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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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Presented in Partial Fulfillment of the Requirements for

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By

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Western Kentucky University
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ABSTRACT

SNUTS is a protein coding gene present in *Drosophila melanogaster* whose product contains conserved domains that are present in a range of eukaryotic organisms. The SNUTS protein is made up of four domains, two Sterile Alpha Motif (SAM) domain, and two Plant Homeodomains (PHD). The biological function of SNUTS or the various domains is largely unknown. One study demonstrated that SNUTS was important for proper development of the stem cell niche. In the present study data from both overexpression and downregulation of *Snuts* and the resulting phenotypes are presented. Data supporting potential mechanisms resulting in the phenotype are also presented. Creation of reagents that would help us better understand the spatial and temporal localization of gene expression using RNA in-situ hybridization are documented as well. Overall this study presents a preliminary characterization of the gene *Snuts*.

Keywords: *Drosophila melanogaster*, basement membrane remodeling, extracellular matrix, JNK pathway, Shrunken Nuts (SNUTS)

Dedicated to my parents

Who instilled in me

a love for learning

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VITA

March 29, 1991	Born – Murray, Kentucky
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PRESENTATIONS

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FIELDS OF STUDY

Major Field: Biology

Minor Field: Biochemistry

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CHAPTER 1

INTRODUCTION

The basement membrane is an extracellular region that plays a crucial role in the development of organisms and is a central focus of research in cancer biology (Furuyama & Mochitat, 2000). Composed of a variety of proteins including collagen, the basement membrane forms a selective barrier between blood vessels and body tissues. Basement membrane plays a role in cellular signaling, differentiation, and angiogenesis (Schwarzbauer, 1999). One of the critical pathways in the maintenance of the basement membrane is the c-Jun N-terminal kinase signaling pathway. This member of the MAP kinase family is activated in response to a variety of signals which regulate a variety of transcription factors which result in proliferation and apoptosis (Bogoyevitch, Ngoei, Zhao, Yeap, & Ng, 2010).

It has been known for some time that basement membrane degradation is a critical step in the process of tumor metastasis (Srivastava & al., 2006). When additional nutrients are required, tumors release angiogenesis releasing factors which start a cascade that ultimately results in the degradation of the basement membrane allowing for the formation of new blood vessels near the tumor (Grant & al, 1994). It is this degradation that allows malignant cells to metastasize to other parts of the body. As the basement membrane is being destroyed, cancerous cells which have lost cell adhesion as well as polarity can become dislodged and flow freely into the body's circulatory system (Figure

1). Metastasis is associated with increased mortality of nearly all cancer cases (Shinichi, et al., 2007).

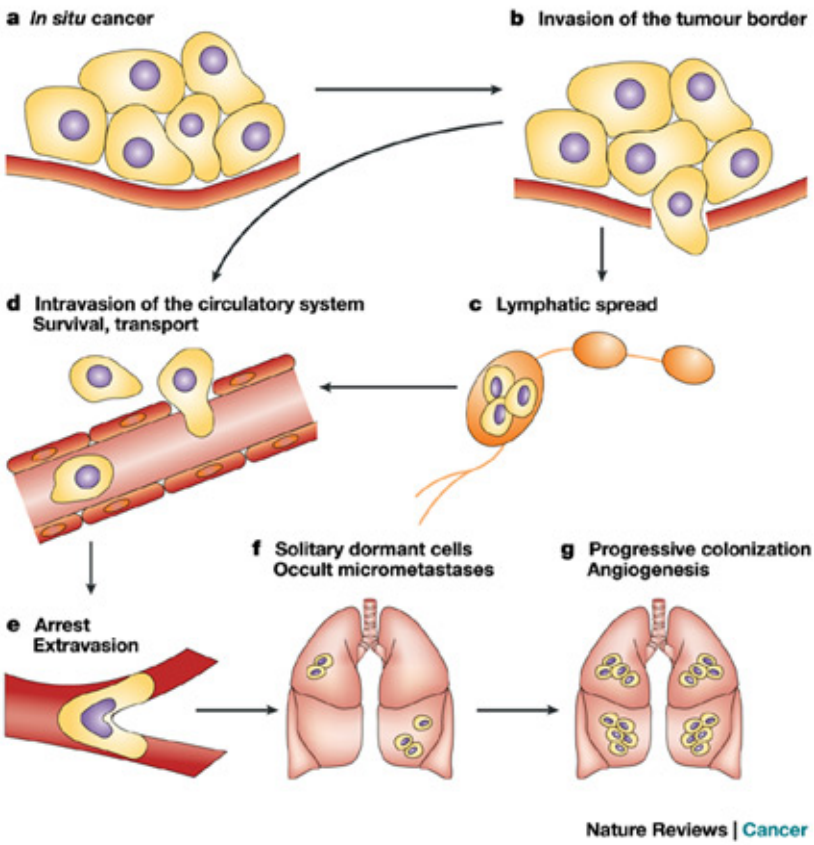


FIGURE 1: Tumor Metastasis

The central focus of my project has been the characterization of a novel gene identified in a genetic screen performed by the Srivastava Lab. Referred to as *Shrunken Nuts* (*Snuts*) this gene's biological functions are a mystery. We hope to gain insight into *Snuts* function by studying the expression, spatial location and the developmental consequence of manipulating its regulation. A previous study showed that SNUTS played a role in the maintenance of the stem cell niche in Drosophila (Bausek, 2007). The stem cell niche is responsible for providing the nutrients as well as directions for cellular differentiation (Li & Xie, 2005). Both utilize similar signaling pathways that regulate cellular division in these undifferentiated cells (Reya & al., 2001). A better characterization of SNUTS' role in development and basement membrane degradation will improve our understanding of how Extracellular Matrix (ECM) remodeling occurs in response to cellular processes (i.e. angiogenesis).

Domain Architecture of SNUTS

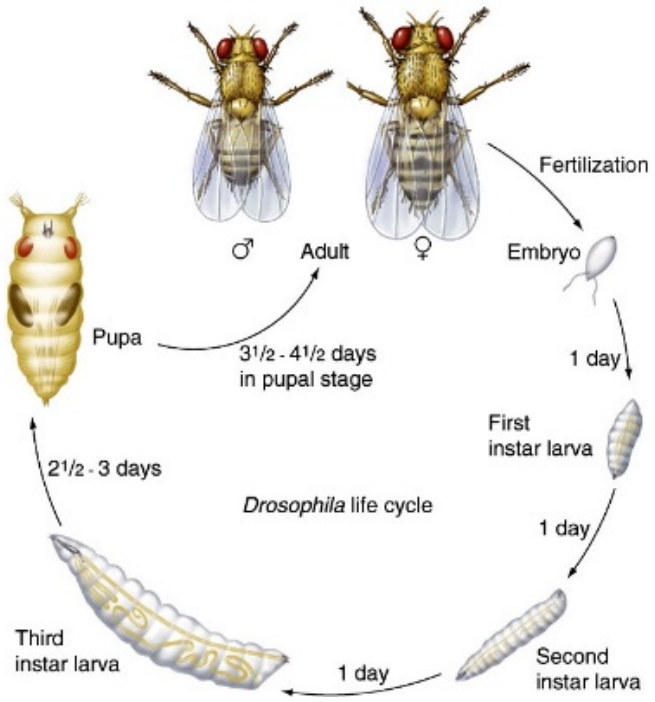
SNUTS is a protein composed of 446 amino acids (Gene Dmel\CG2662, 2014). Database analysis (nucleotide query) has shown two conserved domains are present in the SNUTS protein, with the Plant Homeodomain (PHD) zinc finger being of a particular interest (NCBI, 2014). Zinc fingers enable proteins to bind to nucleic acids and in many cases other proteins, making it one of the most abundant domains in eukaryotic organisms (Laity, Lee, & Wright, 2001). PHD is found in nuclear proteins, often involved in epigenetics and transcriptional regulation (Letunic, Doerks, & Bork, 2011). The other conserved domain is the Sterile Alpha Motif domain (SAM), a domain that has widespread functions in signaling (Letunic, Doerks, & Bork, 2011). Defining the function

of these domains will help us to better understand SNUTS role in development. (see Figure 4 and 5 for representation of SNUTS protein and gene respectively)

Model Organism Drosophila

For all of our experiments we employed the model organism *Drosophila melanogaster*. Since Thomas H. Morgan first used *Drosophila* mutants to discover chromosomes, *Drosophila* continue to expand our understanding of biology (Rubin & Lewis, 2000). *Drosophila* serves as an excellent disease model with nearly 75% of human diseases having an ortholog found in *Drosophila*. *Drosophila* husbandry is easy to learn and a host of powerful genetic tools helps to answer a variety of questions ranging from evolution to biochemistry. *Drosophila* have a short life cycle characterized by distinct stages in development. Starting from embryos, flies develop into larva with 3 instars followed by pupation into the adult (Figure 2) (Drosophila melanogaster, 2013). From embryo to adult takes only 2 weeks at room temperature. They are inexpensive to

Figure 2: *Drosophila melanogaster* life cycle (Drosophila melanogaster, 2013)

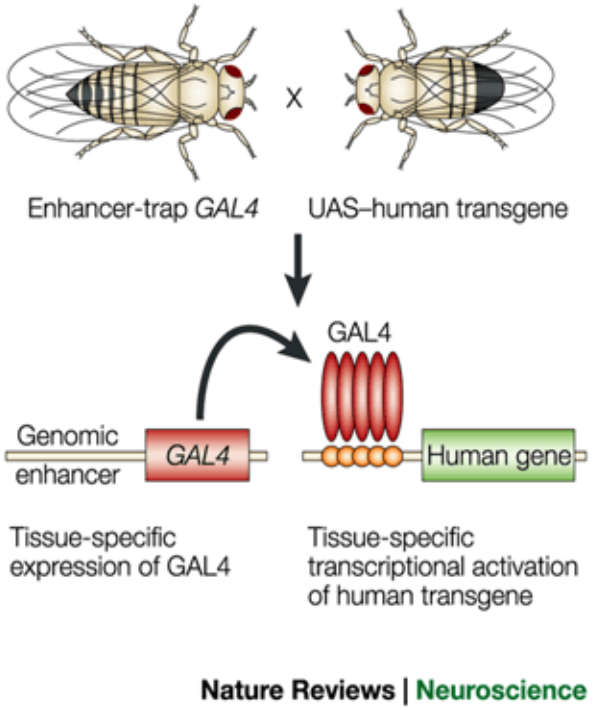


rear and stocks of mutant flies are readily available through stock centers. A wide variety of genetic tools, discussed later, are also available for use with *Drosophila* (Duffy, 2002).

To use *Drosophila* for experiments we must use virgin females to ensure the genotype we want is represented in the progeny. Females can hold sperm in storage which makes them unacceptable for controlled crosses. Performing a cross is as simple as putting virgin females and males in a vial with food. Naturally they will procreate and lay their eggs in the food. Two weeks from conception, adult embryos emerge from pupa and any distinctive phenotypes can be recorded.

The tool which makes *Drosophila melanogaster* especially useful in genetics is the *UAS-Gal4* system (Duffy, 2002). Originally developed in yeast, the *UAS-Gal4* system allows us to control the expression of genes (Brand & Perrimon, 1993). Gal4 is a protein that binds to the UAS (Upstream Activation Sequence) of a gene resulting in the expression of the gene attached to the UAS. Additionally, expression can be further limited to a specific location by attaching the *Gal4* gene to another gene. This causes *Gal4* to be expressed only where and when the attached gene is subsequently expressed (Figure 3). The result is complete control over where and when gene expression happens. *UAS-Gal4* system forms the backbone of all of our lab's work.

Figure 3*: UAS-Gal4* System (M.K. & Feany, 2002) When a *Gal4* fly and *UAS* fly are allowed to mate, the GAL4 protein activates the UAS element resulting in expression of the gene.



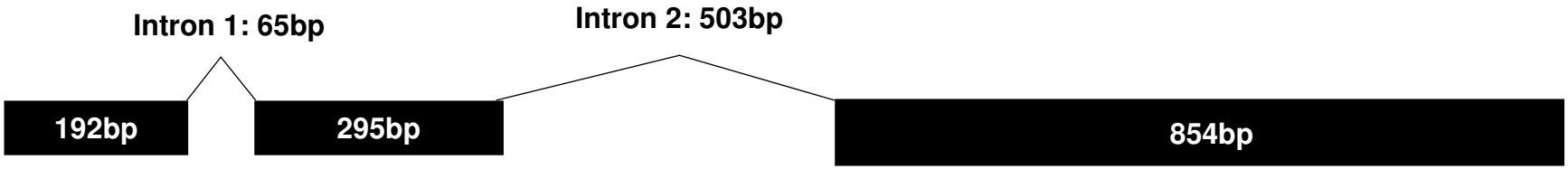
Stocks of flies with specific genes attached to *gal4* maintained in labs and stock centers across the *Drosophila* community. To use the *UAS-Gal4* system we took a fly that has a *uas* attached to a gene of interest and mated it with a fly containing Gal4. After two weeks, we looked at the phenotypes of the progeny. The progeny of these two flies expressed our gene in the same spatial location where the *gal4* gene was expressed, therefore any abnormal phenotypes will manifest in the area where Gal4 is expressed (i.e. wing, eye, etc.).

The gene *Snuts*, which is the focus of my CE/T project, was identified in a genetic screen for potential basement membrane degraders. *Beadex-Gal4* was used to overexpress *Snuts* in the drosophila wing. The resultant phenotype was indicative of a degradation in basement membranes. This phenotype will be discussed later.

In this study a preliminary characterization of *Snuts* was accomplished. Using *UAS-Gal4* and the RNA interference tools, discussed in the material and methods section, *Snuts* was either overexpressed or knocked down in various tissues in *Drosophila*. We found that up-regulation of *Snuts* produces striking phenotypes in the eye and the wing as well as lethality in some lines. When down regulated using RNA interference, no lethality occurred, but some mild phenotypes were observed. We also demonstrated that *Snuts* upregulates the JNK pathway and currently we are working towards making reagents for RNA in-situ hybridization to further our understanding on the developmental consequences of *Snuts* by showing where expression occurs.

Figure 4: Approximate location of domains on SNUTS. Approximate location of conserved domains found on SNUTS, Sterile Alpha Motif Domain (SAM) and Plant Homeodomain (PHD)

Figure 5: Genomic Organization of the *Snuts* gene. Exons are shown as black bar with their length giving relative sizes. Introns are the spaces in between and also show relative sizes.



CHAPTER 2

MATERIALS AND METHODS

2.1 Fly Stocks

Over-expression stocks and *gal4* driver lines were ordered from Bloomington *Drosophila* Stock Center. RNAi stocks were ordered from the Vienna *Drosophila* RNAi Stock Center.

2.2 LacZ Staining on SNUTS

We used a LacZ stain to see the expression of the gene *puckered*, a gene expressed during JNK activation. If SNUTS upregulates the JNK pathway, then we would also expect to the *puckered* expressed as well. Females from the fly-stock Ptc-gal4; UAS-GFP; PucZ/Tm_6Tb were mated with Gal4 males for SNUTS to produce progeny that over-express SNUTS in the wing. Wing disc from 3rd instar larva were dissected in 1X PBS buffer, fixed with fresh 1% glutaraldehyde in 50mM phosphate buffer for 10 minutes, washed with 50mM phosphate buffer, stained with 20µg/mL X-gal in 50mM Potassium ferri-cyanide and 50mM ferro-cyanide, incubated in the dark at 37°C for 8 hours, and mounted on microscope slides (Srivastava, et. al).

2.3 Stocks used for Over-Expression Screen

A variety of gal-4 lines were used to help deduce *Snuts* potential role in development. By over-expressing *Snuts* with these tissue specific gal4 driver, we hope to observe phenotypes that could help us understand *Snuts* role in basement membrane remodeling. We crossed *Snuts* with the gal4 driver lines shown in the table below in order to over-express it in a variety of tissues.

Table 1: Gal4 Drivers Used for Over-Expression Screen

Gal4 Driver Line	Gene	Gal4 Expressed In
Ptc-gal4	*Patched*	Anterior/Posterior compartment border
w; LSP2-gal4	*Larval Serum Protein 2*	Third Instar Fat Body
w; Ey-gal4/CyO	*Eyeless*	Eye disc, ventral nerve cord, brain
w; Sd-gal4	*Scalloped*	Wing
Vg-gal4 BE III	*Vestigial (Boundary Enhancer)*	Wing
yw; Tub-gal4/Tm_3Sb	*Tubulin*	Ubiquitous
W;;Ubx-gal4/Tb	*Ultrabithorax*	Abdomen, halthere, wing
Cg-gal4	*Combgap*	Hemocytes
GMR-gal4	*Glass Multimer Reporter*	Eye
Vg-gal4 QE III	*Vestigial (Quadrant Enhancer)*	Wing
Pnr-gal4/Tm_6Tb	*Pannier*	Dorsal Cells Along length of fly

2.4 RNA interference and Inhibition of SNUTS

RNA inhibition lines were ordered from the Vienna *Drosophila* RNAi Center and crossed with the RNAi driver lines in table 2. Two lines (genes) were available from Vienna, KK106361 and GD33544 which are located on the 2nd and 3rd chromosome respectively. Males from the RNAi lines were crossed with virgin females from RNAi driver lines. Progeny from both sexes were examined for phenotypes.

Table 2: RNAi Driver Lines

RNAi Line	Gene Affected	Expected Phenotype Region
P{UAS-Dcr-2.D}1, w1118; P{GawB}nubbin-AC-62	*Nubbin*	Wing
P{UAS-Dcr-2.D}1, w1118; P{en2.4-GAL4}e16E, P{UAS-2xEGFP}AH2	*Engrailed*	Engrailed Domain
P{UAS-Dcr-2.D}1, w1118; P{GawB}pnrMD237/TM3, Ser1	*Pannier*	Dorsal Cells Along Length of Fly
P{w[+mW.hs]=GawB}elav[C155] w[1118]; P{w[+mC]=UAS-Dcr-2.D}2	*Embryonic Lethal Abnormal Vision*	Nervous System
Actin-gal4/Cyo; UAS-CR-2/Tm$_6$Tb	*Actin*	Ubiquitous
P{UAS-Dcr-2.D}1, w1118; P{bs-GAL4.Term}G1/CyO	*Blistered*	Wing
P{UAS-Dcr-2.D}1, w1118; P{GawB}salmLP39	*Spalt Major*	Wing
w[1118] P{w[+mW.hs]=GawB}Bx[MS1096]; P{w[+mC]=UAS-Dcr-2.D}2	*Beadex*	Wing

2.5.1 Isolation of total RNA from Drosophila

Total RNA was isolated from male adult, 3rd instar larva, and pupae flies utilizing the Qiagen RNeasy kit (Qiagen 74104). The nucleic acid was analyzed using a spectrometer to determine the purity and concentration of the isolated RNA.

2.5.2 Reverse Transcription of Snuts' mRNA

The Qiagen One Step RT-PCR kit (Qiagen 210210) was used to reverse transcribe the RNA into cDNA by following the manufacturer's protocol. As an internal control the *Snuts* RT-PCR primers were designed to amplify a region with an intron between the primer pair. A band that was larger than ~200 base pairs would indicate genomic contamination. Amplification of the cDNA was achieved by using the sequence specific

primer pair 1 in Table 3. The ribosomal protein 49 primers (primer pair 2) which is ubiquitously expressed was used as a positive control for this experiment. The cycling conditions for the RT-PCR reaction are provided in Table 5. The location of the amplified product is shown on Figure 6.

Table 3: RT-PCR Primer Sequences

Primer Pairs	Primer	Sequence (5'- 3')	PCR Length
1	SNUTS-RT Forward	TCGACACGTGCCAGAAGTG TGG	176bp
	SNUTS RT-Reverse	CCCCTCACGTTGCATAACCG GCA	
2	RP49 Forward	AGATCGTGAAGAAGCGCAC CAAG	206bp
	RP49 Reverse	CACCAGGAACTTCTTGAATC CGG	

Figure 6: Genomic Organization of the *Snuts* gene with location of RT-PCR primers. Amplification using our RT-PCR primers (Table 3) for *Snuts* should produce a band ~ 200bp on an agarose gel. If genomic contamination is present in the RNA isolation, a band will be amplified that is ~600bp on an agarose gel.

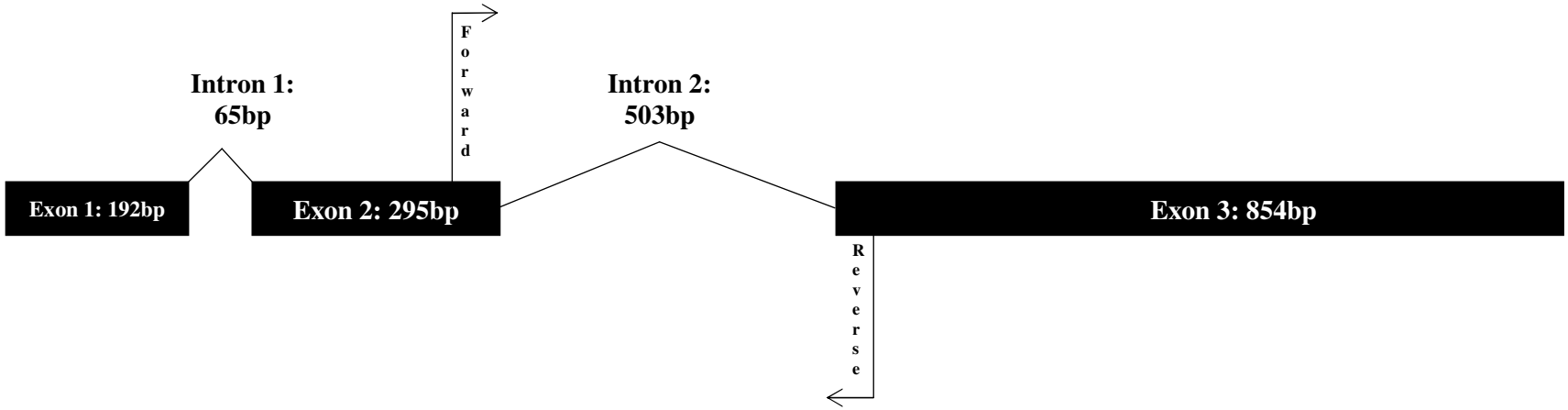


Table 4: Reaction mixture for RT-PCR of *Snuts* expression in wild type flies.

Component	Volume/Reaction	Final Concentration
QIAGEN OneStep RT-PCR Buffer (5X)	10 µL	1X; 2.5 mM Mg2+
dNTP mix (10mM each)	2 µL	400 µM of each dNTP
Forward Primer	3 µL	0.6 µM
Reverse Primer	3 µL	0.6 µM
QIAGEN OneStep RT-PCR Enzyme Mix	2 µL	
Template RNA	Variable	1 µg
RNase Free Water	Variable	
Total Volume	50uL	

Table 5: RT-PCR Thermocycler Conditions of *Snuts* expression in wild type flies.

Step		Time	Temperature
Reverse-Transcription		30 min	50°C
Initial PCR Activation		15 min	95°C
X 35 Cycles	Denaturation	1 min	94°C
	Annealing	1 min	57.5°C
	Extension	1 min	72°C
Final Extension		10 min	72°C

2.5.3 Gel Electrophoresis

The RT-PCR product was resolved on a 1.5% UltraPure agarose gel from Invitrogen at 100 volts for 60 minutes using 1X TAE buffer. 1uL of a 10mg/ul stock solution of ethidium bromide was mixed into each 50mL gel before solidifying. Each sample was mixed with a 10X glycerol loading buffer before being loaded into the wells.

After electrophoresis, the gel was imaged and the size of the products was estimated using the Life Technologies 1Kb plus DNA ladder.

2.5.4 Reverse Transcription of Over Expressed and Inhibited SNUTS

To verify our over-expression and inhibition studies that SNUTS expression is being affected during our experiments we utilized RT-PCR described above to confirm our results being caused by over-expression/inhibition. We isolated RNA from one over-expression line (32443♂ X *Gmr-gal4*♀) and one inhibition line (KK106361♀ X 25754♂). The RNA was analyzed using a spectrophotometer. Using the same conditions as mentioned in 2.5.2, the RNA was transcribed for *Snuts*, and visualized on a 1.5% gel for 60 minutes at 100 volts.

2.6 Creation of DNA construct for probe preparation and use in RNA In-Situ Hybridization

DNA was isolated from wild type male *Drosophila* adults using the Qiagen DNeasy kit (Qiagen 69504) according to the manufacturer's protocol for insects. Using the primers in Table 6 below, PCR was performed with the conditions in Table 8. The products of the reaction were separated on a 1% agarose gel, then extracted using the Qiaex II Gel Extraction Kit (Qiagen 20021). *Snuts* DNA was isolated and then analyzed using the spectrophotometer to determine the concentration and purity of the sample.

Table 6: PCR Primer Sequences for fragment amplification

Primer	Sequence (5’ - 3’)	PCR Length
PCR-*Snuts* Forward	AGAATTCGCTAGCCAAACGTAGACAGCC	300bp
PCR-*Snuts* Reverse	AGAATTCACGCCTCCTTCGGATAG	

Figure 7: Genomic Organization of the *Snuts* gene with location of PCR primers.

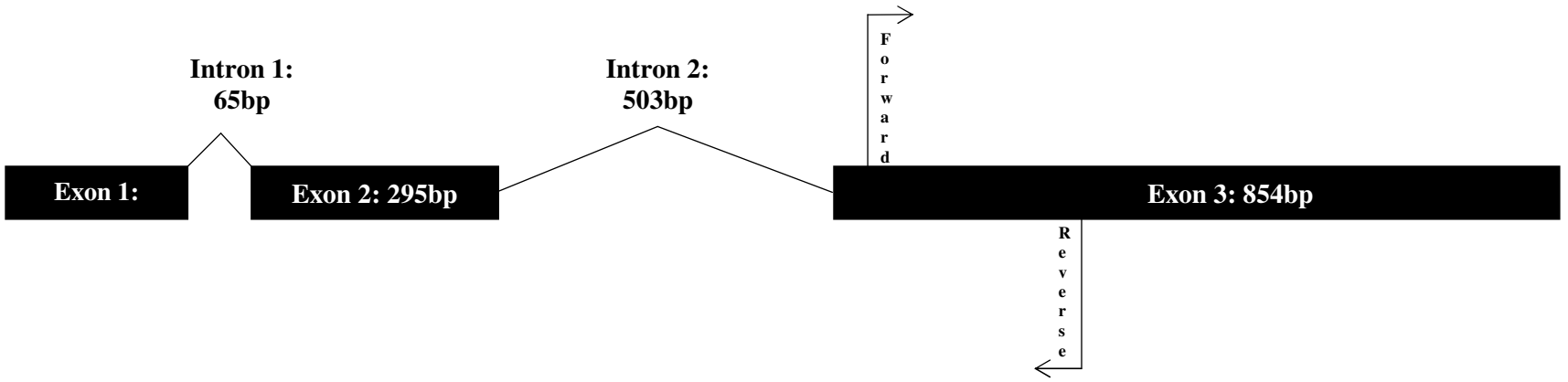


Table 7: PCR Reaction Mix

RT-PCR Component	Volumn	Final Concentration
SuperMix (Invitrogen)	45 µL	1X
DNA (7.6ng/µL)	3 µL	< 500ng
Forward Primer	1 µL	0.5µM
Reverse Primer	1 µL	0.5µM

Table 8: PCR Conditions for *Snuts* fragment amplification

Step		Time	Temperature
Activation		3 min	95°C
X 35 Cycles	Denaturation	0.5 min	95°C
	Annealing	0.5 min	58.5°C
	Extension	1 min	72°C
Final Extension		5 min	72°C

To make a probe for the RNA In-situ hybridization, a fragment was made using PCR on a fragment of *Snuts* using the primers from table 6. The PCR product (location shown on Figure 7) was sequenced to verify the identity of the fragment using a nucleotide alignment database. The SNUTS fragment was ligated to pGEM-t (Figure 8) using the Promega pGEM-t vector system, in accordance to the manufacturer's protocol. Ligated PGEM-t *Snuts* product was transformed in DH5α cells and plated on 100 µL/mL LB-Ampicillin plates. These plates were allowed to incubate overnight at 37°C. Three putative colonies were chosen, mini-prepped with the Qiagen Mini-prep kit (Qiagen 27104), ran on a 1% agarose gel for 60 minutes at 100 volts, with the corresponding band (200bp) extracted using QIAEX II Gel Extraction kit (Qiagen 20021), analyzed for concentration and purity using a spectrophotometer, 1ug of DNA digested with EcoRI

according to manufacturer protocol (Promega), and ran on a 1% agarose gel. To further verify their identities, these ligated products were sequenced using both the PCR primers (Table 6) and the T7 promoter primer.

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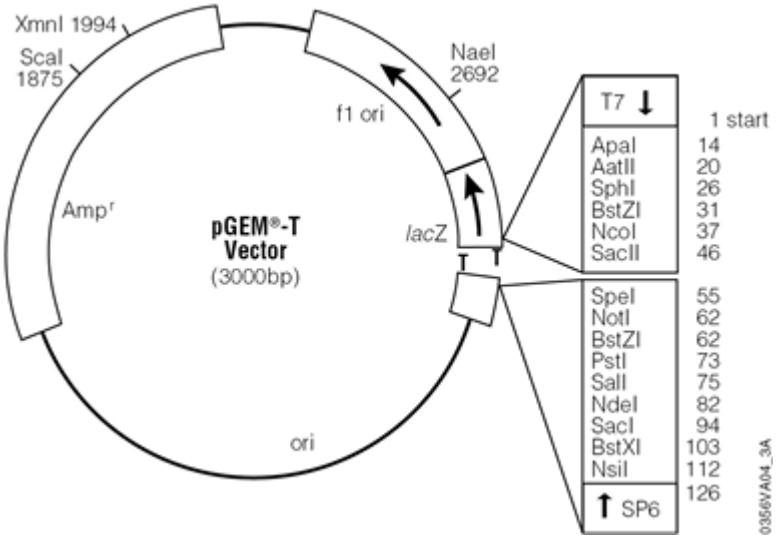
Figure 8: pGEM-t Vector



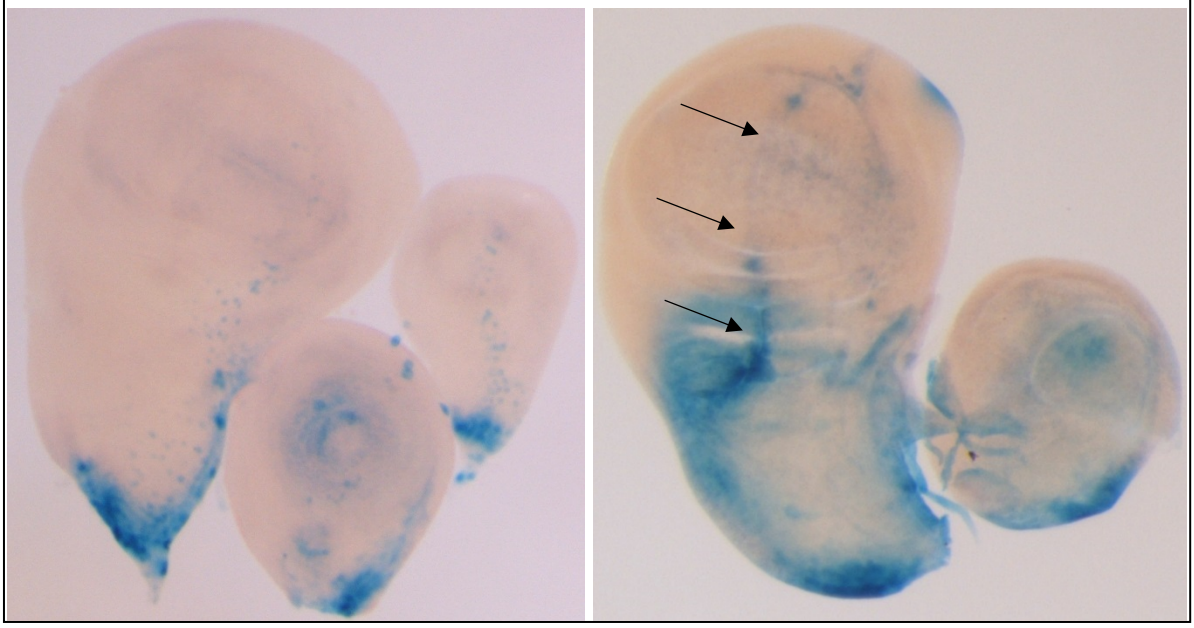
CHAPTER 3

RESULTS

3.1 LacZ Staining of SNUTS

To see if *Snuts* up-regulates the JNK pathway, we performed a LacZ staining. We performed the *Puckered* - LacZ staining on the wing disc of progeny from the cross of *Snuts*♂ and *Ptc-gal4, UAS-GFP/Cyo; PucZ/Tm6Tb*♀. The 3rd instar larva were selected for and stained using x-gal. In the wild type wing disc we saw no expression along the posterior/anterior boundary where Puckered is expressed during JNK pathway activation. In the over-expressed wing disc we

Figure 9: c-Jun N-Terminal Kinase Pathway expression. The image on the left shows the wild type expression of JNK pathway. On the right, the image shows JNK expression when SNUTS is over-expressed. The arrows indicate upregulated *puckered* expression.



saw a blue stain along the posterior/anterior boundary which indicates that the JNK pathway was expressed.

3.2 Over-Expression Screen of Snuts

Puckered (*Puc)* is expressed during JNK activation, and serves as a reporter by which we can determine if our candidate genes up-regulate the JNK pathway. To determine if *Snuts* up regulates the JNK pathway, we performed a cross using the fly line with genes Ptc-gal4; UAS-GFP; PucZ/Tm_6Tb. *Patched* (*Ptc*) is expressed in the anterior/posterior boundary in the larval wing disc. We used a LacZ reporter with x-gal to stain for the presence of *puckered*. The over-expression screen revealed a critically important phenotype that indicates that JNK signaling is up-regulated by *Snuts*. The mutant phenotype seen in *Ey-gal4* and *Gmr-gal4* indicated apoptosis, a process mediated through the JNK pathway. When we looked at these two phenotypes, we saw that the eye lost its organized structure. Additionally, close inspection revealed the ommatidia were clearly no longer present. In addition to these eye phenotypes, the following figure summarizes the results of the over-expression screen.

Table 9: Over-Expression Screen. None indicates that no abnormal phenotype was found, only wild type expression.

	Driver Line	Expected Phenotype Region	18°C	25°C
32443 ♂	w, LSP2-gal ♀	Third Instar Fat Body	None	None
	w, Ey-gal4/CyO ♀	Eye disc; ventral nerve cord; brain	None	Degraded Eye
	Vg-gal4 (BE)/(III) ♀	Wing	Wing Degraded	Wing Degraded
	Ptc-gal4 ♀	Wing Margin	Lethal	Lethal

	w;; ubx-gal4/Tb ♀	Abdomen, haltere, wing	None	None
	yw; Tub-gal4/Tm3Sb ♀	Ubiquitous	None	Lethal
	GMR-gal4 ♀	Eye disc	None	Degraded Eye
	Cg-gal4 ♀	Hemocyctes	None	None
	Pnr-gal4/Tm6Tb ♀	Dorsal Cells along length of the fly	None	None
	Vg-gal4 (QE)/(III)	Wing	Wing Degraded	Wing Degraded
	w; Sd-gal4 ♀	Pattern of the Scalloped Gene	Lethal	Lethal

Gmr-gal4 expresses in the eye disc of developing flies. When we looked at the eye in adult flies we saw that the size was significantly reduced as compared to the wild type. One of the likely causes of apoptosis in the eye is JNK pathway. When activated, or in this case up-regulated, the number of adult cells are reduced, accounting for the small size of the eyes observed in the *Gmr-gal4* flies. The same can be said in the case of *Ey-gal4*, where we not only saw a reduction in the size of the eye, but also a general destruction of eye tissues.

Figure 10: Eye Phenotypes at 25°C. Over-Expression of *Snuts* using gal4 drivers.

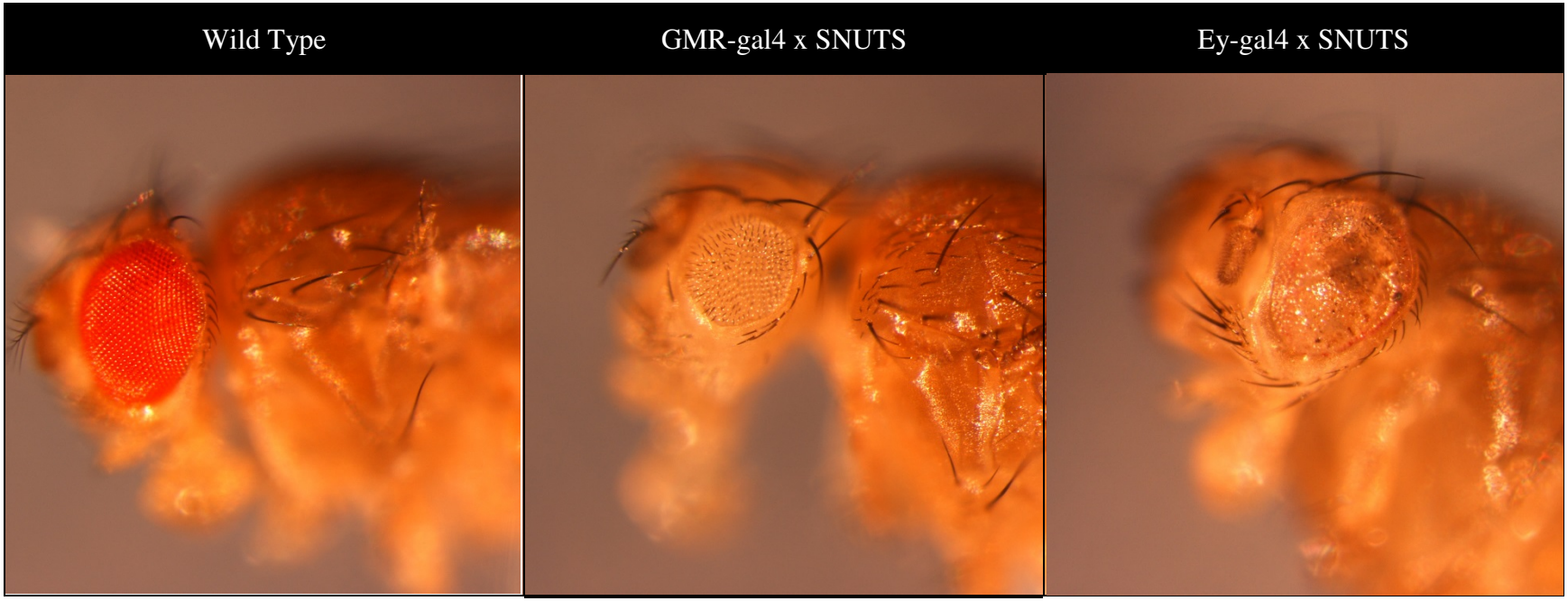


Figure 11: Wing Phenotypes at 25°C Over-Expression of *Snuts* using gal4 drivers.

3.3 Inhibition of SNUTS expression using RNA interference

To better understand *Snuts* function we knockout this gene's function by utilizing RNAinterference. The phenotypes we observe are mild and further experimentation is necessary to ensure that *Snuts'* function is being inhibited. Inhibition of *Snuts* did not produce any striking phenotypes as can be seen in the table below other than the wing phenotype seen in Figure 12. As a means to ensure that we up-regulated *Snuts* as well as inhibited with RNAi we performed Reverse-Transcription PCR, in conjunction with the RT-PCR on wild type flies we executed to confirm that *Snuts* transcripts are present during developmental stages.

Table 10: RNA interference Screen None indicates wild type phenotype. All crosses were performed at 25°C

	RNAi Driver Line	Phenotype Manifests In	Resulting Phenotype
KK106361♀	25754♂	Wing	Uninflated wing
	25752♂	Wing	None
	25758♂	Dorsal Cells Along Fly	None
	25750♂	Nervous System	None
	Actin-gal4/Cyo; UAS-CR-2/Tm6Tb♂	Ubiquitous	None
	25753♂	Wing Disc	Wing not flat
	25755♂	Wing Disc	Wing not flat
	25706♂	Wing Disc	None
GD33544♀	25754♂	Wing Disc	None
	25752♂	Wing Disc	None
	25758♂	Dorsal Cells Along Fly	None
	25750♂	Nervous System	None
	Actin-gal4/Cyo; UAS-CR-2/Tm6Tb♂	Ubiquitous	None
	25753♂	Wing Disc	None

	25755♂	Wing Disc	None
	25706♂	Wing Disc	None

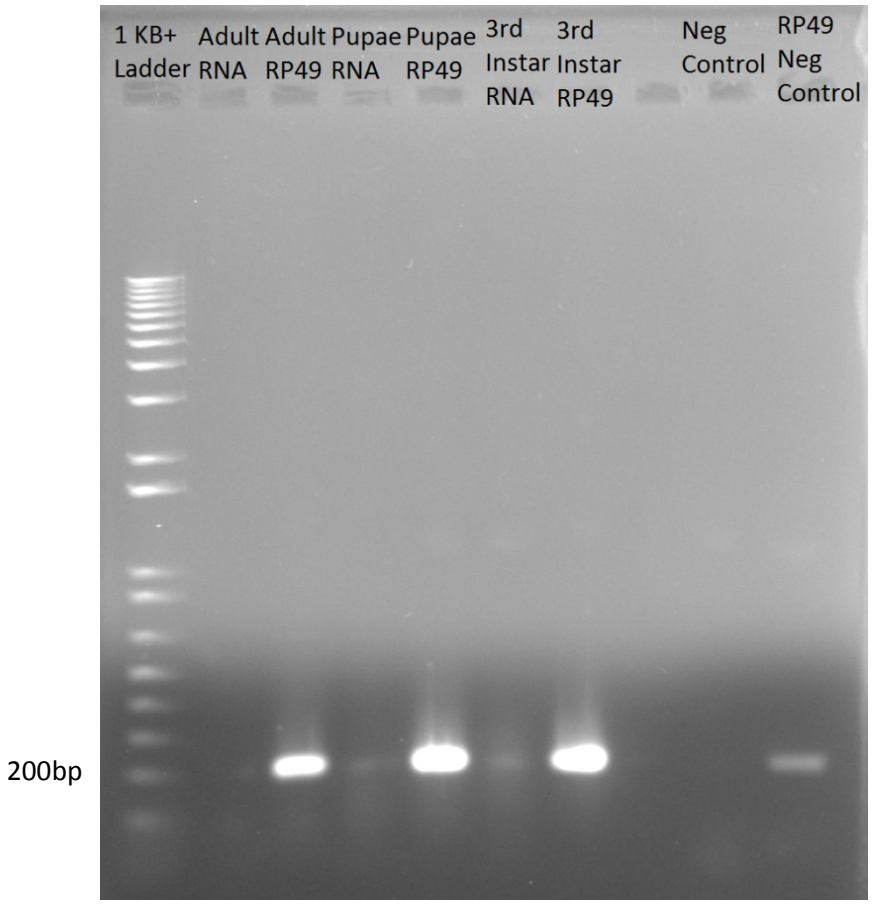
Figure 12: RNAi Phenotypes Knockout of *Snuts* function using RNA interference. The wild type image shows what the wing should look like, while the image on the right shows how the wings have not unfolded after pupation.

Wild Type	25754 X KK106361
	

3.4.1 Developmental RNA expression profile using Reverse Transcription of Snuts' mRNA

To understand the expression of *snuts* we wanted to see during which stages it is expressed and determine a relative expression level in comparison to each stage. The RT-PCR results were analyzed on a 1% agarose gel. We saw expression of *Snuts* at the adult, 3rd instar, and pupae developmental stages. It also appears that the 3rd instar and pupae stages show more expression than the adult stage. The gel was ran on a 1% agarose for 90 minutes at 80 volts.

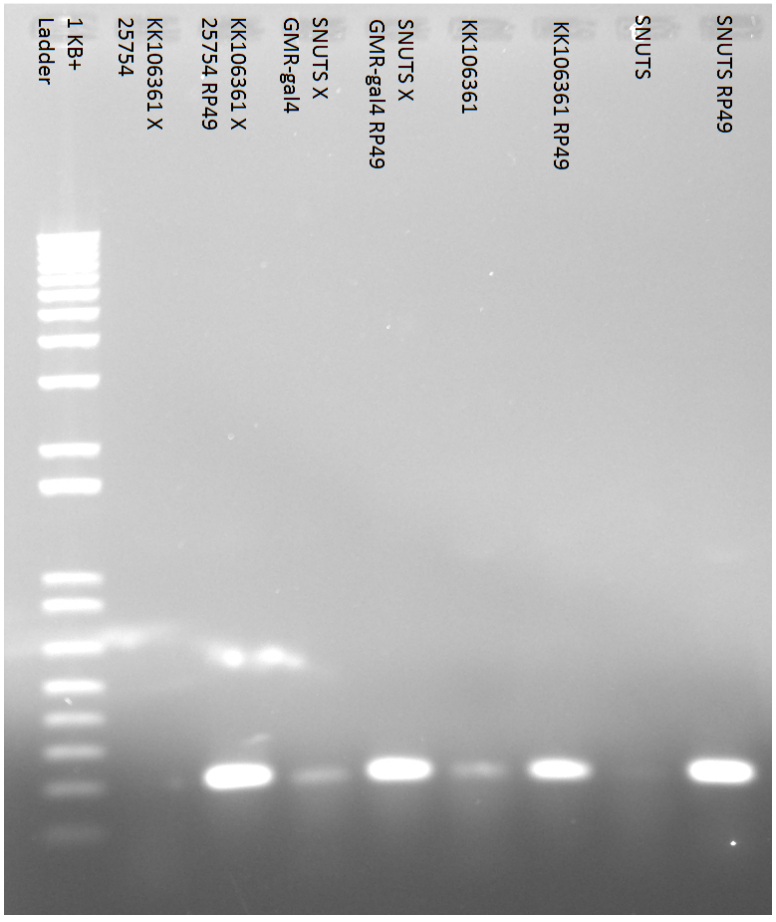
Figure 13: RT-PCR for *Snuts* Expression on 1% Agarose Gel for 90 minutes at 80 Volts. Amplification of the *Snuts* fragment using the RT-PCR primers from table 3 was expected to be 176bp band while RP49 would produce a 206bp band.



3.4.2 Reverse Transcription PCR of Over-Expressed and Inhibited SNUTS expression

To verify that we over-expressed or knocked down *Snuts* expression using the *UAS-Gal4* system we performed RT-PCR to measure the expression levels of *Snuts*. The KK106361 X 25754 line showed reduced expression (weaker band in figure 14) when compared to the wild type KK106361. *Snuts* X *Gmr-gal4* increased (brighter band in figure 14) expression when compared to *Snuts* expression. These results show the expression at the 3rd instar larva stage.

Figure 14: RT-PCR for Over-Expression and Inhibition of *Snuts*. The inhibition cross (KK106361 X 25754) showed reduced expression when compared to the wild type profile (KK106361). The over-expression cross (*Snuts* X *Gmr-gal4*) showed increase expression when compared to wild type (*Snuts*). 1.5% agarose gel ran at 80 volts for 90 minutes.



3.5 Preparation of Reagents for RNA In-Situ Hybridization

The SNUTS gene was cloned into the Promega pGEM-t vector. Use of the pGEM-t vector system allowed efficient cloning. This linear vector has 3' terminal thymidines at each end, preventing the vector from ligating on itself. PCR primers for *Snuts* (Table 6) were used to produce an amplified product (~200bp). Using the manufacturer protocol 15ng of this amplified product was ligated to pGEM-t. The ligated product was transformed with JM109 High Efficiency Competent Cells. A putative positive colony was isolated and sequenced. Sequencing using the PCR primers (table 6) confirmed the identity of the SNUTS sequence. T7 Promoter primer confirmed the orientation of the SNUTS fragment.

Figure 15: Blast Alignment of Sequenced *Snuts*
using *Snuts* PCR Reverse Primer (Table 3)

Drosophila melanogaster CG2662 (CG2662), mRNA
Sequence ID: ref|NM_130662.2| Length: 1647 Number of Matches: 1

Range 1: 974 to 1230 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
457 bits(247)	3e-125	254/257(99%)	2/257(0%)	Plus/Minus

```
Query  5     CAACTTGGA-GACCTGTT-CACGGACCAGGTTGATACTGGATGGCTTCTTCTCTCTACAG  62
             ||| ||||| |||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1230  CAAATTGGACGACCTGTTCCACGGACCAGGTTGATACTGGATGGCTTCTTCTCTCTACAG  1171

Query  63    CTGTGATTGGCGTTAGGCGTAAGGAAAGCGATTCCTGTTCTGGCCCAGGCAGATCGTTAT  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170  CTGTGATTGGCGTTAGGCGTAAGGAAAGCGATTCCTGTTCTGGCCCAGGCAGATCGTTAT  1111

Query  123   TCTCAATCTGCTCAGTCTCCATATCTACCGGCTCGACCTTCTCCACCTTGACTTTTATTT  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1110  TCTCAATCTGCTCAGTCTCCATATCTACCGGCTCGACCTTCTCCACCTTGACTTTTATTT  1051

Query  183   GTGCCGGCTCCTCATCCTCCTCCTTTTTGTACTCTTTCTTCTTTTCTGTTTTCAAGGGCT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1050  GTGCCGGCTCCTCATCCTCCTCCTTTTTGTACTCTTTCTTCTTTTCTGTTTTCAAGGGCT  991

Query  243   GTCTACGTTTGGCTAGC  259
             |||||||||||||||||
Sbjct  990   GTCTACGTTTGGCTAGC  974
```

Figure 16: Sequence using T7 Promoter Primer

Cloning vector pGEM-5Zf(-)

Sequence ID: emb|X65309.2| Length: 3001 Number of Matches: 1

Range 1: 52 to 357 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
566 bits(306)	1e-157	306/306(100%)	0/306(0%)	Plus/Plus

```
Query  295  ATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGC  354
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  52   ATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGC  111

Query  355  ATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGT  414
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  112  ATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGT  171

Query  415  TTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAA  474
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  172  TTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAA  231

Query  475  AGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCAC  534
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  232  AGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCAC  291

Query  535  TGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCG  594
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  292  TGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCG  351

Query  595  CGGGGA  600
            ||||||
Sbjct  352  CGGGGA  357
```

CHAPTER 4

DISCUSSION

The genetic screen that crossed beadex-gal4 with the *Snuts* gene indicates that we have a putative basement membrane degrader, a process that potentially could be controlled by the JNK pathway. Therefore if we up-regulate *Snuts* while *Gal4* is being expressed under the control of the *Beadex* enhancer, any abnormal phenotype we see could be the result of JNK up-regulation. The cross we performed showed a phenotype that indicates up-regulation by process of basement membrane remodeling. The LacZ staining assay further provided evidence that JNK pathway was up-regulated by observing if the gene Puckered was being over-expressed. The next step in the process is to dissect where in the JNK pathway *Snuts* is playing a role.

When *Snuts* is over-expressed in the eye disc of the flies, we suspect that apoptosis occurs resulting in degenerative phenotypes. The over-expression of *Snuts* in the eye (using *Gmr-gal4* and *Ey-gal4*) are indicative of apoptosis, a process coordinated by the JNK pathway (Danny & E. Premkumar, 2008). These phenotypes only occurred when *Snuts* was over-expressed in the eye at 25°C. We did not see any resulting phenotype from the 18°C over-expression experiments. In addition to the eye phenotypes, we saw that when *Snuts* was overexpressed ubiquitously in the flies, a lethal phenotype is present. To ensure that *Snuts* was over-expressed and to what degree it is being over-expressed, we performed RT-PCR using *Gmr-gal4* to show the level of expression is

greater than wild type expression. RT-PCR on the inhibited line (KK106361) using the driver in the wild type fly. The over-expression of SNUTS using *Gmr-gal4* was confirmed by RT-PCR showing *Snuts* being expressed more than the wild type. We can reasonably assume that the eye phenotype we saw is the direct result of over-expressing *Snuts*.

Overall the inhibition of *Snuts* utilizing RNA interference did not reveal any abnormal phenotypes that could indicate any process affected by *Snuts*. The mild phenotypes that we saw were from the KK106361 line, meaning the other line may not be as expressed as much. This gene is located on 2nd chromosome of the fly and future experiments with the inhibition will use this line as it is clear that it more potently expressed the inhibition gene for *Snuts*. To verify that *Snuts* was inhibited during these experiments, we performed RT-PCR to show the relative amount of *Snuts* expression is reduced compared to the wild type expression. For the inhibition of *Snuts*, we see less expression in the KK106361 crossed with 25754 than we do with the KK106361 alone. This means the phenotype we saw is the result of the inhibition of *Snuts*.

Finally the RT-PCR on three stages of *Drosophila* development revealed that *Snuts* expression is ubiquitous during three of the primary stages of development. If *Snuts* plays a role in the JNK pathway, then *Snuts* would be expressed during every stage of development where JNK pathway is expressed. Because JNK pathway is responsible for mediating cellular stress and proliferation, *Snuts* would be expressed during every stage of development as the organism's cells continue to divide. The expression of *Snuts* is greatest during pupae and 3rd instar stages while less during adult stages, This suggests

that *Snuts* is necessary for proper development of *Drosophila*, supporting the data we have already collected.

CHAPTER 5

FUTURE DIRECTIONS

The lab is focused on several different approaches to help understand the role *Snuts* expression has on development and tumor metastasis. One is understanding *snuts* relationship within the JNK pathway. To accomplish this objective we are in the process of planning further experiments which will help us to determine where along the JNK pathway *Snuts* is acting.

A central focus for our lab is expanding the RNA in-situ data available from the Berkeley *Drosophila* Genome Project, as well as the Stone Cold Harbor Labratory. RNA in-situ hybridization binds tags to a gene transcript in a preserved tissue or organism, showing where expression can be found. It can tell us where and when expression occurs for a given gene. The RNA in-situ data from these organizations do not reflect the later stages of embryonic development or in the larval tissue. This information is crucial to understanding *Snuts* expression and spatial location. It is also important to understand where *Snuts* is located within the cell. This will be visualized by using immunohistochemistry which will bind tags to the protein SNUTS. It shows us where the protein is present and at what stages it is expressed.

The key to understanding SNUTS function is to understand the conserved domains, the PHD and the SAM domains. To understand the role that these domains play we are going to clone *Snuts* using the pUAST vector so that it can be inserted into a

Drosophila melanogaster embryo. This allows us a new approach of over-expressing *Snuts* in *Drosophila*. Additionally we can selectively delete each domain in the *Snuts* genome, which will allows us to perform crosses to knockout the function of each domain. Abnormal phenotypes of viability of the progeny should help to reveal the function of the gene as well as when it is expressed.

Given what little is known about the domains on SNUTS, I infer that SNUTS serves as a transcription factor promoting cellular proliferation in response to the JNK pathway since PHD zinc fingers often serve a transcription factors. No signaling sequences have been found that would indicate it to be extracellular or to work in any of the other compartments in the cell. Since the majority of PHD domains serve as nuclear proteins it is likely that SNUTS does as well. All of the conserved domains on SNUTS have been shown to bind to DNA/RNA and/or proteins. This further supports its role as a transcription factor, since transcription factors need to be able to bind to both DNA and proteins.

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