

**ACTIVATION AND MODIFICATION OF SLPR-MEDIATED JNK SIGNALING
DURING DORSAL CLOSURE**

by

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University of Pittsburgh, 2006

Human diseases such as spina bifida are caused by a failure in cell morphogenesis and tissue fusion. Dorsal closure in the *Drosophila* embryo is a model for these tissue closure processes where proper Jun N-terminal Kinase (JNK) signaling is necessary. JNK activity is required in the leading edge cells of the epithelial layer to modulate the cytoskeleton and cell shape, allowing the epidermis to close on the dorsal side of the embryo. The mixed lineage kinase (MLK), Slipper (Slpr), is the JNKKK which is responsible for activation of the pathway during dorsal closure. The pathway components that regulate Slpr, as well as upstream activation signals, are not yet identified. We have examined the involvement of the Ste20-like kinase Misshapen (Msn) to act as the JNKKKK in the JNK pathway during dorsal closure through a direct interaction between Msn and Slpr. By observing phenotypes of recombinant and heterozygous mutants of *slpr* and *msn*, we have examined the genetic interactions. Also, by using a non-biased screen, we have investigated unknown regulators of the Slpr-mediated JNK pathway which have an effect on dorsal closure. These techniques have begun to identify regulatory interactions of molecules within the JNK pathway, and have narrowed down regions of chromosome two which may contain new modifiers further regulating JNK signaling, in order to provide a robust and highly regulated tissue closure event.

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PREFACE

I'd like to thank Dr. Beth Stronach for her superb expertise and patient guidance in this project. Her ability to invoke passion and the need to learn was an invaluable source of motivation. I'd also like to thank the other members of the lab, Becky Gonda, Stephanie Polaski and a former lab member, Lisa Whitney for their encouragement and support.

1.0 CHAPTER 1: INTRODUCTION

Everyday, researchers work diligently to uncover the mysteries behind many types of human maladies. Their goal is to understand how specific biological processes function in order to design treatments and therapies for those affected. The work described here is broadly focused on the cellular processes linked to spina bifida, cleft palate and defects in other developmental processes[1-4]. There are a limited number of active pathways during development which are responsible for proper patterning and maturation of the embryo[5]. Specificity is the key to regulating the signaling process, ensuring appropriate downstream outputs. So how is one pathway able to elicit multiple responses? Overlapping pathways or molecules with promiscuous activity in more than one pathway may be partly responsible. It is also possible to modify core proteins in such a way as to cause a variety of responses. Understanding the regulation of developmental pathways is necessary to find treatments for those afflicted by such early morphogenetic deficiencies.

1.1 CELL SIGNALING

The cell is a highly complex system receiving a barrage of signals and activities in order to operate as part of a tissue, a system, an organism. There are a limited number of signaling pathways which are responsible for controlling development. Because multiple signals activate each pathway, the ability to render distinct and diverse outputs based on efficient signaling depends a great deal on specificity. Determining the mechanisms by which cells mediate this specificity is the key to understanding complex cell behaviors. Multiple ligands may be used to coordinate specificity. This is clearly seen in the different Pvf ligands which may form homo- or

heterodimers to bind to a single Pvr receptor[6, 7]. Ligand dimerization and receptor activation can lead to actin reorganization, prevention of apoptosis or cell growth[6]. Another way pathways achieve multiple outputs could be through homologous family members. Frequently, the core pathway may include one or more proteins which may act at a certain step in the pathway, and this may allow for more specific signaling[1, 8-14]. Further still, scaffolding proteins, branched pathways and multiple transcriptional regulators can all influence the outcome of a “single” signaling pathway[1, 8-12, 15-20].

1.2 MAPK PATHWAY

One pathway which controls many types of cell responses is the Mitogen-Activated Protein Kinase (MAPK) pathway. This pathway plays a role in the regulation of embryogenesis, cell proliferation, cell differentiation and cell death[10]. Activation of the pathway is completed through a series of phosphorylation events beginning with stimulation of a triple kinase, the MAPKK kinase. This in turn activates one or more MAPK kinases, which are dual specificity kinases, meaning they will consequently activate MAP kinases by phosphorylating specific Tyr and Thr residues[2, 8, 10, 12, 21]. This general schematic is seen in two types of MAPK pathways: the ERK (extracellular-signal related kinase) pathway, involving Ras/Raf/MEK/ERK and the Jun-N-terminal Kinase pathway, or the JNK pathway[22]. In *Drosophila*, the ERK pathway is involved in cell differentiation, proliferation and cell cycle progression[23], while the JNK pathway shows activity during embryogenesis, wound healing, and plays a role in immunity and cell cycle control[1, 9, 11-14, 22, 24]. This is a classic example of one core pathway having multiple outcomes. Importantly, this pathway is conserved from yeast to metazoans.

MAPK PATHWAY JNK PATHWAY DORSAL CLOSURE

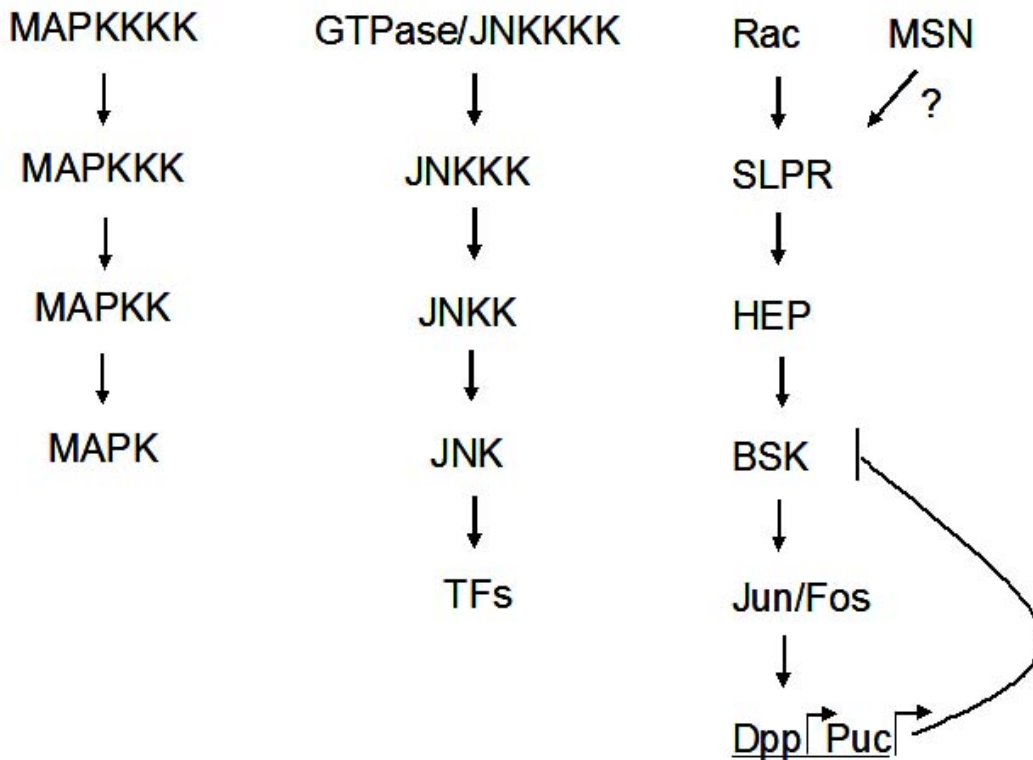


Figure 1: Pathways.

A schematic of the generic MAPK and JNK pathways, and the specific proteins responsible for JNK activation during dorsal closure. Slpr is the triple kinase involved in dorsal closure.

1.2.1 JNK Pathway

The JNK pathway is activated in many types of cells to control many processes during development. Similar to the mammalian stress-activated protein kinase (SAPK) pathway, the JNK pathway has been shown to regulate cell proliferation[25] and differentiation[26], immune response[27, 28], cell morphogenesis[24, 29], planar cell polarity[30, 31], cell death[26, 32] and wound healing[33, 34]. In *Drosophila*, the core signaling molecules include the JNKK Hemipterous (Hep)[35]. Hep activates the single kinase, Basket (Bsk), and Bsk in turn activates Jun. Together with Fos, these two proteins make up the AP-1 transcription factor which is

activated in the majority of processes regulated by the JNK pathway[36]. This core of the JNK pathway transmits signals initiated from all upstream components.

1.2.2 Triple Kinases

There are six JNKK kinases (JNKKK) which can lead to explicit responses downstream. Each triple kinase in *Drosophila* is a homolog of each MAPKKK family in mammalian cells which can be activated via a variety of upstream activation signals. Family members include ASK, DLK, MLK, TAK, ZPK, and MEKK1-4(reviewed in[4]. In *Drosophila*, activation of ASK or TAK stimulates the JNK pathway during apoptosis and immunity responses[37, 38]. DLK is involved in cell differentiation and nervous system development in mice[39, 40]. Other JNKKK's have been implicated in response to various signals such as LPS and oxidative stresses. Mammalian MLKs are implicated in eyelid closure and neuronal apoptosis and the *Drosophila* MLK is required for dorsal closure[4, 9].

1.2.3 Dorsal Closure

This work focuses on dorsal closure and the activity of the *Drosophila* MLK, Slipper (Slpr), during this process. During development of the fly embryo, germband extension and retraction take place after gastrulation, leaving an open hole on the dorsal side of the embryo. A thin sheet of cells called the amnioserosa is left covering the developing embryo[41]. The sides of the epithelium must then be drawn together at the dorsal midline to form a seamless epithelial sheet. In order for dorsal closure to properly occur, JNK signaling is necessary in the leading edge cells of the ectodermal epithelial layer. This causes the cells to elongate and accumulate considerable amounts of actin and myosin, which form a cable-like structure to act as a “purse-string.” The actin cord then pulls the two outer layers of cells toward the dorsal midline[3, 42]. The cells also develop filopodia and begin to pull themselves toward each other[43, 44]. The amnioserosa contracts to help pull the epithelial sheets closer and then deteriorates underneath while the cells fuse to form a seamless dorsal ectoderm.

1.2.1 Dpp and Puc: Two JNK pathway targets

Two genes whose expression within the leading edge cells is dependent upon the JNK cascade are *decapentaplegic (dpp)* and *puckered (puc)*[45, 46]. Both genes are transcriptionally activated by the AP-1 transcription factor. Dpp is a homolog of the TGF- β family member BMP4 which activates Wingless (Wg) signaling[47]. Puc is a MAPK phosphatase which feeds back to negatively regulate the pathway by dephosphorylating Bsk[45]. Both proteins are upregulated in the leading edge cells as a result of JNK signaling. Loss of Puc function causes excessive JNK signal [45], and a puckered midline along the dorsal side of the embryo, reflecting an inability of the JNK pathway to be downregulated. Dpp and Puc expression are both useful reporters of JNK signaling in the leading edge.

1.2.2 Slipper

The JNKKK which activates Hep during dorsal closure is the *Drosophila* mixed lineage kinase, Slipper (Slpr)[13]. Slpr is the only MLK homolog in the fly, and so far the only triple kinase shown to be involved in dorsal closure[9, 13]. Mutant forms of *slpr* cause a loss of Dpp in the leading edge cells of the ectoderm mimicking the effect observed with mutations in *hep* and *bsk*. Furthermore, removing one copy of the JNK phosphatase *puc* can dominantly mask the effect of *slpr* mutant alleles[13]. Defects in the mammalian MLK3 cause epidermal defects at the dorsal midline of mice, although the mechanism behind this deficiency is not yet clear[48]. Upstream activators of Slpr are as yet unknown, but these and other studies show definitively Slpr's role in dorsal closure.

1.2.3 Slpr structure

MLKs are termed as such because of the conservation in amino acids within the kinase domain which is common to both serine/threonine kinases and tyrosine kinases. Their protein structure is characterized by an N-terminal Src-homology 3 (SH3) domain, followed by a kinase domain, a leucine zipper domain (LZ) and a Cdc42-Rac interacting binding (CRIB) motif[4, 9].

Mammalian homologs of Slpr have been shown to interact through this last domain with the Rho-family GTPase Rac[13, 49]. The LZ is necessary for dimerization and auto-phosphorylation in the mammalian MLK3 while the SH3 is necessary for auto-inhibition through association with a conserved proline residue between the LZ and CRIB domains[9]. The active (open) form of Slpr may use the SH3 domain for binding proline-rich domains of an upstream activator[50]. The non-conserved C-terminus may be involved in signaling specificity.

1.2.3.1 Slpr alleles

There are currently three alleles of *slpr* which provide some insight into Slpr's role within the JNK pathway. The most severe allele is *slpr⁹²¹*, which contains a point mutation in the kinase region making this allele an inactive form of Slpr. Flies hemizygous for this allele die early in embryogenesis. A second allele, *slpr^{3P5}*, is an early truncation of the protein near the end of the kinase domain which allows some flies to live to the larval stages, although most still do not make it through embryogenesis and show dorsal open phenotypes. Embryonic lethality associated with these alleles seems to be caused by a dominant-negative effect of the mutant proteins. The null allele, *slpr^{BS06}*, is surprising in the fact that its phenotype is not as severe as our other alleles, allowing some flies to eclose as adults at a reproducible ratio. *slpr* is a maternally deposited gene and it is believed that this contribution is enough to get some *slpr^{BS06}* embryos through the process of dorsal closure. Some adult escapers possess a variety of phenotypes including missing maxillary palps, cleft thoraces, wrinkled wings, and genital rotation and eversion defects in males[51].

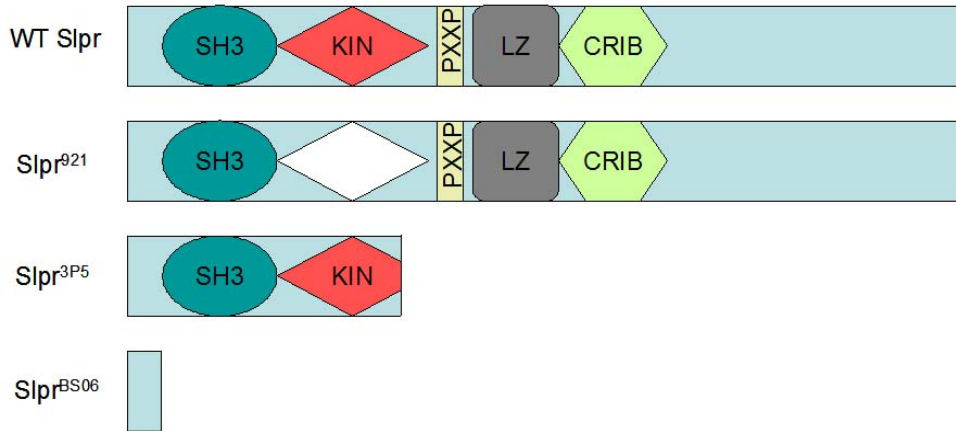


Figure 2: Slpr alleles.

Wild-type Slpr has four conserved domains, and a proline-rich region thought to be involved in auto-regulation. Slpr⁹²¹ is a kinase dead allele. Slpr^{3P5} is a truncation mutant at the end of the kinase domain and Slpr^{BS06} is a very early truncation and acts as a null allele.

1.2.4 Misshapen

Another gene which has been linked to dorsal closure and the JNK pathway is *misshapen* (*msn*). Msn is an Ste20-like kinase originally characterized for its role in photoreceptor axon targeting in the fly eye[52]. Of the two families of Ste20 kinases, Msn is in the SPS1 family, which is composed of kinases lacking a p21Rac- and Cdc42-binding (CRIB) domain in their amino terminus. Mammalian members include germinal center kinase (GCK), NCK-interacting kinase (NIK) and hematopoietic progenitor kinase (HPK)[53]. In addition to its role in dorsal closure and photoreceptor shape control[52, 53], Msn is also involved in nuclear migration in the *Drosophila* eye[54] and acts downstream of the Frizzled receptor in planar cell polarity[30]. Su et al.,(1998), first showed an involvement for Msn in dorsal closure by looking at genetic interactions. Expression of Msn in cell culture shows an upregulation of Jun; consistent with that observation, a dominant negative form of Msn inhibits Jun activation. Embryos homozygous for *msn* mutants or doubly heterozygous for *msn* and *bsk* display a defect in dorsal closure and lack Dpp expression in the leading edge[53]. Nevertheless, the direct substrate for Msn has yet to be defined.

1.2.4.1 Msn Structure

Msn contains only three domains: a kinase domain at the N-terminus, a C-terminal regulatory region and a proline-rich region at the center of the protein. PXXP motifs like those found in Msn have been shown to bind to SH3 domains in other proteins[55-58]. This proline-rich region is now known to bind to the SH3 regions of Dreadlocks (Dock) during axon guidance in the eye[59]. Dock is, however, not required for Msn function during dorsal closure[18]. Interestingly, observations made during a structure-function analysis of Msn revealed that Msn constructs lacking the C-terminal domain only rescued 10% of *msn* mutants indicating a role for both the kinase and C-terminal regulatory domains in dorsal closure[18, 53].

1.3 SIGNIFICANCE

It is clear that although there have been many advances in organizing the role of JNK signaling during dorsal closure, there is still much work to do. This project set out to look for effectors and upstream activators of the pathway and to dissect their direct relationship to Slpr. The first question asked was “Are there proteins which modify Slpr-mediated JNK signaling?” To answer this, a large-scale, non-biased genetic screen was done using *Drosophila* deficiency lines covering the second chromosome. A phenotype-based secondary screen was used to verify potential interactors. The second question addressed was whether Misshapen is an upstream kinase responsible for activating Slpr to elicit JNK signaling during dorsal closure. Pull-down assays and phenotypic evaluations were used to test for interactions.

Attempts to answer these questions allow us to begin to understand what type of regulation is occurring upstream of the pathway in order to elicit the specific responses necessary for completing dorsal closure. Identifying unknown regulators of the pathway may uncover cross-talk between two pathways as well as putative proteins not previously linked to JNK activity. It is the goal of the lab to uncover positive and negative regulators of JNK signaling

during dorsal closure and to understand how that differs from signaling during other JNK-activated processes.

The screen uncovered two regions of chromosome two which gave consistent phenotypic results. Further research will be needed to narrow down the precise genes involved and to characterize an interaction with Slpr. It will also be necessary to observe any defects in dorsal closure, or other JNK-dependent processes.

Studies testing a direct interaction of Msn with Slpr did not lead to any definitive conclusions; however, progress was made providing the laboratory with various Msn constructs and protocols which can be used once reliable resources are available. Phenotypic and biochemical assays have been established and may lead to identification of proteins with a direct effect on Slpr activation.

2.0 CHAPTER 2: A GENETIC SCREEN FOR EFFECTORS OF SLPR-MEDIATED JNK SIGNALING

2.1 BACKGROUND AND SIGNIFICANCE

It has recently been hypothesized that members of the ERK-mediated MAPK pathway may be involved in stimulation of the JNK pathway, or may play a role in compensatory signaling. Embryos double mutant for *slpr*^{BS06} and *pvf1*, a PDGF/VEGF ligand, have dorsal closure defects. Also, mutant *pvr/pvf1* receptor/ligand complexes have been shown to cause genital rotation defects, as seen in *slpr*^{BS06} mutants[7]. One member of the ERK-mediated pathway, Pp2C is a serine/threonine phosphatase which has shown genetic interactions with the JNK signaling pathway as well(Stronach unpublished,[60]). Such examples of cross talk between pathways have more recently come to light[59, 61-71], indicating that regulation of signaling is far more complex than single pathway relay events.

It is logical then, to wonder if such a closely related pathway might be able to compensate for loss of the Slpr-mediated JNK signal, or if both pathways are part of the many signals which provide the cell with a balanced readout to ensure that dorsal closure takes place. Still, other proteins may exist which may potentiate or attenuate the JNK signal and effect dosage or specificity of signaling modifications. If such players exist but are not absolutely necessary for dorsal closure to take place, identifying them may not be so obvious.

In addition to observations regarding the ERK/MAPK pathway, other mutants which share phenotypic defects with Slpr potentially implicate them in JNK signaling pathway regulation. It was previously stated that the *slpr*^{BS06} allele lacks detectable protein production, but with the help of the maternal contribution allows some adults to survive. Interestingly, the number of hemizygous *slpr*^{BS06} males that eclose is consistently observed at 10% compared to their brothers[51]. Therefore, the question was raised “Are there other proteins which can

potentiate or attenuate the *slpr*^{BS06} signal and consequently increase or decrease the number of adult males which eclose?" By reducing the dosage of genes using deficiencies and observing phenotypic changes, we have found a way to look for modifiers of the JNK pathway.

2.1.1 Hypothesis: Modifying the dosage of proteins involved in regulating Slpr-mediated processes or molecules acting in parallel pathways which affect tissue morphogenesis may alter the phenotypes observed in *slpr* mutants.

2.2 EXPERIMENTAL APPROACHES

The Bloomington Deficiency kit was used to remove generous portions of the second chromosome. It was hypothesized that removing one copy of a gene which had some effect on the Slpr-mediated pathway, would affect the amount of JNK signaling and therefore cause a change in the number of *slpr*^{BS06} males which eclosed. There were originally 73 deficiency lines tested which covered most of the second chromosome. Expectations included finding known members of the pathway as well as novel modifiers of JNK signaling during dorsal closure.

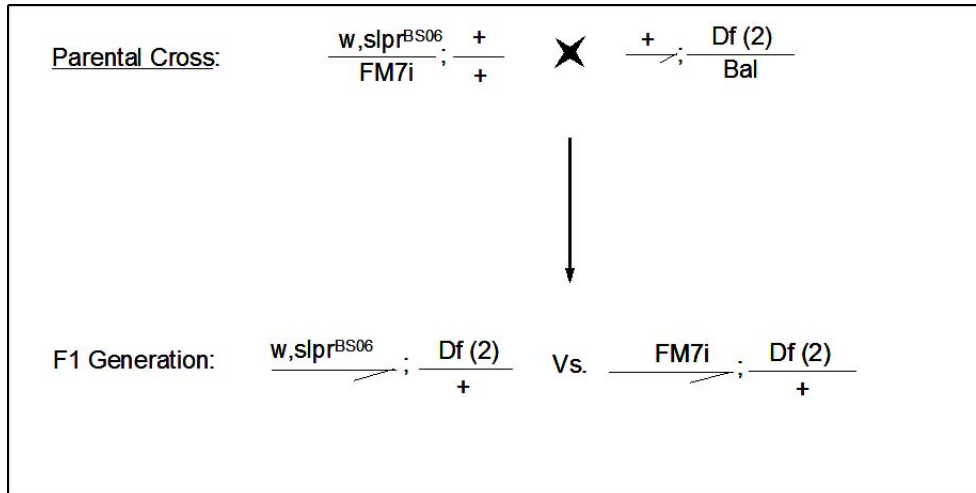


Figure 3: Deficiency cross outline.

Df flies were crossed to $slpr^{BS06}$ flies and the number of males was scored. The percent viable was measured as $slpr^{BS06};Df$ males over $FM7i;Df$ males. $FM7i$ and the w - marker were used to decipher between the two classes of Df-carrying flies. “Bal” stands for the balancer over which the Df was stabilized.

2.2.1 Set up of cross

The original screening process looked at progeny from deficiency lines which remove large regions of the second chromosome, crossed to $slpr^{BS06}$ mutant lines. The F1 male progeny were scored, with the genotypes indicated as a percentage. Normally, 10% of the male progeny with the $slpr^{BS06}$ allele eclose as adults. I chose $\geq 50\%$ eclosion or $\leq 2\%$ eclosion to define putative suppressors or enhancers, respectively.

2.2.2 Results of large deficiencies

Table 1: Deficiency lines from initial screen which alter the number of BS06 males.

$w, slpr^{BS06}/FM7i$ females were crossed to Df/Bal males. The stocks listed here by breakpoints made the first cut by producing more than 50% or less than 2% $w, slpr^{BS06};Df$ males (compared to $FM7i;Df$ males). *stocks were sick and did not produce many progeny; analyzed with caution

Breakpoints	Df stock #	Deficiency	%BS06;Df	#BS06;Df	#FM7i;Df
21D1-2;22B2-3	3084	Df(2L)last2	0.0%	0	30
22D2-3;22F1-2	7144	Df(2L)BSC37	87.50%	14	16
23C1-2;23E1-2	1567	Df(2L)JS17,dpp[d-ho]	50.0%	9	18
23C5-D1;23E2	6875	Df(2L)BSC28	181.25%	29	16
23E5;23F4-5	6965	Df(2L)BSC31,net[1]cn[1]	64.71%	11	17
25D2-4;26B2-5	781*	Df(2L)cl-h3	0.0%	0	6
25F3-26A1;26D3-11	490	Df(2L)E110	50.0%	15	30
29C1-2;30C8-9	2892*	Df(2L)N22-14	0.0%	0	6
32F1-3;33F1-2	3079*	Df(2L)PrI,PrI[1]nub[PrI]	100.0%	7	7
34A3;34B7-9	6999*	Df(2L)BSC30	50.00%	2	4
34B12-C1;35B10-C1	3138*	Df(2L)b87e25	100.0%	2	2
35B4-6;35F1-7	3588	Df(2L)TE35BC-24.b[1]pr[1]pk[1]cn[1]sp[1]	0.0%	0	25
36C2-4;37B9-C1	420*	Df(2L)TW137, cn[1]bw[1]	0.0%	0	1
42A1-2;42E6-F1	1007	Df(2R)nap9	0.0%	0	23
42A2-3;58A4, h42-h43;59E1	749	ln(2R)bw[VDe2L]Cy[R]	0.0%	0	18
49A4-13;49E7-F1	754	Df(2R)vg-C	52.0%	13	25
51D3-8;52F5-9	3518	Df(2R)Jp1	133.3%	12	9
54B17-C4;54C1-4	5680	Df(2R)robl-c	71.4%	25	35
55A;55F	1547*	Df(2R)PC4	0.0%	0	11
56D7-E3;56F9-12	6647	Df(2R)BSC22	0.00%	0	18
56F12-14;57A4	6609	Df(2R)BSC19,cn[1]bw[1]	56.0%	14	25
58D1-2;59A	282	Df(2R)X58-12	76.9%	10	13
60E6-8;60F1-2	3157	Df(2R)ES1,b[1]pr[1]cn[1]wx[wxt]	87.0%	20	23
60F1;60F5	4961	DF92R)Kr10,b[1]pr[1]Bl[1]c[1]	60.0%	6	10

The stocks in Table 1 showed $\geq 50\%$ or 0% *slpr*^{BS06} males after one round of scoring. New deficiencies removing overlapping regions still within the original chromosomal area defined by the deficiencies were tested in a similar fashion for suppression or enhancement of the *slpr*^{BS06} phenotype. This data is shown in Table 2. The stocks in Table 1 are listed as “Parental Dfs” to compare the percentages of *slpr*^{BS06} males eclosing between the two different sized deficiencies.

2.2.3 Results of overlapping deficiencies

Table 2: Overlapping deficiency regions.

These stocks were tested because they were smaller, overlapping regions of the deficiencies from Table 1. These were scored in the same way. "Parental" stands for the larger deficiency (from Table 1) encompassing the same chromosomal region. Stocks producing a similar amount of $w,slpr^{BS06}$ males would help to narrow down the gene which was presumably affecting the pathway. *stocks were sick and produced low numbers; noted with caution

<u>Breakpoints</u>	<u>Df</u>	<u>%BS06;Df</u>	<u>BS06;Df</u>	<u>FM7;Df</u>	<u>Breakpoints</u>	<u>Parental Df</u>	<u>Parental %</u>
	W1118	14.0%	6	43		W1118	5.0%
21D2;21D3	7489	42.1%	8	19	21D1-2;22B2-3	3084	0.0%
21D3;21E3	7490*	50.0%	2	4	21D1-2;22B2-3	3084	0.0%
22A3;22B3	5450	15.4%	2	13	21D1-2;22B2-3	3084	0.0%
22D1;22E1	7493	5.6%	1	18	23C5-D1;23E2	6875	181.2%
22E1;22F3	7783*	0.0%	0	0	23C5-D1;23E2	6875	181.3%
23C4;23D1	7784	6.9%	2	29	23C5-D1;23E2	6875	181.3%
23D2;23E3	4954	22.7%	5	22	23C5-D1;23E2	6875	181.3%
23E5;23F5	7787	6.7%	1	15	23E5;23F4-5	6965	64.7%
34A2;34A7	7823*	0.0%	0	3	36C2-4;37B9-C1	420	0.0%
34A4;34B6	7421	183.3%	11	6	34A3;34B7-9	6999	50.0%
36C;36E-F	6087	31.6%	6	19	36C2-4;37B9-C1	420	0.0%
36E6-F1;36F7-9	343*	100.0%	7	7	36C2-4;37B9-C1	420	0.0%
36F7-9;37B2-7	3781*	20.0%	2	10	36C2-4;37B9-C1	420	0.0%
37B2-8;37C5	5372	7.1%	2	28	36C2-4;37B9-C1	420	0.0%
41A;41A	742	14.3%	4	28	42A2-3;58A4, h42-h43;59E1	749	0.0%
41BC;42A16-B1	4308*	0.0%	0	7	42A2-3;58A4, h42-h43;59E1	749	0.0%
56C4;56D6-10	6866	14.3%	2	14	56D7-E3;56F9-12	6647	0.0%
58B3;59A1	100*	500.0%	5	1	42A2-3;58A4, h42-h43;59E1	749	0.0%
59C1;59C4	7265	7.1%	1	14	42A2-3;58A4, h42-h43;59E1	749	0.0%
59C3-4;59D1-2	6147*	14.3%	1	7	42A2-3;58A4, h42-h43;59E1	749	0.0%

The stocks in both Tables 1 and 2 were retested for accuracy and consistency. There were some stocks which produced offspring with phenotypes uncharacteristic of the known chromosomal markers and were unable to be classified. This was attributed to possible recombination,

nondisjunction or incorrect notation of chromosomal markers. These lines were not dissected further. Some stocks were very sick and were not able to give more than 15 male progeny, even though hundreds of female progeny eclosed. In order to get a more confident percentage and still retain the ease of using deficiencies for screening, we then retested some crosses using a stock of *y,w,slpr^{BS06}* flies, utilizing the yellow body marker. Data from all tables plus the data from the second set of crosses was put into Table 3 as a total collection of all scored flies. Figure 3 is a graphical representation of all flies scored. From here, stocks which produced consistent results were deemed significant. The break points were analyzed for two reasons: (1) to obtain smaller deficiencies for further analysis and (2) to look for specific genes which could be tested directly for an interaction.

2.2.4 Total deficiency data results

Table 3: Total deficiency data.

73 *Df* lines were crossed to *w,slpr^{BS06}/FM7i* or *y,w,slpr^{BS06}/FM7i* flies and *slpr^{BS06};Df* males were compared to *FM7i;Df* males. Some stocks were very difficult to score based on phenotype or were too sick to produce enough progeny for confident analysis and were thrown out. * marks stocks determined to be significant, + marks stocks with too few male progeny, or undetermined genotypes

Breakpoints	Stock number	Deficiency Name	%<i>slpr^{BS06}/FM7</i> (Df)	#<i>slpr^{BS06};Df</i> scored	#<i>FM7i;Df</i> scored
	W1118	None, wild type control	10.34%	66	638
21C3-4;21C6-8	6608	Df(2L)BSC16,net[1]cn[1]	14.29%	3	21
21D1-2;22B2-3	3084*	Df(2L)last2	1.79%	1	56
21D2;21D3	7489	Df(2L)Exel6002,P{w[+mC]=XP-u}Exel6002	22.45%	11	49
21D3;21E3	7490	Df(2L)Exel6003,P{w[+mC]=XP-u}Exel6003	11.54%	6	52
22A3;22B3	5450 ⁺	Df(2L)frtz14,P[ry[+t7.2]=neoFRT]42D cn[1]sp[1]	15.4%	2	13
22D1;22E1	7493	Df(2L)Exel6007,P{w[+mC]=XP-u}Exel6007	5.26%	2	38
22D2-3;22F1-2	7144*	Df(2L)BSC37	34.72%	25	72
22E1;22F3	7783 ⁺	Df(2L)Exel7011,P+PBac{XP5.WH5}Exel7001	0.00%	0	6
23C1-2;23E1-2	1567*	Df(2L)JS17,dpp[d-ho]	40.00%	12	30
23C4;23D1	7784	Df(2L)Exel7014,P+PBac{XP5.RB3}Exel7014	5.26%	2	38
23C5-D1;23E2	6875 ⁺	Df(2L)BSC28	32.14%	36	112
23D2;23E3	4954	Df(2L)S2590	22.7%	5	22
23E5;23F4-5	6965*	Df(2L)BSC31,net[1]cn[1]	37.80%	31	82
23E5;23F5	7787	Df(2L)Exel7016,P+PBac{XP5.WH5}Exel7016	17.92%	19	106
23F3-4;24A1-2	6507 ⁺	Df(2L)drm-P2,p{w[+mC]=lacW}Pds[w[k10101]	16.67%	2	12
24A2;24D4	5330 ⁺	Df(2L)ed1	30.77%	4	13

Table 3 continued...					
25D2-4;26B2-5	781 ⁺	Df(2L)cl-h3	83.33%	5	6
25F3-26A1;26D3-11	490*	Df(2L)E110	40.79%	31	76
26D10-E1;27C1	6374	Df(2L)BSC7	15.79%	3	19
27C1-2;28A	2414	Df(2L)spd[j2],wg[spd-j2]	10.34%	6	58
27E2;28D1	4956 ⁺	Df(2L)XE-3801	22.22%	2	9
28A4-B1;28D3-9	7147 ⁺	Df(2L)BSC41, dp[ov1] cn[1]	15.38%	2	13
28DE	140	Df(2L)Trf-C6R3	6.35%	4	63
28E4-7;29B2-C1	179	Df(2L)TE29Aa-11, dp[*]	5.77%	3	52
29C1-2;30C8-9	2892 ⁺	Df(2L)N22-14, (Raw Df)	10.00%	2	20
30C3-5;30F1	6478	Df(2L)BSC17	10.00%	2	20
30D-F;31F	1045 ⁺	Df(2L)Mdh,cn[1]/Dp(2;2),cn[1]	N/A	Hard to score	--
31B;32A	3366 ⁺	Df(2L)J2 (bsk Df)	42.86%	6	14
32D1;32D4-E1	7143	Df(2L)BSC36	15.38%	4	26
32F1-3;33F1-2	3079*	Df(2L)Prl,Prl[1]nub[Prl]	87.27%	48	55
34A2;34A7	7823	Df(2L)Exel7055,P+ PBac(XP5.WH5)Exel7055	7.98%	15	188
34A3;34B7-9	6999 ⁺	Df(2L)BSC30	27.66%	26	94
34A4;34B6	7421	Df(2L)ED784, P{w+[mW.Scer /FRT.hs3]=3'.RS5+3.3'}ED784	37.93%	11	29
34B12-C1;35B10-C1	3138 ⁺	Df(2L)b87e25	28.57%	2	7
35B4-6;35F1-7	3588*	Df(2L)TE35BC-24.b[1]pr[1]pk[1]cn[1]sp[1]	1.28%	1	78
35D1;36A6-7	1491	Df(2R)r10,cn[1]	10.00%	2	20
36C;36E-F	6087	Df(2L)T317,b[1],pr[1],cn[1],sca[1]	17.24%	5	29
36C2-4;37B9-C1	420 ⁺	Df(2L)TW137, cn[1]bw[1]	2150.00%	43	2
36E6-F1;36F7-9	343 ⁺	Df(2L)M36F-s6	46.67%	7	15
36F7-9;37B2-7	3781 ⁺	Df(2L)TW3,(2)74i[1]	20.00%	2	10
37B2-12;38D2-5	567	Df(2L)pr-A16,cn[1]bw[1]	9.46%	7	74
37B2-8;37C5	5372 ⁺	Df(2L)hk-UC2	21.43%	3	14
38A6-B1;40A4-B1	167	Df(2L)TW161, cn[1]bw[1]	14.93%	10	67
40h35;40h38L	4959 ⁺	Df(2L)C'	50.00%	13	26
41A;41A	739	Df(2R)M41A4	45.00%	9	20
41A;41A	742	Df(2R)r110a,lt[1],rl[10a],cn[1]/ ln(2LR)bw[v1],ds[33k]bw[v1]	12.20%	5	41
41BC;42A16-B1	4308	Df(2R)nan14	1.35%	1	74
42A1-2;42E6-F1	1007*	Df(2R)nap9/ln(2LR)Gla	20.00%	7	35
42A2-3;58A4, h42- h43;59E1	749*	ln(2R)bw[VDe2L]Cy[R]/ ln(2LR)Gla,wg[Gla-1]	0.00%	0	60
42B3-5;43E15-18	1888	Df(2R)ST1,Adh[n5]pr[1]cn[*]	15.00%	6	40
42E;44C	3368 ⁺	Df(2R)cn9	10.00%	1	10
43F;44D3-8	198	Df(2R)H3C1	16.67%	4	24
44D1-4;44V12	201	Df(2R)H3E1	4.55%	1	22
44F10;45D9-E1	3591 ⁺	Df(2R)Np5,ln(2LR)w45-32n,cn[1]	21.43%	3	14
45A6-7;45E2-3	4966	Df(2R)w45-30n,cn[1]	8.33%	3	36
45D3-4;45F2-6	6917 ⁺	Df(2R)BSC29,cn[1]bw[1]sp[1]	23.08%	9	39
46A;46C	1743 ⁺	Df(2R)B5,px[1]sp[1]	7.69%	1	13
46C2;47A1	1702 ⁺	Df(2R)X1,Mef2[X1]	30.77%	4	13
46D7-9;47F15-16	447 ⁺	Df(2R)stan1,P{ry[+t7.2]= neoFRT}42Dcn[1]sp[1]	71.43%	5	7
47D3;48B2	190	Df(2R)en-A	34.00%	17	50
48A3;48C6-8	1145	Df(2R)en30	3.90%	3	77
48C5-D1;48D5-E1	7145	Df(2R)BSC40	40.00%	6	15

Table 3 continued...					
48E;49A	4960 ⁺	Df(2R)CB21	36.36%	4	11
48E1-2;48E2-10	7146 ⁺	Df(2R)BSC40	13.33%	2	15
48E12-F4;49A11-B6	5879	Df(2R)BSC3,w[+mC]unch[k15501] cn[1]bw[1]sp[1]	8.70%	2	23
49A4-13;49E7-F1	754 [*]	Df(2R)vg-C	41.18%	14	34
49C1-4;50C23-D2	442 ⁺	Df(2R)CX1,b[1]pr[1]	28.57%	2	7
50D1;50D2-7	6516 ⁺	Df(2R)BSC18	27.27%	3	11
50E6-F1;51E2-4	6455 ⁺	Df(2R)BSC11	7.69%	1	13
51D3-8;52F5-9	3518 ⁺	Df(2R)Jp1	33.93%	19	56
52F5-9;52F10-53A1	3520 ⁺	Df(2R)Jp8,w[+]	18.18%	2	11
53E;53F11	6404	Df(2R)P803-Delta15,cn[1]	17.65%	6	34
53E4;53F8	6916 ⁺	Df(2R)ED1,P{w[+mW.Scer/ FRT.Hhs3]=3'.RS5+3.3'}ED1	7.69%	1	13
54B17-C4;54C1-4	5680 [*]	Df(2R)robl-c	31.33%	26	83
54C1-4;54C1-4(?)	5574	Df(2R)k10408,P{w[+mC]= acW)mthl3[k10408]	10.14%	7	69
54D1-2;54E5-7	6779	Df(2R)14H10Y-53	22.22%	4	18
54E5-7;55B5-7	6780 ⁺	Df(2R)14H10-35	15.38%	2	13
55A;55F	1547 ⁺	Df(2R)PC4	4.35%	1	23
55E2-4;56C1-11	757	Df(2R)P34	5.56%	4	72
56C4;56D6-10	6866	Df(2R)BSC26	6.35%	4	63
56D7-E3;56F9-12	6647	Df(2R)BSC22	11.54%	3	26
56F12-14;57A4	6609	Df(2R)BSC19,cn[1]bw[1]	19.72%	28	142
56F5;56F15	543	Df(2R)017	47.06%	8	17
56F9-17;57D11- 12;56D-E;58E-F	3467	Df(2R)AA21,c[1]px[1]sp[1]	8.57%	3	35
58B3;59A1	100 ⁺	Df(2R)X58-8,pr[1],cn[1]	14.29%	1	7
58D1-2;59A	282 ⁺	Df(2R)X58-12	65.22%	30	46
59A1-3;59D1-4	3909	Dr(2R)59AD	5.00%	1	20
59C1;59C4	7265	Df(2R)Frd-R1,wg[Sp-1],Pin[2]	5.13%	2	39
59C3-4;59D1-2	6147	Df(2R)twi,b[1],pr[1],cn[1],bw[1]	6.90%	2	29
59D5-10;60B3-8	1682	Df(2R)or-BR6	16.00%	4	25
60E2-3;60E11-12	2471	Df(2R)M60E/ln(2LR)bw[V32g],bw[V32g]	8.11%	3	37
60E6-8;60F1-2	3157 [*]	Df(2R)ES1,b[1]pr[1]cn[1]wx[wxt]	32.09%	43	134
60F1;60F5	4961 ⁺	DF(2R)Kr10,b[1]pr[1]Bl[1]c[1]	60.00%	6	10

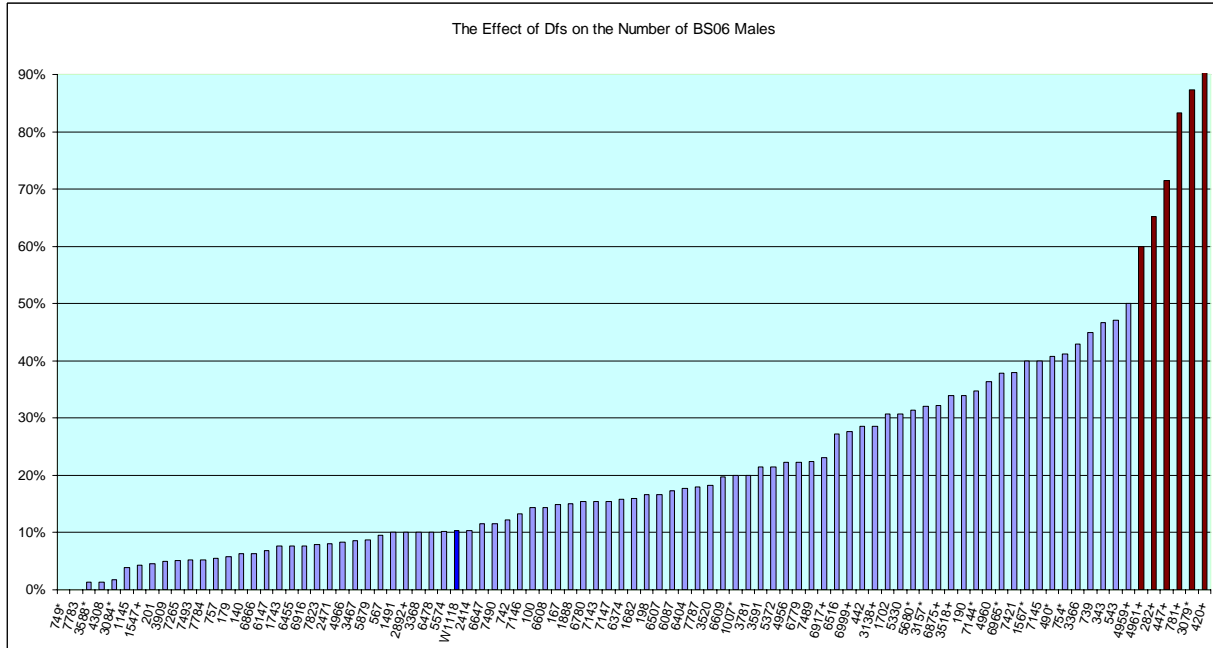


Figure 4: Graphical representation of results from total screen. The bar graph shows the percentages of *slpr^{BS06}; Df* males ranging from 0%-100%. Deficiencies are labeled by stock number. Maroon bars are greater than 50%. The dark blue bar is the control (W1118) at 10%.

2.2.5 Genes in smaller regions

Once the final numbers of F1 progeny were tabulated (Table 3) and analyzed, stocks producing a significant change in male progeny were analyzed by breakpoints. Flybase (<http://flybase.bio.indiana.edu/>) was used to find deficiencies removing smaller regions of the second chromosome which overlapped with the previous lines. These were then crossed again to *y, w, slpr^{BS06}* flies and scored in the same manner. Results are listed in Table 4.

Also, two overlapping enhancer lines showing 0% *slpr^{BS06}; Df* males, both uncovered the kinase Src42A within the deleted region. Src42A has been shown genetically to play some role in dorsal closure, indicated by defects observed in homozygous *src42A* or transheterozygous *src42A tec29* or *src42A src64* mutant embryos[72]. Therefore, it was hypothesized that Src42A was a probable attenuator of the *slpr^{BS06}* signal. Two *src24A* recessive, hypomorphic mutants were obtained; (1) *src42A^{myri}* a myristylation mutant, missing the second amino acid, and (2)

src42A^{El}, a stop codon eliminating the COOH-terminal part of the kinase domain[72]. These were also crossed to *y,w,slpr^{BS06}* flies and scored correspondingly (Table 4).

Table 4: Overlapping Deficiency Stocks.

Deficiencies which showed a significant change in the number of *slpr^{BS06}* males were analyzed by breakpoints. Deficiencies removing smaller regions of the overlapping deficiencies of the chromosome as well as two alleles of the *src42A* kinase were tested using *y,w,slpr^{BS06}*. *W1118* was used as a control cross. * marks stocks causing a significant effect, + marks stocks with a significant but opposite effect as compared to the “parental” (larger) deficiency stock.

Breakpoints	Df Stock #	% BS06:Df	<i>y,w,BS06</i> ; Df	FM7i:Df	Parental Breakpoints	Parental Df	Parental %
33A8-B1;33B2-3	3129	47.0%	31	66	32F1-3;33F1-2	3079	87%
33A1-2;33B1-2	3130	30.0%	18	60	32F1-3;33F1-2	3079	87%
33B2-3;34A1-2	3344	16.1%	9	56	32F1-3;33F1-2	3079	87%
35C2;35C5	3592	21.4%	9	42	35B4-6;35F1-7	3588	1%
41F3-4;42A3-9	4913*	0.0%	0	53	41BC;42A16-B1	4308	1%
32D1;32F1-3	5869+	0.0%	0	64	32F1-3;33F1-2	3079	87%
34C6-7;35B9-C1	6068	14.3%	9	63	35B4-6;35F1-7	3588	1%
35B1-2;35B2-4+35D1-2;35E2	6085	12.2%	5	41	35B4-6;35F1-7	3588	1%
35C5;35D2	7830*	1.4%	1	74	35B4-6;35F1-7	3588	1%
42A13;42E6	8045	17.5%	7	40	42A2-3;58A4, h42-h43;59E1/41BC;42A16-B1	749/4308	0%
41D1-2;42A2-10	8893	7.8%	4	51	41BC;42A16-B1	4308	1%
42A7-10;42B1-2	8896	6.3%	3	48	41BC;42A16-B1	4308	1%
42A6-7	Src42AE1	4.5%	5	111	42A2-3;58A4, h42-h43;59E1	749	0%
42A6-7	Src42Amyri	9.8%	4	41	42A2-3;58A4, h42-h43;59E1	749	0%
	W1118	7.4%	4	54			10%

2.2.6 Conclusions

The purpose of the screen was to pull out modifiers of the JNK pathway by removing one copy of genes on the second chromosome and observing an effect on the number of males with both the deficiency chromosome and the *slpr*^{BS06} allele. Starting with large deficiencies was beneficial in order to cover as much of the chromosome as possible with the least amount of work. Known members of the JNK pathway, *bsk* and *raw*, both have allelic interactions with *slpr*^{BS06}, but deficiencies tested in this screen removing portions of these genes do not cause a significant effect, indicating the possibility to miss effectors. However, four of the smallest deficiencies analyzed show strong enhancement or suppression of the *slpr*^{BS06} number of males. Three of those are enhancers. The *src42A* alleles did not show any change in the number of *slpr*^{BS06}; *Df* males (at 4.5% for *src42A*^{E1} and 9.8% for *src42A*^{mryi}). Stronger alleles should be tested. Also, examination of the results using the smaller deleted regions shows that one of the smaller deficiencies which was deemed significant did not give the same outcome (enhancement or suppression) as their “parental” deficiency. This could be explained by the presence of two or more genes in the large deficiency which may affect one another, or together, may cause an effect on the JNK signal which is different from the effect of removal of only one of those genes. It may also be a result of different genetic backgrounds in which the deficiencies were generated. More thorough investigation and testing of available alleles will help to resolve this anomaly.

2.3 GMR-RAC

A secondary screen using an overexpression of the GTPase Rac (GMR-Rac[73]) in the fly eye was used to check the validity of our putative interacting stocks. Overexpression of Rac using the eye-specific GMR driver leads to an upregulation of the JNK pathway and causes the eye to become smaller and darker. The ommatidia become unordered producing a “rough” appearance in the posterior portion of the eye. When mutant alleles of the JNK pathway are crossed to this line, they reduce the amount of JNK signaling causing a repression of the phenotype – a normal looking eye. Crossing GMR-Rac flies to mutants of the negative feedback regulator Puc hyperactivates the JNK pathway and causes enhancement of the phenotype, making the eyes

much smaller, darker and highly unordered[13]. Consequently, deficiency lines which enhanced the *slpr*^{BS06} phenotype should suppress the rough eye phenotype seen here. Lines which suppressed the *slpr*^{BS06} lethality should enhance the eye phenotype. All Df crosses were compared to crosses with wild-type flies, and to crosses with members of the known JNK signaling cascade

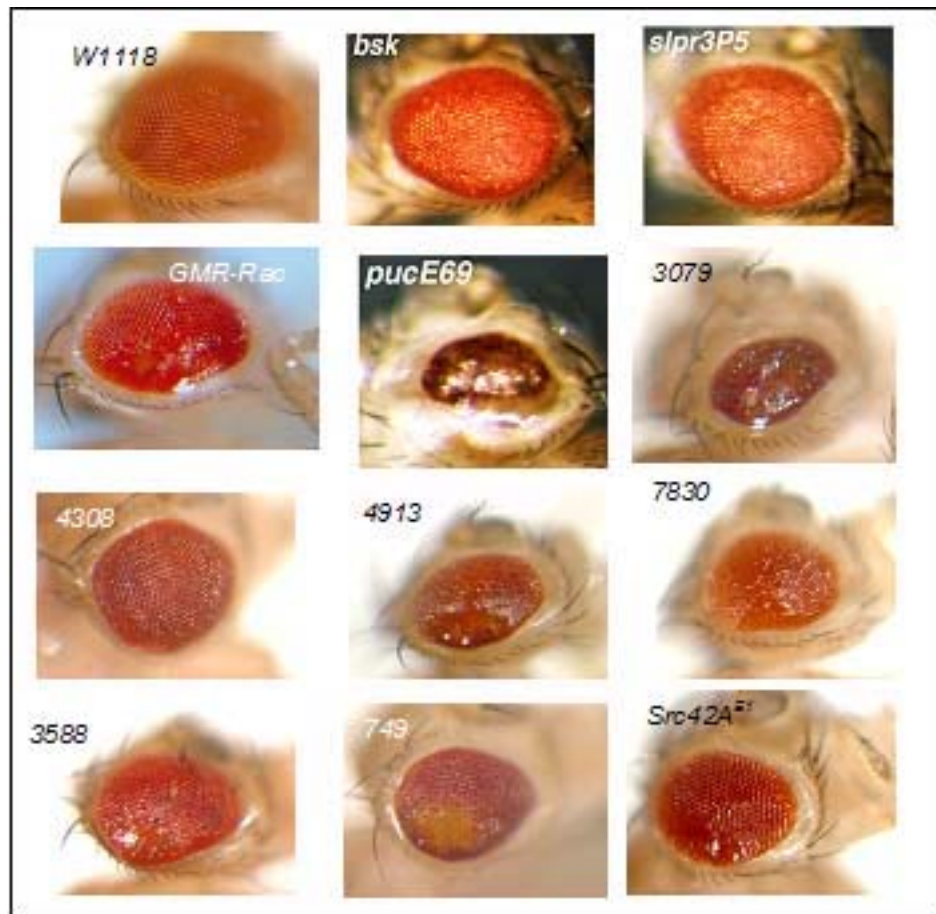


Figure 5: GMR-Rac eye phenotypes.

GMR-Rac flies were crossed to deficiency stocks which had shown an effect on the number of *slpr*^{BS06} male adults. *W1118* is wild-type, GMR-Rac shows the original rough eye phenotype. *Bsk* and *slpr*^{3P5} show how known members of the pathway suppress the phenotype and *puc*^{E69} shows how a negative regulator enhances the phenotype (pictures adapted from Stronach 2002). Stock 3079 (Df(2L)PrI,PrI[1]nub[PrI], breakpoints 32F1-3;33F1-2) is an enhancer, and stock 4308 (Df(2R)nan14, breakpoints 41BC;42A16-B1) is a suppressor. Stocks 4913, 7830, 3588, 749, and the *Src42A* alleles showed no significant change in the phenotype.

2.3.1 Results

Two of the deficiency stocks produced expected results when crossed to GMR-Rac flies. 3079 was a suppressor of the *slpr*^{BS06} phenotype, producing 87% BS06 males. When screened using Rac overexpression, the eye phenotype was enhanced, indicating a negative regulatory relationship on the JNK signal. Similarly, 4308 enhanced the BS06 phenotype, knocking down the number of BS06 males. This line was able to repress the eye phenotype (like *slpr* mutants), indicating a requirement to promote signaling through the JNK pathway. The consistent results strongly suggest that there are modifiers of Slpr-mediated JNK signaling within these regions of chromosome two.

One line (stock 7830) produced curious results, enhancing both the BS06 and the GMR-Rac phenotypes. Also, stock 5869 enhanced BS06 but showed no effect on the GMR-Rac phenotype. Its role in the pathway is questionable, but should still be analyzed further. Both of these results are curious but because of the consistency of their results in the BS06 screen, they should be analyzed further.

2.3.2 Conclusions

The deficiency stocks ordered as a kit are useful for the type of non-biased screen that was done here. The goal of the screen was to discover modifiers of JNK signaling and currently, there are two stocks which have produced consistent results in both screens, and three stocks which produce consistent numbers in the *slpr*^{BS06} screen only. A similar screen using the deficiency kit of the third chromosome has already produced a least one modifier of JNK signaling, the phosphatase Pp2C. Clearly, the screen is able to meet expectations and the work described here has provided the lab with five possible enhancers or suppressors of the JNK pathway. Future work should define the genes within the regions described and may lead to characterization of novel proteins and/or specific pathway interactions.

As discussed previously, when planning the cross itself, it was beneficial to use multiple markers to ensure accurate results and to obtain high enough numbers to be confident with the

results. It was also beneficial to set up multiple crosses to ensure consistent percentages. This screen has revealed three lines which show consistent results in the *slpr*^{BS06} viability screen throughout. One of the small deficiencies is a strong candidate as an enhancer of the *slpr*^{BS06} phenotype, but is different from its larger, “parent” deficiency region. Although the inconsistency is an issue, it is not unexplainable. The larger deficiencies cover vast stretches of the chromosome and remove up to 100 genes; therefore, there may be unknown interactions occurring depending on the genes contained in the deficiency regions. As a result, each of the smaller regions should be studied in depth to rule out multiple genetic interactions.

The GMR-Rac screen is also useful as a secondary screen and is more phenotypically based rather than based on numbers of flies. Indeed, this could also be used as a primary screen itself. This data shows that two of the deficiency interactors act appropriately in the secondary screen, confirming the validity of the Df screen and providing further confidence that the region in question harbors a true modifier of the pathway. Still, results which oppose the Df screen are not necessarily unreliable, as the role the JNK pathway plays in Rac-dependent eye morphogenesis is currently unclear. Similarly, if the molecule in question acts through another pathway, in an upstream role, or in a compensatory function, it may be unaffected by Rac overexpression.

Testing of the *src42A* alleles showed no significant effect on the *slpr*^{BS06} viability. The overlapping region of the two deficiencies will need to be analyzed further to look for other potential modifiers. Interestingly, this region is adjacent to the centromere and that might be the cause of the outcome observed. Further testing is necessary.

Overall the ability of both screens to identify suppressors or enhancers of the Slpr-mediated JNK pathway was reasonable. I improved several problematic issues by using more chromosomal markers and retesting Df lines. More detailed analysis will be necessary to find the gene(s) causing the perceived effect.

Table 5: Genes of interest in *slpr*^{BS06} modifying Dfs.

<u>Breakpoints</u>	<u>Df #</u>	<u>Df Name</u>	<u>Genes of interest</u>	<u>Effector Type</u> <u>(of BS06 phen.)</u>	<u>Effector type</u> <u>(of Rac eye phen.)</u>
			<u>deleted</u>		
h42- h43;42A2-3	749	In(2R)bw[VDe2L] Cy[R]	rl, Src42A	Enhancer	No change
32F1-3; 33F1-2	3079	Df(2L)Prl	unknown	Suppressor	Enhancer
41BC;42A16- B1	4308	Df(2R)Nap14	Src42A, p120ctn, Gprk1	Enhancer	Suppressor
32D1;32F1-3	5869	Df(2L)FCK-20	unknown	Enhancer	No change
35C5;35D2	7830	Df(2L)Exel8034	unknown	Enhancer	No change

2.4 FUTURE DIRECTIONS

The next step would be to analyze the smaller deficiency regions of chromosome two to uncover the interactors. Some potential genes of interest are listed in Table 5. Rl is the MAPK involved in photoreceptor eye differentiation[74], and more severe alleles of Src42A should be tested. P120ctn regulates in adherens junctions, which may influence cell movement[75]. Gprk1 is a G-protein coupled receptor kinase[76]. There are a number of uncharacterized and/or predicted genes in most of the deleted regions which may lead to identification of new protein products. Available alleles of possible effectors should be tested individually in both screens. Phenotypes of the *slpr*^{BS06};Df males should also be noted since the phenotypic defects of *slpr*^{BS06} males are numerous.

GMR-Rac screening should be reanalyzed for consistency. It is extremely important to use comparative analysis during this phenotypic study setting up crosses to wild-type flies and known enhancers and suppressors of Rac overexpression. Also, stocks are sometimes unhealthy and should therefore be done in duplicate and scored everyday for reliable observation.

Furthermore, the GMR driver is temperature sensitive. Analysis of putative modifiers at varying temperatures may be beneficial.

If a known gene is recognized as an enhancer or suppressor through the previous screening processes, it can be tested in a variety of ways to show a direct interaction with the Slpr-mediated JNK signaling. Protein overexpression and rescue experiments can be used to compare phenotypic effects and the presence of dorsal open cuticles. Mutant alleles of other members of the JNK pathway (*bsk*, *puc*, etc.) can be used to test for interactions with uncharacterized proteins as well. If it is an uncharacterized gene that appears to modify the *slpr*-mediated effect, then characterization will be necessary to look for homologs and similar structured proteins. Also, protein production can be measured and analyzed for expression profiles using tagged forms of the protein and immunofluorescence.

The large-scale screen used here was successful in identifying regions of the second chromosome that seemed to have an effect on Slpr-mediated JNK signaling. The remarkably consistent eclosure rate of *slpr*^{BS06} males provided a measurable way to look for modifiers of the JNK signal. At this point, there are approximately four small regions of the chromosome which interact with *slpr*^{BS06} and are ready to be further dissected. Identifying the gene and how it functions in JNK signaling will be exciting future work and may lead to characterization of unknown proteins and /or pathway associations.

2.5 MATERIALS AND METHODS

Fly stocks:

Deficiency stocks were ordered from the Bloomington Stock Center or borrowed from the Campbell lab at the University of Pittsburgh. *Src42A* alleles were obtained from the lab of Brooke McCartney at Carnegie Mellon University.

Crosses:

Preliminary screening was done using two separate crosses of *w,slpr^{BS06}/FM7i* females and *Df(2) / balancer* males and incubated at 25°C. Vials were flipped one time and progeny were scored. *w,slpr^{BS06} ; Df(2)* males were counted as a percentage against *FM7i; Df(2)* males. All other progeny were scored as well. GMR-Rac crosses were raised at 2C and scored based upon visual phenotypes comparing crosses to *W1118* and other members of the JNK pathway.

3.0 CHAPTER 3: EXPLORING THE ROLE OF MSN AS AN ACTIVATOR OF SLPR DURING DORSAL CLOSURE

3.1 BACKGROUND AND SIGNIFICANCE

Because of the many essential roles of the JNK pathway throughout development, understanding the unique set of activation signals and component interactions during each event is imperative. Specificity is likely influenced through distinctive sets of protein-protein interactions, including multiple upstream activators and variable protein complexes. Currently, it is not clear what signals stimulate JNK activity in the leading edge cells during dorsal closure. The molecules which directly activate Slpr to elicit JNK activity, or modifiers of this pathway, either directly or through feedback mechanisms are also largely unknown. This project takes a candidate approach by testing a specific molecule with ties to both JNK signaling and dorsal closure.

3.1.1 Misshapen

One candidate for Slpr activation is Misshapen (Msn), an Ste20-like kinase shown to bind to Dock, an SH3/SH2 adaptor protein. In mammals, Msn's homolog NIK, binds to the homolog of Slpr[16-18, 54]. Genetic evidence also indicates an involvement of Msn in dorsal closure, and this has been shown to operate through the JNK pathway[16-18, 53, 54]. Double heterozygous mutants of *msn* and *bsk* or *hep* show an increased number of dorsal open cuticles[53]. Msn is hypothesized to act as the JNKKK kinase upstream of Slpr, however, a direct connection has yet to be elucidated. Here we began to test Msn's ability to bind to and ultimately activate Slpr.



Figure 6: Msn protein structure.

The middle proline-rich region has been shown to bind to Dock in eye development and is hypothesized to bind to the SH3 domain of Slpr during dorsal closure.

3.1.2 Msn structure

Msn has three domains: a kinase domain on the N-terminus, a regulatory domain on the C-terminus and a middle region rich in PXXP motifs. It is hypothesized that the middle proline-rich region of Msn binds to Slpr. In developing eye discs, the 447 amino acid region, termed here as MsnM, binds to the SH3 regions of Dock[17]. A GST-tagged form of MsnM was used in pull-down assays with Slpr; specifically, a 6xHIS-tagged form of Slpr comprised solely of the SH3 region at the N-terminus was used (a.a.1-114). Two other HIS-tagged constructs of Slpr exist; one with the SH3 and the kinase domain, and another including the LZ/CRIB domains as well. Bacterial expression of the larger constructs yielded insoluble protein so the HIS-SH3 was the only construct used in the pull-down assays described here.

Notably evidence supports a role for the C-terminal portion of Msn in dorsal closure; moreover, expression of Msn lacking amino acids 332-667 (the middle region) was able to almost fully rescue mutant embryos to the pupal stage, indicating that this region is not necessary for dorsal closure[18]. Still, it may play a role in binding Slpr within the full length protein.

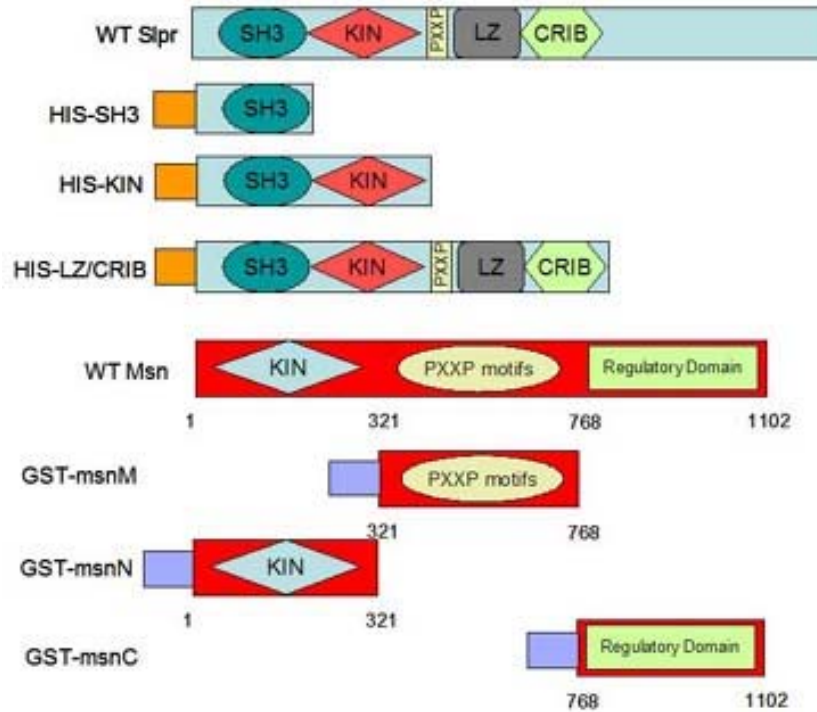


Figure 7: Constructs used in pull-down assays.

Three forms of Slpr are 6x-HIS tagged, and three constructs are GST-tagged Msn proteins. The SH3 portion was tested here for its ability to bind to GST-msnM.

3.1.3 Hypothesis: The middle proline-rich region of Misshapen binds to the SH3 domain of Slpr to act as the JNKKKK during dorsal closure.

3.2 PULL-DOWNS

Pull-down experiments were done utilizing both the HIS and GST tagged forms of the proteins expressed in bacterial extracts. Lysate of one protein was put on either the nickel column (HIS-SH3) or glutathione agarose beads (GST-msnM). The alternate protein was then tested for its

ability to bind. Proteins were then eluted or boiled in sample loading buffer and separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and analyzed for bound protein.

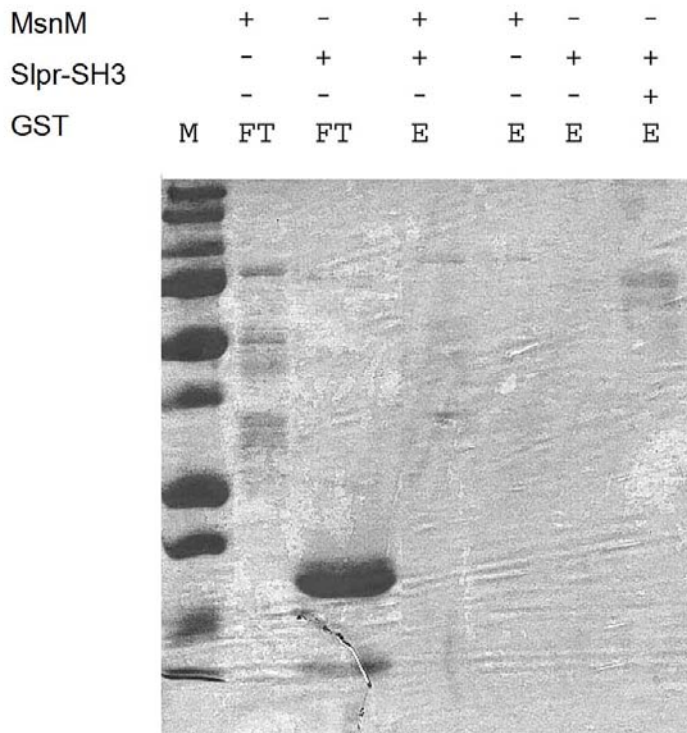


Figure 8: GST pull-down.

Purified GST-msnM was put onto glutathione agarose beads and purified HIS-SH3 was tested for its ability to bind. HIS-SH3 was also tested for its ability to bind to the beads or to GST alone. Because both proteins were purified and left in elution buffer, they do not stick to their respective columns. Most, if not all, loaded protein ran off the beads as seen in the flow through (first two lanes) and HIS-SH3 is not seen in any eluted fractions.

3.2.1 Results

The results of the first pull-down experiment are shown in Figure 7. The GST-msnM protein was bound to glutathione agarose beads and excess was washed off. HIS-SH3 was then mixed with the beads and washed before all protein was eluted from the beads. Controls include both proteins alone mixed with the beads and SH3 and GST alone. The eluates show no binding between GST-msnM and HIS-SH3. The elution fractions show low levels of GST-msnM. It is comparable to the amount seen in the flow-through fraction which was originally put on the beads. Reasons for this will be discussed below.

Clearly, the results are difficult to distinguish. Analysis of this original trial revealed problems with the buffers used and the set-up of the experiment itself.

3.2.2 Problems

The problem with the experiment shown in Figure 7 has to do with the state of the proteins used. In this case, both proteins used in the pull-down assay were purified; the HIS-SH3 on the nickel column and the GST-msnM on the glutathione agarose beads. Both were then eluted from their respective columns and used in the pull-down experiment. This posed a problem for two reasons. First, because the GST-msnM was in elution buffer, this made it very difficult if not impossible to stick to the beads. Second, the HIS-SH3 was also in elution buffer containing imidazole and would not have stuck to the nickel column if a nickel column pull-down was attempted.

In order to fix this problem, bacterial lysates were initially put on the respective columns and purified protein was tested for binding (see Figures 8 and 9).

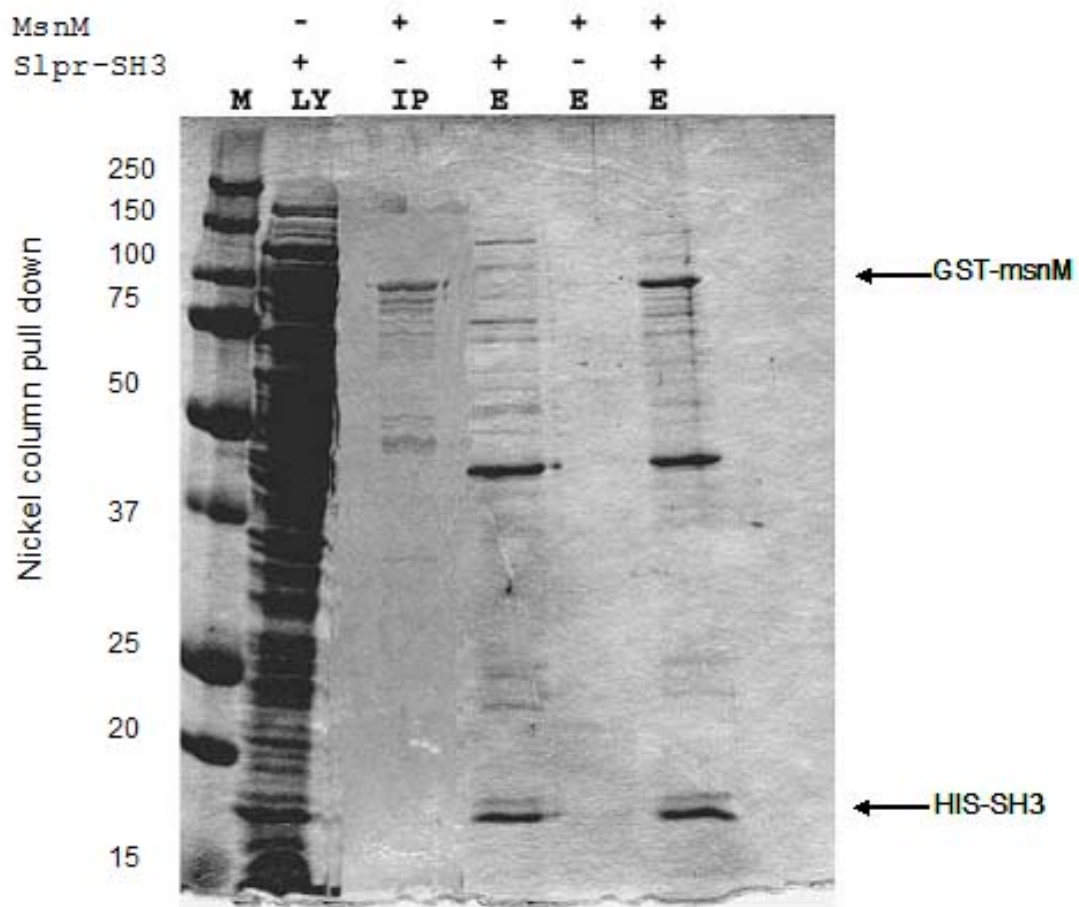


Figure 9: Nickel column pull-down.

HIS-SH3 lysate was put on the nickel column and purified GST-msnM was tested for binding. LY: bacterial lysate expressing HIS-SH3; IP: amount of purified GST-msnM input on column; E: elution fractions. The last three lanes show HIS-SH3 eluted from the column alone, a lack of GST-msnM being eluted from the nickel column, and both proteins present in the elution when both proteins were put on the same column together. Excess proteins are also visible in the elution lanes containing HIS-SH3.

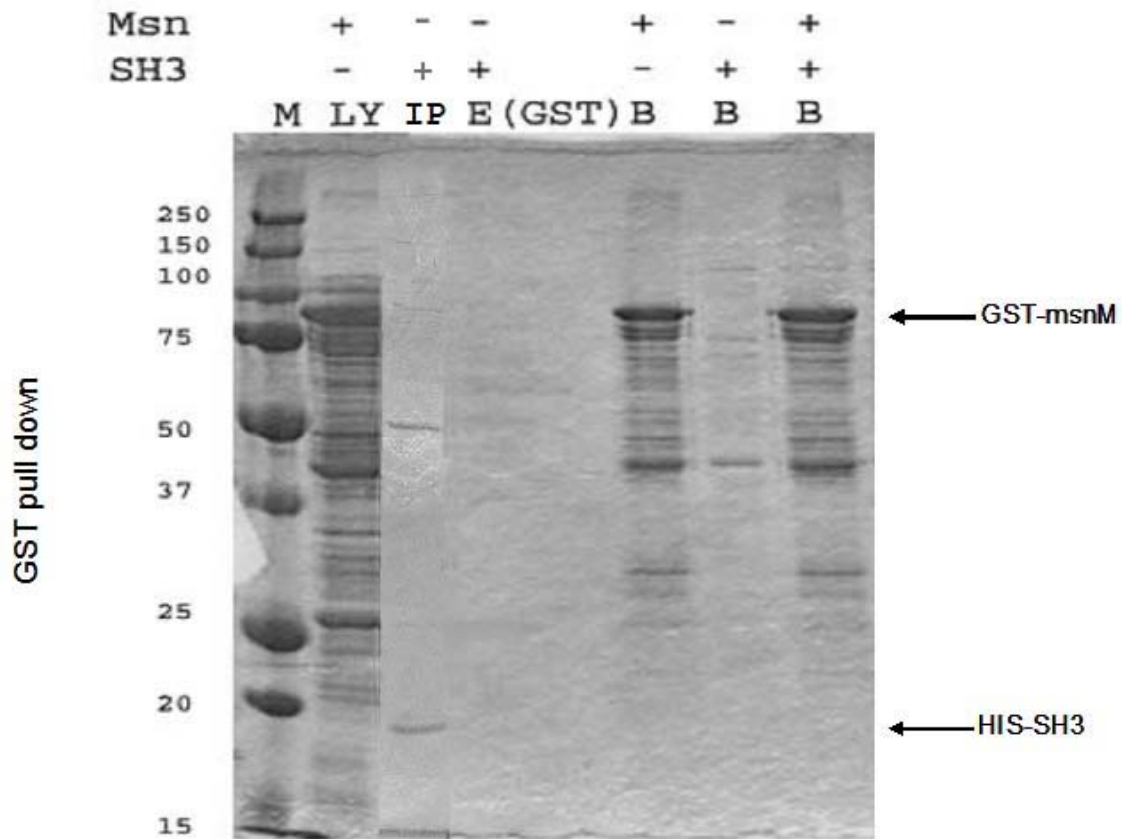


Figure 10: GST-pull down.

GST-msnM lysate was put on glutathione agarose beads and purified HIS-SH3 was tested for binding. LY: bacteria lysate expressing GST-msnM; IP: input of purified HIS-SH3; E: elution fraction; B: beads were boiled to release all bound proteins. The GST eluate shows that no HIS-SH3 bound to the GST alone. In the boiled fractions, GST-msnM is clearly seen at approx. 80kD, but no SH3 is present in any eluate.

3.2.3 Results

The results shown in Figure 8 and Figure 9 were done using original bacterial lysates expressing either GST-*msnM* or HIS-SH3. Purified HIS-SH3 or GST-*msnM* were then added to each bound protein and tested for their ability to bind. Again, when the GST-column was used, no HIS-SH3 was detected in any boiled fraction indicating a lack of binding. However, when HIS-SH3 was bound to the nickel column, GST-*msnM* was able to bind and was seen in the eluate only when HIS-SH3 was present. Together, both pull-downs seem to give ambiguous results; however, it is clear that only the protein with the correct tag binds to its respective column. It is possible that the tags caused differences in folding severe enough to change the binding properties. Still, the purified proteins used were not dialyzed into more physiological buffers from the elution buffers in which they were purified. To be confident of binding, this step would be necessary. Also, the amount of protein originally loaded on each column should be quantified.

3.2.4 Controls

Before attempting to repeat the pull-down experiments, I generated additional controls. Since earlier analysis had shown the SH3 regions of Dock bound the middle region of *Msn*, the Dock cDNA was obtained and its SH3 domains were PCR amplified and cloned into the HIS-tag pET vector to use as a positive control. Also, the N- (a.a. 1-317) and C-terminal (a.a. 763-1102) regions of the *Msn* protein may serve as negative controls to show specificity for amino acids 321-768 and/or binding to these other regions.

The first attempts at generating the other *Msn* constructs were done using RT-PCR on mRNA from embryos. The primers annealed to another region of homology in the mRNA pool and amplified a different gene instead of the C-terminus of *Msn*. Furthermore, the N-terminal portion was difficult to PCR amplify at all. Eventually, the *msn* cDNA was acquired from the Treisman lab at the Skirball Institute of Biomolecular Medicine [18, 53] and PCR was done to

amplify the regions of interest. These were cloned into the pGEX 4T-1 vector to generate an N-terminal GST tag. The C-terminal portion of Msn (called GST-msnC) was tested by cutting at restriction sites engineered in the primers. Induction with IPTG led to expression of protein of the correct size (approx. 66kD). Induction of GST-msnC produced large amounts of protein, but it was highly insoluble. Treatment with 8M urea was unsuccessful to solubilize the protein. Induction of potential N-terminus (called GST-msnN) clones revealed a 26kD expressed protein, the size of GST alone suggesting an early termination of the protein or a frame shift introduced during cloning. Sequencing reactions were not conclusive thus far.

As steps were taken to use the two new controls, GST-msnC and HIS-Dock, GST-msnM ceased to be expressed as before and so the cDNA was used to generate a new construct as well, in order to ensure the correct protein was engineered and tested. Now we wonder whether the cDNA is questionable. For now, these constructs need to be verified through sequence analysis and protein induction, then solubility can be refined. The cDNA must be reanalyzed and the sequences of all constructs verified before further binding experiments can take place.

3.2.5 Conclusions

Pull-down experiments using various tagged forms of proteins are common experiments done in the lab to show an interaction between two proteins. The experiments completed so far show that it is feasible to use this procedure to check for binding between Slpr and Msn. Previous research has shown that SH3 domains often bind to PXXP motifs, and that this is true of Msn and Dock[17]. Therefore, we have made constructs including just the PXXP motifs of Msn and the C-terminal portion as a non-binding control. The HIS-SH3 construct of Slpr expresses well in *E. coli* and is soluble. Fortunately, most of the protein is easily bound to and eluted from a nickel column. Also, the three SH3 domains of Dock, a positive control for Msn binding have been cloned and behave well during expression and purification.

3.3 FUTURE DIRECTIONS

This assay is the first step to establishing if Msn is an upstream activator of Slpr. Pull-downs are often done as initial experimentation assessing an interaction between two proteins. In this case, although the hypothesis suggests binding between the middle region of Msn and the SH3 domain of Slpr, it is possible that this may not be the case. According to genetic rescue experiments, the C-terminal portion of Msn seems to be necessary for rescue of a mutant phenotype and therefore may be necessary for binding as well. The other constructs described here will be able to test for binding of all parts of Msn, albeit separately. A full length, tagged construct can be prepared and tested as well. The ultimate goal is to identify Msn as an activator of Slpr. This, obviously, will require at least the kinase domain, if not the full-length protein to be used in activation assays.

3.3.1.1 Pull-downs

Future directions begin with authenticating each of the Msn constructs in the pGEX plasmid. Sequencing and protein induction tests will confirm the correct sequence and protein size. Protein will then need to be solubilized and tested individually for the ability to bind to glutathione agarose beads. Once each of the constructs are confirmed, purified samples of each Msn construct, the HIS-Dock and HIS-SH3 proteins will be made. Critically, each protein will need to be dialyzed into a neutral buffer so that it can be used for binding in the opposing assay.

Once these steps are completed, the pull-down assays can be completed as described in the experimental methods section. Briefly, GST-tagged protein lysates can be loaded on glutathione agarose beads, rinsed and then purified HIS-SH3 or HIS-Dock can be loaded over top. The beads can then be rinsed and boiled, lysates separated by SDS-PAGE and stained with Coomassie. Subsequently, proteins could be identified by Western blot. Also, HIS-tagged protein lysates can be bound to nickel columns and rinsed. GST-tagged purified proteins can then each be run individually over the columns, rinsed, and finally eluted with 100mM imidazole. Proteins will be identified after running on a gel. Western blotting will be critical to positively identify proteins eluted from the columns or beads.

Similarly, both proteins can be incubated together first, then run over the respective columns and eluted or boiled. Although purifying the proteins is recommended, it would be

possible to use lysates of both proteins in each assay and may be necessary if other molecules or scaffolding proteins are necessary to facilitate binding. Full length constructs of both proteins can be made as well, should binding be absent in all cases. To overcome the problem of purifying insoluble tagged proteins, they can be in vitro transcribed and translated incorporating radioactive methionine to label the protein. Here, only the radiolabelled protein would be visible using a phosphoimager, indicating its ability to bind to the protein on the column. Western blotting could be used to identify all significant proteins present.

3.3.1.2 Kinase Assays

The vital follow-up experiment to the binding assay is a kinase assay testing the ability of Msn to activate Slpr as a phosphorylation substrate. Most likely, the N-terminal kinase domain construct (GST-msnN) would be tested for its ability to phosphorylate Slpr. A full-length Msn protein should also be engineered in order to test Slpr activation. The two longer HIS-tagged forms of Slpr could be utilized, as well as a full-length construct. In vitro transcription/translation and phosphatase assays, as well as [³²P]-labeled ATP could test for the transfer of phosphate groups to Slpr, and such protocols are being currently developed in the lab.

3.4 MSN PHENOTYPES

A second way to test for a relationship between Msn and Slpr is through genetic studies. Previous data have indicated an interaction between the JNK pathway and Msn in the embryo[18, 53]. Genetic data has linked Msn to the JNK pathway during dorsal closure, but Msn has not been shown to interact genetically with Slpr thus far. Cuticle preps of *msn* mutant embryos have revealed obvious holes in the dorsal side of the embryo[18, 53]. In order to relate Msn's role in dorsal closure to Slpr-specific JNK signaling, we set out to look for genetic interactions between the two genes.

3.4.1 Embryonic Viability Screen

It has been shown that heterozygous *msn* mutants have no or weak dorsal open phenotypes but homozygous *msn* mutants and transheterozygous *msn* and *bsk* mutants exhibit holes in the dorsal side of the cuticle[53]. In order to repeat these results and to test for this phenotypic interaction with *slpr* mutants, crosses between multiple *msn* mutants and mutant alleles of *bsk* and *slpr* were set up. Embryo viability and cuticle phenotypes were scored.

3.4.1.1 Experimental Approach

slpr^{BS06} heterozygous flies were crossed to *bsk*² and *msn*^{JIE2}, *msn*¹⁷² and *msn*¹⁰² heterozygous mutant flies. Embryonic lethality was scored and cuticles of dead embryos were analyzed for the presence and severity of dorsal holes. *msn*^{JIE2} is a recessive allele harboring a P-element insertion and *msn*¹⁷² and *msn*¹⁰² are viable inversion mutant alleles[52]. *bsk*² is a dominant EMS mutant. Doubly heterozygous progeny were expected to show an increase in percent lethality and number of dorsal open cuticles. If Msn is involved in dorsal closure mediated by Slpr signaling then doubly heterozygous progeny (*slpr*/+;*msn*/+) are expected to show an increase in lethality and phenotypic severity.

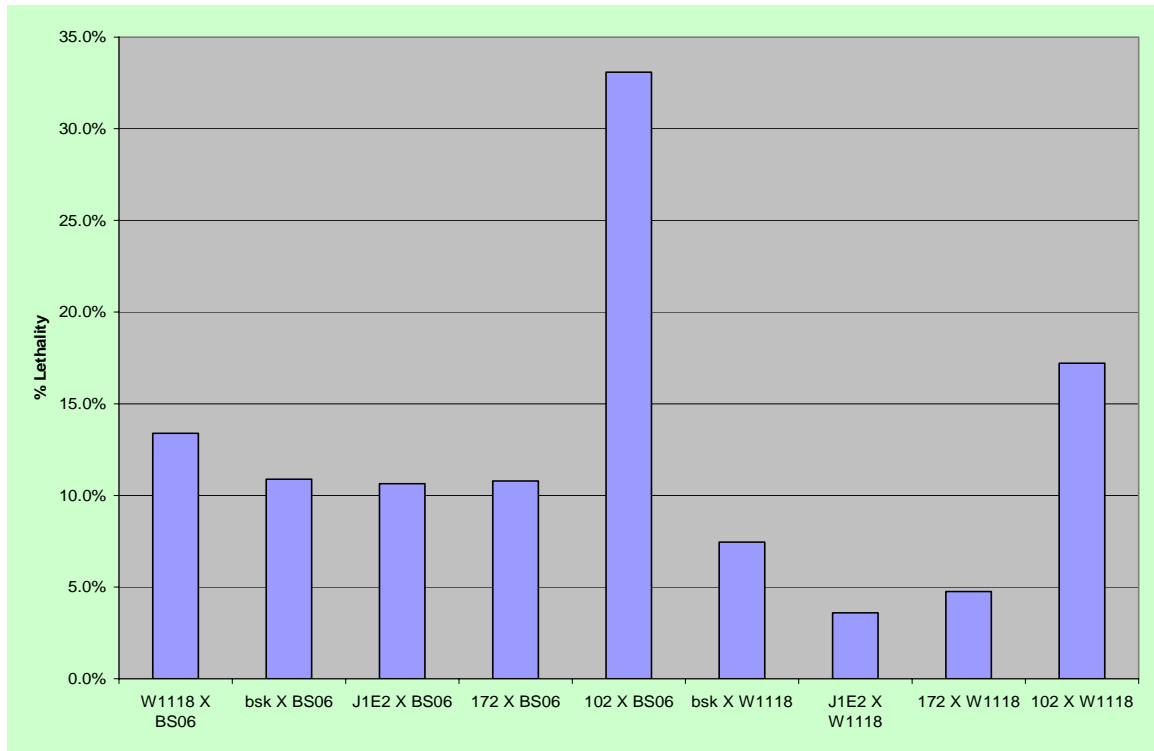


Figure 11: Embryonic viability results.

Heterozygous mutants of *msn* or *bsk* were crossed to both wild-type (*W1118*) and *slpr*^{BS06} heterozygous mutants. Embryos were lined up and dead embryos were scored as a percentage against the total amount of fertilized embryos. Except for *msn*¹⁰², lethality does not seem to be significantly affected by having both *msn* and *slpr* mutations.

3.4.1.2 Results

*msn*¹⁰² was the only allele with any change, having 33% lethality when crossed to *slpr*^{BS06} compared to 17.2% lethality when crossed to *W1118*. The other alleles of *msn* and *bsk* had approximately the same percentage as wild-type.

When cuticles were scored, the amount of embryos scored possessing dorsal holes was unusually high (compared to Su et al. (1998) and other Stronach lab data). This could be a result of phenotypes which were difficult to score, and possibly a problem with the environment of embryos before analysis.

3.4.2 Transgenics

Genomic insertions of Slpr wild-type (*slpr^{wt801}*), a kinase-dead form of Slpr (*slpr^{kd13}*) and wild type Msn (*msn*) exist with Gal4 Upstream Activating Sequences (UAS). Recombinant lines were made harboring two transgenes, *UAS-slpr^{wt801}* and *UAS-msn* (*UAS-slpr^{wt801}*, *UAS-msn*), and also *UAS-slpr^{kd13}* and *UAS-msn* (*UAS-slpr^{kd13}*, *UAS-msn*) transgenes. These lines, as well as the individual UAS lines, were then crossed to various Gal4 drivers in order to look for phenotypic differences during protein overexpression. It was hypothesized that phenotypes observed with the wild-type *slpr* transgene would be exacerbated with the *UAS-msn* present. In addition, phenotypic observations were hypothesized to provide more specific information on the relationship between the two proteins.

3.4.2.1 Experimental Approaches

The approach discussed here looked at phenotypes of the five different transgenic lines: (1) *UAS-msn*, (2) *UAS-slpr^{wt801}*, (3) *UAS-slpr^{kd13}*, (4) *UAS-msn*, *UAS-slpr^{wt801}*, and (5) *UAS-msn*, *UAS-slpr^{kd13}*. These were all crossed to either *pnrGal4* or *pnrGal4*, *puc^{E69}* flies. *pnrGal4* drives expression of the UAS constructs in the *pannier* domain around the dorsal side of the embryo and developing adult thorax. *puc^{E69}* is a P-element insertion disrupting *puc* and harboring a *LacZ* reporter[45]; therefore, the negative feedback mechanism is disturbed and the JNK signal cannot be down-regulated through Puc's phosphatase activity. Moreover, we could assess the extent of JNK signaling in the leading edge in the embryo using Puc as a readout. The adults that eclose from the cross could also be analyzed for phenotypic consequences. Both sets of crosses were incubated at 18°C and 22°C as the Gal4/UAS system is temperature sensitive, eliciting more protein production at higher temperatures.

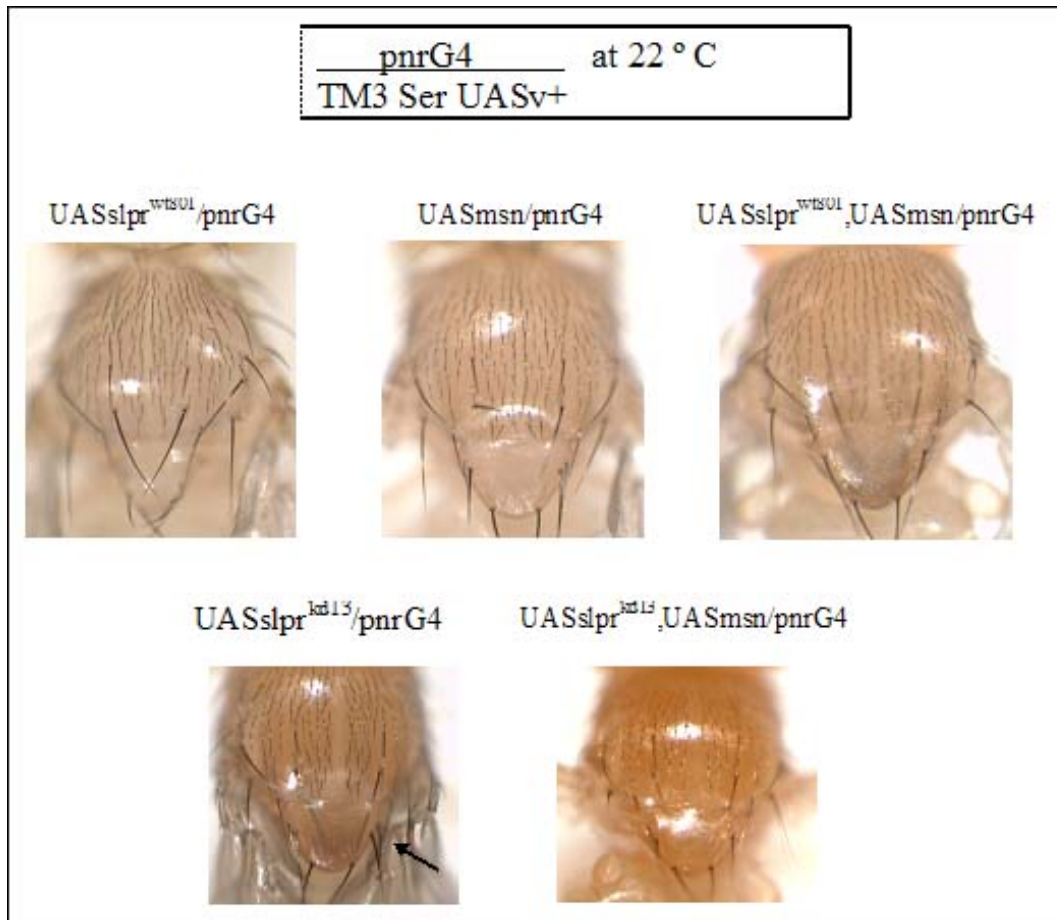


Figure 12: Phenotypes of pnrGal4 at 22°C.

UAS lines were crossed to *pnrGal4/TM3,Ser,UASy⁺* and raised at 22°C. Thorax phenotypes were observed. *UAS-slpr^{wt801}* expression causes a narrowed scutellum. Doubled bristles are also seen on the scutellum of *UAS-slpr^{kd13}* flies (arrow). However, Msn and Slpr coexpression does not lead to an increase in phenotypic severity suggestive of increased JNK signaling. Since *UAS-msn* alone did not give a phenotype, it was difficult to assess whether coexpression of *UAS-slpr^{kd13}* blocked it.

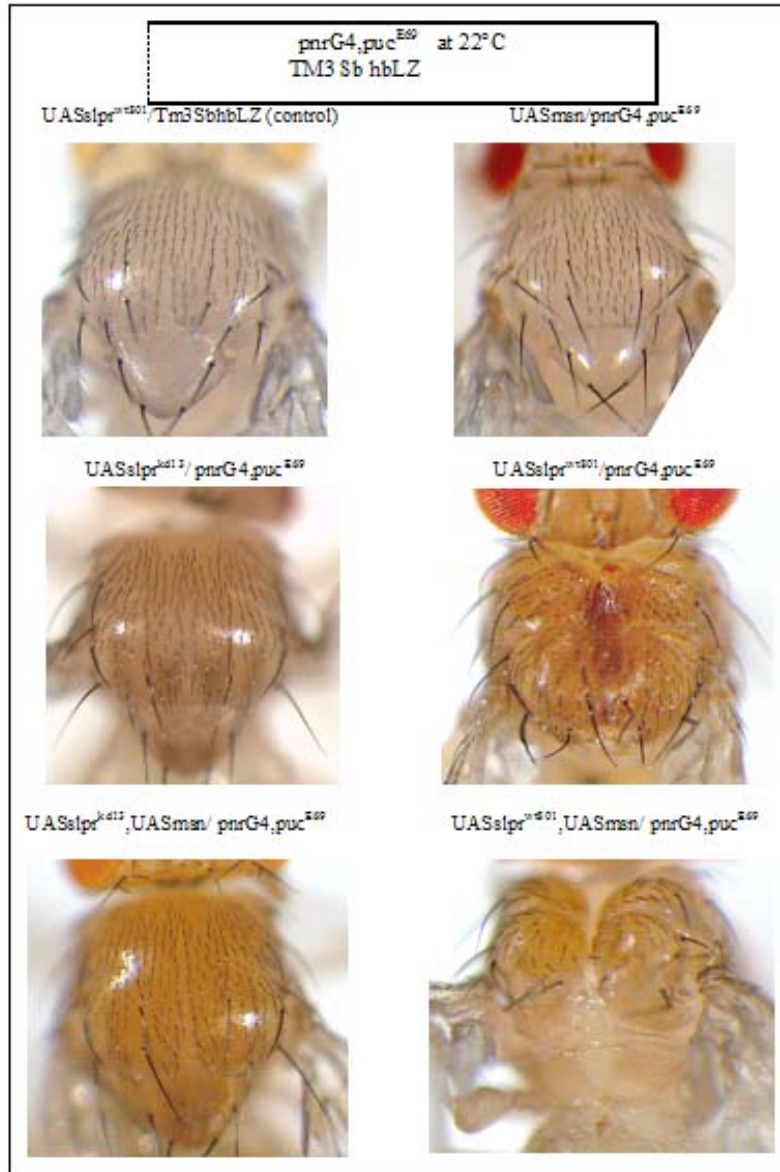


Figure 13: Slpr and Msn overexpression phenotypes.

UAS flies were crossed to *pnrGal4,puc^{E69}* and raised at 22°C. Thorax phenotypes were examined, special notice given to width, number of bristles and size of scutellum. Phenotypes were more severe at 22°C. *UAS-slpr^{wt801}*, with or without *UAS-msn* showed severe cleft thoraces and loss of the scutellum. No phenotype was seen with *UAS-msn* alone.

3.4.2.2 Results: Phenotypes of recombinants

At 18°C, there was no significant effect on thorax phenotype when all five transformant lines were crossed to *pnrGal4* flies (not shown). *UAS-slpr^{wt801}* and *UAS-slpr^{wt801},UAS-msn* flies crossed to *pnrGal4, puc^{E96}* at 18°C showed a highly reproducible severe cleft thorax, and usually loss of the scutellum. Bristles were shortened, kinked or sometimes doubled. As seen in Figure 10, at 22°C, *UAS-slpr^{wt801}* flies showed thinner, more compact thoraces, with a more pointed scutellum when crossed to *pnrGal4* flies. *UAS-slpr^{kd13}* and *UAS-slpr^{kd13},UAS-msn* thoraces were slightly wider, often with doubled bristles on the side of the scutellum. *UAS-slpr^{wt801}* and *UAS-slpr^{wt801},UAS-msn* crosses with *pnrGal4,puc^{E69}* crosses at 22°C showed a severe thorax phenotype as well. It did not appear as though the addition of *UAS-msn* affected the phenotype of the *slpr^{wt801}* alone. Notably, no phenotypes were seen when *UAS-msn* was crossed to either Gal4 driver, at either temperature, indicating no effect of overexpression of the protein in the *pnr* domain, or a lack of expression of the Msn protein. Also interesting was the lack of *UAS-slpr^{kd13}* strong phenotypes.

3.4.3 Conclusions

The *UAS-slpr* constructs (both *wt801* and *kd13*) showed defects in thorax closure. The kinase dead construct did not show very severe phenotypes, which is interesting because the 921 allele of *slpr*, which is also a kinase dead form, shows very early lethality. However, there may be enough wild-type *slpr* in these transgenic flies to make it through to adulthood. Unfortunately, *UAS-msn* did not show any phenotypes, either alone or in combination with the *UAS-slpr* constructs. A new stock harboring the *UAS-msn* transgene was ordered from the Bloomington Stock Center to check for activity, in the chance that our stock had changed, or was not as we had thought it to be. This transgene was mapped to the third chromosome (though it was annotated as mapping to the second chromosome) by following an eye color selectable marker.

Crosses to Gal4 drivers such as *apterousGal4* and *actinGal4* did show slight phenotypes (see Figure 12) suggesting that the transgene was being expressed. Therefore, the phenotypic studies using the *UAS-msn* construct should be repeated. New recombinants between *UAS-slpr^{wt801}* or *UAS-slpr^{KD13}* should also be made with the new *UAS-msn* line.

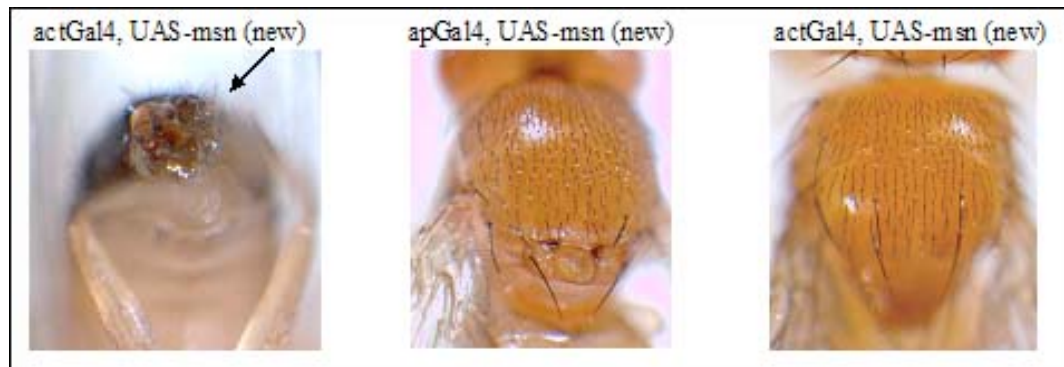


Figure 14: Phenotypes of UAS-msn.

When the new stock of *UAS-msn* was crossed to various Gal4 drivers, some phenotypes were observed. *actinGal4* caused a genital rotation phenotype and a slight narrowing of the thorax. Similar phenotypes are observed in *slpr* mutants. *apterousGal4* caused a severe shortening of the scutellum.

3.5 FUTURE DIRECTIONS

Future studies should include re-engineering the recombinant Msn/Slpr lines and observing any overexpression phenotypes. Lethality screening and cuticle observations should also be repeated for consistency and correct identification of dorsal open phenotypes. Another approach which can quickly be done to look at JNK activity in embryos during dorsal closure is immunofluorescence. The same transgenic lines used in the phenotypic experiments should be crossed to *pnrGal4,puc^{E69}*. Embryos can then be collected, washed and immunostained with antibodies against β -gal and phospho-tyrosine, a general cell membrane marker. *puc^{E69}* harbors a *LacZ* reporter and is a consistent readout of JNK pathway activity. Loss or expansion of

pucLacZ may be predicted if coexpression of *Msn* and *Slpr* changes the balance of JNK signaling negatively or positively.

Also, transgenic lines were recently made to express *UAS-SH3-HA* and *UAS-LZCRIB-HA*. Phenotypic analysis of the individual lines should be completed, as well as with recombinants made between these new lines and the *UAS-msn* line. Antibodies against the HA tag can be used to look for protein expression and localization.

3.6 MATERIALS AND METHODS

Pull-downs:

GST-pull-downs were done using approximately 100uL glutathione agarose beads (Sigma 4510). Approx. 1 mL GST-*msnM* protein lysate or purified HIS-SH3 was put on the beads and incubated with rocking at 4°C for one hour. Each tube was washed with 500uL PBT 10 minutes at 4°C with rocking, spun down 2 minutes at 2000rpm. To the GST-*msnM* containing tube, 1 mL purified HIS-SH3 was added and incubated one hour at 4°C with rocking. The tubes were washed again with PBT and eluted with 100uL elution buffer (5mM reduced glutathione in 50mM Tris-HCl, pH 9.5) for 15 minutes. Boiled elutions were obtained by adding 100uL 2X SDS buffer and boiled at 95°C for 2 minutes.

Nickel-column pull-downs were done under native conditions according to protocols by Qiagen. 600uL of purified GST-*msnM* was added after the second wash step and spun according to protocol. Two more washes were done and then eluted as described.

Lethality Screens and Cuticle preps:

w,slpr^{BS06}/FM7i,GFP females were crossed to *w;msn¹⁷²,FRT80/+*, *w;msn¹⁰²/+*, *yw;p(w+msn^{JIE2})/+*, *bsk² cn¹ bw¹ sp¹/+* or *W1118* males and incubated at 25°C in cages overnight. 125-225 embryos were collected and lined up on apple juice plates and incubated at 25°C overnight. After 24hrs, unhatched embryos were counted and then scored as fertilized or

unfertilized. The total fertilized but unhatched number was recorded as a percentage over total fertilized.

Cuticles were processed from brown, fertilized but unhatched embryos by fixing in acetic acid:glycerin (4:1) for 30 minutes at 60°C and then holding at RT for at least 24 hours. The 4:1 solution was then replaced with CMCP-10 mounting media:lactic acid (3:1) and placed on a slide. The slides were then incubated on a slide warmer (~ 50° C) overnight. Dark field microscopy was used to view cuticles and embryos were scored for the lack or presence of mild, or severe dorsal holes.

Cloning:

UAS-slprSH3-HA and UAS-slprLZ/CRIB-HA: The SH3 domain of Slpr and the LZ/CRIB portions of Slpr were amplified by PCR (primers are listed in Appendix A) and cloned into the pGEM-T Easy vector (Promega). Each PCR construct was then digested out of pGEM-T Easy using Kpn1 and EcoRI restriction sites and ligated into a pBS(SK+) vector containing a C-terminal 2x HA-tag (obtained from the lab of Gerard Campbell, University of Pittsburgh). The SH3-HA and LZ/CRIB-HA tagged constructs were digested from pBS-HA and ligated into pUASp using Kpn1 and Not1 restriction sites.

HIS-Dock: Dock cDNA LD42588 was ordered from DGRC. The three SH3 regions of Dock were PCR amplified and ligated into pET16b (Novagen) to add an N-terminal 6xHIS-tag.

GST-msnN and GST-msnC: mRNA from W1118 embryo lysates was originally used to amplify the N- and C-terminal portions of Msn by RT-PCR. The msn cDNA was also obtained from the lab of Jessica Treisman (New York University Medical Center) and used as a template to PCR amplify both constructs. PCR products were ligated into pGEX-4T-1 (Amersham).

4.0 CHAPTER 4: CONCLUSIONS

4.1 DEFICIENCY SCREEN

In an attempt to efficiently screen the entire genome of *Drosophila* for modifiers of Slpr-mediated JNK signaling, a deficiency “kit” was used to check the entire second chromosome. The large deleted regions, although harboring many genes, covered the majority of the second chromosome. By reducing the dosage of genes using the deficiencies, it was possible to look for modifiers of JNK signaling. The normally consistent viability phenotype of the BS06 allele became a malleable readout of JNK activity. Some problems were evident involving percentage consistency and phenotypic observation. It was very important to set up multiple crosses and to thoroughly research the chromosomal markers for each deficiency stock. Crosses that produced unexpected progeny due to recombination or nondisjunctions were discarded. Eventually some stocks showed consistent results and were analyzed to determine breakpoints. The genes within the noted regions were also analyzed in order to find smaller deficiencies as well as alleles of putative interactors.

Deficiencies are becoming more popular in their value as genetic tools. Consequently, deficiencies exist from Bloomington (which are used here) and are updated frequently. Also, there is a collection from Harvard (Exelixis) which can be used to find smaller deletions. If there is a gene which shows promise as a strong candidate, then alleles of that gene can also be obtained and tested, as was done with the *src42A* alleles. Unfortunately, neither *src42A* allele modified the *slpr*^{BS06} mutant. Also, although it is possible to get no BS06 males (0%) after setting up many crosses, indicating the presence of an enhancer, the ability to pull out suppressors is more efficient.

In order to substantiate the effect seen in the deficiency screen, we performed a secondary assay which enhanced or suppressed the level of JNK signaling. For our screening

purposes, the ease of the assay was beneficial, and because all of the members of the pathway acted accordingly, it was with confidence that this phenotype was legitimately used to check for validity of our original screen. Nonetheless, because the molecular interactions which cause the eye to have a rough appearance are not understood, it is possible that a modifier of *BS06* lethality may not have acted as expected. Therefore, results should be interpreted with caution, especially when looking at specific mutant alleles. One important experimental note is to carefully use comparative analysis when using the GMR-Rac assay. Control crosses should always include wild-type and known enhancers and suppressors of the eye phenotype in order to compare the unknown interactor. Phenotypes are somewhat variable and must be scored as an average of all progeny.

The results of both screens have led to identification of five deficiency stocks which show enhancement or suppression of JNK signaling. Two of these show consistent results in both assays. Future work would include obtaining smaller deficiency lines to further narrow down the region where the gene resides, and testing specific alleles of predicted modifiers. The two stocks which show varying results between the two screens should be retested in both assays and analyzed further.

The prospect of finding possibly four genes which may play some role in the specificity of signaling through the JNK pathway is exciting. However, it is very possible that one of the many uncharacterized genes within the regions of interest may be influencing JNK signaling. Further work will be needed to identify and characterize the involvement of any molecules and to provide the link to Slpr-mediated JNK signaling.

4.2 MISSHAPEN

In addition to looking for modifiers of the pathway, this work also set out to identify upstream signaling components involved in dorsal closure. By taking a candidate approach, the ability to test directly for binding and activation was fairly straightforward. Although the conclusions drawn here cannot say with certainty whether Msn is the JNKKKK acting on Slpr, progress has been made in order to reach that goal.

Based on the hypothesis that Msn is the upstream activator of Slpr, we tested for interactions using pull-down assays with bacterially expressed proteins. Although initial attempts proved inefficient, they were cause for reevaluation of buffer conditions, controls and overall experimental design. The constructs which are currently being tested in the lab include GST-tagged forms of Msn; the middle proline-rich region as well as both termini, and two HIS-tagged constructs; one is the SH3 domain of Slpr and one is the SH3 domains of Dock (which have previously been shown to bind to GST-msnM). Some of these are ready to be used, and others are still being verified.

Because of the intriguing genetic studies using portions of Msn, it will be interesting to see how binding is achieved and through what portions of the protein this is accomplished (if binding is actually taking place). Kinase assays will further establish whether Msn is an upstream component of the JNK pathway and an activator of Slpr.

The genetic analysis presented here were also useful to test for phenotypic detection of overexpressional analysis in vivo. In order to retest and feel confident in the outcome, the new UAS-msn stock should be used to make new recombinant lines and retested in the *pnrGal4* and *pnrGal4puc^{E69}* expression crosses. However, it was clear that *UAS-slpr^{wt801}* and *UAS-slpr^{kd13}* showed some type of thorax developmental defects, related to the defects seen in *Mlk3* *-/-* mice midline phenotypes. Testing the ability of *UAS-msn* to affect these phenotypes should further the data regarding Msn's effect on JNK signaling. The phenotypes seen with the new UAS-msn transgenic line are interesting and open the door to further research, especially considering the genital rotation defect observed in approximately 5% of the male progeny when expressed using the *apGal4* driver. This is the same phenotype seen in *slpr^{BS06}* mutants and with mutant *Pvf1/Pvr* ligand/receptor complexes.

4.3 CONCLUSION

Overall this work was directed at identifying molecules that influence Slpr-mediated JNK signaling, specifically those that affect the process of dorsal closure. It has narrowed down four regions of chromosome two which likely encompass enhancers or suppressors of the pathway. Additional assays are necessary to examine protein-protein interactions between Slpr and Msn,

which have previously been implicated through genetic experiments. Further phenotypic analysis can be used to explain the role of Msn as a stimulant of the pathway and may lead to an understanding conditions which lead to activation of JNK signaling, specifically the conditions necessary for dorsal closure to occur.

This is just one pathway which affects a small number of processes during embryo development. But understanding this sequence of events can provide volumes about cell signaling in all types of processes. The specificity required for correct, robust cellular responses is embedded in the network of protein-protein interactions and the intersection of molecules between pathways, cells, and tissues. It is the hope that further work on this project may eventually lead to medical contributions for those affected with developmental and wound healing defects.

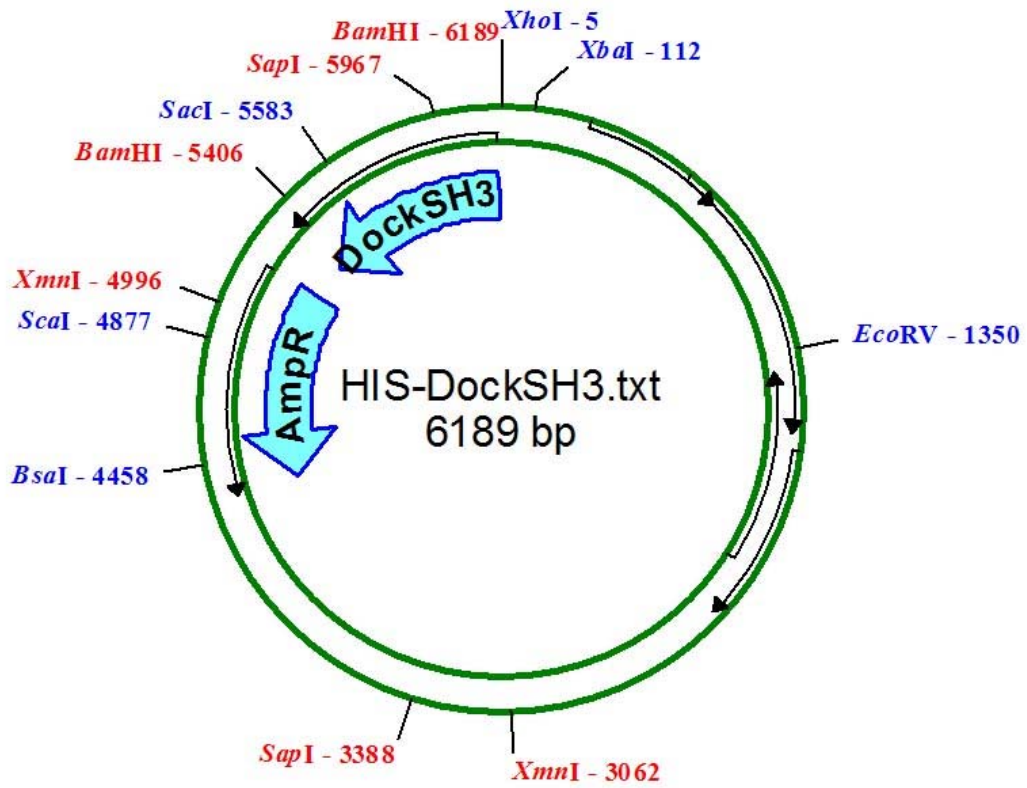
APPENDIX A

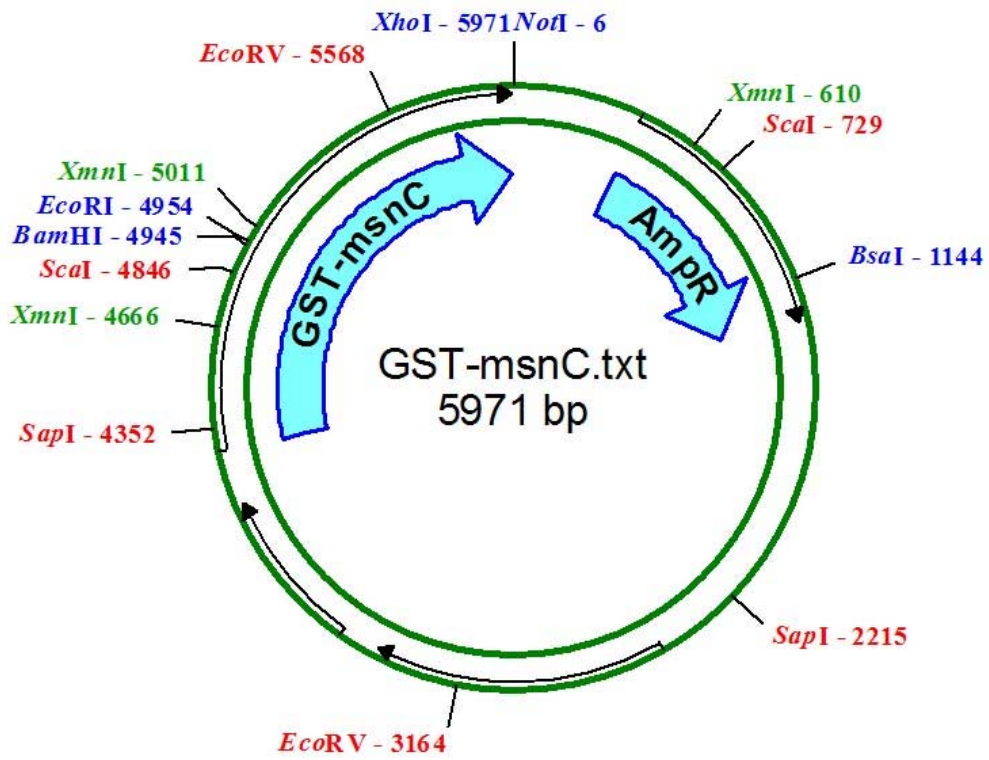
PRIMERS USED FOR CLONING

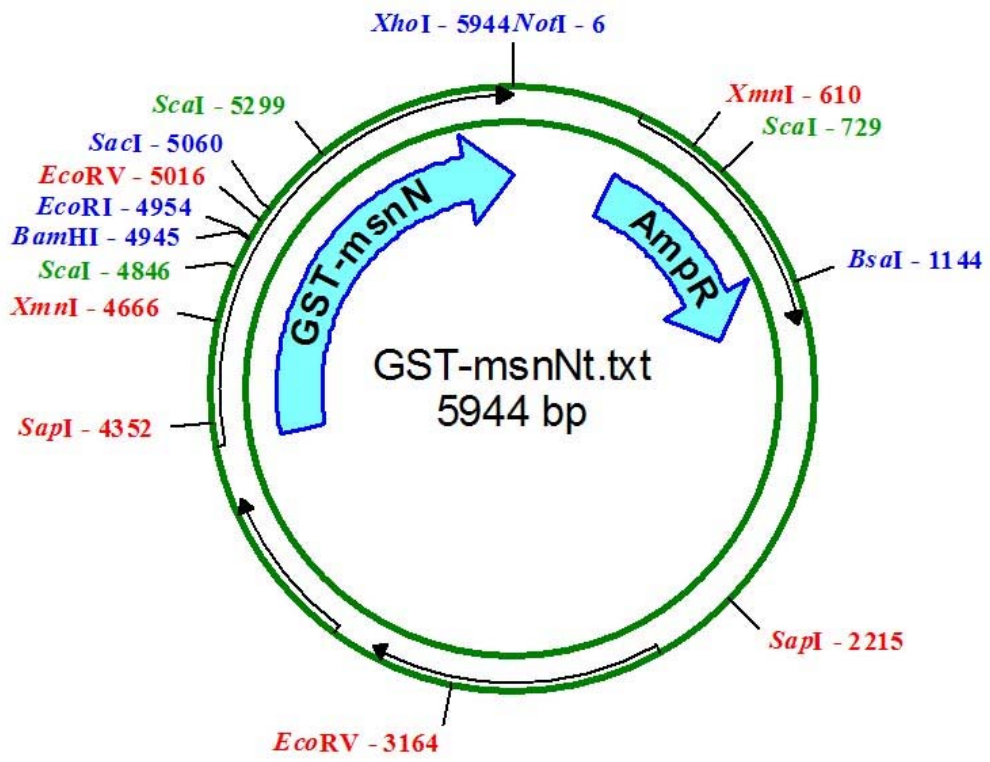
<u>Construct</u>	<u>Primer Name</u>	<u>Restriction Site</u>	<u>Primer Sequence (5' - 3')</u>
UAS-SH3-HA	CRSH3f	Kpn1	GGATGGGGTACCCCCAACATGCTG
	CRSH3R	EcoR1	GATGTCGCCGAATTCGGACGAGAC
UAS-LZCRIB-HA	CRLZf	Kpn1	GCCCGGGGTACCCCTTCACTACATG
	CRCRIBr	EcoR1	GATGCGGAATTCGGAGAAGGAAGG
HIS-Dock	dockSH3FBam	BamHI	CACAGGATCCGGGCAACATGAAGCAC
	DOCKSH3ref	BamHI	GATCGGATCCGCACTGGCACTCGCATTACG
GST-msnN	MSN Nfor	EcoRI	GATCGAATTCATGGCGCACCAGCAGCAAC
	MSN Nrev	XhoI	GATCCTCGAGATAGTCCTCGCGCTCCTTC
GST-msnC	MSN Cfor	EcoRI	GATCGAATTCACCTCCCACGAGGCGGCC
	MSN Crev	XhoI	GATCCTCGAGTTACCAATTGGCCATGC
GST-msnM	MsnMRev	XhoI	CGTACTCGAGGAGGTGGGCGTTACATTGACA
	NEWMsnMFor	EcoRI	GATCGAATTCCAGGAGAAGGAGCGCGAG

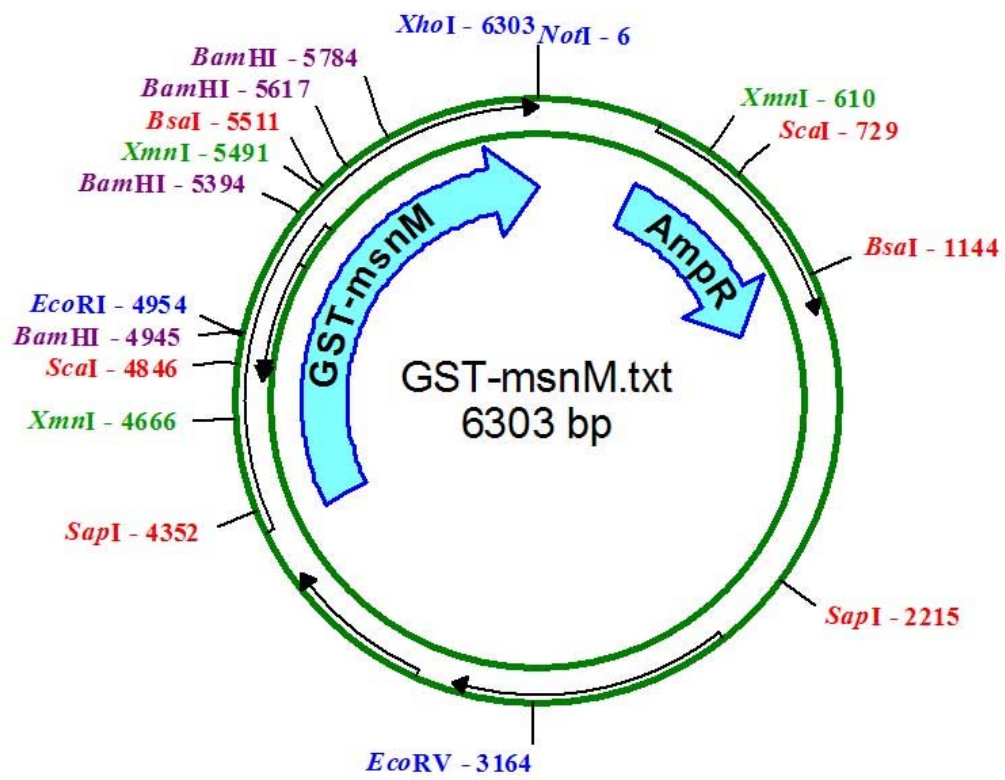
APPENDIX B

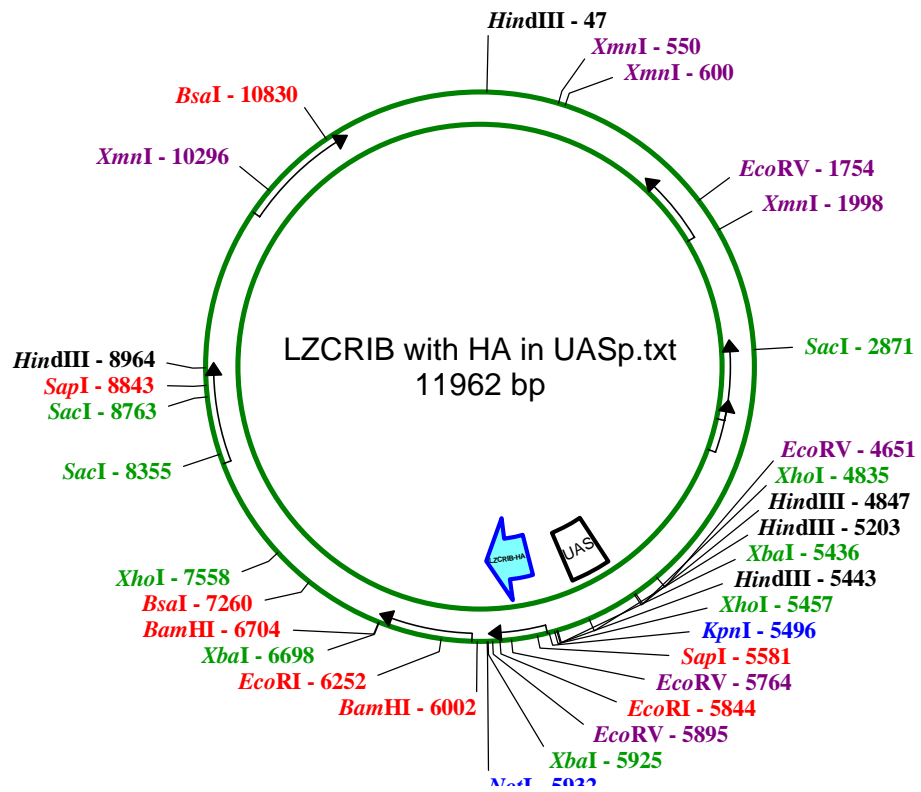
PLASMID MAPS

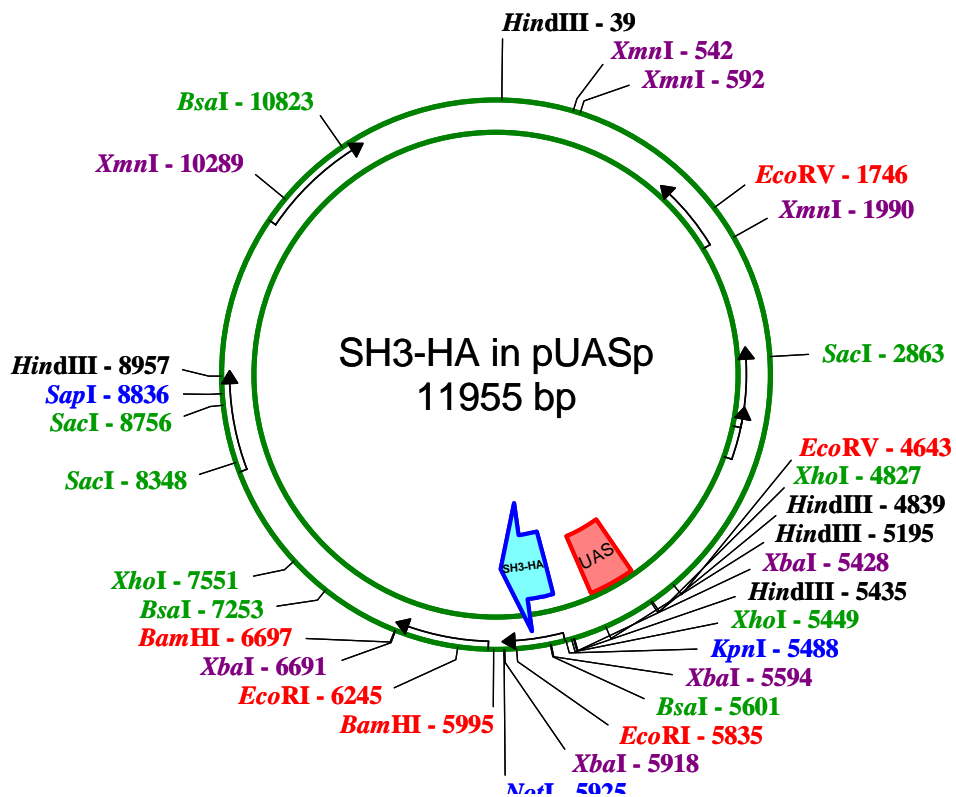












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