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Functional and Expression Analysis of a Novel Basement Membrane Degrader in Drosophila Melanogaster

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FUNCTIONAL AND EXPRESSION ANALYSIS OF A NOVEL BASEMENT MEMBRANE DEGRADER IN *DROSOPHILA MELANOGASTER*

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FUNCTIONAL AND EXPRESSION ANALYSIS OF A NOVEL BASEMENT MEMBRANE DEGRADER IN *DROSOPHILA MELANOGASTER*

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This thesis would not have been possible without the support of many individuals.

My parents who raised me to love learning and to question what I knew.

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FUNCTIONAL AND EXPRESSION ANALYSIS OF A NOVEL BASEMENT MEMBRANE DEGRADER IN *DROSOPHILA MELANOGASTER*

Christopher Fields August 2016 58 Pages

Directed By: Ajay Srivastava, Cheryl Davis, Rodney King

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The Srivastava Lab is focused on the identification and characterization of genes that play a role in basement membrane remodeling. Previously, we identified putative basement membrane degraders through a genetic screen. One such gene has been suggested to play a role in the maintenance of the stem cell niche in *Drosophila melanogaster*, but no other information about the role this gene plays in development or disease has been published. Here, data are presented from experiments utilizing *Drosophila* genetics and immunohistochemistry that provide important insights on the biological role of this gene.

Collagenase activity was up-regulated upon overexpression of this gene, confirming it as a basement membrane degrader. Additionally, RNA in-situ hybridization experiment results showed expression in the developing imaginal discs of the 3rd instar larva tissues. Overexpression and knockdown studies further demonstrated morphological defects in a number of tissues, including the wing and the eye, and are suggestive of apoptosis. Acridine orange staining confirmed that cell death occurred when the gene was overexpressed and a cleaved caspase antibody staining indicated that process to be caspase-mediated apoptosis.

1 Introduction

1.1 Basement membrane and its role in tumor metastasis

Basement membrane (BM), or basal lamina, is a specialized form of extracellular matrix found in nearly all tissues throughout the body (Yurchenco, 2011). Composed of type IV collagen, laminin, and various other proteins, the basement membrane forms a barrier that regulates passage of nutrients through various tissues and provides structure for surrounding cells. Forming half the weight of the basement membrane, the backbone of the basement membrane is composed of interlocking pieces of collagen IV (Yurchenco, 2011). This scaffold has been implicated in processes such as signaling, differentiation, and angiogenesis (Schwarzbauer, 1999).

Basement membrane degradation is necessary for tumor metastasis, a central hallmark in the genesis of cancer (Srivastava et al., 2007 and Hanahan and Weinberg, 2000). During tumor formation, malignant cells become starved for nutrients and release proteases and signals for angiogenesis which stimulate the destruction of the nearby basement membrane and facilitate tumor invasion. Metastasis makes cancer difficult to treat and is associated with high mortality in nearly all cancer cases where metastasis occurs (Sleeman and Steeg, 2010).

1.2 *Drosophila* as a model organism for studying basement membrane degradation

In this study, we utilize the powerful genetic tools present in *Drosophila* along with advanced molecular biology techniques. Like Thomas Hunt Morgan, we use *Drosophila* because they are small, easy to care for, have short distinct life stages, and their genetics are simple and well understood (Miko and LeJeune, 2009). *Drosophila*, share approximately 75% known disease causing genes with humans, and serves as an excellent genetic model for studying disease (Reiter et al., 2001 & Lloyd and Taylor, 2010). Additionally, genetic tools such as the UAS-Gal4 system allow for the controlled expression of genes using simple *Drosophila* mating schemes (Busson and Pret, 2007).

The speed of the *Drosophila* life cycle is a major advantage for using the model system to study genetic concepts. Figure 1 shows the stages of the *Drosophila* life cycle. Following fertilization, *Drosophila* embryos develop into 1st instar larva in about 1 day. It takes another day for the 1st instar larva to develop into a 2nd instar larva and another day to a 3rd instar larva. They will spend about 2 days as motile 3rd instar larvae eating food, storing energy, and preparing to pupate. Once a pre-pupa has formed the *Drosophila* will take approximately 4 days to emerge as an adult.

Drosophila imaginal discs serve as a genetic model for basement membrane degradation as well as tissue invasion (Srivastava et al., 2007). The imaginal discs are composed of an outer peripodial epithelium and stalk (PS) that breakdown the basement membrane between the PS and larval epithelium

during metamorphosis (Pastor-Pareja et al., 2004). Upon breaking the basement membrane, the PS invades the larval epithelium, and forms the adult structures. This process allows us to design experiments for observing the effects genes have on BM.

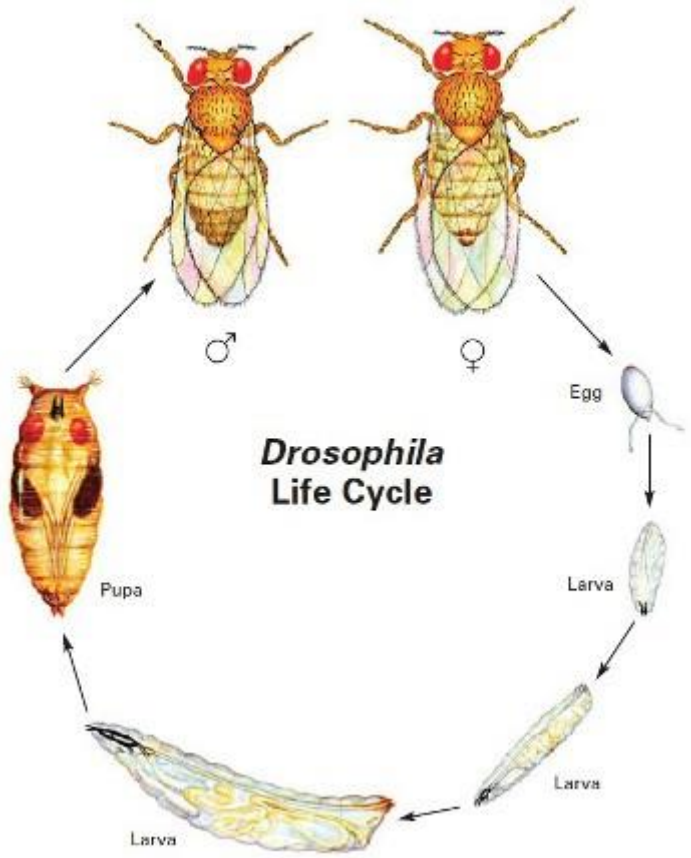

Figure 1: Life Cycle of *Drosophila* melanogaster.

The *Drosophila* life cycle occurs over 2 weeks at 25°C and longer at lower temperatures. Following fertilization, the embryo begins to divide and differentiate over 24 hours reaching the 1st instar stage. Over the course of two days it develops into the 2nd instar and 3rd instar larva. After 2 days as a 3rd instar larva it pupates and remains a pupa for 4 days. Adults flies emerge from the pupa fully developed and will mature sexually within a day. (Image Source: Raymond Flagg, Carolina Biological Supply Company)

Traditionally mutants were generated in *Drosophila* as a means to study gene function. While many of the fundamental concepts of genetics were studied using this classical system, modern genetic tools have expanded our ability to understand gene expression. When mutated, some genes result in lethality; which demonstrates the importance of a gene, but limits our ability to study the gene's function in vivo. For example, a mutation of a homeobox gene is typically lethal in early development. Many of these genes have functions in later development that can't be studied using classical genetics. This limitation can be addressed by using the UAS-Gal4 system and RNA interference (RNAi) technology. The UAS-Gal4 system allows us to manipulate when and where genes are expressed.

Originally identified in yeast, the UAS-Gal4 system allows for the targeted expression of genes (Griggs and Johnston, 1993 and Duffy, 2002). In this system, Gal4 is a transcriptional activator which binds to the upstream activation sequence (UAS) fused to a gene of interest. The Gal4 protein is under the control of another gene enhancer, such as actin, and is produced wherever and whenever the endogenous gene is expressed. This allows the UAS-Gal4 system to be controlled temporally and spatially using tissue specific gal4 drivers. Various gal4 drivers are used in this approach and are available from the Bloomington Drosophila Stock Center. Figure 2 below describes this process.

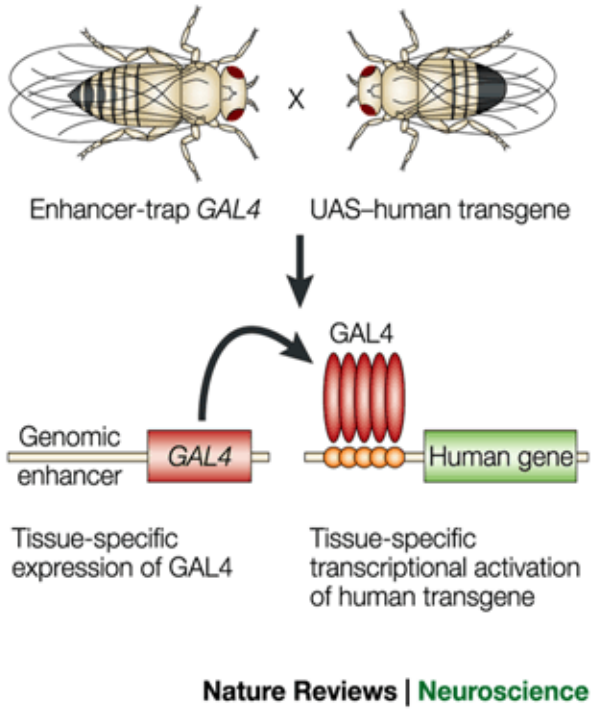

Figure 2: UAS-gal4 System

Enhancer trap GAL4 fly is mated with a fly bearing a *UAS*-gene X. Progenies possessing both elements will express the gal4 protein in the pattern specific to the enhancer. The gene of interest (*UAS-gene*) will be expressed along the same pattern, providing tissue specific expression. (Muqit and Feany, 2002)

RNA interference technology (RNAi) is an innovative system that in combination with the UAS-gal4 system in *Drosophila* provides a powerful tool to knockdown gene expression in a tissue specific manner (Kennerdell and Carthew, 2000; Martinek and Young, 2000; Kalidas and Smith, 2002). The advantage of using RNAi as opposed to knockout mutations is the ability to knock down gene expression in a tissue specific manner with the UAS-gal4 system. This targeted gene knockdown results in better control over lethality resulting in better understanding of a gene's role in the development of specific tissues. The process of RNAi is detailed in Figure 3.

Another tool that has allowed *Drosophila* to be such a powerful and versatile model is that its genetics are simpler in comparison to vertebrate models. Many of the components involved in basement membrane degradation, such as matrix metalloproteinases (MMPs) have multiple mammalian orthologs (~24 MMPs), whereas *Drosophila* contain only two MMPs (Page-McCaw et al., 2007). This is further simplified by the fact that one of the MMPs is extracellular and the other is intracellular. There are many examples where *Drosophila's* simplified genetics have allowed us to better understand the role genes play without the complicated interactions present within mammalian models.

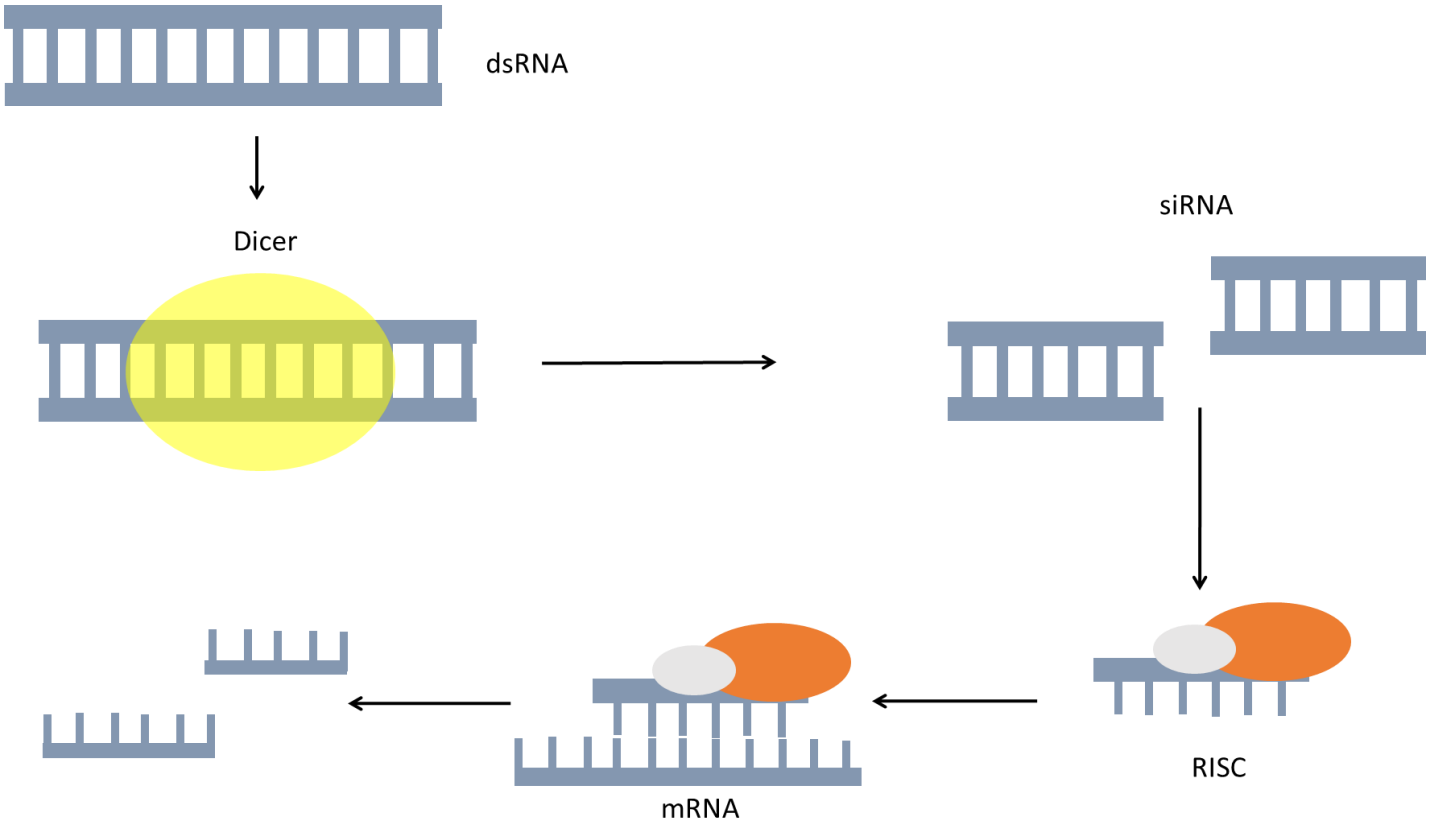

Figure 3: RNA Interference

Double stranded RNA (dsRNA) is cleaved into short segments by the protein DICER forming small interfering RNA (siRNA). The anti-sense strand is incorporated into the RNA-induced silencing complex (RISC). The resulting complex binds to target mRNA and cleaves it, thereby silencing expression.

1.3 *snuts* as a putative basement membrane degrader

While the clinical focus of basement membrane degradation is centered on tumor metastasis, the remodeling of the basement membrane is a critical aspect of normal development (Kalluri, 2003). Specifically, the genetics that control this process are not well understood and the focus of this study is on one of several genes our lab has identified as being a putative basement membrane degrader. Of those we identified in the initial screen, we found that many have no known function associated with their expression and for many their role in development is unknown. One such novel gene is known as *shrunken nuts* or *snuts.*

snuts is a novel gene that has received little attention; therefore, its role in basement membrane degradation as well as normal development is not understood. *snuts* is a 2.3kb gene that encodes a 446 amino acid protein product (Figure 4). The structure for this protein is unknown, but the sequence is predicted to contain 2 Plant Homeodomains (PHD) and a Sterile Alpha Motif Domain (SAM). High throughput data have shown that *snuts* is expressed throughout *Drosophila* embryonic development and our data have shown that it is expressed in 3rd instar larvae, pupa, and adult flies (Fields, 2014, Lecuyer et al., 2007; and Tomancak et al., 2007). Bausek et al. (2007) found that *snuts* is important in the maintenance of the stem cell niche, a region responsible for providing nutrients, support, and signaling for stem cells. They reported that mutations in *snuts* resulted in the shrinkage of male testis.

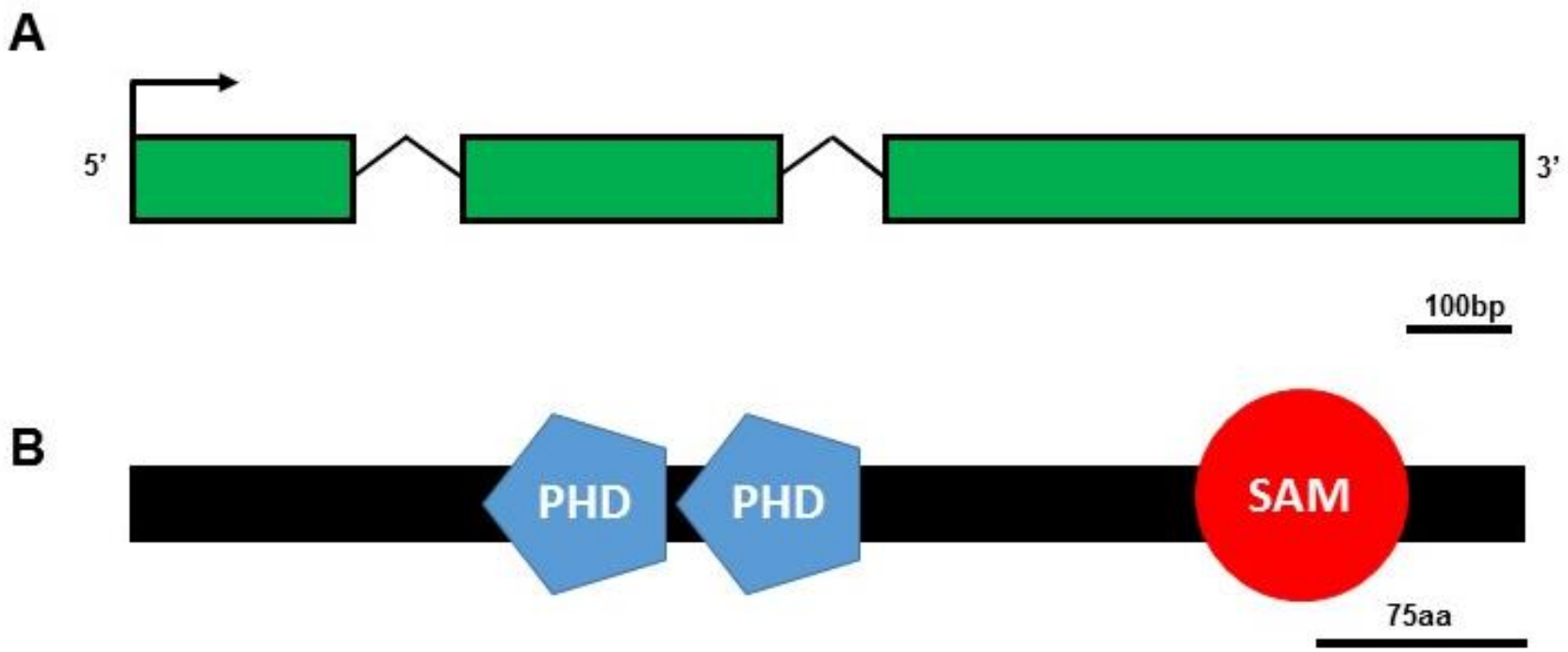

Figure 4: Repersentation of *Snuts*' Gene Organization.

Panel A shows the genomic representation of *snuts*, which is composed of three exons and two introns.

Panel B shows the primary structural representation of the SNUTS protein, composed of two Plant Homeodomain (PHD) and a Sterile Alpha Motif (SAM).

1.4 Domain architecture suggests SNUTS function

Two different conserved domains are predicted to exist in *Snuts* based on evolutionary conserved sequences, a PHD domain and a SAM domain (Altschul et. al. 1990). The role that the PHD and SAM domains play in the function of *snuts* as well as in other genes is not well understood, despite high degree of conservation. They are found throughout the Eukaryotic domain, but show a diverse range of functions that have made understanding their roles elusive (Capili et. al., 2001; Kim and Bowie, 2003). A description of these domains and their function is provided below.

Plant Homeodomain containing Proteins

PHD domains are composed of a Cysteine – Histidine – Cysteine motif and have been suggested to work in chromatin-mediated transcriptional regulation (Aasland et al., 1995). These domains are composed of a ~65 residue effector that is commonly found in chromatin-remodeling proteins. (Musselman et al., 2011). The role PHD domains play in chromatin-mediated transcriptional regulation may facilitate or repress gene expression depending on the other proteins. Indeed, as Aasland et al. (1995) reported, PHD domains are commonly found in transcription factors. Several PHD domain containing proteins are described below as are the roles their PHD domains play in their function.

The ubiquitously expressed *Drosophila* gene *Pygopus* is a PHD domain containing protein that is required for wingless signaling throughout development (Parker et al., 2002). Common with other chromatin remodeling factors, the PHD

domain is found on the C-terminus while a nuclear localization signal is found on the N-terminus of *Pygopus*. Mutations in *Pygopus* almost exclusively result in disruption of *Wingless* signaling; a single amino acid change in the PHD domain is sufficient to disrupt *Pygopus* function. (Kessler et al., 2009; Belenkaya et al., (2002).

Another group of PHD domain containing proteins in the mammalian gene family, *Polycomb-like proteins (PCL-1-3)*, a family of gene repressors that have vital roles in embryonic development, stem cell differentiation, and cellular proliferation (Sauvageau and Sauvageau, 2011 and Lanzuolo and Orlando, 2012). *PCLs* contain a PHD domain in their N-terminal region that facilitates their ability to bind to p53 (Yang et al., 2013). Without the PHD domain, *PCLs* are unable to bind to p53, preventing them from initiating cellular quiescence (Brien et al., 2015). *PCLs* typically have both chromatin dependent and chromatin independent functions that allow it to regulate pathways, such as the p53 pathway.

A PHD domain containing protein that exemplifies chromatin remodeling is *Rhinoceros,* a protein that antagonizes Ras signaling in the *Drosophila* eye (Voas and Rebay, 2003). This protein possesses a PHD domain in its N-terminus that when mutated, resulted in loss of *Rhinoceros* function. Furthermore, Voas and Rebay, (2003) found that this gene is a nuclear protein likely involved in a chromatin-remodeling complex. They further emphasized the importance of the PHD domain in *Rhinoceros* which is conserved between the *Drosophila* and human homolog.

Other studies have suggested that PHD domains by themselves don't bind nucleic acids, but instead facilitate protein-protein interactions (Ragvin et. al., 2004; Shi et. al., 2006). Mansfield et. al. (2011) showed that PHD domains could play a role in the epigenetic modification of histones by binding to two separate histones. This contributes to evidence that PHD domains are important for the proper remodeling of chromatin.

Sterile Alpha Motif containing Proteins

SAM domains show a more diverse range of functions than PHD domains. Structurally, they share a compact globular fold of six helices (Grimshaw et. al., 2004). Like PHD domains they are capable of facilitating protein interactions, and have also been shown to interact with mRNA. SAM domains contain a conserved tyrosine residue that often plays a role in cell-cell signal transduction (Schultz et. al., 1997). The diversity of functions between SAM domains in different proteins has made generalized inferences regarding domain function difficult. Despite this, we can state that SAM domains serves as a regulator of gene expression by facilitating binding between the SAM containing protein and another protein. A summary of SAM containing proteins and their functions is provided in the following paragraphs.

Kim et al., (2002) reported that SAM domains are important in the *Polycomb* family of proteins which are required for the repression of homeotic genes. *Polycomb* inhibits transcription through a mechanism that is not understood. Two of the *polycomb* genes in this family share a SAM domain which is heavily conserved in sequence and structure between the two, which

suggest that the function is shared between the two even if they have different targets. The conservation of the SAM domain between these two genes illuminates the importance of the SAM domain.

The protein p53 is well known for its control of the cell cycle, DNA repair, and tumor suppression. Mutations in p53 are found in a diverse range of cancers (Holstein and Sidransky, 1991). While p53 does not have a SAM domain, its two closely related family members, p63 and p73, contain SAM domains. Both p63 and p73 function in a manner similar to p53, although they are rarely found mutated in cancers (Levrero et al., 2000). These SAM domains are required for their respective functions. The p63 and p73 proteins can alternatively splice the SAM domain and have been shown to be ancestral to p53, suggesting that p53 has lost its SAM domain over time (Dotsch et al., 2010).

SAM domains often mediate protein-protein interactions, but also have been shown to bind with mRNA. For example, the SAM domain containing protein *Smaug* controls *nanos* expression by binding to its mRNA (Green et. al., 2003; Knight et. al., 2011). *Smaug* has been shown to be important in *Drosophila* maternal to zygotic gene expression (Benoit et al., 2009). The *nanos* mRNA is recognized through the SAM domain of *Smaug*. The SAM domain in *Snuts* may share similar functions.

1.5 Known information from preliminary characterization of *snuts*

Degradation of the basement membrane is key in both normal development as well as in tumor metastasis (Liotta et al., 1980). One means by which *snuts* may promote the breakdown of BM is through matrix metalloproteinases (MMPs). MMPs are zinc-dependent endopeptidases that cleave collagen IV and other components of the basement membrane. Srivastava et al., (2007) demonstrated a connection between the c-Jun N-Terminal Kinase (JNK) pathway and MMP function. They found that JNK activity regulates MMPs. Furthermore, it was shown that BM degradation is controlled by expression of JNK signaling.

The JNK pathway is a central regulator of different cellular activities including growth, stress, and apoptosis. JNK activation follows a MAP kinase scheme where JNK (*Basket*) is activated by JNK Kinase which is also activated by a JNK Kinase Kinase (Karin and Hunter, 1995) (Figure 5). With regard to normal development in flies, the JNK pathway is necessary for the metamorphosis of larval tissues into adult structures (Srivastava et al., 2007). In addition, it was demonstrated that overexpression of *snuts* activates the JNK pathway, through an unknown mechanism. Figure 5 details the JNK pathway in *Drosophila*. The JNK pathway is part of the MAP kinase family of signaling pathway. It is activated by extracellular signals (i.e. TNF, Rac, radiation, and other stress inducing signals). Upon activation the MAPKKK TGF-β activated kinase (dTAK) is phosphorylated, which in turn phosphorylates Hemipterous (HEP). Upon activation, HEP phosphorylates the JNK Basket (Bsk). Basket is

then transported to the nucleus where it can activate numerous transcription factors.

When *snuts* is overexpressed by crossing a *UAS-snuts* male to a *Ptc-gal4, UAS-GFP PucZ/Tm6Tb* female we observed up-regulated expression of *puckered* along the anterior/posterior boundary of the 3rd instar wing disc. *Puckered* is a downstream product in the JNK pathway and serves as a reporter for JNK activity. We suspect that *snuts* interacts with the JNK pathway to modulate basement membrane remodeling, but how it interacts with the pathway is not clear.

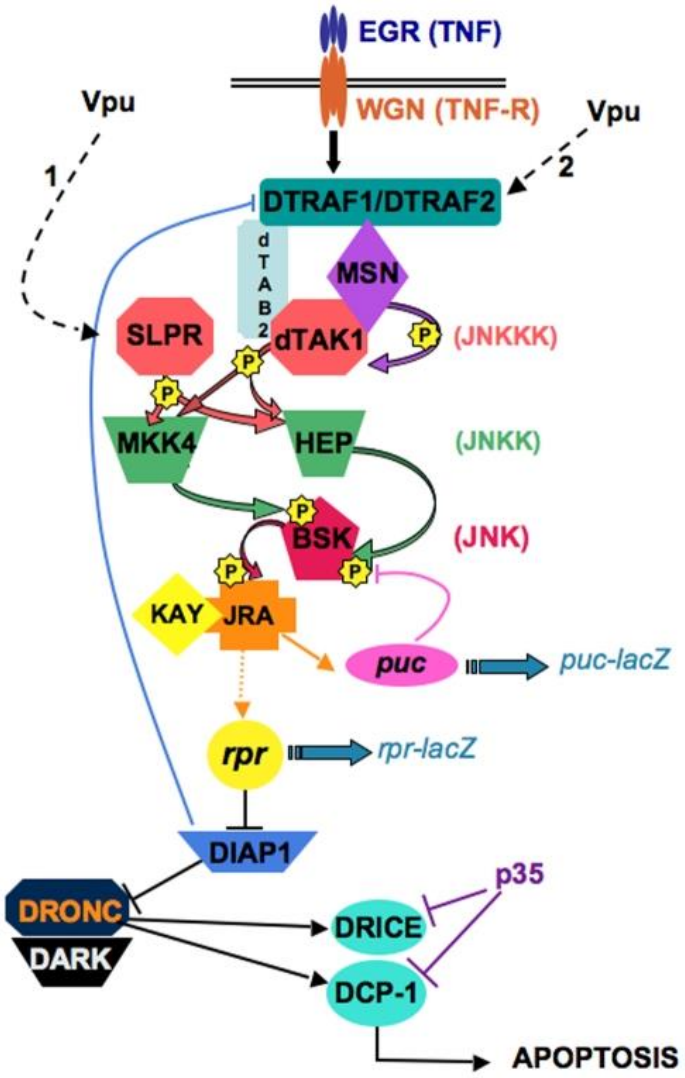

Figure 5: *Drosophila* JNK pathway

The JNK pathway follows a MAP kinase cascade. A signal such as TNF begins the activation and results in phosphorylation of TGF β (dTAK). Activation of dTAK phosphorylates the JNKK, Hemipterous (HEP). Following, the JNKK phosphorylates the JNK *Basket (Bsk)* resulting in its localization to the nucleus and subsequent activation of transcription factors. Other molecules of interests include *puckered* (*Puc*) which we used as a reporter for JNK activity as well as *Death-associated inhibitor of apoptosis 1* (*DIAP1*) which is an apoptosis inhibitor. Both *Puc* and *DIAP1* negatively regulated the JNK pathway. (Marchal et al. 2012)

Previously, we utilized an assortment of gal4 drivers to overexpress *snuts* in a variety of tissues and observed various phenotypes. We found that when *snuts* is overexpressed in the eye (*GMR-gal4* and *Ey-gal4*), phenotypes suggestive of apoptosis result. *GMR-gal4* is expressed posteriorly to the morphogenetic furrow in the developing eye while *Ey-gal4* is expressed anteriorly to the morphogenetic furrow (Song et al., 2000 and Lai and Rubin, 2001). When expressed using *Ey-gal4*, the number of ommatidia is visibly reduced while in *GMR-gal4* the eye forms the rough eye phenotype. These phenotypes indicate an upregulation of apoptosis. One aim of the current study is to understand the relationships between apoptosis and *snuts* expression.

Apoptosis is a key regulator in the development of *Drosophila*, as well as other Eukaryotes (Abrams et al., 1993). The JNK pathway activates apoptosis as a response to cellular stresses (Lee et al., 2005). JNK activation leads to the inhibition of the protein DIAP1, an inhibitor of apoptosis (Liu and Lin, 2005). Overexpression of *snuts* in the eye results in apoptotic phenotypes while in the wing it results in structural defects, or in the worst case, lethality. When *snuts* is overexpressed in the wing using *Ptc-gal4*, lethality occurs at the adult stage at 25°C. At 18°C lethality is repressed, but the wing shows morphological defects in the hinge as well as along the edge of the wing.

Expression of *snuts* is needed for proper unfolding of the wing following eclosion from the pupa stage. When *snuts* is downregulated using an RNAi driver, *UAS-Dcr-2; Nubbin-gal4*, the wing does not unfold at 25°C. When the

same cross is conducted at 18°C, wing folding is restored with minor defects to the wing veins.

2 Materials and Methods

2.1 Drosophila stocks and culture

Fly crosses were setup at 25°C, unless stated otherwise, in *Drosophila* media (Lab Express) using standard procedures. *UAS-GFP, Ptc-gal4; PucZ/Tm6Tb* was used to over express *snuts* (FBst0032443) along the *Patched* pattern. *Ptc-gal4; UAS-srcRFP/CyO* was used to overexpress *snuts* in the wing for the collagenase assay, caspase staining, and the acridine orange staining.

Table 1: Stocks Used in This Study

STOCK	Purpose
w*, UAS-snuts	Used to overexpress *snuts*
w, UAS-Dcr-2; nubbin-gal4	RNAi driver used to downregulate *snuts* wing pouch
KK106361	RNAi Line for *snuts*
Ptc-gal4; UAS-srcRFP/CyO	Used to overexpress *snuts* along the anterior/posterior boundary of the wing
GMR-gal4	Used to overexpress *snuts* in cells anterior to the morphogenetic furrow in the developing eye
w; Ey-gal4/CyO	Used to overexpress *snuts* in cells posterior to the morphogenetic furrow in the developing eye
UAS-BskDN; Sp/CyO	Used to downregulate the JNK pathway
GMR-gal4/CyOActGFP	2nd chromosome GMR-gal4 used with transposase (Δ2-3)
w;; Dr/TM3SBΔ2-3	Transposase used to move GMR-gal4 onto the X-Chromosome
w; Sco/CyO; Sb/TM6Tb	Double Balancer Line with white eyes

2.2 RNA In-Situ Hybridization

Probe Generation

Probes for the *snuts* RNA in-situ assay were generated using the Roche SP6/T7 in-vitro transcription kit (Roche 10999644001). The template used in the reaction was generated as previously described in Fields (2014) from a linearized cDNA clone with T7 and SP6 promoters on the upstream and downstream regions respectively or from a cDNA template (RE68603) that was PCR amplified using primers with T7 and SP6 promoter sites. RNA probes were generated from the template which incorporates digoxigenin-dUTP into the sequence. The reaction setup is detailed in Table 2.

Probes were purified using ethanol precipitation as specified by Doroquez, (2003). 2.5 µL 4M Lithium chloride and 50 µL of pre-chilled (-20°C) 100% ethanol was added to each sample and incubated at -80°C for 30 minutes. Samples were subjected to centrifugation at 14,000 rpm for 15 minutes at 4°C. The ethanol was decanted, washed with 50 µL 70% pre-chilled ethanol, and spun at 14,000 rpm for 5 minutes at 4°C. The ethanol was decanted and the pellet was suspended in 100 µL fresh RNase-free Hybridization Buffer. Probes were verified by gel electrophoresis and stored at -20°C (Figure 6).

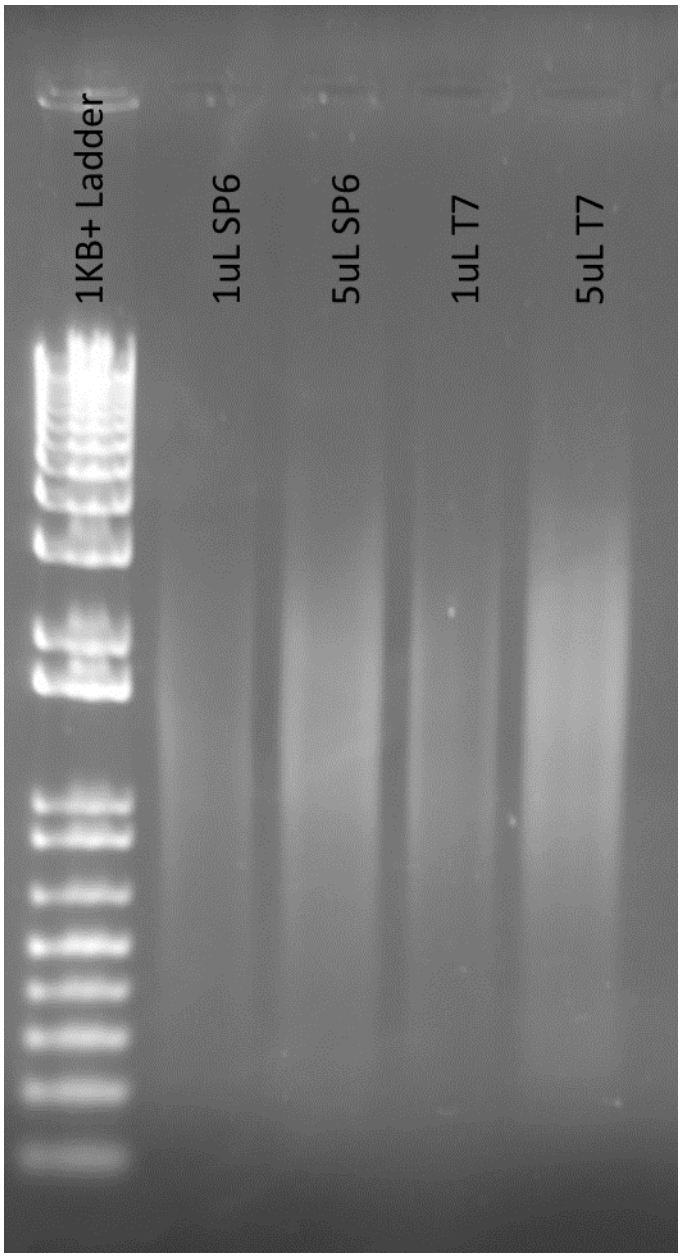

Figure 6: Verification of *snuts* Probe.

SP6 is the sense probe and T7 is the anti-sense probe. RNA probe samples ran on a 1% agarose gel at 120 volts for 60 minutes. The smear is typical for RNA run on an agarose gel.

Table 2: DIG-RNA Labeling Setup

Component	Amount
10x NTP Labeling Mixture	2 µL
10x Transcription Buffer	2 µL
Protector RNase Inhibitor	1 µL
RNA Polymerase (T7 or SP6)	2 µL
Template	X µL
Water	Up to 20 µL
Incubate for 2 hours at 37°C	
DNase I, RNase-free	2 µL
Incubate 15 minutes at 37°C	
Stop reaction by adding 2 µL 0.2 M EDTA (pH 8.0)	
Proceed to Ethanol Precipitation	

Hybridization of probe to snuts mRNA

Approximately 20 3rd instar larvae were inverted and washed in 1X PBS. Larvae were placed in a fixative solution (refer to Table 3) for 45 seconds. Larvae were post fixed in 4% paraformaldehyde for 20 minutes. Samples were rinsed 4 times with 1X PBT (Table 3). Larvae were digested with 1X proteinase K for <1 minute then the reaction was stopped with ice cold 2mg/mL glycine/PBT and incubated for 2 minutes at room temperature. The proteinase K/glycine solution was removed and the sample was rinsed for another 2 minutes with 800uL 2mg/mL glycine/PBT. Following this, the sample was rinsed 2X in PBT. The sample was post-fixed in 1mL 4% paraformaldehyde/PBT for 20 minutes, rinsed 4X with PBT, washed with 1:1 PBT:RNA hybridization solution for 10 minutes, and washed in hybridization solution for 10 minutes. The tissue was pre-hybridized for 2 hours at 50°C. Five microliters of the probe was diluted in 200uL of RNA hybridization solution, denatured it at 80°C for 3 minutes, and allowed it to cool briefly on ice. Tissues were transferred to a new tube and the probe was added to the sample. The probe was allowed to hybridize overnight at 50°C for 12-16 hours.

Development of Signal

The hybridization mixture was discarded and the sample was washed 3X with RNA hybridization solution at 50°C. The tissues were washed in 1:1 PBT:RNA hybridization solution for 20 minutes at 50°C and rinsed 4X with PBT at 50°C. The sample was allowed to cool to room temperature and was then washed with PBT for 10 minutes. The tissues were incubated with a 1:2000

dilution of anti-DIG-AP/PBT for 2 hours at room temperature. Following incubation, the tissue was rinsed 4X in PBT. The sample was transferred to a glass dish well and incubated in alkaline phosphatase buffer (AP) containing NBT and BCIP (SIGMA*FAST*™ BCIP®/NBT Sigma B5655 tablet in 10 mL water according to manufacturer's instruction). After sufficient signal was produced the reaction was stopped using PBT and discs were mounted in Vectashield on a slide with a cover slip. Samples were viewed using a Leica stereomicroscope. Reagents used in the RNA in-situ assays are described in Table 3. All steps prior to incubation in AP buffer were shaken in a gyro shaker at room temperature, or in the hybridization oven for incubation/wash steps at 50°C.

Table 3: RNA In-Situ Reagents

RNA In-Situ Reagents	Description
Snuts_RNAINSITU_SP6_F	ATTTAGGTGACACTATAGGCTAGCCAAACGTAGACAGCC
Snuts_RNAINSITU_T7_R	GAATAAATACGACTCACTATAGGGAGAACGTTCGCCTCCTTCGGATAG
RNase Free Hybridization Buffer	• 2.37 mL RNase-free water (Thermo Fisher 10977015) • 5 mL Formamide (Sigma Aldrich F9037) • 2.5 mL SSC (20X) (Sigma Aldrich S6639) • 20 µL Heparin (50mg/mL) (Fisher Scientific BP252450) • 100 µL Sonicated Salmon Sperm DNA (10 mg/mL) (Thermo Fisher 15632011) • 10 µL Tween-20 (100%) (Sigma Aldrich P9416)
0.3% PBT	• 150 µL Triton X-100 (ICN 807426) • Fill to 50 mL w/ 1X PBS (Gibco 70013)
4% Paraformaldehyde	• 1.25 mL 16% Paraformaldehyde (EMS 15700) • 3.75 mL PBT
Fixative Solution	• 500 µL Heptane (Fisher Chemical H350) • 312.5 µL 16% Paraformaldehyde (EMS 15700) • RNase-free water (Thermo Fisher 10977015) • 10X PBS (Gibco 70013)
1:2000 Anti-Dig-AP	• 0.2 µL anti-DIG-AP (Roche 13680324) • 400 µL PBT
1X Proteinase K (50 mg/mL)	• 10 µL 100X Proteinase K (5 mg/mL) (Sigma Aldrich P2308) • 990 µL PBT
2 mg/mL Glycine/PBT	• 20 mg Glycine (BioRad 161-0718) • Fill to 10 mL PBT

2.3 Immunohistochemistry

Immunohistochemistry was performed as described previously in Srivastava et al., (2007). The cleaved caspase primary antibody was used at a 1:100 dilution while the secondary antibody, anti-rabbit conjugated Alexa 488, was diluted at 1:600.

2.4 Scanning electron microscopy (SEM)

Overexpression and downregulated phenotypes were observed using standard scanning electron microscopy. Adult wing phenotypes were mounted on carbon tape, sputter coated with silver particles to prevent charging, and visualized using the SEM at 20kV. Adult eye samples were dehydrated in ethanol for 12 hours each treatment (25%, 50%, 75%, 2X 100%). Samples were critical point dried in accordance with the manufacturer's protocol. Fly heads were removed and placed on carbon tape, sputter coated with silver, and viewed at 20kV using a JEOL 5400LV SEM equipped with a tungsten filament.

2.5 Transmission electron microscopy (TEM)

The ultrastructure of the overexpression eye phenotypes was analyzed using standard transmission electron microscopy techniques as adapted from Mishra and Knust (2013). *Drosophila* heads were removed using a sharp razor blade. Additionally, the proboscis was pulled away and removed. Heads were incubated in a fixative solution (2.5% glutaraldehyde & 2% paraformaldehyde in 0.2M phosphate buffer) overnight at 4°C. Following incubation, the heads were washed three times in 1X PBS for ten minutes each and fixed with 2% osmium tetroxide in 1X PBS for two hours in darkness. The heads were washed three

times in 1X PBS for ten minutes each. The samples were subjected to a dehydration series for 10 minutes each (50%, 70%, 90%, 95%, 2X 100%) and washed with acetone two times for ten minutes each. In a chemical hood, the heads were infiltrated with acetone:resin mix in the following ratios 3:1, 1:1, 1:3, and pure resin for 2 hours, overnight, 3 hours, and 3 hours respectively. Heads were mounted in molding blocks, filled with pure resin, and placed in an 80°C oven for 24 hours. The blocks were trimmed first with a razor blade and then with the EM trim. Blocks were mounted on an ultra-microtome and 100nm sections cut using a glass knife. Samples were collected on copper mesh grids and viewed at 80kV using a JEOL 120-CX TEM with a LaB6 gun.

2.6 Collagenase assay

Collagen IV is the main component of the extracellular matrix (and basement membrane). Collagenase is an enzyme known to degrade collagen IV and serves as an indicator of basement membrane degradation. Samples were prepared as previously described in Dong et al., 2015. Third instar larvae were dissected in cold 1X PBS, incubated in a staining solution (100ug/mL DQ Gelatin in 1X PBS) for 90 minutes, then incubated in 4% paraformaldehyde fixative for 30 minutes, and washed two times in PBTA (1X PBS, 0.1% Triton X100, 1% bovine serum albumin, 0.01% sodium azide) for 20 minutes at room temperature. Samples were mounted in a drop of Vectashield-DAPI and imaged using a Carl Zeiss Axioplan 2 Imaging Fluorescent Microscope.

2.7 Acridine orange staining

Acridine orange staining was adapted from Wolff and Ready, 1991. Acridine orange is a fluorochrome dye which can enter dying cells, but not living cells. Inside the cell, it intercalates between the base pairs of DNA and fluoresces green under blue light. *snuts* was overexpressed using the *Ptc-gal4; UAS-src RFP/CyO* driver line, and 3rd instar larvae expressing red fluorescent protein were selected. Third instar larvae were dissected in cold 1X PBS, briefly rinsed in 1X PBS, and incubated in $1.6x10^{-6}$M acridine orange for 5 minutes. The reaction was stopped with the addition of 1X PBS and washed. Wing discs were dissected, mounted on a microscope slide, and covered with a cover slip. Images were obtained immediately using a Leica stereo-microscope under blue light.

3 Results

3.1 Overexpression of *snuts* degrades the basement membrane

Our previous genetic screen identified *snuts* as a putative basement membrane degrader (Srivastava et al., Unpublished). To confirm our suspicion that *snuts* acts as a basement membrane degrader, we performed a collagenase assay (Dong et al., 2015). *snuts* was overexpressed using *Ptc-gal4; UAS-srcRFP/CyO*. The results confirmed that *snuts* is a BM degrader as indicated by Figure 7 below. Collagenase activity was up-regulated along the patched pattern in the wing pouch of 3rd instar larva.

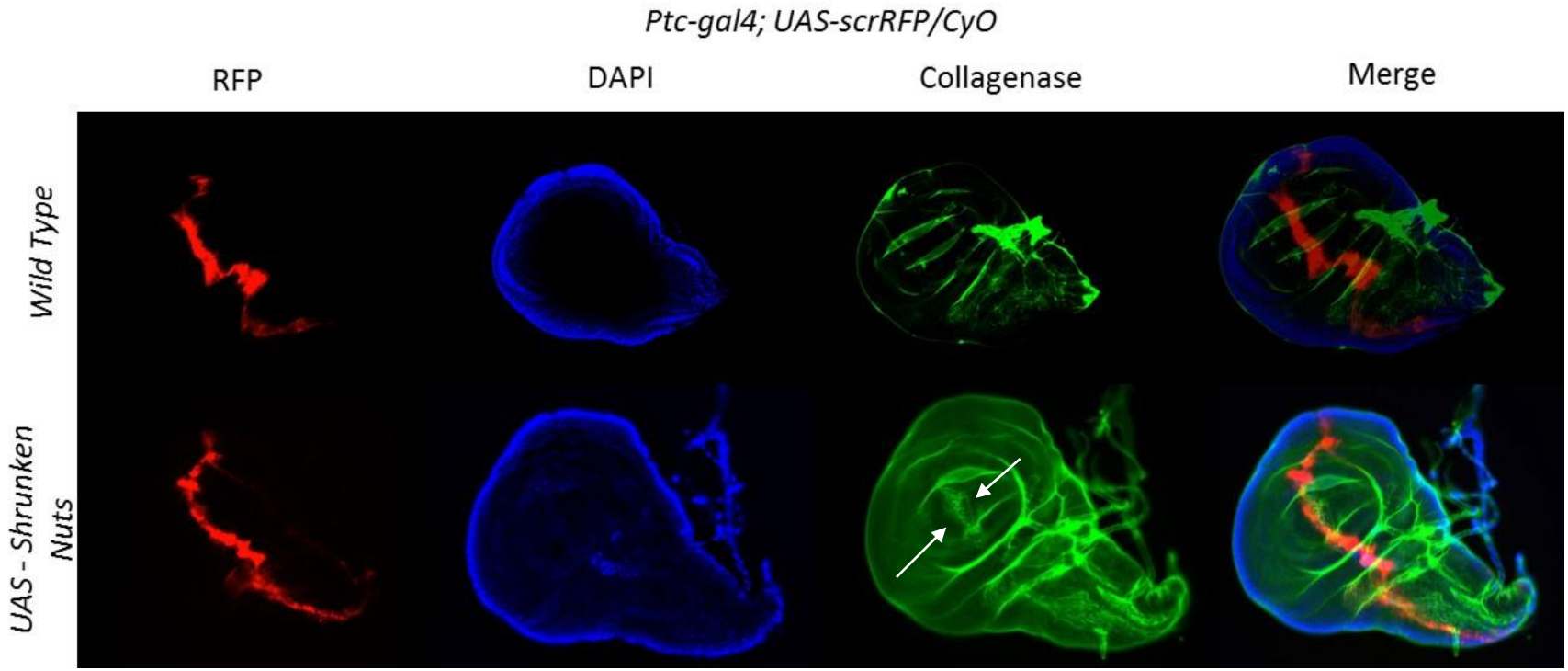

Figure 7: Collagenase Assay of snuts

Overexpression of *snuts* up-regulates collagenase expression in the wing pouch along the *ptc* pattern. Collagenase activity is indicative of basement membrane degradation. Notice that the entire wing disc stains green, which is expected as the developing wing continually breaks down and rebuilds the BM as a developmental tool. The upregulation is indicated by the arrows.

3.2 *Shrunken nuts* expression profile

The focus of this study was to understand both the function and expression of *snuts* in development and in the process of tumor metastasis. With the exception of high-throughput embryo expression, little is known about *snuts* expression. We were interested in understanding the normal expression of *snuts* during another important developmental stage of development, the third instar larva. Utilizing RNA in-situ hybridization, we determined the normal spatial expression of *snuts* in wild type flies. Our previous results suggested that *snuts* is a basement membrane degrader; therefore, we speculated that the expression would be ubiquitous throughout the larval imaginal discs, sites of intense basement membrane remodeling.

Indeed, *snuts* was expressed in all third instar larval discs (Figure 8). In the wing disc we noticed increased expression in the wing pouch region compared to the rest of the wing disc (Figure 8C). The haltere and leg disc showed even staining throughout, while the genital disc in males and females showed similar expression with more on the ends than in the middle (Figure 8E, I-K). Expression between males and females was the same with the exception of the female genital disc showing no expression through the middle of the disc. The spatial expression in the eye-antenna disc was uneven through-out (Figure 8D). Expression of *snuts* in the eye appeared to mimic expression pattern of *wingless* as indicated by the arrows. *Wingless*, a gene product critical in development, is known to interact with the JNK pathway (Swarup and Verheyen, 2012).

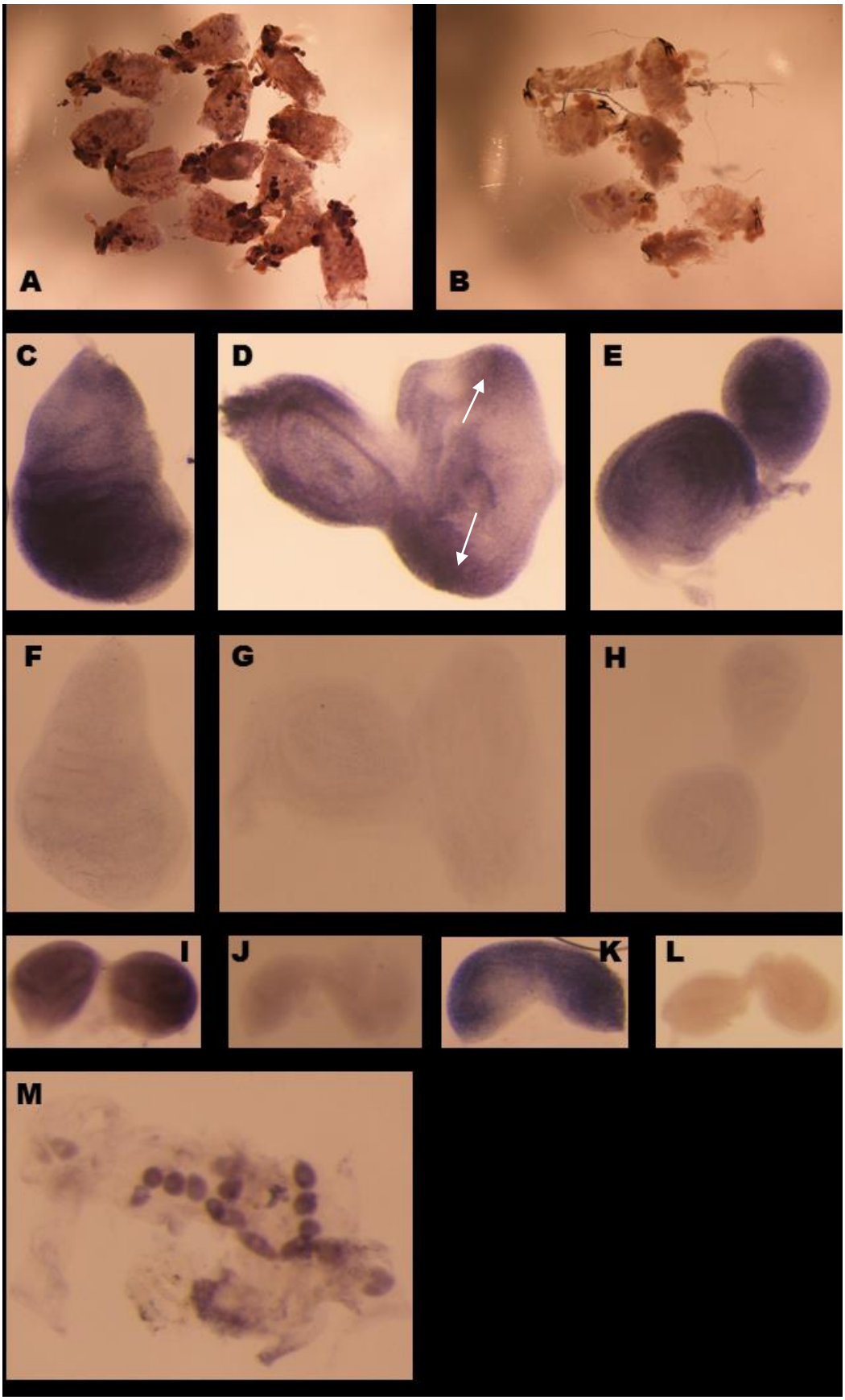
A
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Figure 8: RNA In-Situ Hybridization using snuts Probe

A *snuts* probe was generated to perform RNA in-situ hybridization in 3rd instar larval disc. Expression was found throughout all disc assayed. Expression was greater in the wing pouch and an expression profile similar to *wingless* was observed in the eye disc (as indicated by the arrows).

A: *snuts* anti-sense probe on 3rd instar larva

B: *snuts* sense probe on 3rd instar larva

C: *snuts* anti-sense probe on 3rd instar female wing disk

D: *snuts* anti-sense probe on 3rd instar female eye antenna disk.

E: *snuts* anti-sense probe on 3rd instar female haltere and leg disc

F: *snuts* sense probe on 3rd instar female wing disc

G: *snuts* sense probe on 3rd instar female eye antenna disk

H: *snuts* sense probe on 3rd instar female haltere and leg disc

I: *snuts* anti-sense probe on 3rd instar male genital disc

J: *snuts* sense probe on 3rd instar male genital disc

K: *snuts* anti-sense probe on 3rd instar female genital disc

L: *snuts* sense probe on 3rd instar female genital disc

M: *snuts* anti-sense probe on 3rd instar male hemocytes

3.3 Overexpression of *snuts* Induces Apoptosis

Previously we found phenotypes that suggest *snuts* utilizes apoptosis during adult eye development in *Drosophila* when *snuts* was overexpressed using eye specific gal4 drivers. In addition, we have shown that *snuts* overexpression upregulates the JNK pathway (Fields, 2014). As the JNK pathway induces apoptosis under cellular stress, we believe that *snuts* utilizes the JNK pathway to activate apoptosis as part of its developmental function. To determine if overexpression of *snuts* results in an increase in cellular death we performed acridine orange staining, a stain that serves as an indicator of cell death. When we overexpressed *snuts* using *Ptc-gal4; UAS-srcRFP/CyO,* we found an increase in cell death along the *ptc* pattern (Figure 9).

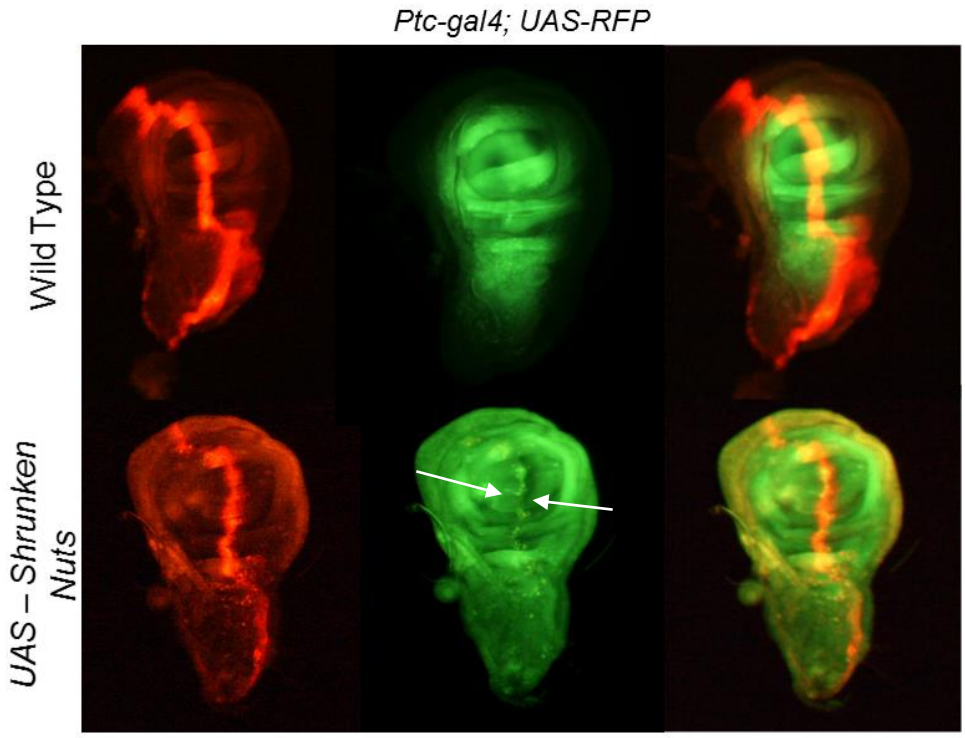

Figure 9: Acridine Orange Staining

Overexpression of *snuts* using *Ptc-gal4; UAS-srcRFP/CyO*. When *snuts* is overexpressed using *Ptc-gal4*, cell death is upregulated along the *patched* pattern. Images were taken at 11.5X magnification using a conventional Leica fluorescent Microscope. Upregulation of cell death is indicated by the arrows.

Acridine orange is only an indicator of cell death and cannot distinguish between programmed cell death (apoptosis) and necrosis. To clarify whether this was programed cell death or simply the necrotic death of cells, we performed immunohistochemistry using a cleaved caspase antibody. Caspase is activated in the apoptosis pathway. Therefore, we overexpressed *snuts* using *Ptc-gal4; UAS-srcRFP/CyO* and used a cleaved caspase antibody to indicate upregulation of apoptosis along the *ptc* pattern (Figure 10). As expected, there was an upregulation of caspase present along the *ptc* pattern when *snuts* was overexpressed.

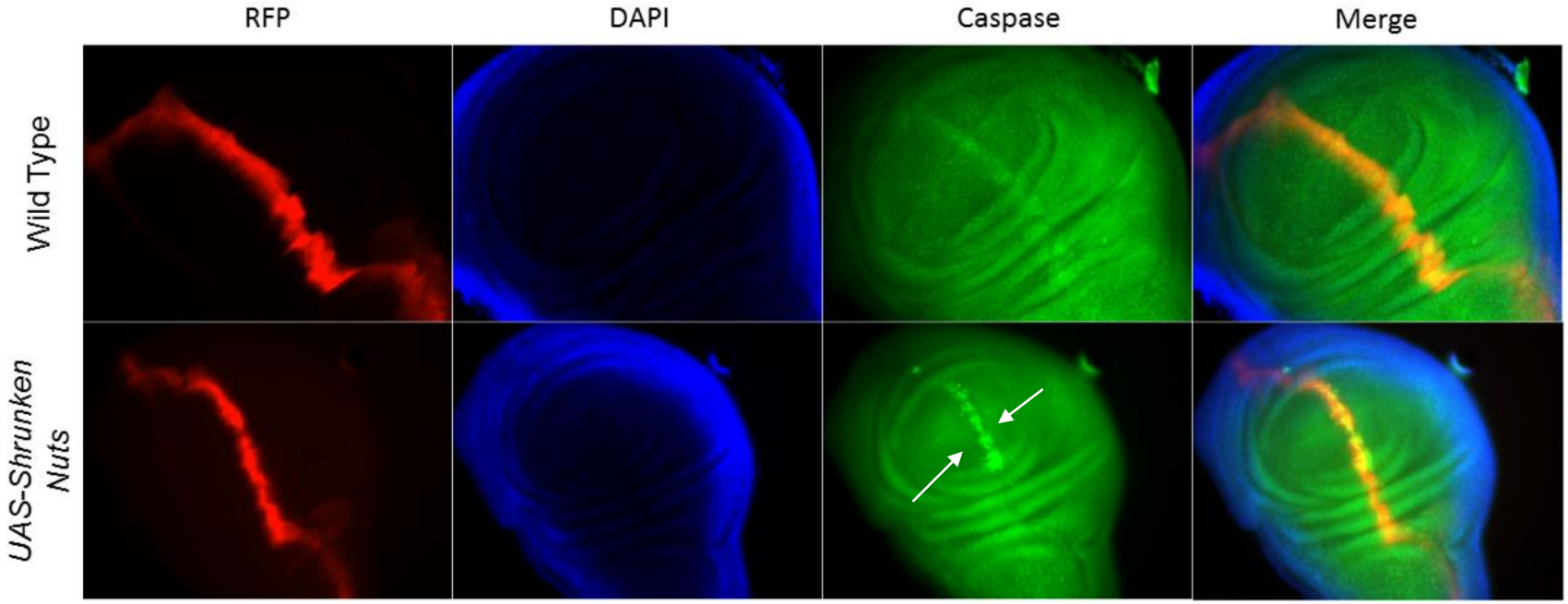

Figure 10: Cleaved Caspase Immunohistochmistry

snuts was overexpressed using *Ptc-gal4; UAS-srcRFP/CyO*. Upregulation of caspase along the *ptc* pattern shows apoptosis is being upregulated when *snuts* is overexpressed. Upregulation of caspase is indicated by the arrows.

To further examine the effects of *snuts* expression we utilized standard TEM techniques to look at the ultrastructure of the eye phenotypes. An ultrastructure analysis allowed us to observe the state of the individual cells and draw conclusions from structural deviations. As the *Drosophila* eye is a tightly organized unit of cells called ommatidia, deviation in the structure could have significant consequences in the function of the eye. Each of the ommatidia is composed of eight photoreceptor cells called rhabdomeres, a structure which contains the photoreceptor elements for the eye. We were curious to know whether the increased apoptosis in the eye changed the ultrastructure, particularly of the rhabdomeres. We expected to see missing rhadomeres or cells in varying degrees of degeneration.

TEM thin sectioning showed that when *snuts* was overexpressed using *w; Ey-gal4/Cyo*, the number of rhabdomeres was the same as seen in wild type (Figure 11). While the SEM image showed a clear reduction in the number of ommatidia in *snuts* overexpressed with *Ey-gal4*, the ultrastructure appeared to be intact, while the organization of the rhabdomeres was severely disrupted. The spacing between the rhabdomeres was greatly increased.

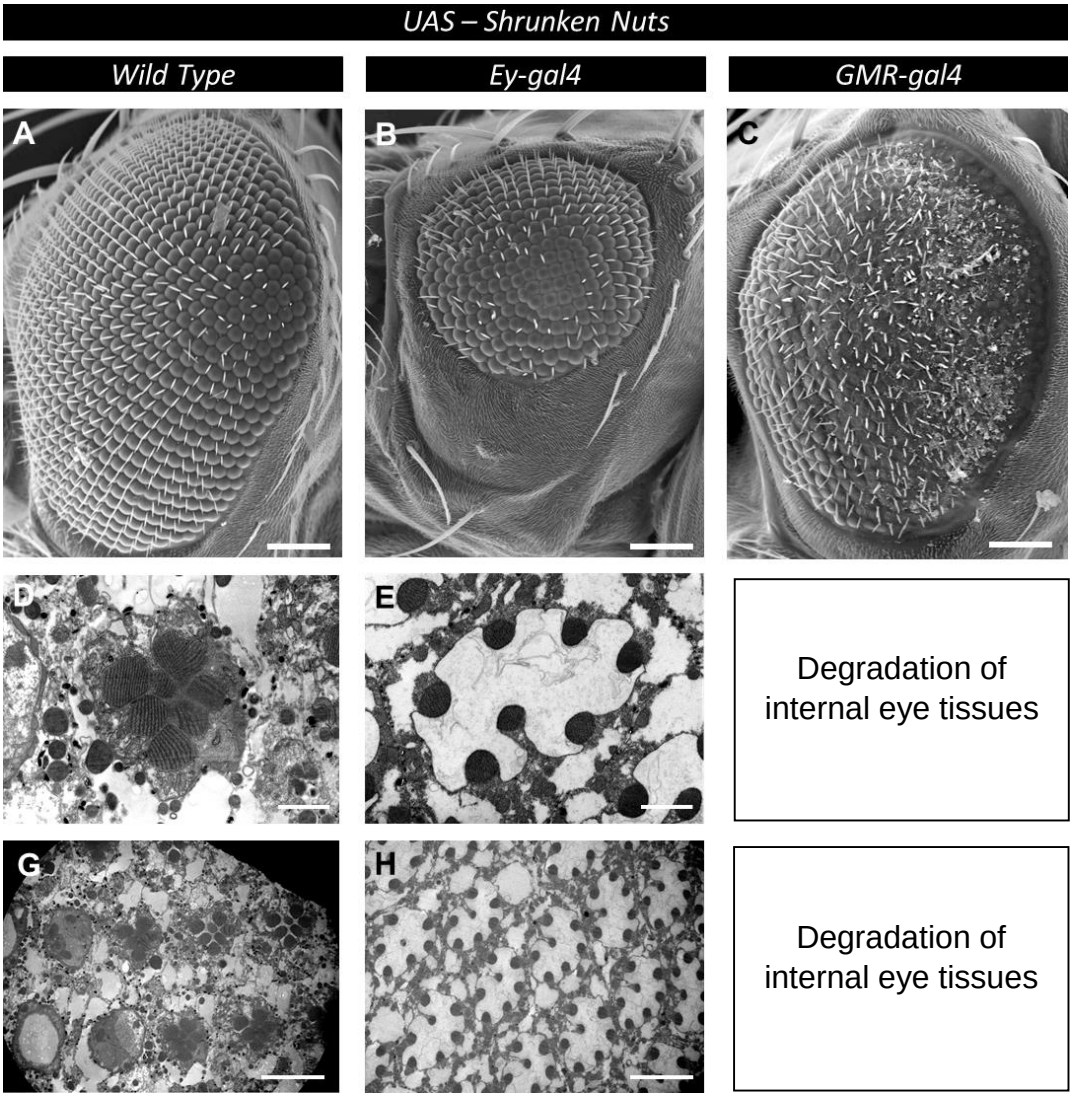

Figure 11: Ultrastucture Analysis of *snuts* Overexpressed Eye

Images A-C are from Fields, 2014. When *snuts* is overexpressed using *Ey-gal4*, the regular spacing between the rhodomeres of each ommatidia is dispersed. (A-C) Scale markers represent 100 µM and images taken at 200X magnification. (D-F) Scale markers represent 1 µm and images taken at 2000X magnification. (G-I) Scale markers represent 4 µm and images taken at 600X magnification.

3.4 Downregulation of the JNK pathway restores wild type phenotype in eyes overexpressing *snuts*

Previous results from this lab showed that when overexpressed, *snuts* up-regulates the JNK pathway. When *snuts* is overexpressed in the eye, we observed phenotypes that suggested apoptosis. This was confirmed using cleaved caspase antibody. To determine if the phenotype is due to *snuts* upregulating the JNK pathway, we hypothesized that knocking down JNK expression would restore the wild type phenotype even as *snuts* is being overexpressed.

We generated a *basket* dominant negative genotype along with a *GMR-gal4* to see if the downregulation of *basket* will restore the wild type phenotype when *snuts* is overexpressed. When the gene *basket* was downregulated while *snuts* was overexpressed using *GMR-gal4*, wild type eyes were restored as compared to *snuts* overexpressed using *GMR-gal4*.

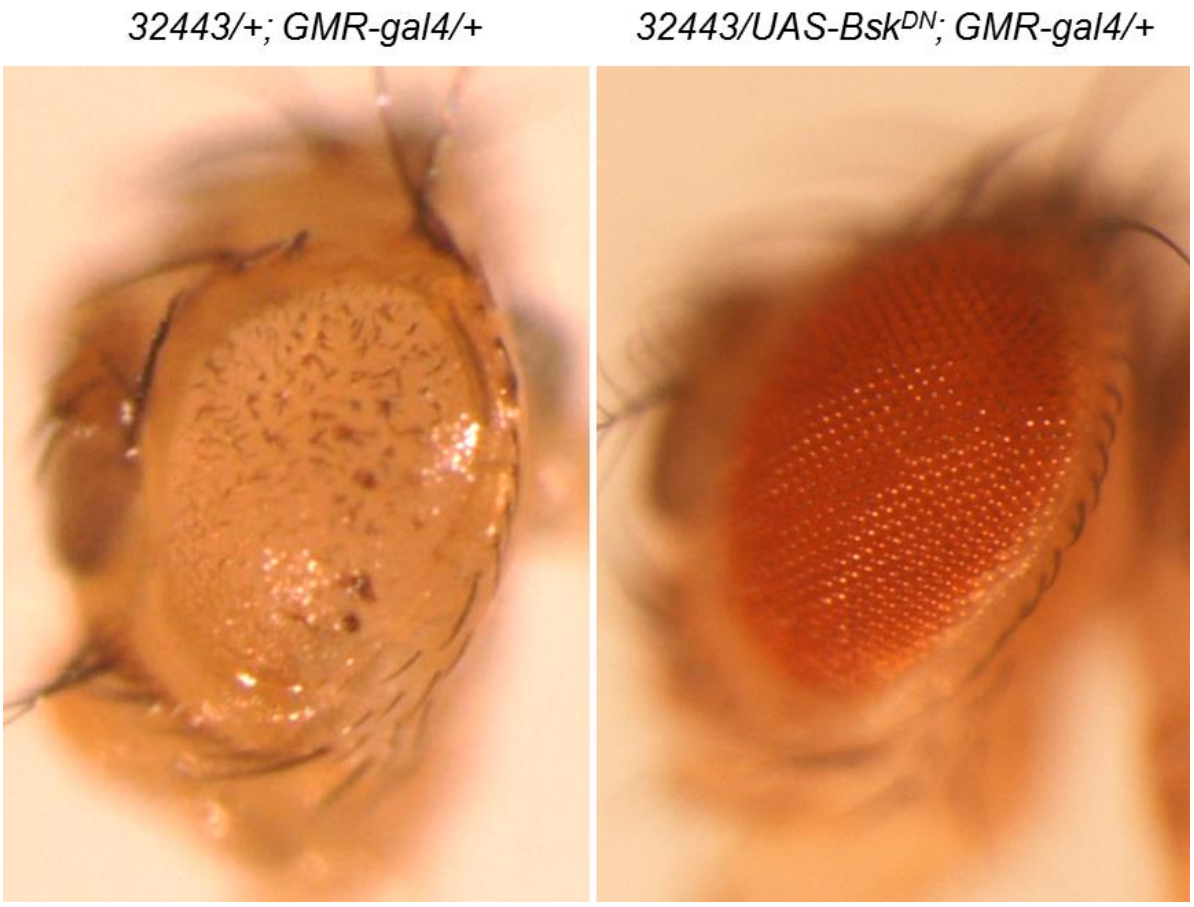

Figure 12: Rescue of Rough Eye Phenotype

Mutant phenotype in *Drosophila eyes (32443, GMR-gal4)*. To confirm this, we downregulated the JNK *basket* while overexpressing *snuts* using *GMR-gal4*. Images were taken with 6.5X magnification using a Leica light microscope. Downregulation of JNK *basket* rescues mutant eye phenotype.

3.5 Generation of reagents for epistasis experiments

To determine where *snuts* operates along the JNK pathway it is important to perform epistasis experiments. We decided to generate reagents to aid in performing these epistasis experiments. We transposed *GMR-gal4* onto the X-chromosome where *snuts* is located to create a permanent phenotype (*GMR-gal4, 32443*) that we could attempt to rescue (or worsen) by overexpressing genes involved in the JNK pathway. We mobilized *GMR-gal4* using transposase and removed it in the following generation. We crossed each individual fly and screened it for *GMR-gal4* on the X-chromosome. Positive flies were crossed with *snuts*. The progenies of this line were crossed with a white eyed line and progenies showing recombination (the rough eye phenotype) were selected and inbred to create a stock. The full schematic of the movement of *GMR-gal4* onto the X-chromosomes is shown in Figure 13.

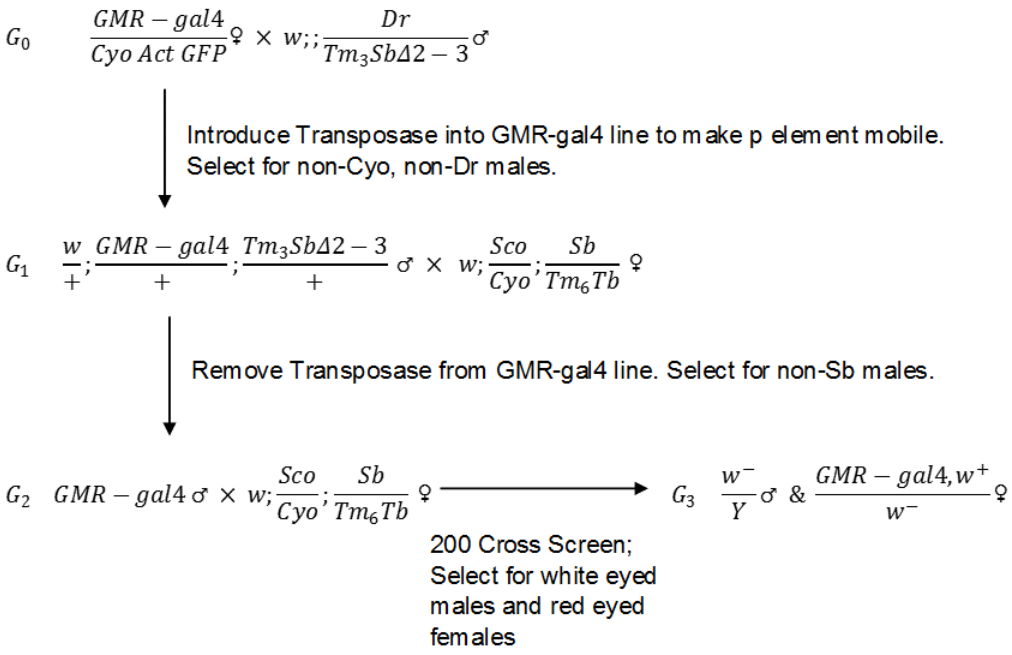

Figure 13: Translocation of GMR-gal4 onto the X-Chromosome

Schematic showing the movement of *GMR-gal4* onto the X-Chromosome utilizing transposase (Δ2-3) and classical *Drosophila* genetics. Flies bearing *GMR-gal4* on the X-Chromosome were self-crossed until the stock was homozygous.

3.6 Downregulation of *snuts* in the wing pouch, suggests important role in wing development.

We previously found that when *snuts* is downregulated in the wing, the wing did not properly unfold after eclosion (emerging from pupa). Consequently, we wanted to look at the expression of *snuts* in the wing disc using RNA in-situ hybridization. When *snuts* was downregulated using *Nubbin-gal4; Dicer2*, expression was downregulated in the anterior portion of the wing disc (Figure 14). When dicer expression was reduced with temperature, wild type wing phenotype was restored (Fields, 2014). Closer examination however revealed that while wing folding is restored, structural abnormalities persist in the hinges of adult wings (Figure 15).

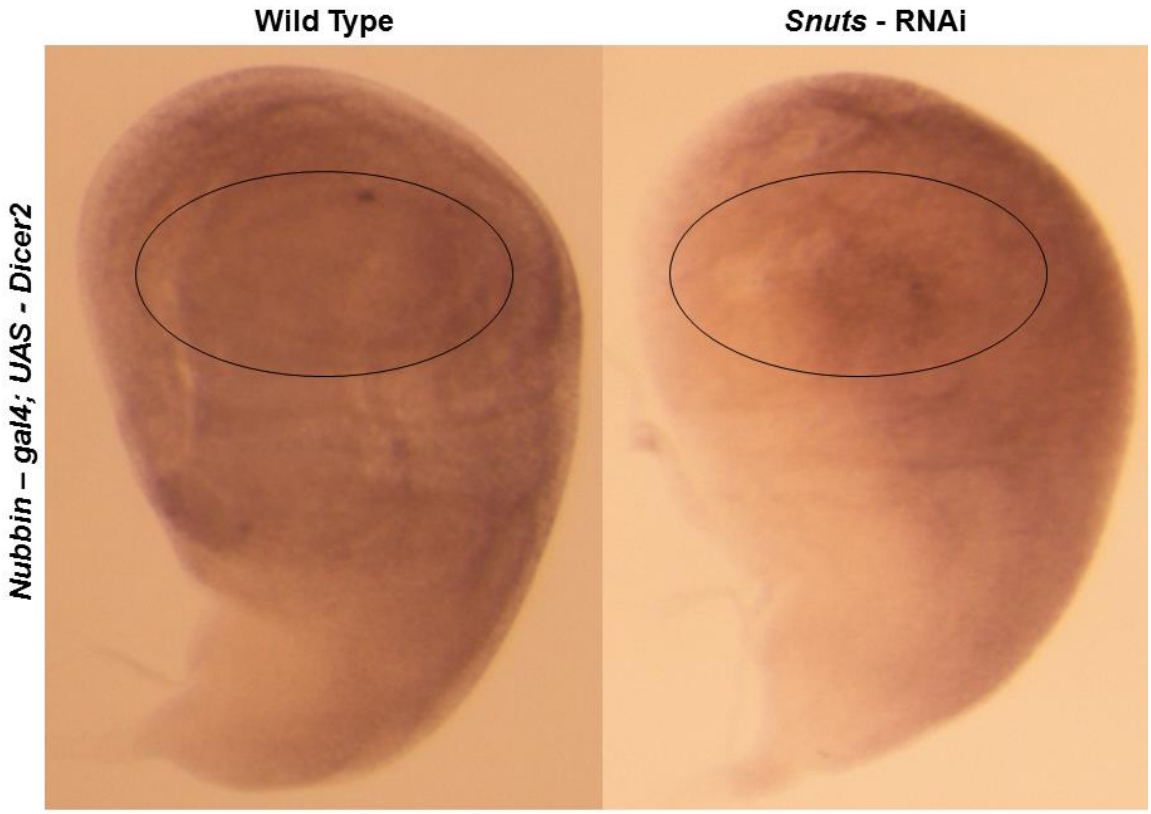

Figure 14: *snuts* Downregulation in Third Instar Wing Disc

snuts was downregulated using *Nubbin-gal4; UAS-Dcr-2* which is expressed in the wing pouch as indicated by the circle. The wing pouch later forms the adult wing and is a site for intense basement membrane remodeling. A reduction in the wing pouch area was observed, suggesting a mechanism by which the adult wings have defects/unfolding abnormalities.

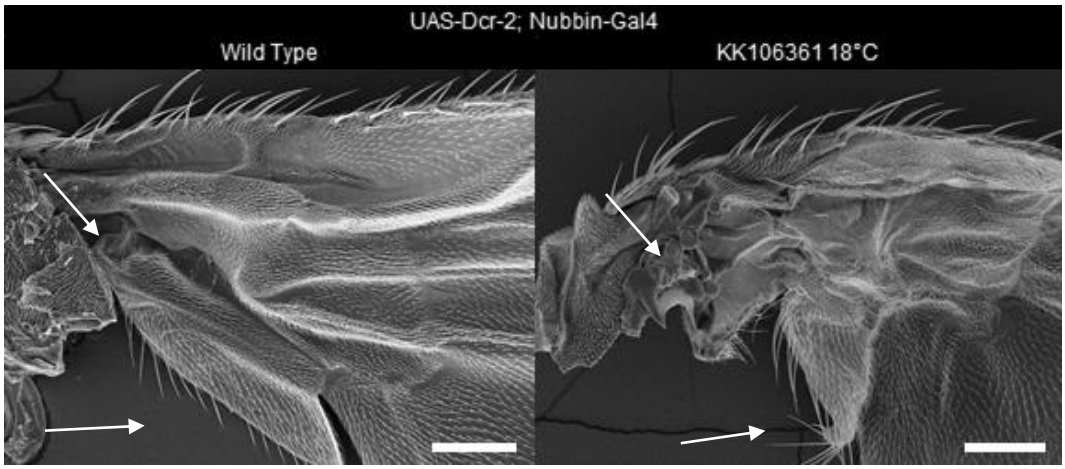

Figure 15: Wing Hinge Defects

SEM image of adult *Drosophila* wing hinges when *snuts* is downregulated using *UAS-Dcr-2; Nubbin-gal4*. These data suggest *snuts* plays a role in proper wing development. Image taken at 200X magnification and the scale marker represents 100 µm. Arrows indicate areas of interest.

4 Discussion and Future Directions

Our experiments have confirmed that *snuts* is a basement membrane degrader, when overexpressed it upregulates collagenase activity. We have also demonstrated that expression of *snuts* occurs in the larval discs of developing flies, which are areas of intense BM remodeling. *snuts* expression also appears to be important in wing and eye development; and as Bausek et al., (2007) stated, in stem cell niche maintenance as well. A *snuts* antibody will be useful in understanding how the *Snuts* protein interacts with other proteins and influences development.

Previously, we showed that overexpression of *snuts* activates the JNK pathway, a strongly conserved signaling cascade that results in BM degradation as well as apoptosis. In this study, we showed that the *snuts* overexpressed eye phenotype can be rescued by inhibiting *basket* expression which suggest *snuts* operates upstream along the JNK pathway. We confirmed that when overexpressed, *snuts* up-regulates caspase mediated apoptosis. The JNK pathway is known to activate apoptosis in response to stress. The eye phenotypes suggestive of apoptosis could be due to *snuts* activating the JNK pathway. While other signaling pathways are known to activate apoptosis, our data strongly suggest that it is the JNK pathway that is inducing apoptosis as we have previously shown that *snuts* upregulated the JNK pathway.

We speculate that *Snuts* acts as a transcriptional regulator because it too possesses SAM and PHD domains. To better understand how these domains help *snuts'* function, we propose to make transgenic flies in which the various

domains in *Snuts* have been deleted. This would allow us to determine the function of each domain and to see if *snuts*, when overexpressed without a specific domain, generates the same phenotypes we have previously observed. To accomplish this, we will clone each domain deletion into a pUAST vector that will be utilized to create transgenic flies.

One of the main priorities for our future research with *Snuts* is to generate an antibody that will allow us to perform direct immunohistochemistry. This will allow us to better assess the final location of expressions (nuclear, intercellular, extracellular, etc.). It will also allow us to perform a pulldown assay to determine what proteins *Snuts* interacts with, such as histone proteins. One future experiment we are interested in performing is to understand *snuts* expression in the gonads of adult flies, as our previous data indicates *snuts* plays an important role in these structures.

It is known that male testis development is controlled by the JAK/Stat pathway. If *snuts* plays a critical role in testis development, it could mean that *snuts* might interact with this pathway as well. Given what Bausek et al., (2007) found, it is highly likely that *snuts* directly or indirectly interacts with the JAK/Stat pathway. Further RNA-in situ analyses could reveal changes in *snuts* expression when JAK/Stat is downregulated. It would also be interesting to understand what role *snuts* might play in the maintenance of the stem cell niche as described in Bausek et al., (2007).

Another area of interest is the role that *snuts* plays in the development of the eye and wings of flies. Although the scope of this thesis did not the address

the role of *snuts* in wing or eye development beyond its effect on apoptosis, our RNAi data (both histological and in-situ analysis) strongly suggest it plays a role. What role this could have on wing development is not clear and is an interest for future study. In light of the RNA in-situ data, this does suggest that *snuts* is important for wing development, at least in the unfolding of the wings.

To spearhead further investigation into the possible role of *snuts* in eye development, the Srivastava lab is looking to collaborate with another lab that specializes in studying eye development. Furthermore, it would also be interesting to perform behavioral assays to assess whether the mutant eye phenotypes affect adult vision. Without a behavioral assay it is not possible to determine if the spacing of the rhabdomeres has any effect on the function of the eye, although personal observation has shown that these flies function normally (i.e. can mate). For example, one could examine the courtship behaviors and time to copulation in wild type versus experimental flies as instructed from a protocol by Nichols et al., (2012).

The gene *wingless* is important in wing and eye development. Our in-situ data for *snuts* shares the same expression as *wingless* in the eye. *Wingless*, a gene critical in development, is known to interact with the JNK pathway (Swarup and Verheyen, 2012). This suggests there might be a relationship between the JNK pathway, the *wg* pathway, and *snuts*. Further experiments will be required to better understand how *snuts* interacts with the JNK pathway.

We would also like to explore the role of *snuts* in tumor metastasis, especially in regards to its role in basement membrane degradation. To begin we

could perform an RT-PCR using a wild type tumor induced line to see if *snuts* is being upregulated. Following this, we could perform *snuts* overexpression and downregulation experiments to see what effects this has on tumor growth/migration.

There is still much work that needs to be done to understand the role of *snuts* in development and tumor metastasis. Its novelty provides us several possible directions of study. The generation of an anti-*Snuts* antibody will greatly expand the avenues we can explore, including its role with stem cells as well as its role in metastasis. In conclusion, the results of this study have given us a better understanding of where *snuts* is expressed as well possible functions, but there is much more to explore.

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ABBREVIATIONS

BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CyO	*Curly*
Ey	*Eyeless*
GFP	Green Fluorescent Protein
GMR	*Glass Multiple Reporter*
JNK	C-Jun N-Terminal Kinase Pathway
MMP	Matrix Metalloproteinases
NBT	Nitro blue tetrazolium
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Saline w/ Triton X-100
PHD	Plant Homeodomain
Ptc	*Patched*
PucZ	*Puckered LacZ*
RNAi	RNA Interference
RFP	Red Fluorescent Protein
SEM	Scanning Electron Microscopy
snuts	*shrunken nuts*
SAM	Sterile Alpha Motif
Tb	*Tubby*
TEM	Transmission Electron Microscopy
UAS	Upstream Activator Sequence
Vg	*Vestigial*