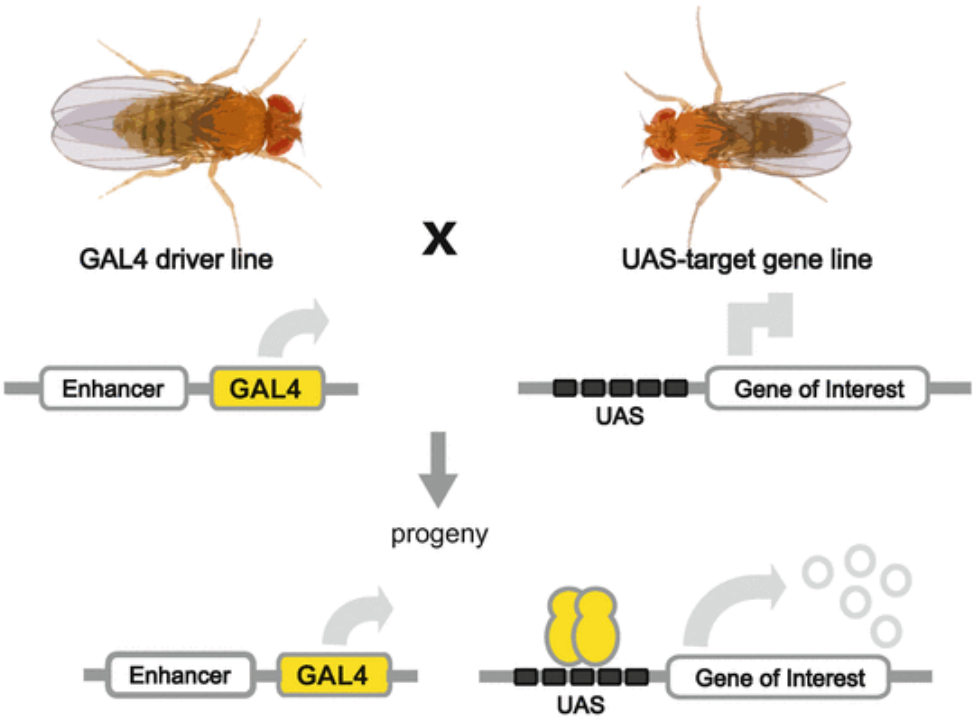

Figure 4: **Overview of UAS/GAL4 system in *Drosophila melanogaster*** (Caygill & Brand, 2016)

Immunohistochemistry

Immunohistochemistry is a serological technique that utilized antibodies to visualize the presence of target cells in a section of tissue. The technique involves the use of a primary and a secondary antibody on a tissue. For this experiment, monoclonal antibodies that were produced by hybridomas in immortalized cell lines were utilized. The antibodies were sourced from the from the University of Iowa's Developmental Studies Hybridoma Bank. The antibodies used in this study are listed in Table 2, which are specific to wing development in *Drosophila* larvae. For our experimentation, the host species utilized for the primary antibody was the mouse. This treatment results in the target cells to be marked with the primary antibody that can be treated with the secondary antibody for visualization.

Table 2: **Primary antibodies utilized**

Antibody Name	UNIProt ID	Function	Cell type stained
4d4	P09615	Wingless protein	Wg-expressing cells
Apa 1	P18502	*Drosophila* patched	Anterior compartment cells
4D9 Anti-engrailed/invected	P02836	Both engrailed and invected gene	Border wing cells
DK 1A4	Q24247	Integrin AlphaPS1 (multiple edematous wing)	Dorsal epithelial cells
2B10	P10180	Cut homeobox	Peripheral neural cells

The wingless gene is a recessive mutation and results in the wingless condition. It has been observed that third instar larvae with the wingless condition have underdeveloped thoracic imaginal discs where the anterior region was normal, but the posterior region develops into the wingless condition (Sharma & Chopra, 1976). The patched (Ptc) gene produces a gatekeeper that prevents abnormal Hedgehog signaling activation (Fan et al., 2019). The *engrailed (en*) and *invected (inv*) gene both encode a homeodomain-containing protein that serves as an important regulator in *Drosophila* development. The genes appeared to be functionally linked and play a role in the development of the posterior compartment in the imaginal discs (Cheng et al., 2014).

The homeobox gene encodes for a globular domain that functions as a DNA-binding domain (Bürglin & Affolter, 2016). The Cut homeobox genes belong to a family with tumor suppressor properties and are required for the development of various structures in *Drosophila* such as wing margin, external sensory organs and antenna (Javeed et al., 2015). The mew gene (multiple edematous wing) encodes for the AlphaPS21 subunit of integrin, which are cell surface receptors for extra-cellular matrix components (Brower et al., 1995). The AlphaPS1 integrin is initially present on all the epithelial cells of the wing imaginal disc, but becomes restricted to the dorsal region in the final larval instar (Wilcox et al., 1981).

The secondary antibody utilized was the AlexaFluor® goat anti-mouse 488nm antibody. The secondary body is created by using the primary antibody as an antigen in a larger host organism, for example a goat. These antibodies are also labelled with fluorophores to allow visualization using fluorescent microscopy. This will stain the mosaic clones on the imaginal discs and visualize them as green at 488nm. The imaginal discs were then mounted on a DAPI Fluoromount-G®. This mount stains all the nuclei of the cells, including those without clones. As the color can be changed, the DAPI Fluoromount-G® was visualized under fluorescence as blue to contrast the clones.

It has been observed that silencing of genes does result in the change of development or lethality in specimens. The present research examined the effect of multiple genes responsible for development in the imaginal discs of *Drosophila melanogaster*. Specifically, this examined the RNA turnover processes and cell-fate specification that can be utilized in the fields of regenerative and stem cell biology.

Chapter 2: Methodology

2.1: Maintenance and Breeding

Drosophila melanogaster stocks were purchased from the Bloomington Drosophila Stock Center run by the Indiana University Bloomington. The stocks were kept in plastic vials sealed with cotton plugs at 25°C. Each vial contained 10ml of fly food prepared using a standard laboratory protocol. The flies were maintained in the vials for 2-3 weeks before being transferred to a new vial containing fresh food. The transfer is conducted by anesthetizing the flies with a blowgun dispersing Carbon dioxide. When the flies are sufficiently anesthetized, they are placed on the Fly pad before being transferred to a new vial. Prepared fly food was maintained at 14°C.

Five RNAi lines were selected and ordered from the Bloomington Drosophila Stock Center. The RNAi lines selected are listed in Table 1. A fly line for Eq-GAL4 was also utilized. These fly lines were allowed to grow and breed in plastic fly vials. Upon breeding, the newly eclosed female virgins from each RNAi line were collected by anesthetizing, pushing, and placing in separate vials. An equal number of male flies were selected from the EqGAL4 line and placed in the tube with the female virgins for breeding. These flies were maintained for 3-4 days until eggs were visible on the surface of the fly food in the vials. The adult flies were then transferred to a new vial.

2.2: Dissection

The vials containing the RNAi x EqGAL4 crosses as well as EqGAL4 were monitored until there were multiple Stage 3 larvae present in the vial. The vials were anesthetized with Carbon dioxide and any adult flies were discarded in a bottle containing NaOH. The food in the vial was sprayed with the Carbon dioxide blowgun. After around 2 minutes, the Stage 3 larvae were collected utilizing forceps and placed into a glass dissection dish containing 2ml of 1x PBS

solution. Another dissection dish containing 1x PBS was placed under a dissection microscope. Using forceps, a single larva was placed in the dissection dish. Dissection was conducted using two forceps. The body of the larvae was held in the middle and the other forceps was used to hold the mouth hooks. The mouth hook was slowly pulled, pulling the brains out of the body cavity. The imaginal discs were detected on the brain structure and carefully removed using the forceps. The discs were then subjected to the immunohistochemistry process.

2.3: Immunohistochemistry

The dissected imaginal discs were suspended in an Eppendorf tube containing 200µl of 4% weight by volume PFA in 1x PBS solution for 20 minutes. The PFA solution was removed and disposed, ensuring the imaginal discs remain in the tube and are not disposed. The imaginal discs are resuspended in 1ml of 0.1% TritonX-100 1x PBS Solution. The tube was placed on a laboratory rocker for five minutes. The solution was removed and discarded, and the process repeated three more times, ensuring the imaginal discs were not disposed. After the fourth wash cycle, the wash solution was disposed. 100µl of 0.1% TritonX-100 1x PBS Solution and 5µl of Goat Serum was added to the tube to create a 5% Goat serum solution and allowed to block for 30 minutes on the rocker at room temperature. 5µl of primary antibody was added and tubes were incubated on a rocker overnight at 4°C.

Upon removal, a repeat wash cycle with 0.1% TritonX-100 1x PBS Solution was conducted four times. 500µl of 0.1% TritonX-100 1x PBS Solution and 1µl of the secondary antibody, AlexaFluor® Goat anti-Mouse 488nm antibody was added to the tube and placed on the rocker in darkness for 60 minutes at room temperature. Upon completion of blocking, the wash cycle with 0.1% TritonX-100 1x PBS Solution was repeated six times. After washing,

800µl of the PBS solution was removed and discarded. The imaginal discs in the tube are ready to be mounted on microscope slides.

2.4: Slide Preparation and Imaging

A glass microscope slide was cleaned with 70% ethanol. Using a transfer pipette, a drop of DAPI Fluoromount-G® was placed on the slide. Using a P1000 Pipette, the solution in the tube was mixed to resuspend the imaginal discs. The discs were transferred from the tube to slide using the pipette. The slide was placed under the dissection microscope and focused. Using a paper towel, excess solution was blotted ensuring the imaginal discs are not removed. Utilizing two pins, any excess tissue was removed from the imaginal discs and discarded. The imaginal discs were moved to the center of the microscope slide. A coverslip was obtained, and a spot of petroleum jelly was added to the corners. The cover slip was placed over the solution, ensuring the imaginal discs are covered and sealed with nail polish. The slides were allowed to dry and stored in a dark box at 4°C until imaged.

Imaging was conducted utilizing the Zeiss LSM 510 UV/META Confocal Microscope. The microscope was set to Plan-neofluar 40x/1.3 Oil DIC objective and oil was added to the lens. The slide was place inverted on the scope and adjusted. The Argon 488nm and 514nm Lasers were switched on. Three channels were set including the Bright Field, green fluorescence, and blue fluorescence. Utilizing the Bright Field and eyepiece, the imaginal discs were located on the slide. The program was then set to Acquisition and the Bright Field became live. The imaginal disc was positioned within the frame using the manual adjustment. The Acquisition was changed to the 488nm channel to visualize green. Using the manual focus, the disc was focused to visualize the green on one end which was set on the Z-stack. The disc was focused until the green on the other end was visualized and this was set as the other boundary for the Z-stack. The

live Acquisition was then switched off and the Z-stack was run for all three channels. The Z-stack images were then processed using the Maximum Intensity Projection for the DAPI and GFP stacks. These images were analyzed.

2.5: Morphological Slide Preparation and Imaging

Two male and two female adult flies were selected from each RNAi x EqGAL4 cross lines. The flies were placed in an Eppendorf tube and placed in -20°C for 20 minutes. A microscope slide was cleaned, and four spots of petroleum jelly were added 1cm apart from each other. The flies were placed on the petroleum jelly using forceps with their back facing upwards. The fly wings were removed using sharpened forceps. The flies were then imaged using Leica M165 FC Stereomicroscope ensuring the back was clearly visible and focused.

Chapter 3: Results

3.1: Immunohistochemistry Results

Immunohistochemistry was conducted on the RNAi crosses as well as the control EqGAL4 with each of the five selected antibodies.

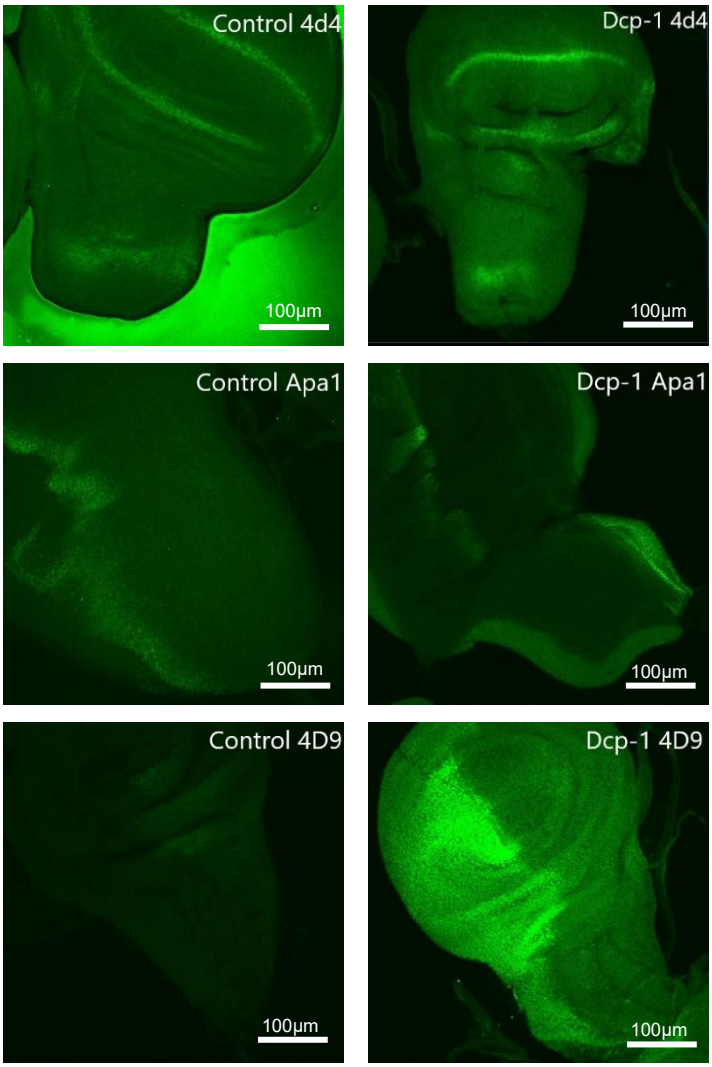

Figure 5. Immunohistochemistry comparison of Dcp-1xEqGAL4 imaginal discs to EqGAL4 control. Fly lines were created by crossing Dcp-1 RNAi line and EqGAL4 line. Imaginal discs were dissected from Stage3 Dcp-1xEqGAL4 and EqGAL4 (control) larvae and treated with immunohistochemistry utilizing 3 primary antibodies (4d4, Apa1, & 4D9) and AlexaFluor® Goat anti-Mouse 488nm secondary antibody. Imaginal discs were mounted and imaged using confocal microscopy. Images displayed were imaged at 488nm to display cells stained by primary antibody. Images display that Dcp-1 RNAi has resulted in hyperproliferation of cells and change in location of protein expression.

In Figure 5, it can be seen that the Dcp-1 gene has changed the expression of various proteins. It has increased the proliferation of the engrailed and invected gene (4D9). The Apa1 patched gene is expressed along the borders too. However, the wingless protein (4d4) displays less proliferation with a smaller band and disc. As Dcp-1 is a caspase that exercises control over programmed cell death. Thus, switching off the Dcp-1 has prevented apoptosis of cells resulting in a hyperproliferation of cells in the imaginal discs.

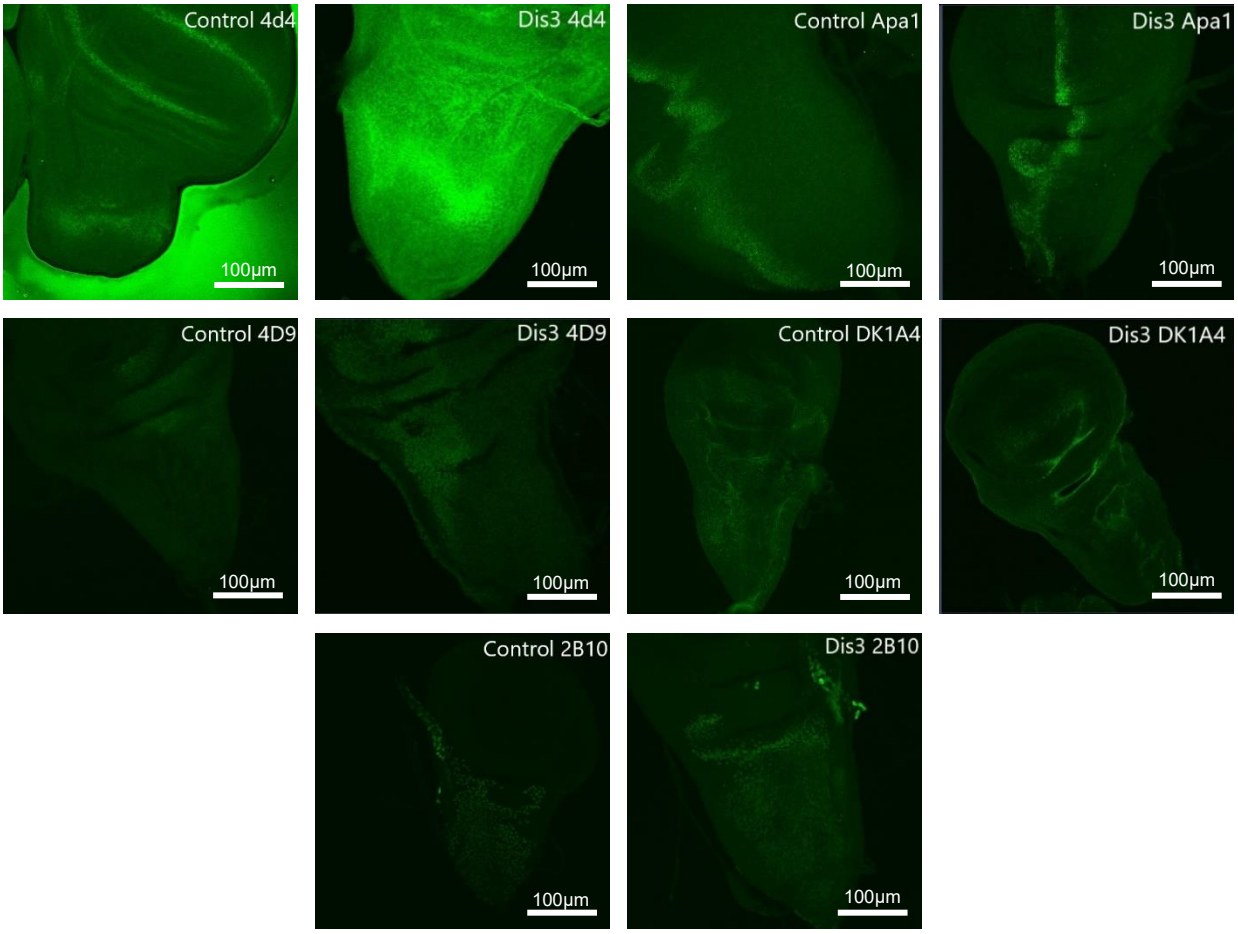

Figure 6. Immunohistochemistry comparison of Dis3xEqGAL4 imaginal discs to EqGAL4 control. Fly lines were created by crossing Dis3 RNAi line and EqGAL4 line. Imaginal discs were dissected from Stage3 Dis3xEqGAL4 and EqGAL4 (control) larvae and treated with immunohistochemistry utilizing 5 primary antibodies (4d4, Apa1, 4D9, DK1A4, and 2B10) and AlexaFluor® Goat anti-Mouse 488nm secondary antibody. Imaginal discs were mounted and imaged using confocal microscopy. Images displayed were imaged at 488nm to display cells stained by primary antibody. Images display that Dis3 RNAi has resulted in hyperproliferation of cells for certain proteins, but some remained consistent.

The analysis of the Dis3xEgGAL4 line indicated an overproliferation of various cells. There was highly increased proliferation in the invected/engrailed gene as well as some increase in the wingless protein and cut homeobox gene. The other proteins appeared to remain consistent. Dis3 is a 3'-5' exoribonuclease. Thus, switching off the gene has affected the 3' pathway of mRNA turnover resulting in lack of mRNA decay. This has resulted in the overproliferation of cells.

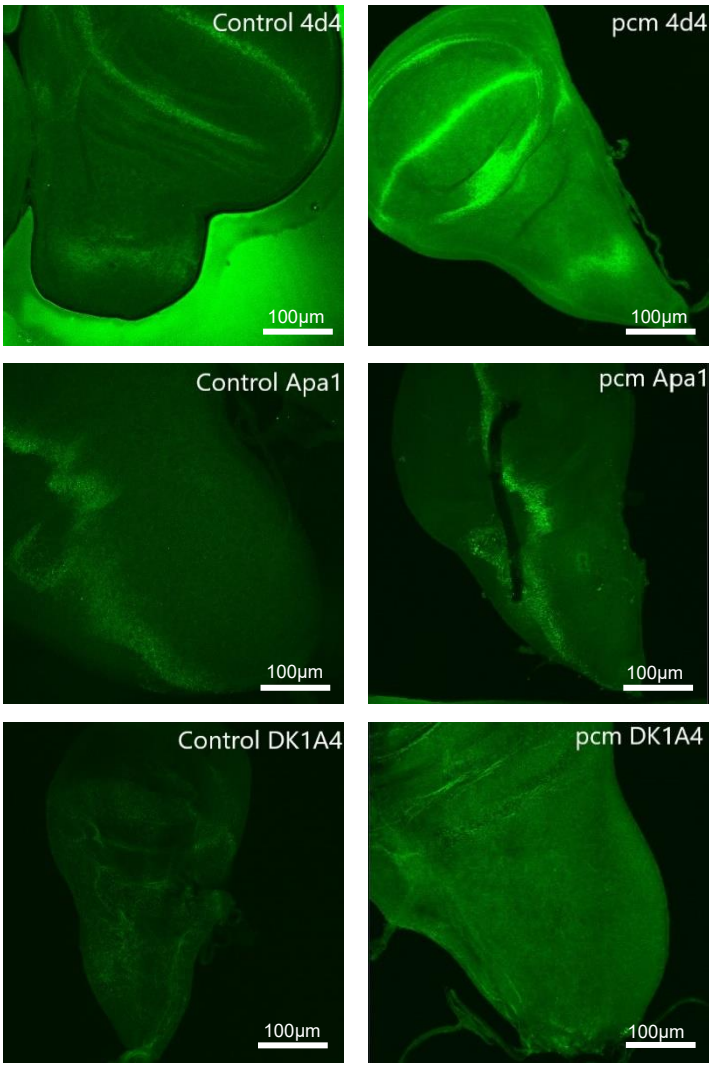

Figure 7. **Immunohistochemistry comparison of pcmxEqGAL4 imaginal discs to EqGAL4 control.** Fly lines were created by crossing pcm RNAi line and EqGAL4 line. Imaginal discs were dissected from Stage3 pcmxEqGAL4 and EqGAL4 (control) larvae and treated with immunohistochemistry utilizing 3 primary antibodies (4d4, Apa1, & DK1A4) and AlexaFluor® Goat anti-Mouse 488nm secondary antibody. Imaginal discs were mounted and imaged using confocal microscopy. Images displayed were imaged at 488nm to display cells stained by primary antibody. Images display that pcm RNAi has resulted in a slight increase in proliferation of cells.

The pcmxEqGAL4 lines exhibited an increase in proliferation with a significant increase in the engrailed/invected gene. Increases were also visible in patched (Apa1) and Integrin AlphaPS1 (DK1A4). pcm is a 5'-3' exoribonuclease which also affects mRNA stability. Thus, switching off the gene has affected the 5' pathway of mRNA turnover resulting in lack of mRNA decay. This has resulted in the over-proliferation of cells.

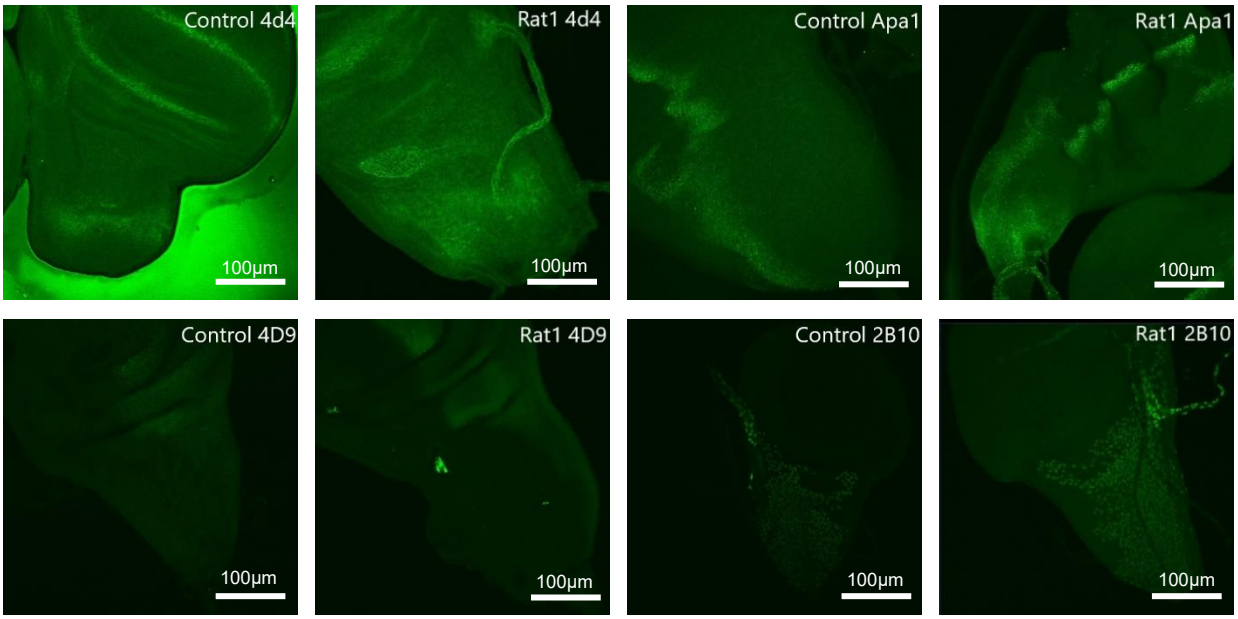

Figure 8. Immunohistochemistry comparison of Rat1xEqGAL4 imaginal discs to EqGAL4 control. Fly lines were created by crossing Rat1 RNAi line and EqGAL4 line. Imaginal discs were dissected from Stage3 Rat1xEqGAL4 and EqGAL4 (control) larvae and treated with immunohistochemistry utilizing 4 primary antibodies (4d4, Apa1, 4D9, & 2B10) and AlexaFluor® Goat anti-Mouse 488nm secondary antibody. Imaginal discs were mounted and imaged using confocal microscopy. Images displayed were imaged at 488nm to display cells stained by primary antibody. Images display that Rat1 RNAi has resulted in hyperproliferation of cells and change in location of protein expression.

The Rat1xEqGAL4 crosses display an increased proliferation with a significant visual increase in the engrailed/invected gene and the bottom of the imaginal disc in the Patched (Apa1) subject. The wingless protein displays bright spots of cell proliferation compared to the control. Rat1 is a 5'-3' exoribonuclease that plays a significant role in mRNA turnover. Thus, switching off the gene has affected the 5' pathway of mRNA turnover resulting in lack of mRNA decay. This has resulted in the overproliferation of cells.

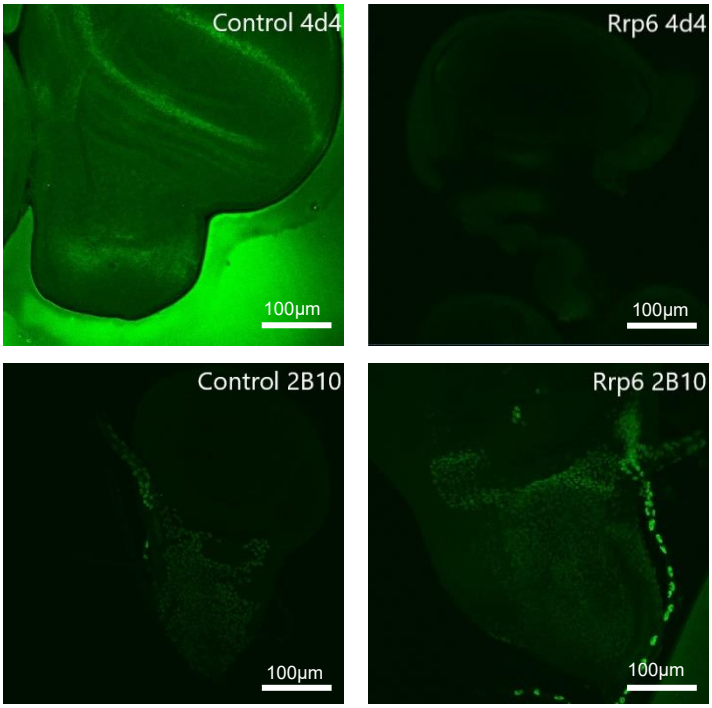

Figure 9. **Immunohistochemistry comparison of Rrp6xEqGAL4 imaginal discs to EqGAL4 control.** Fly lines were created by crossing Rrp6 RNAi line and EqGAL4 line. Imaginal discs were dissected from Stage3 Rrp6xEqGAL4 and EqGAL4 (control) larvae and treated with immunohistochemistry utilizing 2 primary antibodies (4d4 & 2B10) and AlexaFluor® Goat anti-Mouse 488nm secondary antibody. Imaginal discs were mounted and imaged using confocal microscopy. Images displayed were imaged at 488nm to display cells stained by primary antibody. Images display that Rrp6 RNAi has resulted in apoptosis of cells as well as change in location of protein expression.

The Rrp6xEqGAL4 line displays a loss of cells with a dramatic cell apoptosis in the engrailed/invected subject. This apoptosis is not as severe in the cut homeobox line but is present on the sides. Rrp6 is an exonuclease with a role in mRNA biogenesis. When it is switched off, there is a lack of mRNA biogenesis leading to an inability to mature resulting in apoptosis of cells as seen in Figure 9.

3.2: Morphology Analysis

Upon analysis of the morphology of the sensory organs, significant differences could be observed in the crossed flies, visualized in Figure 11.

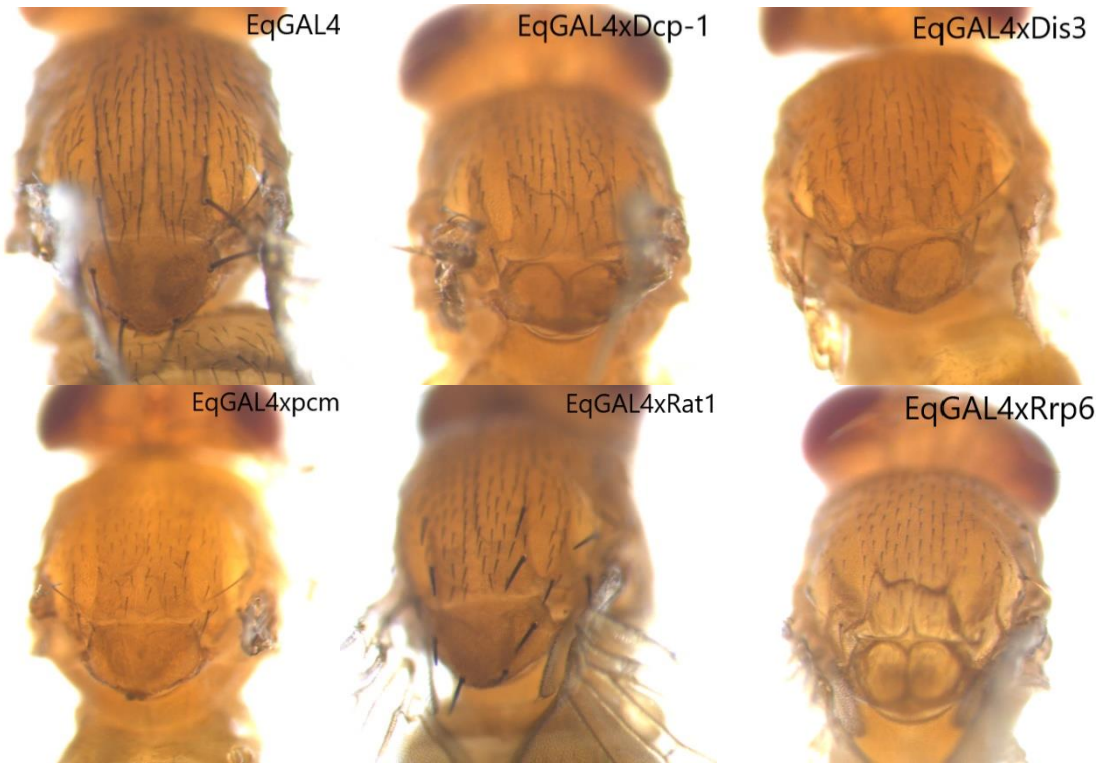

Figure 10. **Morphological images of sensory organs formed from imaginal discs on *Drosophila melanogaster* notum.** Fly lines were created by crossing five RNAi lines (Dcp-1, Dis3, pcm, Rat1 and Rrp6) and EqGAL4 line. RNAixEqGAL4 and EqGAL4 (control) adult flies were obtained and frozen. The flies were then mounted and imaged with a dissecting microscope. The images indicate that in comparison to the control, the RNAi crosses have shorter and thinner notum with some lacking bristles.

In this analysis, the bristles of the control EqGAL4 was compared to those of each cross. In EqGAL4 both the notum and macrochaetes are visible and orderly with the bristles being thick and long. However, RNAi crosses do not display these structures consistently and most display thinner and shorter notum and macrochaetes. The EqGAL4xDcp-1 line displays thinner notum and shorter macrochaetes with bald patches on the sides. The EqGAL4xDis3 displays thinner and shorter notum and macrochaetes as well, but does not have prevalent bald spots. The EqGAL4xpcm displays extremely thin notum and macrochaetes. There are also bald spots visible on the sides. EqGAL4xRat1 possesses thick macrochaetes but they appear short like stubble. Finally, EqGAL4xRrp6 notum and macrochaetes are barely visible as they are extremely

thin. Of the flies imaged, the severity of the changes had a range relating to the penetrance of the phenotype.

Chapter 4: Discussion

The results of this study indicate that there is an effect of mRNA turnover on the signaling pathways leading to cell specification. As mRNA turnover is responsible for the decay of mRNA, interruption of this process results in persistence of the mRNA in the cell, which leads to excessive production of proteins (Meyer, Temme, & Wahle, 2004). This resulting protein production leads to the hyperproliferation visible in RNAi lines that interfered with the mRNA turnover process. However, as certain RNAi lines played a significant role in cellular development, switching them off resulted in the apoptosis of the cell.

As RNA are involved in the maturation of other forms of RNA, the lack of decay and resulting lack of maturation can lead to apoptosis or overproliferation of cells. Dcp-1 can induce cell death displayed by the fact that the expression of Dcp-1 is sufficient to induce apoptosis (Song, McCall, & Steller, 1997). Thus, in this experiment when Dcp-1 was switched off utilizing RNAi, it lacked the ability to induce apoptosis. This lack of apoptosis resulted in an extreme hyperproliferation of cells that is visible in the experimental results. Rrp6 is responsible for the maturation of rRNA, snRNA and snoRNA in the nucleus (Fox et al., 2015). They perform a variety of vital functions. rRNA carries out protein synthesis in ribosomes, snRNA affects splicing, and snoRNA is responsible for chemical modifications such as methylation and pseudouridylation (Allmang et al., 1999). As these are vital in regulation and development, switching off this gene results in cell apoptosis.

It appears that both cytoplasmic and nuclear exonucleases are displaying similar effects on the cell signaling. Although both pcm and Rat1 are 5’-3’ exoribonucleases, pcm is a cytoplasmic exonuclease and Rat1 is a nuclear exonuclease (Krzyszton et al, 2012). However, they both have a function in the degradation of lncRNA. Research has indicated that there may be two separate

yet redundant pathways for lncRNA decay, a cytoplasmic pcm pathway and a nuclear Rat 1 pathway (Giesler et al., 2012). As lncRNA serves to regulate the transcription, lack of this decay results in accumulation of RNA and overproliferation of cells.

Dis3 serves a similar method as a nuclear and cytoplasmic 3'-5' exoribonuclease with a role in RNA quality control and mRNA decay (Robinson et al., 2015). Dis3 plays a role in miRNA regulation and can been observed to degrade mature miRNA as a means of regulation (Robinson et al., 2015). As this miRNA plays a role in RNA turnover, switching off the Dis3 gene allows the miRNA to be unregulated resulting in a lack of turnover and an increase in cell proliferation.

Morphologically, these changes in cell signaling have resulted in tissue degeneration causing the thinner and shorter notum and bristles. As the changes in signaling have led to a change in the cells by hyperproliferation or apoptosis, during pupation the cells cannot successfully metamorphosize and organize into the tissues present in the notum (Gunage et al., 2017). This indicates that despite the overproliferation of cells, these changes have resulted in tissue degeneration and incomplete development.

This research on the effect of mRNA turnover on cellular signaling can be further studied by utilizing more developmental genes including dDcp1 (Lin et al., 2006), dDcp2 (Lin et al., 2008), and lbdr (Okamura et al., 2007). The antibodies utilized for wing proteins can also be expanded to include antibodies targeting Hedgehog (Strigini & Cohen, 2000), Decapentaplegic (Strigina & Cohen, 2000) and Cubitus interruptus (Ramirez-Weber et al., 2000).

Cell signaling plays a vital role in regulation and development. Despite the conserved pathways, there is still a large lack of knowledge regarding the multiple factors that influence cell signaling. In this study, the effect of mRNA turnover on cell signaling indicates that this controls the proliferation of cells as well as the tissue generation and morphological development

of adult structures. As errors in cell signaling can result in a variety of diseases such as cancer, diabetes and more, further understanding of the cell signaling regulatory process may provide insight to these conditions (Berg, Tymoczko, & Stryer, 2002). Understanding the regulatory errors could help provide therapeutic solutions for these diseases and allow for the development of artificial tissues by controlling the cellular signaling mechanism.

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