Phosphorylation of the *Drosophila* JNKKK Slipper is essential for proper morphogenesis and heat shock response

by

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Bachelor of Science, Westminster College, 2004

Submitted to the Graduate Faculty of

Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

SCHOOL OF ARTS AND SCIENCES

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Signal transduction pathways rely on proper protein activation and regulation to elicit appropriate responses to extracellular cues. Kinases within these pathways are commonly activated through a multi-step mechanism, which involves phosphorylation. Additionally, regulatory modifications can occur to modulate signals, ensuring that the proper intensity and duration of signaling is achieved in the correct context. I have identified two modes of regulation of the *Drosophila* JNKKK, Slipper. First, investigation into kinase domain phosphorylation reveals that conserved putative phosphorylation sites are required for Slpr function. Site-directed mutagenesis converting three serine/threonine residues to alanines (SlprAAA) renders the protein inactive, and SlprAAA behaves as a dominant negative during several Slpr-mediated processes. Transgenic flies expressing SlprAAA display phenotypes associated with a loss of JNK signaling such as dorsal open embryos that lose JNK pathway marker gene expression, cleft thorax indicative of a loss of JNK signaling during thorax closure, and a failure to rescue *slpr* mutants. Importantly, double alanine mutant analysis (TST mutated to AAT, ASA, and TAA) reveals that T295 is crucial for Slpr signaling as the two double mutants that contain alanine mutations at that residue are nonfunctional while SlprAAT retains some Slpr function.

An additional phosphorylation site outside of the kinase domain was identified at a conserved MAPK consensus site, PXSP. Though flies expressing a non-phosphorylatable PXAP

develop normally and have no defects in Slpr-dependent functions in morphogenesis, both embryos and adults are sensitive to heat shock. Conversely, a phospho-mimetic version, PXEP, confers thermoresistance. Biochemical assays implicate the Jun kinase, Bsk, as the MAPK required for this signaling to PXSP. These results suggest that Bsk phosphorylates Slpr in a positive feedback loop during heat stress to maintain homeostasis.

Together, these experiments demonstrate the need for Slpr phosphorylation in two circumstances. Not only is phosphorylation required to maintain Slpr-dependent JNK signaling in morphological processes through proper protein activation, but it is also essential in a context-specific manner for a previously unidentified role of Slpr and the *Drosophila* JNK pathway in heat shock response.

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PREFACE

I must start by thanking my advisor, Beth Stronach, who has been a constant source of knowledge, support, and guidance. I am forever grateful for everything throughout the years. You have pushed me to be a better thinker and scientist, and I couldn't have asked for anything more from my graduate experience. Your passion, work ethic, and drive are inspiring, and I have learned so much not only from the guidance you have given me directly, but also by the example you lead. We have come a long way together…I will miss you!

Thank you also to my committee: Dr. Debbie Chapman, Dr. Jeff Hildebrand, Dr. Paula Grabowski, and Dr. Jon Minden. I am truly grateful for your direction and support. Thank you for the critical discussions and help over the years; I am a better scientist because of your guidance.

I need to also thank everyone who has helped me with teaching and the teaching minor. Valerie Oke, Nancy Kaufmann, Lew Jacobson, and Alison Slinsky Legg; thanks for supporting me during all of my teaching endeavors and helping me spread my love of science to students!

I want to also acknowledge Stronach lab members, past and present, for making this a great place to come to work every day. Lisa, Christy, Stephanie, Becky, Luke, Ashley, Rachel, and Alyssa…I had a blast! Also, thank you to all of the undergrads who I mentored, Sara, Josh,

James, and John, as well as past rotation students, for help with my projects and helping me hone my mentoring skills.

The Department of Biological Sciences is a great, supportive place to work. From giant lab meetings, to shared equipment and reagents, to the awesome ladies in the Main and Fiscal offices… thanks for making the past several years go as smoothly as possible! Also, I have made some amazing friends over the course of my graduate career. Former and current graduate students-you are awesome! I will look back fondly at all the fun times.

Debby-thank you for being my best friend. I could have never made it without you. It's rare to find a friend who shares the same quirky sense of humor! I am so lucky to have found that, and so much more, in you. Your constant support, listening ear, shoulder to cry on, and goofy puns are irreplaceable. I'm also so grateful to be a part of Dahlia and Rosella's lives, and I'm looking forward to watching them grow up.

Last, but certainly not least, I want to thank my family. Liz, Mary, and Dan-thank you for your support, loving teasing, and times when we laugh till we cry. Mom and Dad-I would not be where I am today without you. Your support and encouragement have meant the world to me. I am so grateful for everything.

1.0 INTRODUCTION

Cells face an abundance of signals and insults that they must interpret and respond to. Signal transduction pathways are commonly used to relay the signals to generate appropriate responses typically at the level of gene expression. It is critical for the cell to ensure that the proper signaling levels are achieved to respond properly. Understanding how proteins within these pathways are regulated is a key problem in the field. Without appropriate protein regulation, excess or reduced signal can have deleterious effects. *Drosophila melanogaster* is a useful model to study such conserved pathways and monitor how perturbations in signaling can have an effect on the organism. Specifically, our lab is interested in the mechanism of signaling through *Drosophila* Slipper, a member of the Jun N-terminal Kinase pathway.

1.1 SIGNAL TRANSDUCTION

1.1.1 How cells achieve appropriate responses to signals

One way in which organisms respond to cues at the cellular level is by using signal transduction networks. Extracellular signals are transduced through the cell and alter cellular behavior in response to the signal. A common example of a mechanism utilized by cells is a phospho-relay, where kinases are activated to phosphorylate their substrate. Eventually, a transcription factor is

activated, which leads to upregulation of target genes, which are specific for different cellular responses to the signal the cell received.

Signal specificity, strength and duration as well as spatial control of signals can affect the target genes expressed, and thus signal transduction pathways are fine-tuned to generate specific responses through various mechanisms.

1.1.1.1 Signal specificity

Signal transduction pathways are inherently complex, as one kinase can have multiple substrates. For the cell to respond appropriately, the correct proteins must be activated, otherwise inappropriate activation could result in erroneous outcomes. For example, mitogen-activated protein kinase (MAPK) signaling is required for many diverse processes such as apoptosis, differentiation, morphogenesis and homeostasis (Ishibe et al., 2004; Ishibe et al., 2003; Jiang et al., 2011; Park et al., 2002; Park et al., 2001). To achieve such outcomes, MAPK pathways contain multiple proteins that can signal to downstream effectors. For instance, there are numerous MAPKKKs whose activation can lead to signaling through two MAPKs, JNK and p38 (see Figure 1), each with specific outputs. Therefore, it is necessary that the correct MAPK is activated, and one way this is achieved is through scaffolding proteins (Roberts et al., 2007). Scaffolds bind multiple proteins within a signaling pathway to assemble the proteins into a complex to ensure specificity and enhance efficiency. Scaffolds also serve to insulate the pathway by protecting kinases from inactivation or degradation (Locasale et al., 2007). For instance, the JIP family of scaffolds coordinates signals among the Jun N-terminal Kinase (JNK) and p38 pathways (Whitmarsh, 2006). In addition to scaffolds, another way to regulate specificity is differential phosphorylation of common substrates. For example, in *Drosophila*, the MAPKs Bsk and ERK phosphorylate the transcription factor Fos at different sites, resulting

in distinct responses such as tissue closure or neuronal differentiation and wing vein patterning, respectively (Ciapponi et al., 2001).

1.1.1.2 Signal strength and duration

The intensity and temporal control of a signal transduced through the cell also has an effect on signal specificity. That is, sustained signaling can lead to one cellular outcome, whereas a low level of signaling results in a different outcome. An illustration of this phenomenon is in *Drosophila* neuronal cells. Graded activation of the JNK pathway transcription factor complex AP-1 affects axon stability in which sustained signal leads to increased stability whereas milder signaling results in axon overextension, and complete loss of JNK signal directs axon degeneration (Rallis et al., 2010). Another example comes from murine fibroblast cells. The timing and duration of tumor necrosis factor-induced JNK signaling affects cell survival or apoptosis. Short duration signal leads to cell survival, whereas prolonged signal results in apoptosis (Ventura et al., 2006).

1.1.1.3 Spatio-temporal control

Not only does the timing of signaling affect the outcome, but also the subcellular localization of signaling networks can result in distinct cellular responses. One example of proper spatio-temporal control is localization of the MAPK, ERK, to the nucleus to control gene expression (Caunt et al., 2006; Ebisuya et al., 2005; Murphy and Blenis, 2006). The timing of ERK localization is important in determining which genes are transcribed. Immediate-early genes are transcribed upon ERK nuclear translocation, and if ERK persists in the nucleus, other genes will be transcribed. Therefore, the presence of ERK in the nucleus dictates transcriptional output, and the temporal control of that localization determines the specific cellular response.

1.1.1.4 Modes of regulating signal complexity

Proper maintenance or attenuation of protein activation within signaling pathways is critical to generate a specific and controlled response to external stimuli. This elegant control of signal relays is achieved through interplay of various pathways as well as feedback from downstream proteins to generate a specific amplitude and/or duration of signal for the cell to respond properly. Together, this network of protein regulation among many signal transduction pathways within a cell allow it to appropriately respond to the barrage of signals it receives.

Pathway redundancy and crosstalk

It is common for kinases to be involved in signaling of multiple pathways. This is especially true in two stress activated MAPK response pathways, p38 and JNK. These pathways each respond to extracellular stresses, and thus utilize some of the same proteins to achieve proper signaling. For example, the Map Kinase Kinase Kinase (MAP3K) family of Mixed Lineage Kinases (MLKs) signals downstream to p38 in addition to JNK (Gallo and Johnson, 2002; Kim et al., 2004; Tibbles et al., 1996). Another, MAP3K, Tak1, is implicated in both JNK and p38 signaling in response to pro-inflammatory stimuli (Lee et al., 2000; Shim et al., 2005). Additionally, the Map Kinase Kinase (MAP2K) MKK4 has also been shown to activate both JNK and p38 (Derijard et al., 1995). Furthermore, crosstalk occurs within MAPK signaling pathways to integrate signals. For instance, phosphatases that are activated by the p38 and JNK branches of MAPK signaling abrogate Erk signaling to suppress survival cues and promote apoptosis in stressful conditions (Junttila et al., 2008). Thus, crosstalk allows the cell to generate a specific response that perhaps would not be achieved through single linear pathways, but which can contribute to synergy or antagonism within more complex networks to generate precise cellular outputs.

Positive and negative feedback

Feedback is prevalent in MAPK pathways to further regulate signaling. For instance, activated MAPK JNK has been shown to phosphorylate the MAPKKK MLK3 as a means of positive feedback. JNK phosphorylation of the serine within a conserved PXSP motif regulates the distribution and activity of MLK3 (Schachter et al., 2006). The hypophosphorylated form of the protein is reversibly localized to a detergent-insoluble fraction of the cell and is inactive, whereas activation of the pathway and subsequent phosphorylation by JNK maintains a signaling competent pool. Thus, positive feedback from JNK to MLK is critical to amplify the signaling response. In contrast, negative feedback in signaling pathways is commonly achieved by transcriptional induction of an inhibitor of the pathway. In *Drosophila* for example, JNK pathway activity upregulates Puc phosphatase expression, which leads to subsequent dephosphorylation of the *Drosophila* JNK, Bsk, to attenuate JNK signaling (Martin-Blanco et al., 1998). These examples of positive and negative feedback within the JNK pathway are ways in which signaling output is dynamically modulated to maintain a homeostatic environment.

1.1.2 *Drosophila* as a model organism to study signal transduction

The fruit fly, *Drosophila melanogaster*, is a useful model organism to study signaling pathways. *Drosophila* were first utilized in genetic studies by Thomas Hunt Morgan in the early twentieth century. Morgan identified a sex-linked gene, White, through observation of eye color inheritance among a number of generations of *Drosophila* (Morgan, 1910). Since then, flies have traditionally been used for genetic research due to a number of observable phenotypes and ease of genetic manipulation. For instance, *Drosophila* contain transposable elements called P-elements that are used to introduce transgenic DNA into the fly genome (Rubin and Spradling,

1982). Site-specific recombination can be achieved through the Flp-FRT system, which utilizes the Flip recombinase to recombine at specific FRT sites within the genome (Golic and Golic, 1996; Xu and Rubin, 1993). Additionally, overexpression of transgenes is easily achieved through the Gal4-UAS system, which provides spatial and temporal control of ectopic gene expression (Brand and Perrimon, 1993).

The *Drosophila* genome has been sequenced and encodes about 13,000 genes (Adams et al., 2000), which is about half the size of the human genome. Though smaller than the human genome, the *Drosophila* genome shares a high degree of conservation among mammalian genes, and many of the mammalian gene families likely arose from duplications during vertebrate evolution (Gu et al., 2002). Due to the high level of similarity, *Drosophila* are a useful model for human diseases. Of 289 human disease genes studied, 177 have *Drosophila* orthologs (Rubin et al., 2000). This conservation allows for an extensive yet rapid system to investigate the processes of human diseases by taking advantage of *Drosophila* research.

Drosophila have proven to be an excellent model system to study development, during which many signal transduction pathways are active. Thus, the knowledge and tools available from studying *Drosophila* development are conducive to research that investigates the intricacies of signal transduction. A forward-genetics screen performed in 1980 identified genes required for segmentation during *Drosophila* embryogenesis (Nusslein-Volhard and Wieschaus, 1980). Importantly, the genes and signaling pathways identified are highly conserved in mammalian development. Thus the research done in *Drosophila* provides insight into human development.

Drosophila genetics allows researchers to investigate epistasis among signaling proteins to order components and determine their functional dependence. The mechanisms of numerous signaling pathways have been elucidated through *Drosophila* research including the Toll, Hippo,

Wnt (Wingless) Hedgehog, MAPK, and JAK/STAT pathways (Cabrera et al., 1987; De Gregorio et al., 2002; Grusche et al., 2011; Harrison et al., 1998; Mohler, 1988).

Small perturbations in signaling can effectively be assayed in *Drosophila*. For example, the JAK/STAT pathway plays a role in organ and tissue size. This is evident in genetic studies of fly eye development (Luo et al., 1999; Zeidler et al., 1999). Assays that vary the dosage of a negative regulator of STAT result in varying eye sizes; as the amount of JAK/STAT signaling decreases, the size of the eye decreases (Betz et al., 2001). The ability to link signal dosage to eye size has allowed scientists to study how various modifications to this pathway affect eye size, and thus the amount of signal transduced through the JAK/STAT pathway. For example, it was discovered that CycD-CDK4 cooperates with JAK/STAT signaling to regulate eye growth, and a balance of activity is required for proper organ development (Chen et al., 2003).

1.1.3 MAPK signaling

Mitogen-Activated Protein Kinase (MAPK) pathways are a family of highly conserved signal transduction pathways that respond to various signals to regulate many cellular processes during development and throughout an organism's life to maintain homeostasis (Derijard et al., 1995; Rossomando et al., 1989). MAPK signaling is comprised of the extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 pathways (Figure 1). Each MAP kinase is a serine/threonine kinase that contains a canonical TXY dual phosphorylation motif (Canagarajah et al., 1997; Payne et al., 1991). Though the MAP kinases share sequence similarity, they are activated by different upstream proteins and generate unique outcomes.

The Erk pathway responds to extracellular growth factors to control cell division and differentiation (Brunet et al., 1999; Yao et al., 2003). Unlike Erk, the JNK and p38 pathways are

stress-activated pathways, responding to stresses such as UV radiation, high osmolarity, heat shock, X-rays and reactive oxygen species, and inflammatory cytokines. JNK signaling is responsible for cell morphogenesis (Noselli and Agnes, 1999), immune response (Park et al., 2004; Sluss et al., 1996), apoptosis (Lin, 2003; Shaulian and Karin, 2002), and cell proliferation (Shaulian and Karin, 2001), while the p38 pathway contributes to cell proliferation (Khiem et al., 2008; Maher, 1999), inflammatory response (Lee et al., 1994), and apoptosis (Bulavin et al., 1999).

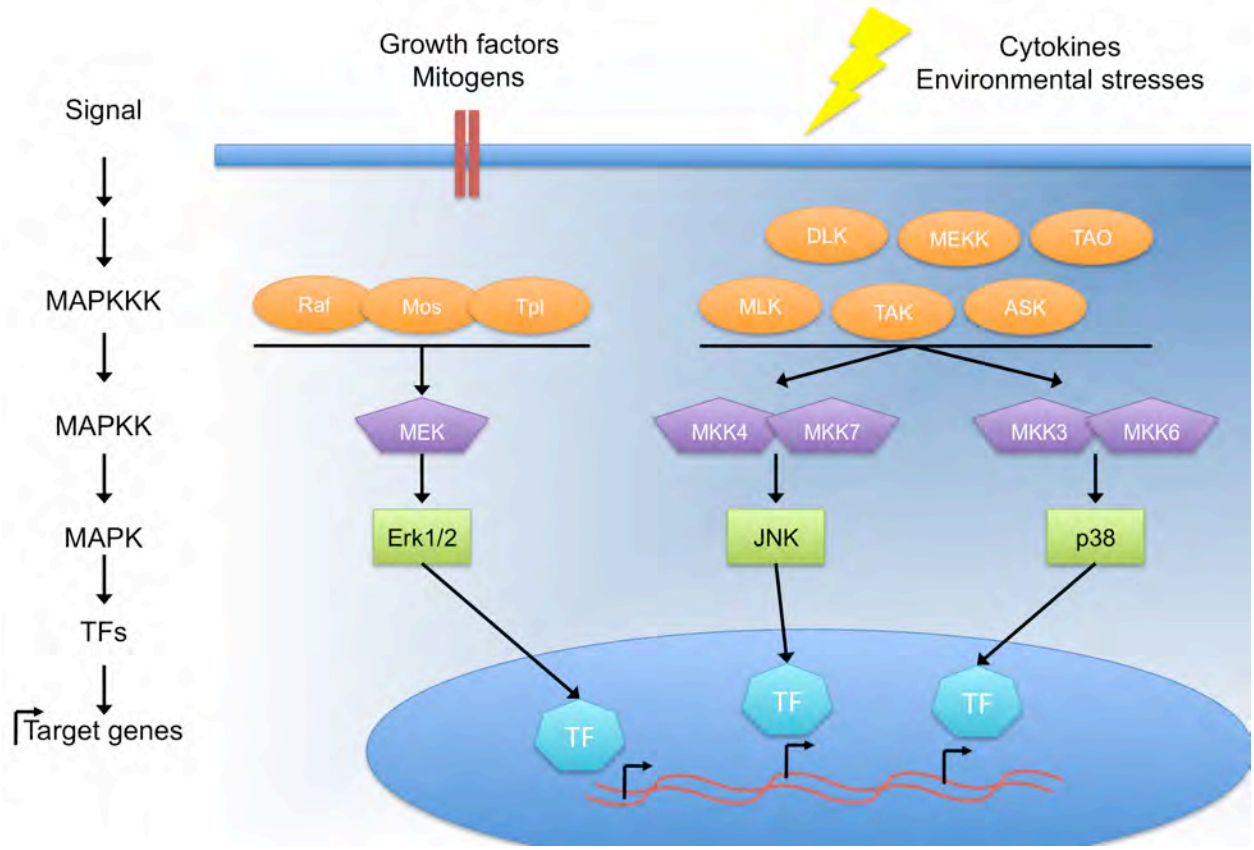

Figure 1: MAPK signaling pathways

The three main MAP kinase signaling pathways are depicted. Each pathway receives a signal and transmits that signal through MAPKKKs and MAPKKs before activating the MAP kinase. Once the MAPK is activated, it in turn activates transcription factors, which regulate the expression of specific genes based on the pathway and signal received.

1.1.3.1 The core MAP Kinase signaling pathways

ERK1/2

ERK1/2 signaling is linked to cell motility, proliferation, differentiation, and survival, and thus tight control of the pathway must be maintained, as ERK pathway members are known oncogenes (Chen et al., 2001; Dhillon et al., 2007; Johnson and Lapadat, 2002; Lewis et al., 1998; Raman et al., 2007; Yoon and Seger, 2006). Receptor tyrosine kinases, ion channels, G-protein coupled receptors, and integrins are stimulated by a variety of cues such as growth factors and mitogens. Adaptor proteins transduce the signal to a GTPase (eg. Ras), which then activates a MAP3K. Raf, Mos, and Tpl are common MAP3Ks that are responsible for activating MEK, the MAPKK. This leads to ERK1/2 activation, which promotes transcription factor activation and subsequent target gene expression (Figure 1) (McKay and Morrison, 2007).

Rolled (Rl) is the *Drosophila* ERK (Biggs et al., 1994; Brunner et al., 1994). Upon activation by the upstream MAPKK, D-MEK, Rl translocates to the nucleus to regulate target gene expression. The involvement of ERK signaling in the development of the *Drosophila* eye has been well studied. Communication between two photoreceptors within each ommatidium via ERK signaling lends to proper eye development. A ligand of the pathway is presented on one photoreceptor, which interacts with a receptor-tyrosine kinase, inducing signaling through Rl (Yamamoto, 1994). Further analysis of ERK signaling in *Drosophila* reveals a role in cell fate during head development, cell proliferation, apoptosis and cell migration (Bergmann et al., 1998; Carmena et al., 1998; Dickson et al., 1992; Kurada and White, 1998).

JNK

The Jun N-terminal Kinase (JNK) pathway can respond to developmental cues as well as extracellular stresses to regulate diverse cellular responses including apoptosis, control of lifespan, wound healing, inflammation, and cell proliferation in many multicellular organisms. There are three mammalian JNK genes, JNK1, JNK2, and JNK3, whose primary target is cJun (Pulverer et al., 1991; Yang et al., 1997), which dimerizes with Fos forming the AP-1 transcription factor. Upstream of JNK are the MAPK kinases MKK4 and MKK7 (Lawler et al., 1998; Tournier et al., 1999). Pathway complexity increases at the level of the MAPK kinase kinase. There are numerous JNKKKs that can activate JNK signaling including MLKs, TAK1, TAO, MEKK, ASK, and DLK (Figure 1). The specific signals and transducers to particular JNKKKs are not well understood, though some MAP4Ks have been identified in JNK signaling such as NIK and PAK4 (Davis, 2000; Su et al., 1997).

In *Drosophila*, a single JNK, Basket (bsk), is activated by the MAP2Ks Hemipterous (Hep) and Mkk4 (Glise et al., 1995; Yang et al., 1997). There are several MAP3Ks that correspond to the mammalian pathway members and act upstream in the pathway. These are Mekk1, Ask1, Wallenda (dDLK), Tak1, Takl2, and Slipper (dMLK) (Stronach, 2005). JNK signaling in *Drosophila* controls development, morphogenesis, and stress responses. For instance, JNK pathway activity has been implicated in embryonic dorsal closure, adult thorax closure, nervous system development, wound healing, and immune response as well as controlling apoptosis and cell proliferation (Adachi-Yamada et al., 1999a; Agnes et al., 1999; Glise and Noselli, 1997; Noselli, 1998; Noselli and Agnes, 1999; Riesgo-Escovar et al., 1996; Sluss et al., 1996; Zeitlinger and Bohmann, 1999).

p38

The p38 branch of MAPK signaling was first identified in hyperosmotic yeast as the HOG1 pathway (Brewster et al., 1993), but it is clear that p38 signaling is evolutionarily conserved in plants and animals to respond to diverse environmental stresses such as heat stress, oxidative stress, and immune stimulation (Chang and Karin, 2001; Jiang and Song, 2008; Shinozaki and Yamaguchi-Shinozaki, 1997; Torres and Forman, 2003). p38 signaling is transduced by the MAPK kinases MKK3 and MKK6. Upstream, p38 shares many of the same MAPKKKs as JNK signaling (Figure 1).

While mammals have four p38 genes, p38α, p38β, p38γ, and p38δ, the *Drosophila* genome encodes three p38 MAPK genes, *p38a (Mpk2), p38b* and *p38c*, which are activated by the MAPK kinases Mkk3/*licorne* and Mkk4 (Han et al., 1998a; Han et al., 1998b). Further upstream, two *Drosophila* MAPK kinase kinases, Tak1 and Mekk1, have been implicated in p38 signaling (Inoue et al., 2001; Moriguchi et al., 1996). p38 pathway mutants have few developmental defects, however individual members are sensitive to various stresses such as high osmolarity, heat shock, oxidative stress, UV radiation, and immune stimulation (Craig et al., 2004).

1.1.3.2 JNK and p38 in stress response

The stress-activated MAPK pathways, JNK and p38, are highly conserved transducers of cellular information in response to a variety of distinct signals. Cumulative data show that these pathways are used during development, yet they must also be inducible to reestablish homeostasis in an unstable environment (Davis, 2000; Ono and Han, 2000).

The JNK pathway responds to UV and gamma irradiation, heat stress, free oxygen radicals, hyperosmolarity and inflammatory cytokines in mammals (Gotoh and Cooper, 1998; Kasibhatla et al., 1998; Kyriakis and Avruch, 2001; Raingeaud et al., 1995). *Drosophila* JNK signaling has been linked to innate immunity, UV radiation, osmotic stress, and oxidative stress. Another stress response pathway, p38, is required for similar functions in *Drosophila* such as immunity, osmotic shock and oxidative stress (Han et al., 1998a; Han et al., 1998b). Additionally, p38 is implicated in heat shock response and starvation (Craig et al., 2004).

Misregulation of stress signaling can have deleterious effects. Without proper control of the stress response pathways, numerous diseases such as neurodegenerative disorders, immune deficiencies, cancer and metabolic diseases such as diabetes result (Manning and Davis, 2003; Zarubin and Han, 2005). Therefore, accurate maintenance of signaling, especially during highly dynamic stress signaling, is critical.

1.2 KINASE ACTIVATION

1.2.1 Steps required for kinase activation

Kinases require reversible activation, which serves to control the amount of signal a cell receives. Activation is achieved through protein interactions and modifications of the kinase, primarily phosphorylation. A highly orchestrated step-wise activation is common among many kinases. A classic example is the activation of human Src tyrosine kinase. The inactive version of Src is held in a closed conformation due to intramolecular interactions between its SH3 domain and a proline-rich sequence near the SH2 domain (Sicheri and Kuriyan, 1997; Sicheri et

al., 1997; Young et al., 2001). This autoinhibition is relieved through interactions with proteins that bind to its SH3 domain to disrupt its interaction with the polyproline region (Arias-Salgado et al., 2003; Luttrell et al., 1999; Moarefi et al., 1997; Sun et al., 2002). The protein, in its open confirmation, it can trans-autophosphorylate a residue within the Src activation loop (Cooper and MacAuley, 1988; Harrison, 2003; Sun et al., 2002; Yamaguchi and Hendrickson, 1996; Young et al., 2001) and become fully active, whereby it can bind and phosphorylate its downstream substrates.

The steps required for activating the MAPKKK MLK, have been elucidated and are similar to Src activation (Figure 2). The N-terminal SH3 domain is required for protein-protein interactions and autoinhibition. It binds intramolecularly to a downstream proline-rich region between its leucine zipper and CRIB domains (Zhang and Gallo, 2001). Upon GTPase binding to the CRIB domain, intramolecular binding is disrupted and MLK activation is initiated (Bock et al., 2000; Burbelo et al., 1995). Following the relief of autoinhibition, the protein homodimerizes at the leucine zipper domain (Vacratsis and Gallo, 2000). Next, the catalytic kinase domain containing an activation loop is phosphorylated through both autophosphorylation and by upstream kinases (Du et al., 2005). The protein is now active and can phosphorylate its substrates to perpetuate MAPK signaling.

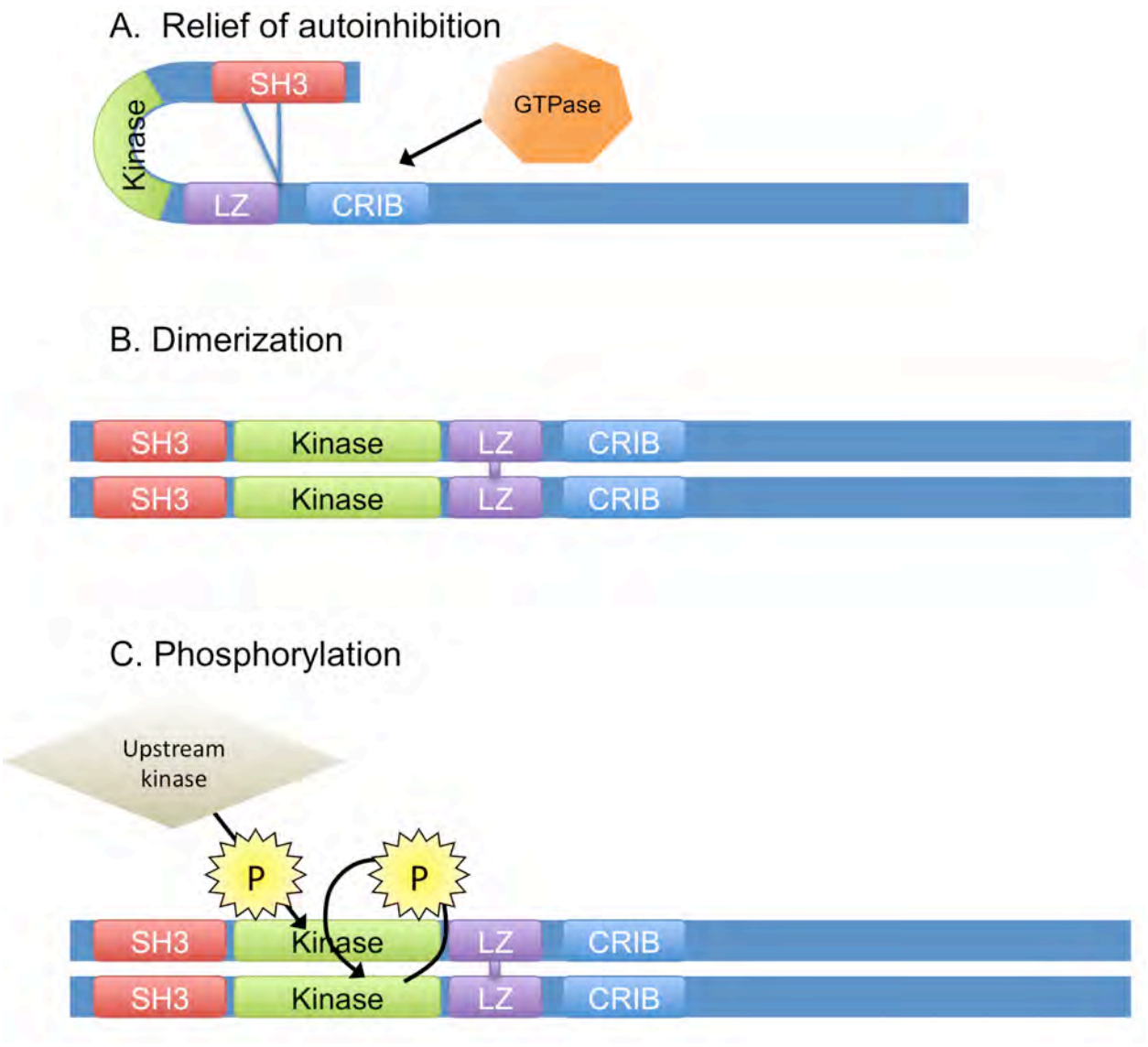

Figure 2: Model of MLK activation

The steps of MLK activation are displayed, with the first step being the relief of autoinhibition by binding of a GTPase to the CRIB domain (A). This is followed by homodimerization (B) and subsequent phosphorylation, both through autophosphorylation and phosphorylation by upstream kinases (C).

1.2.2 Activation segment

The activation segment of the kinase domain is critical for protein activation. The 20-35 residue sequence is flanked by two conserved motifs (DFG…APE) and contains the magnesium binding loop, the activation loop, and the P+1 loop (Figure 3) (Nolen et al., 2004). The magnesium-binding loop is responsible for binding the N-terminus of the activation segment and chelating a magnesium that positions a phosphate for phosphotransfer (Adams, 2001). C-terminal anchoring begins in the middle of the P+1 loop, where the kinase binds its substrate (Brown et al., 1999; Hubbard, 1997; Madhusudan et al., 1994). This leaves a flexible activation loop, and its conformational change upon phosphorylation results in protein activation. Without proper activation loop positioning, the kinase would not be able to bind and phosphorylate its substrate. Thus, phosphorylation within the activation loop is required for kinase activation.

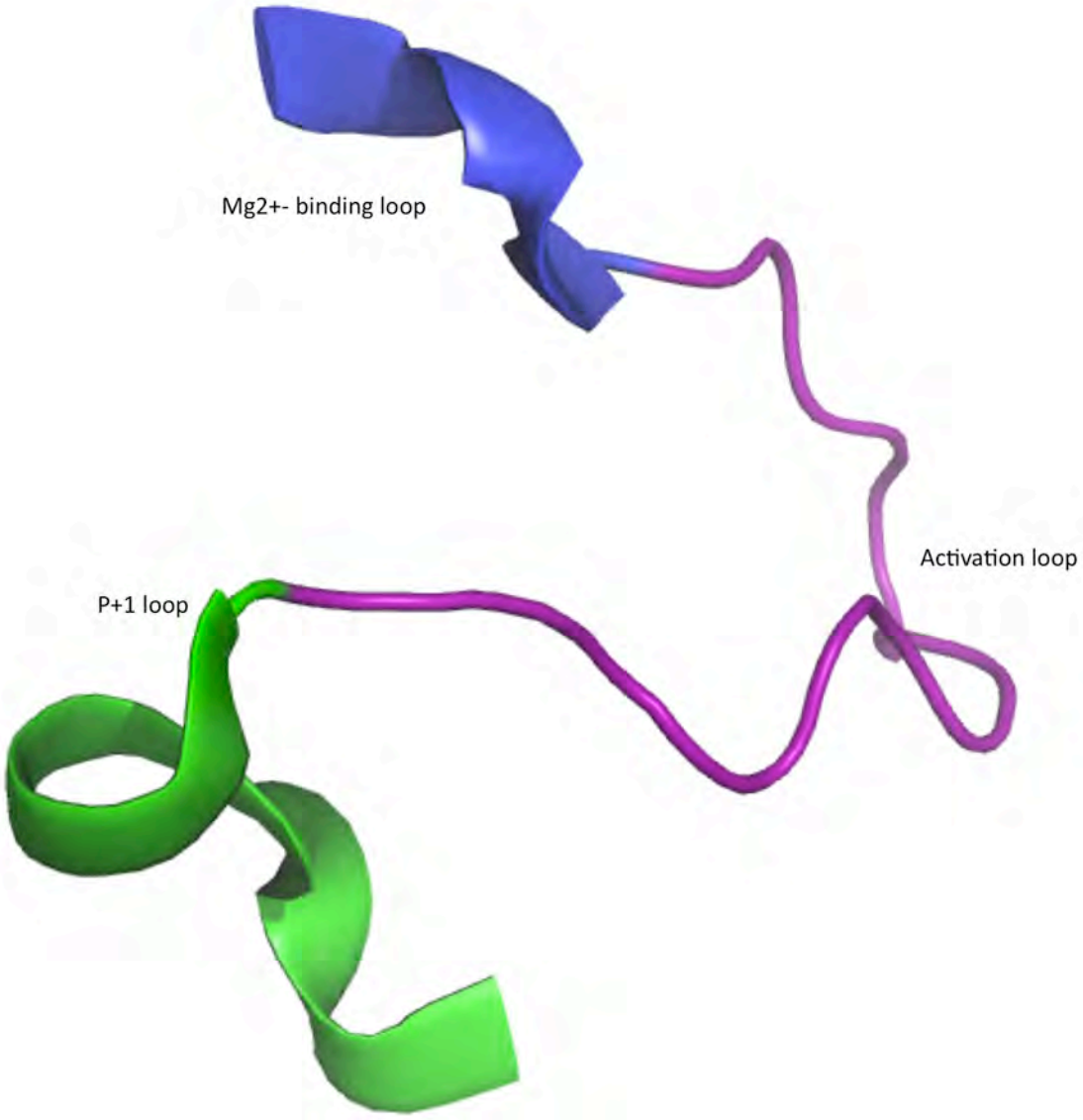

Figure 3: Activation segment of kinase domains

The activation segment of protein kinases contains three main loops: the magnesium-binding loop (blue), the activation loop (magenta), and the P+1 loop (green). Shown here is the kinase domain of CDK2. Though unrelated to MLK, the kinase domain is similar among kinases.

1.3 *DROSOPHILA* SLIPPER

1.3.1 Identification and role in dorsal closure

During embryogenesis, *Drosophila* embryos have a large dorsal hole that is covered by a transient tissue called the amnioserosa. As embryogenesis progresses, the two lateral sheets of epithelial cells migrate and fuse at the dorsal midline in a process called dorsal closure. The first row of epithelial cells, termed the leading edge, elongate and contain an actin cable which serves as a "purse string" to aid in the epithelial movement (Edwards et al., 1997; Jacinto et al., 2000; Kiehart et al., 2000). The JNK pathway is activated in the leading edge cells to coordinate their activity, and loss of this signaling results in a loss of cell elongation and actin cable formation (Glise et al., 1995; Riesgo-Escovar et al., 1996). Ultimately, JNK mutant embryos fail in dorsal closure, signified by a dorsal open phenotype.

Among the numerous JNK kinase kinases in *Drosophila* is the MLK homolog, Slipper (Slpr). Slpr was first identified as the JNKKK required for the JNK-mediated process of dorsal closure (Stronach and Perrimon, 2002). *slpr* mutants are embryonic lethal with a dorsal open phenotype that is typical of a failure of dorsal closure. Moreover, the JNK target genes, *puc* and *dpp*, are absent at the leading edge in such mutants (Stronach and Perrimon, 2002). This evidence suggests that Slpr is required for JNK signaling during dorsal closure.

The N-terminal half of Slpr shares up to 56% sequence identity with mammalian MLK proteins over four conserved functional domains (Figure 4) (Stronach and Perrimon, 2002). At the N-terminus of the protein is the SH3 domain, which is required for protein-protein

interactions. This is followed by the catalytic kinase domain. Immediately downstream of the kinase domain is the leucine zipper region, required for dimerization. Finally, the CRIB (Cdc42/Rac Interacting Binding) motif is a site for GTPase-mediated protein activation. Based on homology to MLK proteins, we can infer roles for Slpr in JNK signaling and for mechanisms of Slpr activation. *Drosophila* is a useful model to study Slpr signaling because we can use a wealth of genetic tools to examine small or large perturbations in signaling, which are evident and easily observed in this organism.

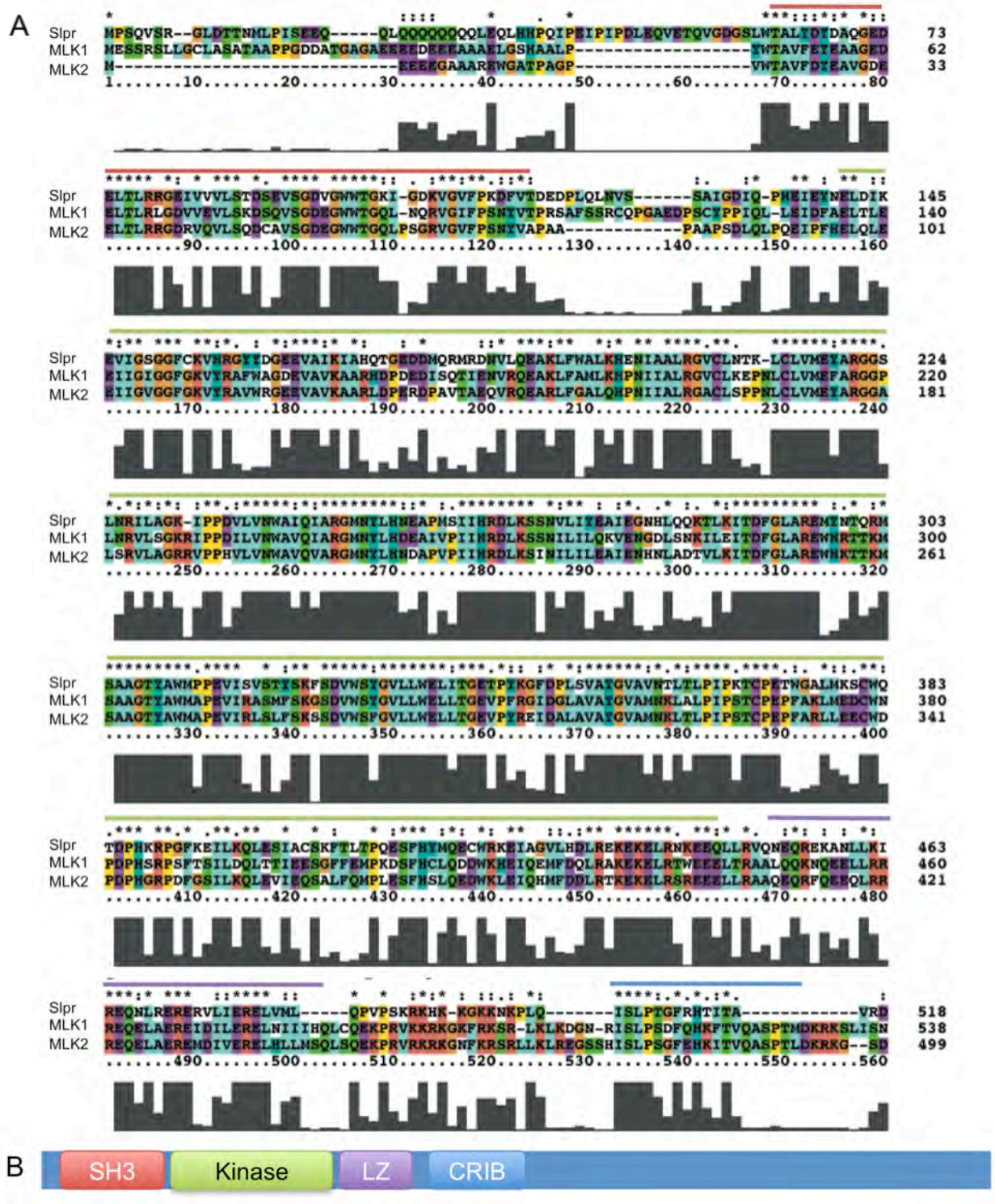

Figure 4: Slpr sequence alignment and domain architecture

A. Slpr shares sequence identity with mammalian MLK proteins; shown is the alignment of Slpr's N-terminal half with human MLK1 and MLK2 (sequences aligned using ClustalX). Consensus is indicated as follows: (*) indicates identical residues, (:) indicates conserved substitutions, and (.) indicates semi-conserved substitutions. Lines above alignment correspond to the domain color in the schematic in (B).

1.3.2 Genetic analysis of Slpr signaling in morphological events

The JNK pathway is required for a number of morphological events during development in addition to its role in dorsal closure, and it has been shown that Slpr mediates JNK signaling in many of these events. Slpr mutant adults display a host of defects consistent with a loss of JNK signaling (Polaski et al., 2006). For instance, thorax closure, in which the two wing imaginal discs fuse during pupation to form the adult thorax, is a JNK-dependent process such that loss of JNK signaling results in a cleft thorax in the adult fly (Agnes et al., 1999; Zeitlinger and Bohmann, 1999). Indeed, *slpr* mutants display a cleft thorax indicative of a loss of JNK signaling. Additionally, proper rotation of the adult male genitalia is regulated by JNK signaling (Macias et al., 2004; McEwen and Peifer, 2005). *slpr* mutants have defects in male genital rotation and eversion, linking Slpr to this JNK-mediated process.

In addition to mutant analysis, Slpr transgene overexpression reveals a role in JNK-mediated processes. Initial experiments confirmed that Slpr lies upstream of JNK in dorsal closure by upregulation of the JNK target gene, *puc*, and a puckered phenotype indicative of upregulated JNK signaling upon SlprWT overexpression (Polaski et al., 2006). Expression of kinase dead versions of the protein results in a dominant negative phenotype in many assays such as loss of *puc* expression in the ectoderm, dorsal open embryos, and cleft thorax in adults (Garlena et al., 2010b; Polaski et al., 2006). To analyze the role of each domain of Slpr, transgenes encoding Slpr's domains were tested in structure/function studies. The SH3 domain was found to inhibit Slpr, presumably through binding a PVP motif between the leucine zipper and CRIB motifs. Also, the C-terminus is essential for proper subcellular localization. Finally,

genetic and biochemical analysis demonstrate that both Rac and Msn interact with Slpr and are upstream in JNK signaling (Garlena et al., 2010b). By studying how the Slpr molecule behaves *in vivo*, it is clear that tight control of the protein is required, and this control is regulated at numerous levels such as intramolecular inhibition to prevent aberrant signaling, proper spatial distribution, and upstream signal-responsive activators binding to stimulate Slpr.

1.3.3 A role for Slpr in stress response?

A role of Slpr in development and morphogenesis has been established. However, less is known about whether Slpr is required for stress response. A recent report links Slpr to p38 signaling in response to microbial insult (Chen et al., 2010). There is more evidence in mammals that MLKs play a role in stress response such that MLKs mediate metabolic-, cytokine-, and ceramide-induced MAPK signaling (Jaeschke and Davis, 2007; Korchnak et al., 2009; Sathyanarayana et al., 2002). Therefore, it is possible that Slpr plays a role in such responses in *Drosophila* in addition to its requirement during development.

1.4 AIMS OF DISSERTAION RESEARCH

By understanding the mechanisms involved in regulating kinases, we gain insight into the complexity of the tight control of signal transduction the cell maintains to generate a specific response to an array of signals. To this end, my dissertation research has focused on the hypothesis that phosphorylation regulates Slpr function in different signaling contexts.

My first aim was to characterize if phosphorylation within the kinase domain regulates Slpr activity. We hypothesized that phosphorylation of conserved residues within the activation loop are required for full activation of the protein. Transgenic flies that express mutations in these conserved residues were utilized in rescue assays, analysis of JNK signaling during dorsal closure, and observation of their effects during thorax closure.

The second aim of my dissertation work was to identify and characterize additional post-translational modifications regulating Slpr function. I identified the serine within a conserved PXSP consensus site as a residue phosphorylated in response to heat stress. I then tested whether phosphorylation at this site was required for known Slpr-mediated functions and tested its effects in stress response by using non-phosphorylatable and phospho-mimetic versions of Slpr.

2.0 MATERIALS AND METHODS

2.1 FLY STOCKS

All stocks were obtained from Bloomington Stock Center unless noted otherwise. w^{1118} was used as a control genotype. *UAS-p38b*^{DN} (Adachi-Yamada et al., 1999b), *Mpk2*^{1} (*p38a* null) (Craig et al., 2004), *UAS-bsk*^{K53R} BL#9311, *hep*^{1} (Glise et al., 1995), *UAS-hep.CA* BL#6406, *slpr*^{BS06}, *Tak1*^{2527} (Polaski et al., 2006), *lic*^{GG01785} BL#19989, *puc*^{E69} *(puc-lacZ) (Ring and Martinez Arias, 1993)*

Gal4 lines: *arm-Gal4 (P{w*$^{+mW.hs}$ *= GAL4-arm.S}11)* BL#1560, *pnr-Gal4 (P{w*$^{+mW.hs}$ *= GawB}pnr*^{MD237}*)* BL#3039, *69B-Gal4 (P{w*$^{+mW.hs}$ *=GawB}69B)* BL#1774, *elav-Gal4 (P{w*$^{+mW.hs}$ *= GawB}elav*^{C155}*) BL#458*

2.2 SLPR TRANSGENES AND GENETIC RESCUE

UAS-SlprAAA, *UAS-SlprAAT*, *UAS-SlprTAA*, and *UAS-SlprASA* were created using site-directed mutagenesis by overlap extension (Ho et al., 1989). Overlapping primers incorporated an alanine in place of T287, S291, and/or T295. *UAS-SlprPXAP* and *UAS-SlprPXEP* were created in a similar manner, but with an alanine or glutamic acid codon in place of serine 512 codon. The

mutant segment was subsequently swapped with the analogous segment of *UAS-SlprWT* (Garlena et al., 2010b) in the UASp vector backbone. Transgenic lines were established after injection of DNA by Genetic Services, Inc. (Sudbury, MA). *UAS-SlprWT* has been described (Garlena et al., 2010b). For rescue of *slpr* mutants to adulthood, we crossed y^{93j} w^{1118} $slpr^{BS06}$*/FM7;arm-G4* females by w^{1118}*/Y;UAS-slpr-Tg* males and raised the progeny at 21±0.5°C for moderate transgene expression. Among the progeny, mutant and *FM7* males were counted to quantify relative eclosion rate as an indication of transgene rescue. To avoid inadvertently including non-*FM7* males that arise from non-disjunction of the maternal X chromosome, we counted only $yellow^-$, non-*FM7* males. A minimum of three transgenic lines of each genotype was used for the rescue experiments.

2.3 THORAX DEVELOPMENT ASSAY

Slpr transgenes were overexpressed using *pnr-Gal4*, and the progeny of this cross were raised to adulthood at 25°C. At least 50 adults were scored based on severity of thorax cleft and imaged with a Leica DC300F camera mounted on an MZ16 setero microscope.

2.4 *IN VITRO* TRANSCRIPTION, TRANSLATION, AND LABELING

slpr DNA sequences were cloned into the pcDNA3.1 vector and the T7 promoter was used for transcription initiation. ^{35}S-methionine-labeled proteins were produced *in vitro* using the TNT

coupled rabbit reticulocyte or wheat germ transcription/translation systems (Promega) according to manufacturer's instructions. Labeled proteins were analyzed by SDS-PAGE and autoradiography. To achieve separation of the SKLC bands, a 10% gel was loaded and run at 70V until the samples entered the separating gel, at which point the voltage was increased to ~150V.

2.5 PHOSPHATASE TREATMENT

In vitro translated protein was incubated with λ-phosphatase at 30°C for 90 minutes in PMP buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 % Brij 35) supplemented with 1mM $MnCl_2$ (New England Biolabs). Proteins were subsequently visualized using SDS-PAGE and autoradiography, and dephosphorylation was detected by shifts in electrophoretic mobility.

2.6 MAPK INHIBITORS

JNK (SP600125), p38 (SB203580), and MEK (U0126) inhibitors were obtained from Calbiochem. Each was prepared in DMSO and used at a final concentration of 50μM when added to the *in vitro* translation reactions.

2.7 IMMUNOFLUORESCENCE

Immunofluorescence of embryos was performed as described (Rothwell and Sullivan, 2000). Affinity-purified rabbit anti-Slpr (Polaski et al., 2006) was used at 1:400 and mouse anti-fasciclin III (7G10, DSHB) was used at 1:40. Secondary antibodies (goat anti-rabbit TxRed and goat anti-mouse FITC; Jackson ImmunoResearch Laboratories, Inc.) were used at 1:200. Images were captured using laser scanning confocal microscopy (Biorad Radiance 2000) on a Nikon E800 compound microscope and assembled in Photoshop.

2.8 HEAT SHOCK

For adult heat shock, 30-50 flies were collected in vials with food, aged three to five days, and placed in a 37°C circulating water bath. At each time point, the vials were individually removed from the water, gently tapped on the side of the vial, and the flies were scored according to their response. Flies were scored as either immobile (not moving but responsive when the vial was tapped) or unresponsive. The vial was then immediately returned to the water bath. For embryo heat shock experiments, overnight egg collections were aged three hours and then placed in a 37°C dry incubator for three hours. After an additional 24 hours at 25°C, the number of dead embryos was recorded. Three trials with at least 100 adults or embryos were used for each heat shock experiment and statistical significance was determined with the Student's T-test.

2.9 WESTERN BLOTS

Unless otherwise noted, western blots were performed on embryonic lysates. Overnight collections of embryos expressing the transgenes were dechorionated and homogenized in 2X Sample Buffer (100mM Tris pH6.8, 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Lysates from heat shocked embryos were prepared in the same manner after a three-hour heat treatment at 37°C. Homogenates were boiled for 7 minutes and loaded onto a 10% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membrane and Western immunoblots were performed using the SNAP ID system (Millipore). Mouse anti-HA (16B12, Covance) and mouse anti-β-tubulin (DSHB) were diluted to 1:300 and 1:90, respectively, in 3ml of 0.2% milk/TBST block. Sheep anti-mouse HRP secondary antibody (Amersham) was used at 1:3,000.

2.10 LIMITED PROTEOLYSIS

Embryonic lysates that were heat shocked for one hour were prepared as described above, however the protease inhibitors were omitted in these samples. The lysates were then incubated at room temperature. For each timepoint, an aliquot of the lysate was taken, mixed with protease inhibitors, and placed in the freezer. Samples were analyzed by SDS-PAGE and Western immunoblot (see above). Anti-Slpr antibody (Polaski et al., 2006) was used at 1:180 in 3ml of 0.2% milk/TBST block. Donkey anti-rabbit HRP secondary antibody (Amersham) was used at 1:3,000.

2.11 2D GEL ELECTROPHORESIS

To analyze transgenic protein by two-dimensional gel electrophoresis, embryonic lysates were prepared in Phosphosafe buffer (Novagen) or 1X PMP buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01 % Brij 35) supplemented with 1mM $MnCl_2$ (New England Biolabs). 100µg of protein was sent to the Kendricks lab (Madison, WI), and subsequent blots of the 2D gels were received back for blotting. Western blots were performed as above.

2.12 CHX TREATMENT

S2 cells were incubated at 21°C. Cyclohexamide (CHX) was added (250 µg/ml) and approximately the same number of cells were isolated at each time point. Lysates were made and endogenous Slpr was visualized by Western blot.

2.13 RT-PCR

mRNA was isolated from embryos using TRI reagent (Applied Biosciences). Briefly, embros were homogenized in the solution and centrifuged to remove lipids and proteins. The mRNA was isolated via cholorform extraction and used for RT-PCR. The SuperScript system (Invitrogen) was used for cDNA synthesis using random primers for first strand synthesis. Standard PCR was then performed using gene-specific primers.

3.0 PHOSPHORYLATION WITHIN THE ACTIVATION LOOP OF SLPR

This chapter is adapted from published material in *Journal of Cell Science*, Volume 123, pages 3177-3188 (Garlena et al., 2010b).

3.1 INTRODUCTION

A tightly controlled multi-step mechanism is often necessary for full protein kinase activation (Nolen et al., 2004). The mechanism of mammalian MLK activation has been elucidated, and each step of activation as been established. MLK is autoinhibited through binding of its N-terminal SH3 domain to a downstream proline. Upon relief of autoinhibition by a GTPase binding to the adjacent CRIB domain, the protein homodimerizes and autophosphorylates at a residue in its activation loop. It is also phosphorylated at a second activation loop residue by an upstream kinase (Bock et al., 2000; Leung and Lassam, 2001; Vacratsis and Gallo, 2000; Zhang and Gallo, 2001). This renders the protein active and able to phosphorylate its substrates. Based on the homology between Slpr and MLKs, we predicted that Slpr would be activated in a similar manner. We want to elucidate the mechanisms of Slpr activation to gain an understanding of how Slpr behaves in the context of a multi-cellular organism. Through knowledge of Slpr activation, we can generate tools (ex. phospho-specific antibodies) that are useful in experiments testing Slpr signaling dynamics in various contexts.

Structure/function analysis of the main domains of Slpr reveals properties of Slpr that correspond to MLK activation (Garlena et al., 2010b). Slpr autoinhibition is apparent through the use of two constructs. First, overexpression of a transgene that lacks the SH3 domain, Δ-SH3, results in hyperactive JNK signaling above that of SlprWT in the leading edge during dorsal closure, suggesting that the SH3 domain normally provides some inhibitory function. To address whether this inhibition is through intramolecular interaction, a second construct was made in which two proline residues between the leucine zipper and CRIB domains, which corresponds to SH3 binding in MLK proteins, were mutated to alanine (SlprAVA) to prevent SH3 binding. Transgenic animals overexpressing SlprAVA display phenotypes that correspond to the highest level of JNK signaling of all transgenes tested. These data suggest that the SH3 domain of Slpr binds a downstream PVP motif to inhibit Slpr signaling. To relieve autoinhibition, it was hypothesized that a GTPase binds the CRIB domain to initiate activation. Indeed, biochemical and genetic assays reveal a role for Rac in Slpr activation (Garlena et al., 2010b).

The final step in protein activation is phosphorylation (Nolen et al., 2004). Within the kinase domain of Slpr are three residues that we hypothesized could be phosphorylated through autophosphorylation or phosphorylation by an upstream kinase. Transgenic mutants were used to assay the requirement of phosphorylation for Slpr-mediated JNK signaling. By elucidating the residues within the activation loop that are phosphorylated upon activation, we can better understand dynamic signaling in various contexts through phospho-specific antibodies to identify and observe an “active” pool of Slpr kinase.

3.2 ALANINE MUTATIONS WITHIN THE ACTIVATION LOOP REVEAL POTENTIAL PHOSPHORYLATION SITES

Phosphorylation within the activation loop is required for maximum catalytic activity (Nolen et al., 2004). The activation loop is identified by two flanking amino acid sequences (DFG…APE) that are separated by 20-35 amino acids. Within this region, there are serine and/or threonine residues that are phosphorylated resulting in a conformational change, making the protein active (Nolen et al., 2004). Within Slpr's activation loop, there are three conserved candidate residues that are phosphorylated in various combinations in homologous MLK proteins; T287, S291, and T295 (Figure 5B). To determine if these residues are required for Slpr function, a non-phosphorylatable mutant was made in which all three of these sites were changed to alanine (*SlprAAA*) (Figure 5C). If phosphorylation of these residues is required for Slpr function, then preventing phosphorylation would render the protein catalytically inactive. Transgenic flies were generated to assay the requirement of activation loop phosphorylation in normal Slpr-dependent processes.

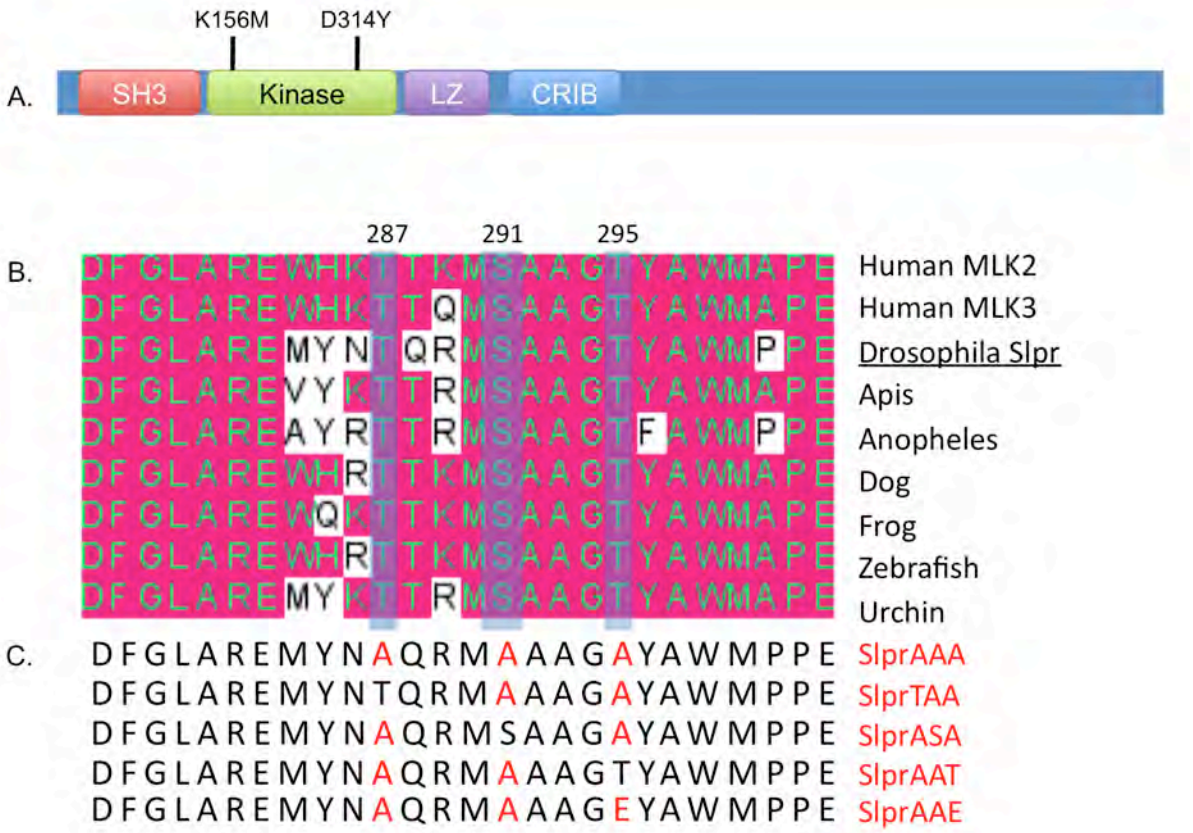

Figure 5: Activation loop alignment and mutations

A. Schematic of SlprKD mutations. SlprKD contains two mutations, each outside of the activation segment. The mutation at K156 is a traditional "kinase dead" mutation in which phosphate transfer is abolished. The D314Y mutation is at an invariant residue that renders the protein inactive. B. Alignment of MLK activation loops from various species; *Drosophila* MLK (Slpr) is underlined. The conserved putative phosphorylated threonine and serine residues are highlighted. C. Alanine mutations generated in Slpr's activation loop in the context of the full-length protein. Mutated residues are in red.

Rescue assays were used to test the requirement of Slpr phosphorylation. First, the ability to rescue *slpr* mutants was investigated. We hypothesized that if activation loop phosphorylation is required for Slpr activation, then ubiquitously overexpressing SlprAAA in a mutant background would not rescue the mutants. Three mutant backgrounds were tested, $slpr^{BS06}$, $slpr^{921}$, and $slpr^{3P5}$, and no adults were recovered in any of these backgrounds compared to overexpressing SlprWT-HA, which recovers between 20-50% of adults (Figure 6, Table 1) (Garlena et al., 2010b). This shows that the non-phosphorylatable *SlprAAA* is inactive in these flies.

Due to the inability of *SlprAAA* to rescue, we wanted to confirm that this was a result of a loss of JNK pathway activity in the developing embryo. *SlprAAA* was overexpressed within the *pnr* domain (dorsal third of the embryo) in embryos with a JNK pathway reporter, puc-lacZ, in the background. JNK activity is evident in the leading edge of wildtype flies (Figure 7F) and overexpressing SlprWT leads to an expansion of JNK signaling beyond the leading edge (Figure 7A'). However, embryos expressing *SlprAAA* lost β-gal staining (Figure 7 B'), suggesting that *SlprAAA* is a dominant negative form of Slpr that blocks JNK pathway activation during dorsal closure. Consistent with the notion that *SlprAAA* behaves as a dominant negative, we observed dorsal-open embryos when *SlprAAA* was overexpressed within the *pnr* domain (Figure7 B) (Garlena et al., 2010b). These data indicate that SlprAAA blocks JNK signaling in the leading edge during dorsal closure.

JNK signaling is required for thorax closure (Agnes et al., 1999; Zeitlinger and Bohmann, 1999), and loss of JNK signaling results in a failure of proper thorax closure, indicated by a cleft in this tissue. Previous experiments implicate Slpr in this process (Polaski et al., 2006). Overexpression of SlprWT results in narrowing of the scutellum, tissue just posterior to the

thorax, and loss of scutellar bristles. Conversely, overexpressing a kinase-dead version of the protein that is mutated in a conserved lysine to prevent phosphate transfer ("SlprKD"; K156M, Figure 5A) results in a cleft thorax. In addition to the phenotypic effects during dorsal closure, we observe *SlprAAA* behaving as a dominant negative in thorax closure as expression results in a cleft thorax in adults (Figure 8). These data suggest that *SlprAAA* blocks JNK signaling in Slpr-mediated thorax closure.

Table 1: Transgenic rescue of *slpr* mutants to adulthood.

*slpr**/FM7; *arm-Gal4* X UAS-Tg	$slpr^{BS06}$/Y	FM7/Y	Relative eclosion	$slpr^{921}$/Y	FM7/Y	Relative eclosion	$slpr^{3P5}$/Y	FM7/Y	Relative eclosion
no Tg	20	396	5%	0	157	0%	0	52	0%
SlprWT HA	228	421	54%	62	300	21%	44	194	23%
SlprAAA	0	257	0%	0	236	0%	0	156	0%
SlprTAA	6	325	2%	0	290	0%	0	256	0%
SlprASA	1	445	0%	0	385	0%	0	312	0%
SlprAAT	17	264	6%	5	282	2%	2	220	1%

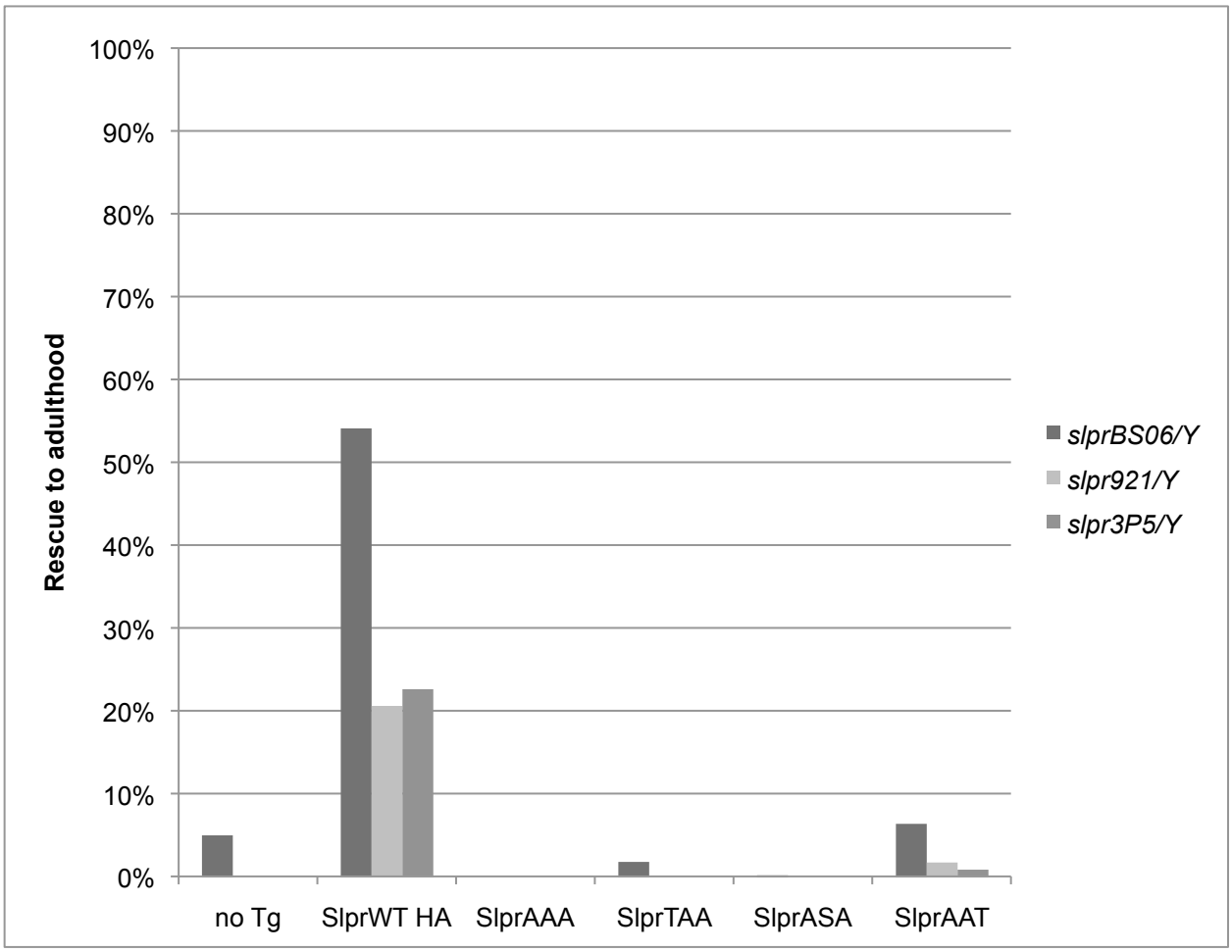

Figure 6: Transgenic rescue of Slpr mutants

Transgenic proteins were overexpressed using the *arm-Gal4* ubiquitous promoter in three different mutant backgrounds; $slpr^{BS06}$, $slpr^{921}$, and $slpr^{3P5}$. While SlprWT is able to rescue the mutants to adulthood, SlprAAA is unable to rescue. SlprAAT has mild rescuing ability.

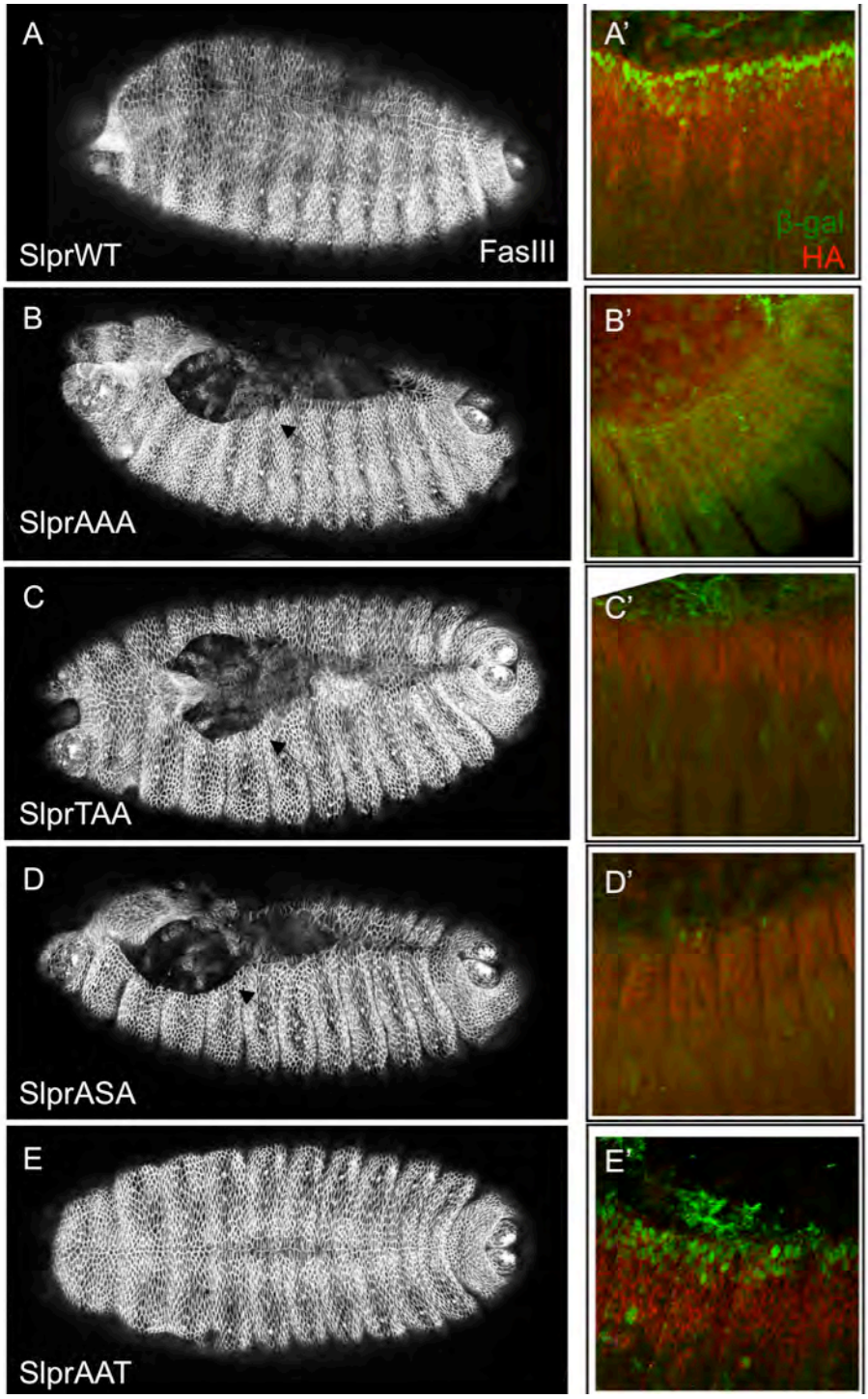
A
SlprWT
FasIII
A'
β-gal
HA
B
SlprAAA
B'
C
SlprTAA
C'
D
SlprASA
D'
E
SlprAAT
E'
F
w1118
β-gal

Figure 7: Overexpression of the activation loop mutants in embryos

Left panel (A-E): Late stage embryos stained with Fasciclin III to visualize the ectoderm. Black arrows indicate dorsal holes in B, C, and D. Right panel (A'-E'): *lacZ* expression in the leading edge of stage 13 embryos overexpressing the transgenes using *pnr-Gal4*. SlprAAA, SlprTAA, and SlprASA lack β-gal staining, indicating a loss of JNK pathway activity in these cells. F. lacZ expression in w^{1118} embryos crossed to pnr-Gal4, puc^{E69}. The reporter is evident in the leading edge of the ectoderm.

3.3 T295 IS REQUIRED FOR FULL SLPR ACTIVATION

To tease apart the requirements for each putative phosphorylation site within the activation loop, double mutants were made in which two of the three putative sites were mutated to alanine (Figure 5C). If two residues must be phosphorylated for full Slpr activation, then the double mutants would pinpoint which two are required. One of these mutants would completely abolish phosphorylation within the activation loop, and thus this mutant would be nonfunctional. Alternatively, if one of these residues is primarily responsible for Slpr activation, then the double mutants would provide insight into which is required, as the two double mutants that contain an alanine at that location would be inactive while the double mutant that retains the wildtype residue would be functional.

Two of the double mutants phenocopied *SlprAAA* in various assays. Both *SlprASA* and *SlprTAA* failed to rescue *slpr* mutants (Figure 6, Table 1). Also, JNK activity was lost at the leading edge during dorsal closure when both of these mutants were overexpressed using *pnr-Gal4* (Figure 7 C', D'), which resulted in dorsal holes (Figure 7 C, D). Additionally, a cleft thorax was observed at varying severities in adult flies overexpressing both *SlprASA* and *SlprTAA*, which resemble *SlprAAA* overexpression. The cleft was scored as either Class 1: a

noticeable interruption of midline bristles, Class 2: a widening of the cleft beyond that of Class 1 flies and gain of scutellar bristles, or Class 3: severe cleft in which the scutellum also fails to fuse (Figure 8). *SlprASA* had a large proportion of moderate to severe clefting and minimal Class 1 adults, which was similar to *SlprAAA*. SlprTAA adults had more of a range of phenotypes, with the majority of these flies having mild to moderate clefts.

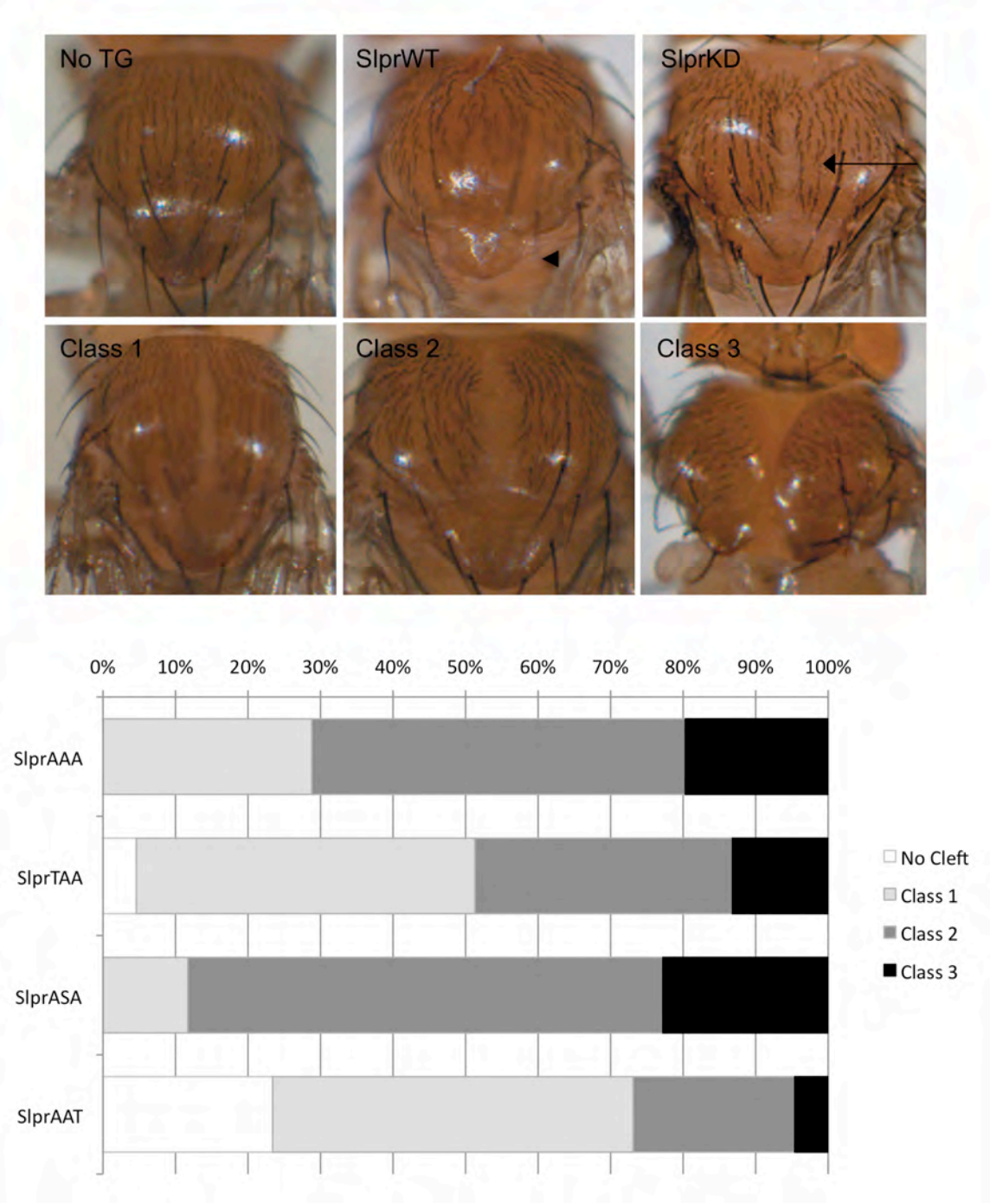

Figure 8: Slpr kinase domain mutants disrupt thorax closure

Thorax closure is used to monitor variable levels of JNK signaling in adults. SlprWT overexpression with *pnr-Gal4* leads to upregulated JNK activity, which is evident in a reduced scutellum and loss of scutellar bristles (arrowhead). Conversely, SlprKD overexpression blocks JNK-dependent thorax closure and results in a cleft thorax (arrow). There is a range of phenotypes associated with loss of Slpr activity varying in the degree of cleft present (Classes 1-3). Adult thoraxes that expressed SlprAAA, SlprTAA, SlprASA and SlprAAT were scored according to class, and the percentage of each class is presented in the graph (N>50 for each genotype).

Interestingly, the *SlprAAT* mutant retained Slpr activity in these assays. Though *SlprAAT* did not rescue as well as *SlprWT*, embryos expressing this mutant maintained JNK signaling at the leading edge and were able to undergo proper dorsal closure (Figure 7 E, E'). Additionally, *SlprAAT* overexpression resulted in a larger proportion of flies with less severe clefting in the thorax closure assay (Figure 8). Notably, about 25% of *SlprAAT* adults did not have a cleft thorax, indicating that there was no loss of JNK signaling in these flies. Since overexpression of the two mutants that contain an alanine mutation at this residue results in dominant negative phenotypes while *SlprAAT* behaves more like *SlprWT*, these data suggest that T295 is required for Slpr activity. Whether phosphorylation at this site is essential was investigated by making a phospho-mimetic version where T295 was mutated to glutamate while the other two putative phosphorylation sites, T287 and S291, were mutated to alanine (*SlprAAE*; Figure 5). Preliminary results suggest that *SlprAAE* cannot rescue *slpr* mutants (data not shown), indicating that phosphorylation at T295 alone is not sufficient to activate Slpr. Further tests on *SlprAAE* must be performed to determine the extent of JNK activity in flies expressing this mutant.

3.4 DISCUSSION

These data reveal the requirement for Slpr's activation loop phosphorylation in proper protein activation. Alanine mutations within the activation loop at T287, S291, and T295 confer a dominant negative effect when the *SlprAAA* mutant protein is overexpressed in various contexts. Interestingly, *SlprAAT* retains signaling capabilities, while *SlprASA* and *SlprTSA* phenocopy *SlprAAA*, indicating T295 is essential for Slpr function.

Previous reports show that activation loop phosphorylation is often achieved first by autophosphorylation, which is followed by phosphorylation at a secondary residue by an upstream kinase (Nolen et al., 2004). Sequence alignment of homologous MLK proteins revealed three conserved putative phosphorylation sites: two threonines and one serine. Indeed, two of these three sites are phosphorylated in varying combinations in the other MLK proteins. For example, mammalian MLK-3 is autophosphorylated at the first threonine and the serine is subsequently phosphorylated by an upstream kinase (Leung and Lassam, 2001). In contrast, MLK-1 is primarily phosphorylated at the second threonine followed by phosphorylation of the serine residue (Durkin et al., 2004).

Based on sequence homology, we hypothesized that two of these three residues are also phosphorylated in Slpr. By eliminating phosphorylation at all three residues, *SlprAAA* behaves as a dominant negative, similar to SlprKD (Polaski et al., 2006), suggesting that one or more of these residues is required for Slpr function. While the double mutant analysis does not definitively identify which two of the three residues are phosphorylated in Slpr, it does reveal the necessity of T295.

T295 lies in a part of the activation segment called the P+1 loop, which is responsible for substrate binding (Nolen et al., 2004). Perhaps phosphorylation of this residue is critical for the conformational change associated with protein activation, ensuring proper protein interactions. Alternatively, mutating T295 may alter the P+1 loop so that it can no longer bind its substrate, and thus this residue is critical for substrate binding but is not phosphorylated. The phosphomimetic version, *SlprAAE*, would reveal whether phosphorylation at this site is sufficient for protein function. Preliminary results suggest that *SlprAAE* cannot rescue mutant *slpr*, and therefore phosphorylation of this residue alone may not be enough to confer active Slpr

signaling. These data imply that the alanine mutation at T295 may abolish substrate binding, and Slpr is not phosphorylated at this residue. An alternative explanation is that the loss of rescue is due to hyperactivity resulting from constitutive Slpr signaling with the *SlprAAE* mutant. The overactive JNK signaling would result in puckered embryos, and these embryos cannot survive embryogenesis (Martin-Blanco et al., 1998). Further tests must be performed to elucidate the role of phosphorylation at T295, though we do conclude that this residue is necessary for Slpr function.

3.5 CONCLUSIONS AND FUTURE DIRECTIONS

Based on this study, we conclude that phosphorylation within the activation loop of Slpr is required for proper protein activation. Eliminating phosphorylation at three putative phosphorylation sites results in a dominant negative form of the protein. Double mutant analysis reveals the requirement for T295, though it is still undetermined whether this is the primary site of phosphorylation or if this residue is required for substrate binding in the P+1 loop.

Single alanine mutants would elucidate the requirement for phosphorylation at each threonine or serine in the activation loop. Here, we could compare the relative JNK activity in each of the single mutants to determine whether one phosphorylation site is preferred over another. Conversely, individual phosphomimetic transgenes can be generated to assay the potential gain of function with each residue. Additionally, it would be beneficial to further explore the *SlprAAE* transgenic flies to determine whether phosphorylation at T295 is required in dorsal closure and morphogenesis.

4.0 PHOSPHORYLATION OF SLPR AT A CONSERVED MAPK MOTIF IS REQUIRED FOR RESPONSE TO HEAT STRESS

The following has been submitted for publication.

4.1 INTRODUCTION

Cellular responses to environmental cues require the appropriate spatial and temporal coordination of signaling events. In fact, the sensitivity, amplitude, and duration of signaling activity in response to external stimuli can dictate distinct cellular outputs (Rallis et al., 2010; Ventura et al., 2006). A classic example is the regulation of cell proliferation versus differentiation by the duration of Mitogen-Activated Protein Kinase (MAPK) signaling in PC12 cells in response to Epidermal or Nerve Growth Factor, respectively (discussed in (Marshall, 1995)). In such kinase-based signal transduction pathways, dynamic modulation of the phosphorylation state of transducers and their substrates is one mechanism to tune a particular response. This can be achieved in part by additional network inputs in the form of feedback or crosstalk. Here, we identify a modulatory phosphorylation site in the *Drosophila* mixed lineage kinase, Slipper (Slpr), which appears to function in positive feedback to maintain Jun NH_2-terminal Kinase (JNK) signaling during environmental stress.

The stress-activated MAPK pathways, JNK and p38, are highly conserved transducers of cellular information in response to a variety of distinct signals. Cumulative data show that these pathways are used during development, yet they are also inducible to reestablish homeostasis in an unstable environment (Davis, 2000; Ono and Han, 2000). The p38 branch of MAPK signaling was first identified in hyperosmotic yeast as the HOG1 pathway (Brewster et al., 1993), but it is clear that p38 signaling is evolutionarily conserved in plants and animals to respond to diverse environmental stresses (Chang and Karin, 2001; Jiang and Song, 2008; Shinozaki and Yamaguchi-Shinozaki, 1997; Torres and Forman, 2003). The *Drosophila* genome encodes three p38 MAPK genes, *p38a (Mpk2), p38b* and *p38c*, which are activated by the MAPK kinases Mkk3/*licorne* and Mkk4 (Han et al., 1998a; Han et al., 1998b). Further upstream, two *Drosophila* MAPK kinase kinases, Tak1 and Mekk1, have been implicated in p38 signaling (Inoue et al., 2001; Moriguchi et al., 1996). p38 pathway mutants have few developmental defects, however individual members are sensitive to various stresses such as high osmolarity, heat shock, oxidative stress, UV radiation, and immune stimulation (Chen et al., 2010; Craig et al., 2004).

The JNK pathway has also been implicated in diverse cellular responses including apoptosis, wound healing, inflammation, and cell proliferation in many multicellular organisms. In *Drosophila*, a single JNK, Basket (*bsk*), is activated by the MAPKKs Hemipterous (*hep*) and Mkk4 (Glise et al., 1995; Yang et al., 1997). There are several MAPKKKs that can act upstream in the pathway. These are Mekk1, Ask1, Wallenda, Tak1, Takl2, and Slpr (Stronach, 2005). We have characterized Slpr, the *Drosophila* homolog of mammalian mixed lineage kinases (MLKs), as the JNK kinase kinase required for embryonic dorsal closure (Stronach and Perrimon, 2002) and other JNK-dependent morphogenetic events throughout development (Polaski et al., 2006).

Whether Slpr plays a role in environmental stress response is still an open question, but mammalian MLKs have been shown to be activated in response to neurotoxic, metabolic, and inflammatory stresses (Jaeschke and Davis, 2007; Korchnak et al., 2009; Sathyanarayana et al., 2002) and to signal downstream to p38 in addition to JNK (Gallo and Johnson, 2002; Kim et al., 2004; Tibbles et al., 1996). Recently, *Drosophila* Slpr has been implicated in p38 signaling in cultured cells, but this link has not been investigated *in vivo* (Chen et al., 2010).

Mammalian MLK3 has been shown to be phosphorylated by JNK as a means of positive feedback (Schachter et al., 2006). In particular, JNK phosphorylates the serine within a conserved PXSP motif, regulating the distribution and activity of MLK3. Specifically, the hypophosphorylated form of the protein is reversibly localized to a detergent-insoluble fraction of the cell where it is inactive, whereas activation of the pathway and subsequent phosphorylation of MLK3 by JNK maintains a signaling-competent pool. Thus, positive feedback from JNK to MLK allows amplification of the signaling response, though it is not clear under what physiological conditions this feedback is employed. In this report, using multiple in vivo assays, we identify a context in which this feed forward loop is critical. Namely, phosphorylation at the PXSP regulatory site in Slpr is dependent on functional Bsk/JNK activity to maintain Slpr-dependent JNK signaling during heat shock.

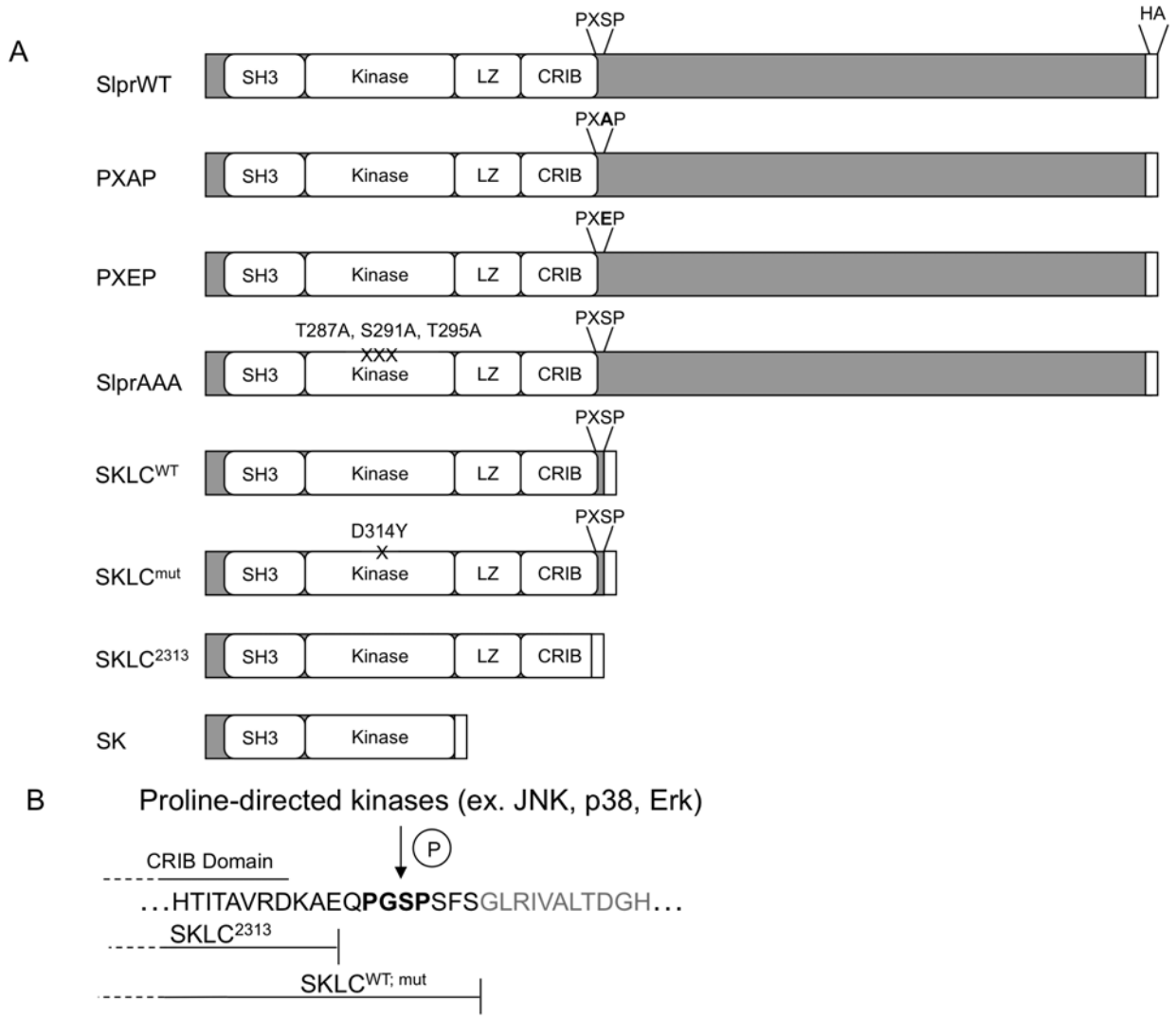

Figure 9: Slpr domain architecture and variant slpr constructs

A. The Slpr protein consists of an N-terminal SH3 domain, the catalytic kinase domain, a leucine zipper domain, a CRIB domain, and a long C-terminal tail with no recognizable domains. Constructs encoding four main domains (SKLC) or the SH3 and kinase domains only (SK) were used for *in vitro* experiments. $SKLC^{mut}$ contains the mutation, D314Y, within the kinase domain rendering it nonfunctional. $SKLC^{2313}$ is truncated immediately downstream of the CRIB domain and lacks the PGSP site. SlprAAA contains three mutations (T287A, S291A, and T295A) within the activation loop of the kinase domain that prevent full Slpr activation. PXAP and PXEP are full-length forms of the protein with a serine mutation within the PXSP motif to either abolish or mimic phosphorylation. Each construct has a C-terminal HA tag indicated. B. Sequence difference between $SKLC^{WT}$ and $SKLC^{2313}$. $SKLC^{WT}$ contains the PXSP site, a canonical substrate for proline-directed kinases, such as the MAPKs JNK, p38, and Erk.

4.2 IDENTIFICATION OF A PHOSPHORYLATION SITE OUTSIDE OF THE KINASE DOMAIN OF SLPR

Kinase activation often requires phosphorylation within the catalytic domain, but many kinases have additional regulatory sites. Given that mammalian MLK family members are extensively phosphorylated (Vacratsis et al., 2002) and that phosphoproteomic studies have identified numerous phosphorylated Slpr peptides (Bodenmiller et al., 2007; Zhai et al., 2008), we hypothesized that Slpr is regulated by other phosphorylation events outside the kinase domain. In this work, we have identified a PXSP motif downstream of the CRIB domain (Figure 9) conserved among the MLK family members (Schachter et al., 2006). We tested the functional consequences of phosphorylation at this site in vivo through the use of mutant transgenic constructs.

Using one-dimensional gel electrophoresis to reveal potential modifications on the Slpr protein, we observed that an *in vitro* translated form of Slpr, called SKLC, comprising the N-terminal SH3, Kinase, Leucine Zipper, and CRIB domains (Figure 9A), migrated as two distinct bands by SDS-PAGE (Figure 10A). To determine whether the difference in electrophoretic mobility of the two bands was due to phosphorylation, the *in vitro* translated and labeled sample was treated with lambda phosphatase prior to electrophoresis. Indeed, the doublet collapsed down to a single band upon phosphatase treatment (Figure 10A), indicating that the slower migrating form was phosphorylated. Kinases often undergo multi-step activation in which intramolecular inhibition must be relieved before dimerization or phosphorylation by upstream

kinases fully activate the protein (Bock et al., 2000; Burbelo et al., 1995; Du et al., 2005; Vacratsis and Gallo, 2000; Zhang and Gallo, 2001). Given that SKLC contains the kinase catalytic and leucine zipper domains, we reasoned that homodimerization, via the zipper motifs, might result in kinase autophosphorylation within the activation loop of the kinase domain. To test whether the phospho-form of SKLC was dependent on dimer-mediated autophosphorylation, two additional Slpr constructs were analyzed: SK, a shorter form of Slpr lacking the leucine zipper and CRIB domains, and $SKLC^{mut}$, containing a mutation in an invariant residue of the kinase domain (D314Y) (Figure 9A). In the context of the full length Slpr protein, the D314Y mutation renders the protein nonfunctional and is presumed to inactivate the kinase (Polaski et al., 2006). *In vitro* translated SK migrated as a single band that remained unchanged upon phosphatase treatment (Figure 10A). Contrary to expectations, $SKLC^{mut}$ behaved similarly to $SKLC^{WT}$, appearing as a doublet before phosphatase treatment. Together, these results suggest that the phosphorylation of $SKLC^{WT}$ is not due to dimerization-induced kinase autophosphorylation, but rather that a kinase in the *in vitro* translation reaction might phosphorylate SKLC. We observed similar results using an alternative *in vitro* translation system (Figure 10A).

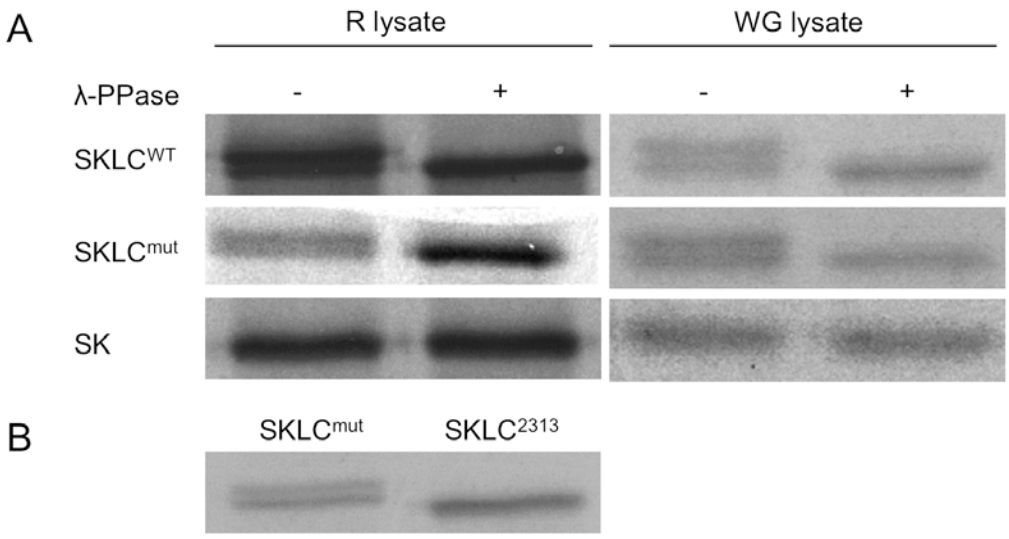

Figure 10: SKLC is phosphorylated *in vitro*

A. SKLC and SK were translated and labeled *in vitro* with ^{35}S-methionine in either a rabbit reticulocyte (R) or a wheat germ (WG) lysate. SKLC^{WT} and SKLC^{mut} proteins produced in either lysate migrate as a doublet on SDS-PAGE and collapse to a single band when treated with λ-phosphatase. The SK protein runs as a single band and remains unchanged when treated with phosphatase. B. The SKLC^{2313} protein, which lacks the PXSP motif, runs as a single band, compared to SKLC^{mut}, a catalytically inactive form of the protein.

The phosphorylation of SKLC but not SK spurred us to look for putative kinase recognition motifs in the nonoverlapping region, consisting of the leucine zipper and CRIB domains (LC). Here we identified a PXSP motif, matching the consensus for proline-directed kinases, just downstream of the CRIB domain (Figure 9B). Instances of this motif are conserved among the mammalian MLK proteins and it has been shown to positively regulate MLK3 function upon phosphorylation by JNK (Schachter et al., 2006). Additionally, recent phosphoproteomic studies identified this motif in Slpr as an abundant phosphopeptide (Bodenmiller et al., 2007; Zhai et al., 2008). Therefore, we tested whether the PXSP motif accounted for the phosphorylation we had observed in SKLC. To this end, we generated the $SKLC^{2313}$ form, which is shortened by eight amino acids including the PXSP motif (Figure 9B). *In vitro* translated $SKLC^{2313}$ migrates as a single band by SDS-PAGE similar to the lower, non-phosphorylated form of $SKLC^{WT}$ (Figure 10B). The absence of apparent phosphorylation upon deletion of the PXSP motif implies that the serine within this site might be phosphorylated within the context of $SKLC^{WT}$ or that a binding site important for phosphorylation elsewhere in the protein has been eliminated.

4.3 CONTEXT-SPECIFIC ACTIVITY OF PXSP PHOSPHORYLATION

Slpr has previously been implicated in JNK-dependent morphological events, such as male genital rotation, maxillary palp formation, adult thorax closure, and embryonic dorsal closure. (Polaski et al., 2006; Stronach and Perrimon, 2002). To determine whether modification of the PXSP motif is required for Slpr during development, we generated nonphosphorylatable

(*UAS-PXAP)* and phosphomimetic (*UAS-PXEP*) transgenes in the context of the full length Slpr protein. As a functional assay for Slpr activity, the progress of dorsal closure was observed upon expression of transgenes in a wildtype background to determine loss or gain of signaling activity (Garlena et al., 2010b). Using *pnr-Gal4* as an embryonic driver line, we compared the effects on dorsal closure from overexpression of the following Slpr transgenes: *UAS-SlprWT*, *UAS-PXAP*, *UAS-PXEP* and *UAS-SlprAAA* (a dominant negative form of the protein (Garlena et al., 2010b), Figure 9A). Fasciclin III-stained embryos were staged according to the extent of their gut development, and the ability to undergo dorsal closure was determined by the presence or absence of a dorsal hole (Figure 11A). Embryos expressing SlprWT display a seamless ectoderm by stage 16, similar to control embryos (Figures 11Aa,b), though they are not completely wildtype due to mild upregulation of JNK signaling, which we confirmed using a JNK target gene reporter (Figure 11B and (Polaski et al., 2006)). In contrast, when SlprAAA is overexpressed, the embryo has a large dorsal-anterior hole at stage 16, indicative of a failure of dorsal closure (Figure 11Ac). Expression of PXAP does not induce a failure of closure by stage 16, nor did we observe any delay in closure; rather, PXAP-expressing embryos resemble those expressing SlprWT (Figure 11Ad). Like PXAP, expression of the phosphomimetic form, PXEP, promotes dorsal closure (Figure 11Ae), but with mild puckering at the midline consistent with upregulation of JNK signaling. Indeed, SlprWT, PXAP, and PXEP overexpression stimulates ectopic JNK pathway signaling, as visualized by reporter gene expression (Figure 11B). Furthermore, overexpression of PXAP allows the recovery of more adult $slpr^{BS06}$ null mutants (Polaski et al., 2006), compared with a no transgene control. This increase in rescuing activity, however, is not as substantial as that observed with expression of SlprWT, while the dominant negative SlprAAA provides no rescuing function whatsoever (Figure 11C). PXEP expression

provides significant rescue of *slpr* mutants to adulthood (Figure 11C). Altogether, these data suggest that phosphorylation at this motif is largely dispensable for Slpr-dependent dorsal closure. More importantly, these data demonstrate that the ability to upregulate JNK signaling upon overexpression is not perturbed by the loss of PXSP phosphorylation.

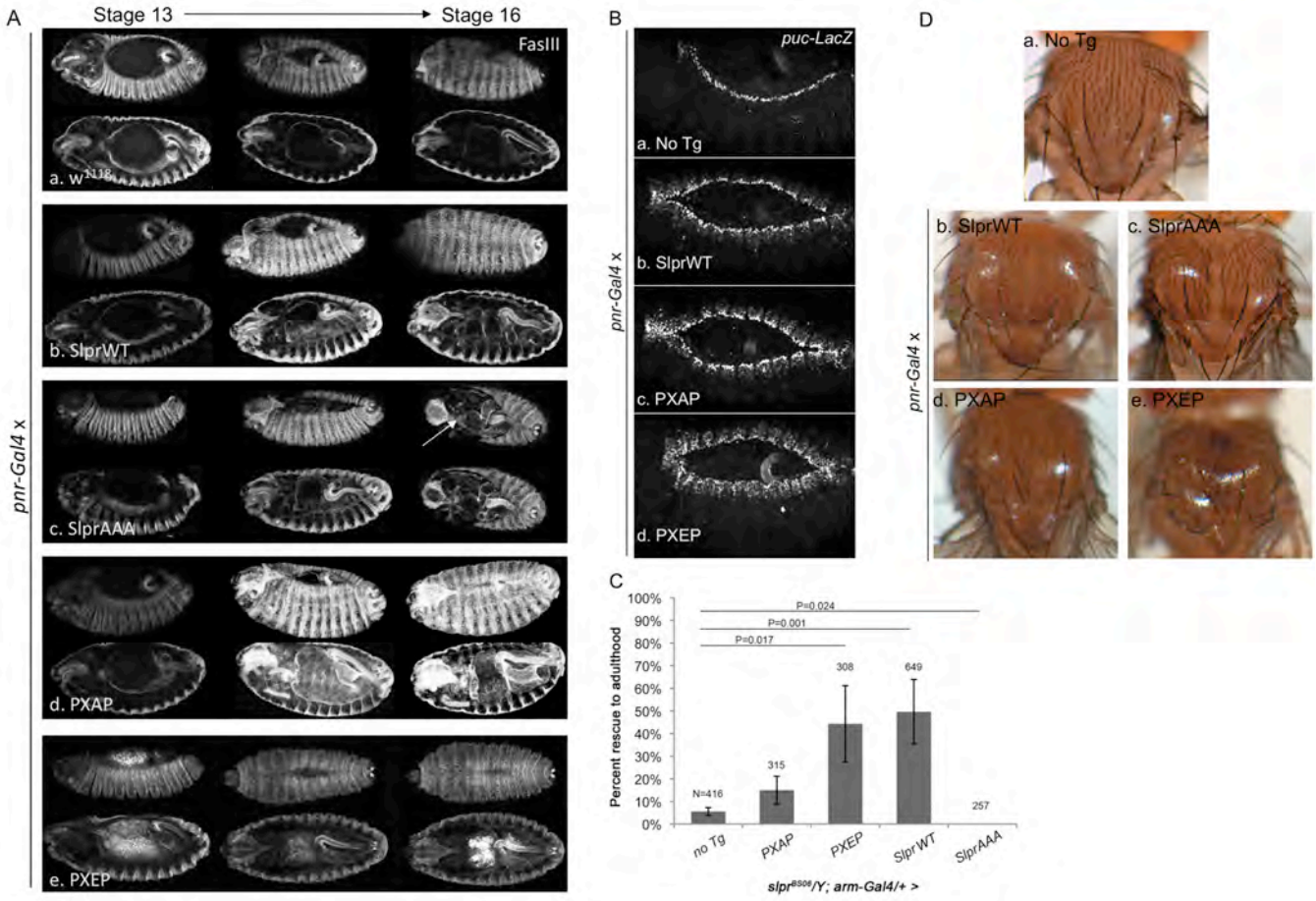

Figure 11: Mutant PXSP transgenes retain signaling function in development

A. Immunofluorescence staining for Fasciclin III in the dorsal ectoderm (upper row) allows visualization of the progress of dorsal closure in embryos expressing the indicated Slpr transgenes with *pnr-Gal4* compared with w^{1118} control embryos. Fas III also labels the developing gut endoderm (lower row) to identify the stages of embryogenesis. Expression of SlprAAA dominantly inhibits dorsal closure resulting in a large dorsal anterior hole (arrow), whereas overexpression of SlprWT, PXAP, or PXEP, promotes dorsal closure, indicative of functional Slpr signaling. B. JNK target gene expression, monitored by the *puc-LacZ* reporter, is upregulated in the *pnr* domain of the dorsal ectoderm upon expression of the indicated Slpr transgenes. C. Rescue of $slpr^{BS06}/Y$ mutants to adulthood with expression of the indicated Slpr transgenes is displayed as a percentage relative to *FM7/Y* siblings and compared to a no transgene control. Dominant negative SlprAAA is unable to rescue, while all of the other transgenes provide varying degrees of rescuing function. Significant p-values are given, based on Students *t*-test. Total numbers of flies scored is given above each column. D. Thorax closure is used to monitor variable levels of JNK signaling in adults. SlprWT overexpression with *pnr-Gal4* leads to upregulated JNK activity, which is evident in a reduced scutellum and loss of scutellar bristles. Conversely, SlprAAA overexpression blocks JNK-dependent thorax closure and results in a cleft thorax. Overexpression of both PXAP and PXEP leads to reduced scutellum and loss of scutellar bristles, indicating upregulated JNK signaling. PXEP appears more severe, suggesting more constitutive signaling.

Next, we asked whether phosphorylation at the PXSP motif was required for Slpr function in the context of adult morphogenesis, namely the JNK-dependent process of thorax closure (Agnes et al., 1999; Zeitlinger and Bohmann, 1999). Using the *pnr-Gal4* driver, its possible to target transgene expression to the presumptive thorax. Recovery of adults revealed a similar trend to what we had observed with embryonic dorsal closure. SlprWT, PXAP, and PXEP expression resulted in mild to moderate narrowing of the scutellum and variable loss of scutellar bristles (Figure 11D), phenotypes that are associated with activation of JNK signaling (Baril et al., 2009; Garlena et al., 2010b). Reduced JNK signaling was inferred by the cleft thorax phenotype seen upon overexpression of SlprAAA. These results imply that PXAP and PXEP are functioning to stimulate JNK signaling, like the WT form, in the additional context of adult tissue morphogenesis, similar to what we observed during dorsal closure.

Since PXSP phosphorylation does not appear to be required for the developmental functions of Slpr, we hypothesized that this modification might play a role in a non-developmental context. To date, Slpr has not been implicated in stress response for example, but the roles of distinct MAP3Ks have not yet been elucidated for many JNK- and p38-dependent processes in *Drosophila*. Moreover, new functions for the JNK and p38 pathways are continuing to be defined with the development of new mutants and assays. In mammals, both the JNK and p38 pathways have been implicated in thermoregulation (Adler et al., 1995; Kano et al., 2004; Kyriakis and Avruch, 1996; Murai et al., 2010), but in *Drosophila*, only a few members of the p38 pathway have been associated with heat shock response. Consequently, we chose to probe the role of Slpr, and in particular PXSP phosphorylation, in response to heat stress. To this end, adult flies were subjected to a sustained 37°C heat shock to assess their ability to withstand an environmental insult, with *Mpk2* (*p38a)* mutant and wildtype flies for comparison (Craig et al.,

2004). Figure 12 shows the results. Control w^{1118} flies initially withstood the stress, but by two hours, approximately 55% were immobile, yet responsive to tapping the vial. Conversely, *p38a* null flies rapidly became catatonic and unresponsive, with over 70% dead by two and a half hours. To test whether Slpr is required for this stress response, $slpr^{BS06}$ null mutants were also subjected to the heat stress and showed sensitivity similar to *p38a* mutants.

Given that the JNK pathway has not been implicated previously in thermostress signaling in flies, we tested another member of the JNK pathway, the JNK kinase, Hep. Like *slpr* mutants, hep^{1} mutants were sensitive to heat shock with nearly 80% of the flies catatonic or dead after two hours of exposure compared to 20% of control flies. One hundred percent of the $p38a^{1}$, $slpr^{BS06}$, and hep^{1} mutants showed reduced locomotion by one and a half hours, whereas a three-hour sustained heat treatment was required to observe a similar effect on all of the control animals. These data imply that both the p38 and JNK pathways are required for response to heat stress and mutants with impaired signaling functions succumb more rapidly to the stress. Notably, Tak1, a JNK kinase kinase that is capable of activating both the JNK and p38 pathways (Lee et al., 2000; Yamaguchi et al., 1995), was not required for this stress response, revealing selective use of Slpr in this context.

Given that *slpr* mutants show increased sensitivity to heat shock, we predicted that overexpressing Slpr might confer some resistance to thermal stress. In addition, this assay would allow us to examine the consequences of phosphorylation for heat shock response upon expression of the other mutant transgenes. Using *arm-Gal4* as a ubiquitous driver, we found that flies overexpressing SlprWT were better able to withstand the heat stress than control flies, indicated by a reduction in the proportion of immobile or unresponsive flies at all time points (Figure 12). Expression of the dominant negative SlprAAA protein conferred slight sensitivity

to heat shock, primarily at early timepoints, though this was not as dramatic as the *slpr*^{BS06} null mutants, which might reflect the relative amount or spatial distribution of transgenic versus endogenous protein. Interestingly, PXAP overexpression also resulted in mild heat sensitivity, with a greater overall percentage of affected flies at each time point compared to control. Specifically, after two hours, there was a noticeable increase in the number of flies showing the terminal phenotype. For example, nearly 70% of adults expressing PXAP appeared catatonic/dead at two hours relative to 22% of control flies. This suggests that phosphorylation within the PXSP motif is necessary for heat stress response, though it appears to be less important for Slpr signaling under normal physiological conditions.

As shown already, SlprWT overexpression provided a mild resistance to heat shock. Interestingly, flies expressing the phosphomimetic PXEP transgene were able to withstand the heat stress even better than SlprWT. At all time points, expression of PXEP provided substantial protection from heat stress relative to control flies. Not only was there a smaller fraction of flies showing any effects from the heat (maximally 74% at 3 hours vs. >95% for all other genotypes), but there were far fewer dead animals as a proportion of the total (13% at 3 hours vs. >70% of w^{1118}), indicating thermoresistance. Altogether, these data indicate that Slpr is required for a heat shock response and that mutations associated with the PXSP motif are sufficient to modulate this response.

It is interesting to note that the transgenic flies were able to recover after 24 hours released from heat shock (Table 2), similar to control flies. MAPK pathway mutants, however, were unable to recover, suggesting that the dominant negative effect that the transgenes have on endogenous Slpr is reversible, and a subset of the flies that appear unresponsive are paralyzed and not dead.

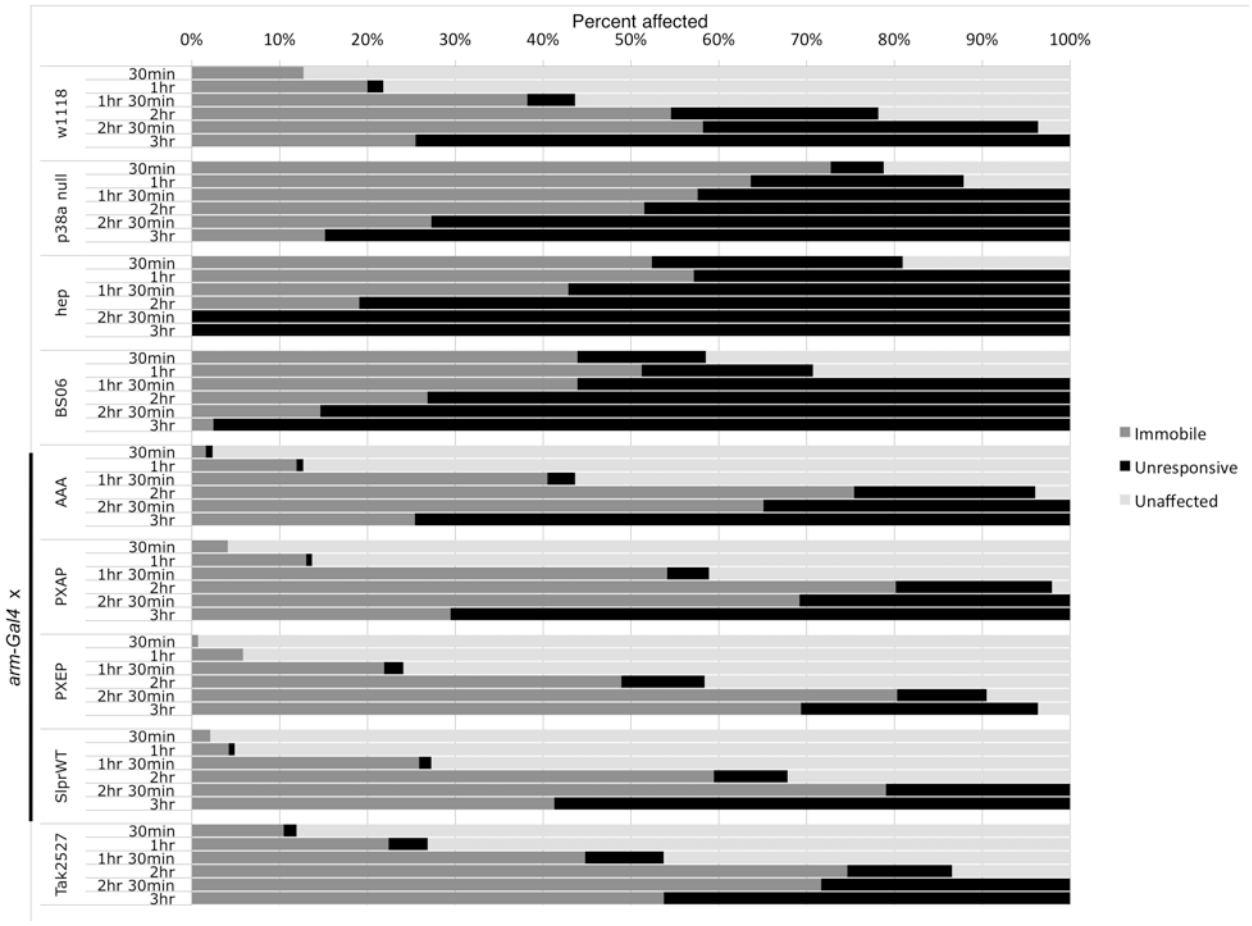

Figure 12: Slpr PXSP mutant transgenes show differential effects in response to heat shock

Adult flies were subjected to continuous heat shock and scored at each time point as either 'immobile' (not crawling, but twitch when the vial is tapped, grey) or 'unresponsive' (black). Transgenes were expressed under the control of a*rm-Gal4* as indicated. N≥300 for each genotype.

Table 2: Ability of adult flies to recover from 3 hour heat shock after 24 hours at room temperature

Genotype	Recovery[a]
w^{1118}	++
p38a^{1}	-
hep^{1}	-
slpr^{BS06}	-
SlprAAA	+
PXAP	+
PXEP	++
SlprWT	+
Tak^{2527}	++

[a] Recovery was scored according to how many flies are scored as unaffected after 24 hours released from heat shock. (++): more than half of the "affected" flies at the 3hr time point recover; (+): 10-50% of the "affected" flies recover; (-): <10% of the "affected" flies recover.

4.4 EFFECTS OF SLPR PXSP PHOSPHORYLATION IN RESPONSE TO HEAT SHOCK IS NOT MEDIATED SOLEY BY RESPONSES IN THE NERVOUS SYSTEM

Overexpression of PXAP using the ubiquitous driver *arm-Gal4* led to sensitivity to heat stress, but it is not clear in which tissue Slpr is required for this stress response. We have observed strong transgene expression in the larval (Garlena et al., 2010b) and adult nervous system using the *arm-Gal4* driver (Beth Stronach, not shown), where it has been demonstrated that the JNK pathway is upregulated in response to stress (Broughton et al., 2005; Karpac et al., 2009; Rulifson et al., 2002; Wang et al., 2005). We therefore hypothesized that PXAP behaved as a thermosensitive dominant negative in the nervous system. Using the neuronal-specific driver, *elav-Gal4*, to overexpress the transgenes, we repeated the adult heat shock assay to directly test whether Slpr is required in the nervous system. While SlprWT and PXEP provided mild resistance to adults during heat shock, SlprAAA and PXAP profiles resembled those of control flies (Figure 13). While selective expression of the dominant negative forms of Slpr did not worsen the response as might be predicted, neuronal specific expression of PXEP was sufficient to evoke mild resistance. Thus, it is likely that the systemic response to heat treatment in the adult requires Slpr-dependent signaling in multiple tissues, not just in the nervous system.

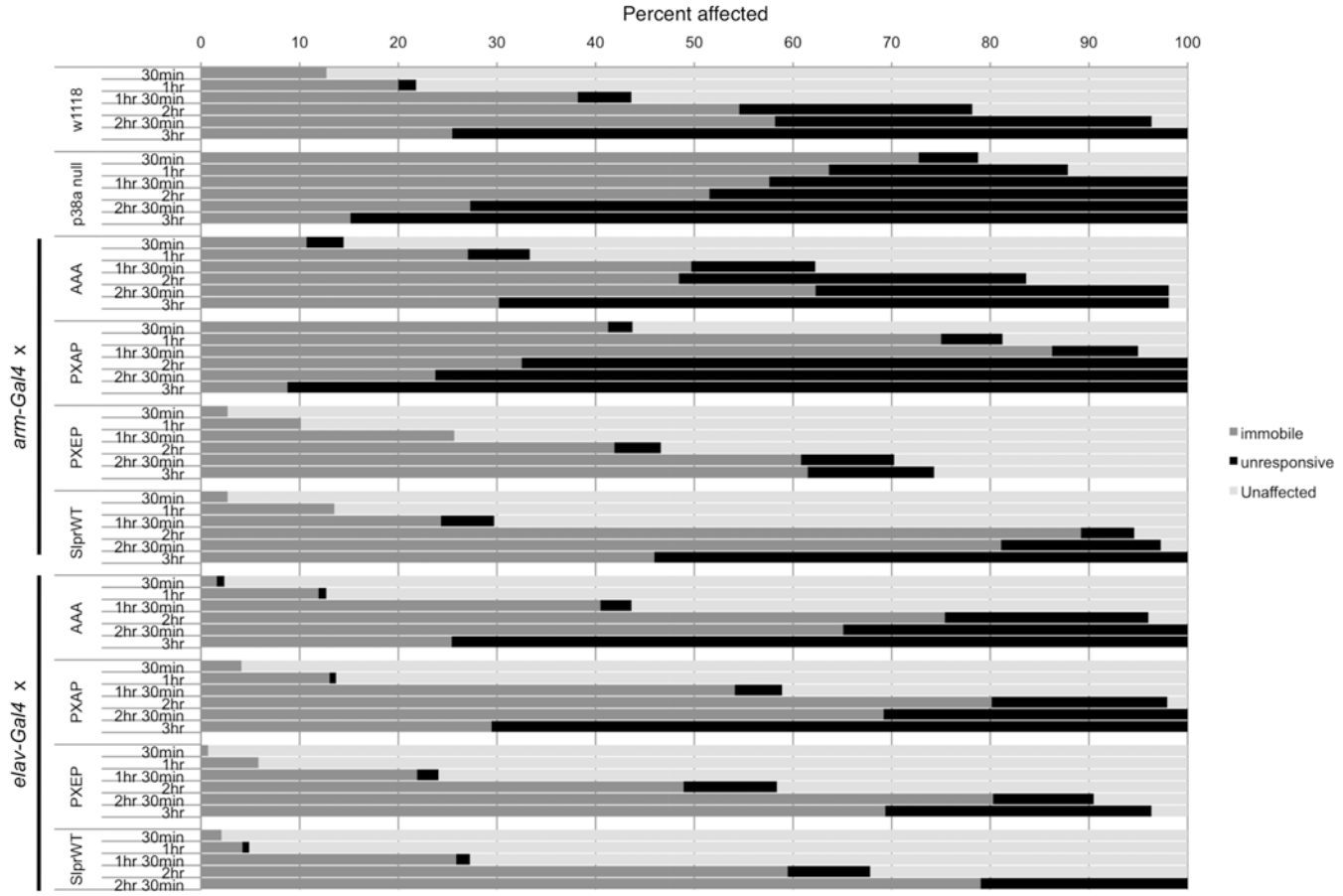

Figure 13: The effect of heat shock on adult flies with neuronal transgene expression

Adult flies were subjected to continuous heat shock as in Figure 12. Slpr transgenes were selectively expressed in post-mitotic neurons using *elav-Gal4*. Adults expressing transgenes with *arm-Gal4* are shown for comparison. While SlprAAA and PXAP are not as susceptible to heat stress using *elav-gal4*, SlprWT and PXEP provide some protection when expressed selectively in the nervous system.

4.5 HEAT SHOCKED PXAP EMBRYOS HAVE DORSAL CLOSURE DEFECTS

We observed that expression of the PXAP mutant transgene confers sensitivity to environmental stress, so we reconsidered whether embryos expressing PXAP would close dorsally under stressful conditions. If PXSP phosphorylation is required for Slpr to propagate signaling during stress, we predicted that loss of this phosphorylation may lead to reduced signaling and an increase in dorsal closure defects. To this end, we collected embryos ubiquitously overexpressing PXAP with the *arm-Gal4* driver and exposed half the population to heat shock. Among the stressed group, we observed a statistically significant increase in the number of dead embryos compared to non-heat-shocked siblings (Figure 14). In the other individual genotypes, SlprWT and PXEP, heat treatment of embryos resulted in no significant increase in lethality compared with their untreated siblings. Yet, between genotypes, PXAP is significantly more sensitive to the heat stress as measured by overall lethality than the stressed SlprWT or PXEP expressing embryos. We also noted that among the dead embryos, cuticles from the stressed PXAP collection displayed dorsal holes and head defects consistent with reduced JNK signaling (similar to those published in (Garlena et al., 2010b)). Together, these data demonstrate that the inability to phosphorylate the PXSP motif results in reduced Slpr function in response to heat shock, although it has minimal impact in the absence of environmental stress. This suggests that PXAP behaves as a thermosensitive dominant negative protein, while the phosphomimetic PXEP form protects Slpr from losing signaling activity under stress.

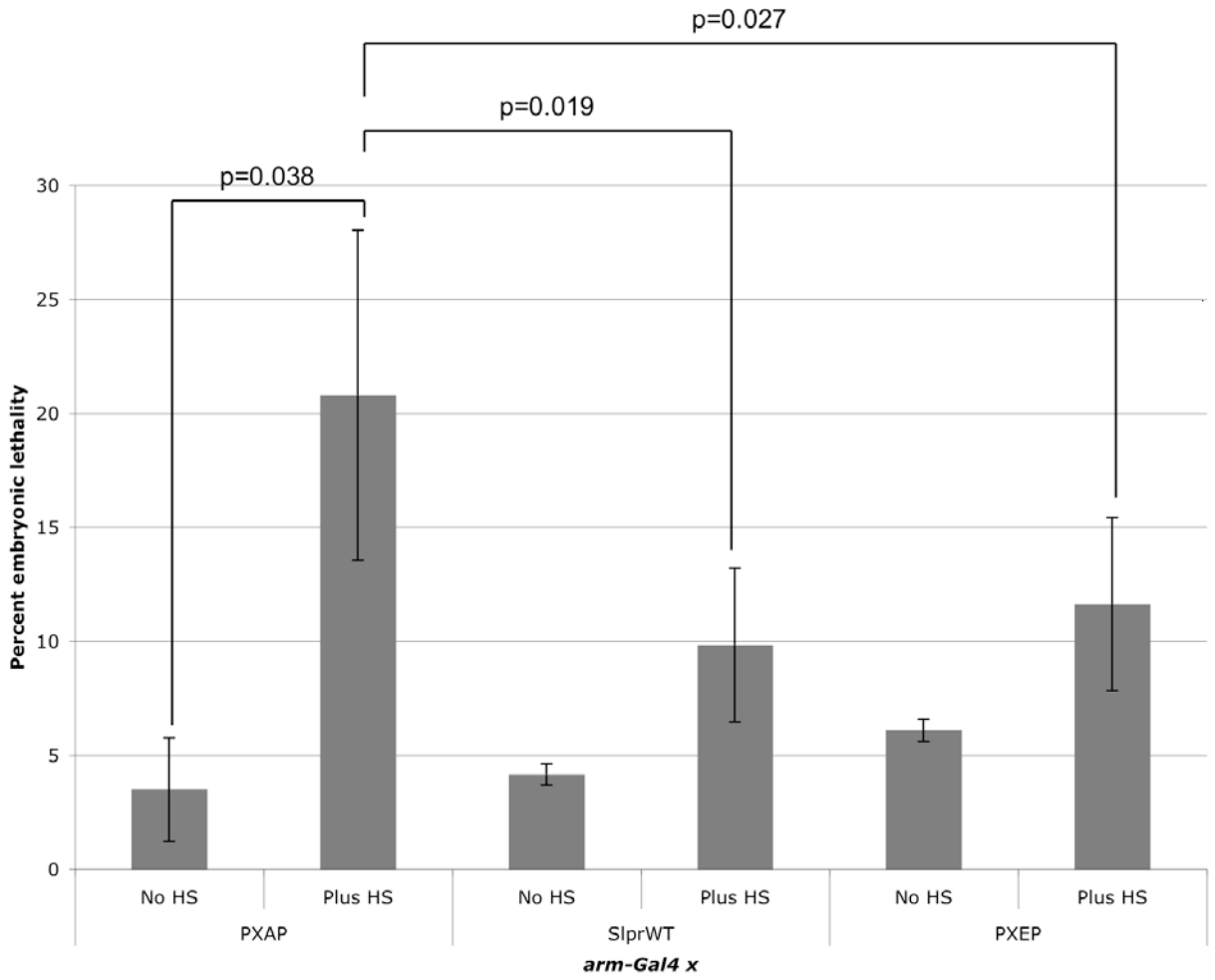

Figure 14: Effect of Slpr PXSP mutant transgene expression in embryos subjected to heat stress

Embryos with the indicated genotypes were either untreated (No HS) or subjected to a three-hour heat shock (Plus HS). Lethality was assessed after 24 hours and plotted as a percentage of the total. There was a significant increase in lethality of PXAP embryos after heat shock compared to untreated siblings. This increase was also significantly different than the slight increase in lethality of the other genotypes subjected to heat shock. Significance is indicated with p-values, determined using Student's *t*-test.

4.6 PXSP PHOSPHORYLATION DOES NOT AFFECT PROTEIN FOLDING

Since the loss of phosphorylation at PXSP results in thermosensitivity, we wondered how this phosphorylation event changes Slpr so that it is responsive to environmental stress. We therefore took a biochemical approach to probe the properties of the wildtype versus the nonphosphorylatable alanine mutant and phosphomimetic PXEP mutant. Western blot analysis of embryonic extracts revealed increased levels of PXAP protein compared to SlprWT, SlprAAA and PXEP; however, RT-PCR of transcripts derived from each transgene showed equivalent signals for SlprWT and PXAP, indicating that the abundance of PXAP is likely a result of post-transcriptional events and not merely a difference in transgene expression (Figure 15). Analysis of three transgenic lines for each genotype confirmed these results.

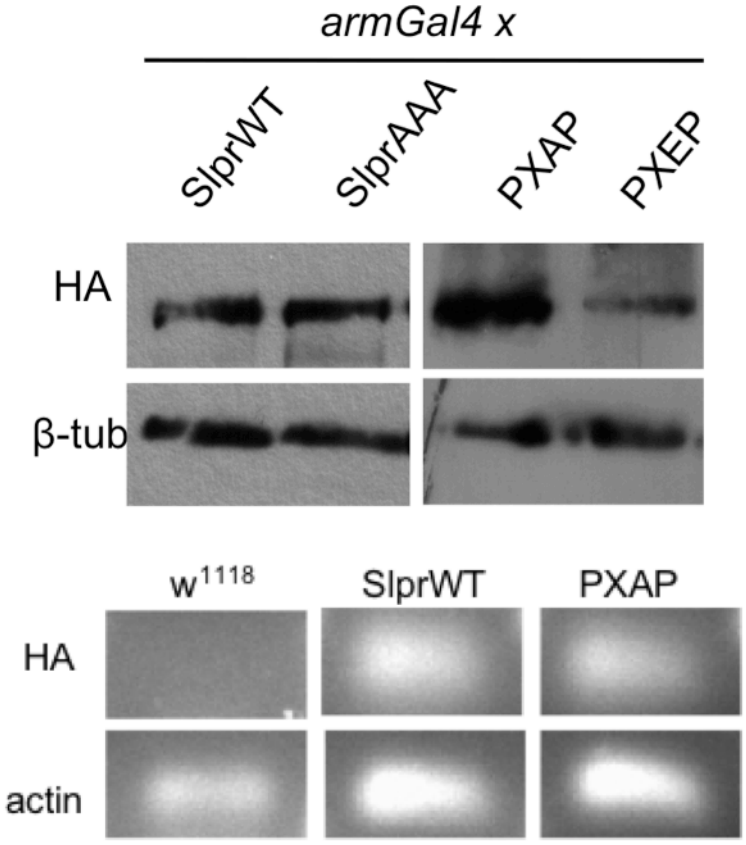

Figure 15: Slpr transgene expression in embryos

A. Western blot of lysates from embryos expressing the Slpr transgenes with the *arm-Gal4* driver. Slpr transgenic protein is identified with anti-HA antibody. Anti-β-tubulin is used as a loading control. PXAP is slightly more abundant than the other forms of Slpr. B. RT-PCR of Slpr transgenes reveal similar mRNA expression levels of SlprWT and PXAP (HA). As expected, there was no amplification using the HA primer on w^{1118} embryos. Actin was used as a control.

Next, we wanted to compare SlprWT and PXAP stability over time as an indication of differences in conformation or accessibility to proteases. We expect that if PXAP is in a different conformation than SlprWT, it could be cleaved at different locations within the protein resulting in variable fragment sizes by western blot. Additionally, we can compare the relative stability of the proteins by observing how quickly proteolysis has an effect on each protein. Therefore, embryonic lysates of heat-shocked SlprWT and PXAP were incubated at room temperature and their degradation patterns were observed over time via western blot. We did not observe variable size degradation products between SlprWT and PXAP (Figure 16), which suggests that these two proteins share similar folding properties and can be similarly processed. Consistent with these results, the unfolded protein response was not induced upon PXAP expression, indicated by the lack of splicing of XBP (Figure 17), whose mRNA transcript is spliced in a Ire1-dependent manner during ER stress (Plongthongkum et al., 2007).

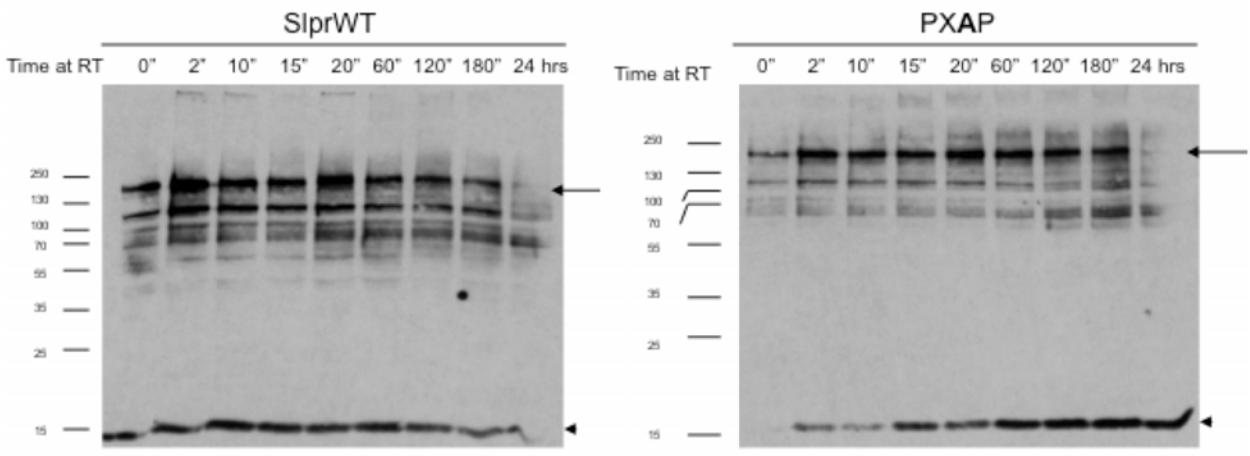

Figure 16: Limited proteolysis of SlprWT and PXAP

SlprWT and PXAP lysates were made in RIPA buffer and were incubated at room temperature. Samples were taken at each time point. Full-length protein is indicated by the arrow. Both SlprWT and PXAP appear stable over time, with most degradation seen after 24 hours. Blots were performed using the Slpr SH3 antibody. A 15 kD fragment appears in both samples (arrowhead), but does not correspond to the N-terminal SH3 domain, as this band appears with samples lacking the SH3 domain (Δ-SH3 and C-term, not shown).

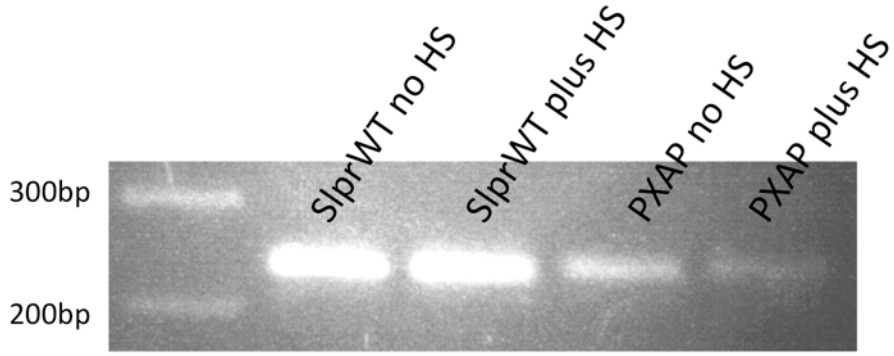

Figure 17: The lack of XBP splicing indicates UPR is not induced with PXAP overexpression

If XBP mRNA is spliced upon ER stress due to the unfolded protein response (UPR), we would expect to see two bands at 240 bp and 220 bp. Here, we only observe the unspliced version (240 bp), indicating that the UPR is not induced under any of these conditions. This suggests that PXAP is not misfolded under normal and heat shock conditions.

4.7 BSK IS REQUIRED FOR PXSP PHOSPHORYLATION *IN VIVO*

Because phosphorylation within the PXSP motif of Slpr provided some protection against heat stress, we hypothesized that the responsible kinase must be active in stress conditions to propagate the appropriate response. We turned our attention to the MAPKs JNK, p38, and Erk, which are all proline-directed kinases that could phosphorylate the serine within the PXSP motif of Slpr as a means of feedback or crosstalk. Indeed, in the *in vitro* translation assay, addition of MAPK inhibitors individually, revealed a requirement for JNK or p38, but not ERK activity (Figure 18A). To identify the kinase that phosphorylates PXSP in the fly, *UAS-SKLC*^{WT} was expressed in the embryonic ectoderm using *69B-Gal4*. Similar to the *in vitro* expression system, SKLC from embryonic lysates migrated as a doublet by SDS-PAGE, suggesting that it was phosphorylated *in vivo* (Figure 18B, lane 1; plotted in Figure 19). If either JNK or p38 kinase is responsible for SKLC phosphorylation, then it is expected that a corresponding change in the mobility of SKLC will be observed with activation or inhibition of each kinase (or their upstream activators, Hep and Lic, respectively). Initially, under normal environmental conditions, we did not observe a loss of the phosphorylated form of SKLC protein upon coexpression with dominant negative JNK (*UAS-bsk*^{K53R}) or p38b, or in a homozygous *p38a* mutant background (Figure 18B, lanes 2,3,5; Figure 19). These data suggest that other MAPKs might compensate when one is inactive, or that there is another kinase phosphorylating SKLC *in vivo*. Additionally, there were no dramatic changes in phosphorylation upon overexpression of Hep or Lic kinases (Figure 18B, lanes 4,6; Figure 19).

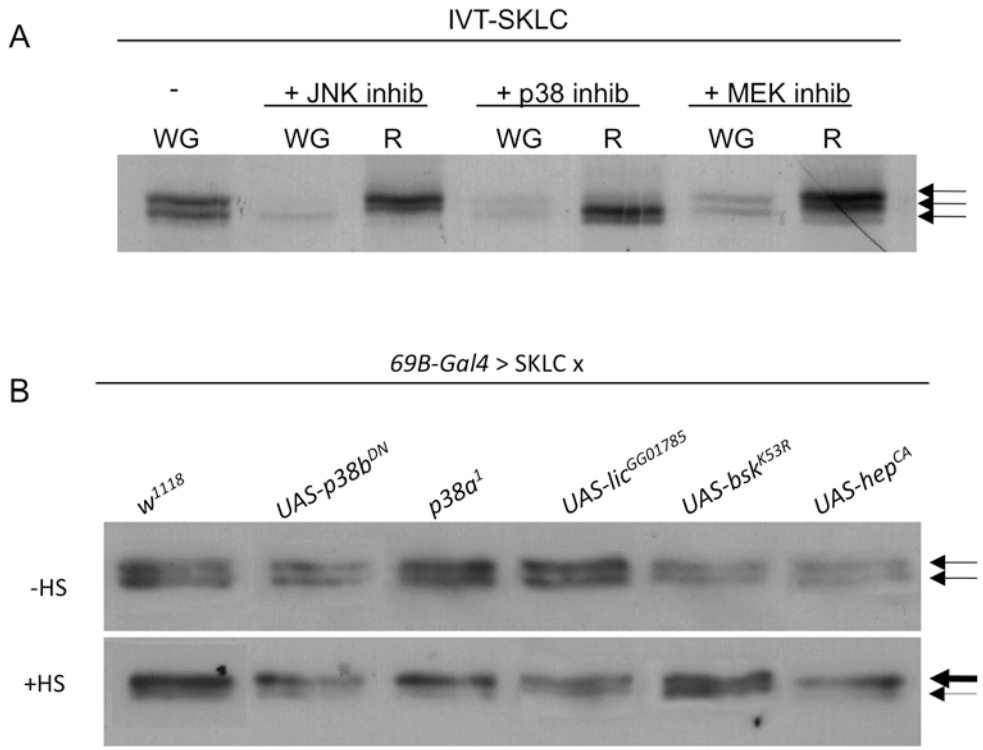

Figure 18: SKLC is differentially sensitive to JNK and p38 inhibitors and phosphorylation is primarily dependent on JNK under conditions of heat stress in vivo

A. MAPK inhibitors were added individually to SKLC in vitro translation reactions. Three forms of SKLC are evident in the reticulocyte (R) lysate (arrows), while two forms are seen in the wheatgerm (WG) lysate (lane 1). SKLC is differentially sensitive to the JNK and p38 inhibitors in the different lysates, while the MEK inhibitor had little effect in either system. B. *69B-Gal4* was used to drive expression of *UAS-SKLC^{WT} in vivo*. Western immunoblot with anti-HA revealed that in embryonic protein lysates, SKLC^{WT} runs as a doublet (arrows, -HS), regardless of coexpression with dominant negative Bsk or p38b^{DN}, or expression in a *p38a* homozygous mutant background. There is an enrichment of the upper phosphorylated form (heavy arrow) after a 3-hour heat shock (+HS) in all samples except with coexpression of Bsk^{K53R}.

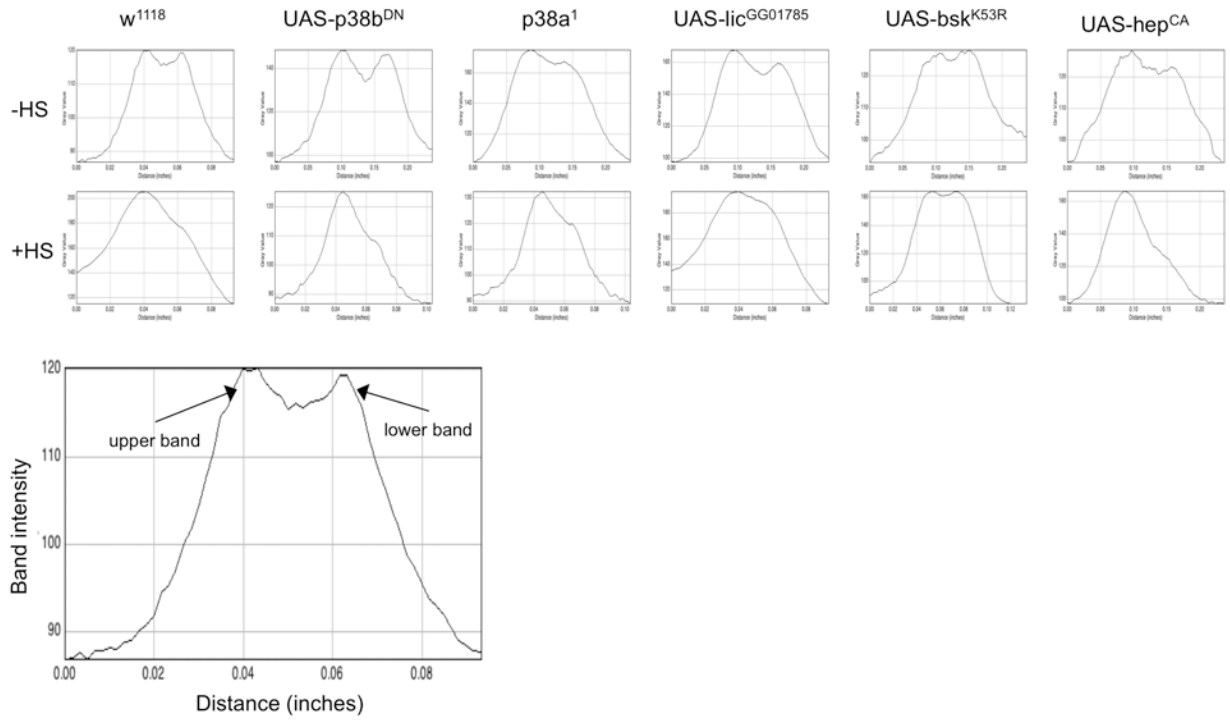

Figure 19: Intensity profiles of SKLC bands from western immunoblot

The intensity (grey value) of the SKLC bands was measured and plotted using ImageJ. A doublet is evident by two peaks of intensity in the untreated samples. Upon heat shock, with the exception of Bsk^{K53R}, profiles indicate one prominent peak corresponding to the upper phosphorylated form of SKLC. An enlarged example shows how each peak is measured; the distance of each peak is plotted along the *x*-axis with the upper band on the left. The peaks signify increased pixel intensity corresponding to each band on the western blot.

Given that SKLC phosphorylation is required during thermostress, we reasoned that exposure of embryos to heat shock might increase the activity of the putative PXSP kinase and enrich for the phosphorylated form of SKLC. Indeed, the SKLC doublet was enriched for the upper band after exposure to heat, indicating an increase in phosphorylation (Figure 17B, quantified in intensity profiles, Figure 18). The preference for the phosphorylated form was evident in all the experimental genotypes with the exception of coexpression with dominant negative bsk^{K53R}. In this sample, the basal level of phosphorylation was maintained, but was not increased (Figure 17B, lane 5). This result suggests that while p38 can sustain basal SKLC phosphorylation when Bsk signaling is depleted, it might not compensate for Bsk during stress. Thus Slpr requires active Bsk signaling to become hyperphosphorylated under heat shock conditions.

4.8 DISCUSSION

In this report, we describe a previously unidentified regulatory mechanism for Slpr activity. While the role of Slpr in JNK signaling during dorsal closure has been well studied, there has been no evidence to date that Slpr is required for stress response *in vivo*. This is the first demonstration that Slpr is heat shock regulated. Specifically, phosphorylation within a conserved PXSP motif of Slpr is enriched upon heat treatment and modulates organismal response to thermostress.

Signal feedback is used by organisms to fine tune signaling in response to specific stimuli. With environmental stress, it is possible that upstream kinases may be phosphorylated

by activated downstream kinases in order to rapidly amplify a response. Bsk is required for phosphorylation of Slpr at the regulatory PXSP motif to maintain proper homeostasis during heat stress. Without this feedback, the basal level of Slpr signaling is not sufficient for the adult fly to withstand a heat shock. Additionally, exposing embryos overexpressing PXAP to heat shock results in a significant increase in embryonic lethality, whereas PXAP overexpression has no dominant negative effect on dorsal closure in untreated embryos. Together with the observation that a nonactivatable Slpr construct, SlprAAA, behaves as a dominant negative, blocking dorsal closure and rendering adult flies heat sensitive, these data suggest that PXAP behaves as a thermosensitive dominant negative protein. That is, under normal conditions, PXAP is functional for JNK signaling, but with thermostress, the inability of the protein to be phosphorylated at this site results in a form that is impaired in signaling and presumably interferes with endogenous Slpr signaling. We see a more dramatic effect in adults, where flies expressing PXAP phenocopy *p38* mutant flies, which are known to be sensitive to heat shock (Craig et al., 2004).

SDS-PAGE reveals a propensity towards the phosphorylated form of SKLC after exposure of embryos to heat stress, consistent with the notion that heat shock causes an increase of phosphorylation at the PXSP site. While *p38a* mutants did not abrogate this phosphorylation, expression of dominant negative Bsk blocked the increase in phosphorylation above basal levels observed in control embryos. We propose that under normal conditions, there is a basal level of feedback by Bsk, in which about 50% of Slpr is phosphorylated at the PXSP site. When Bsk is inhibited, another MAPK, presumably p38, can compensate for the lack of Bsk activity to maintain the steady state level of phosphorylation. However, when exposed to heat shock, the animal requires both JNK and p38 signaling to respond appropriately (Figure 12). Increased p38

signaling during heat shock might be required selectively for specific downstream output during stress. As such, p38 activity may become limiting and insufficient for Slpr phosphorylation in the absence of Bsk activity. Therefore, Slpr does not receive feedback to intensify signaling in response to stress, causing these animals to be thermosensitive (Figure 20).

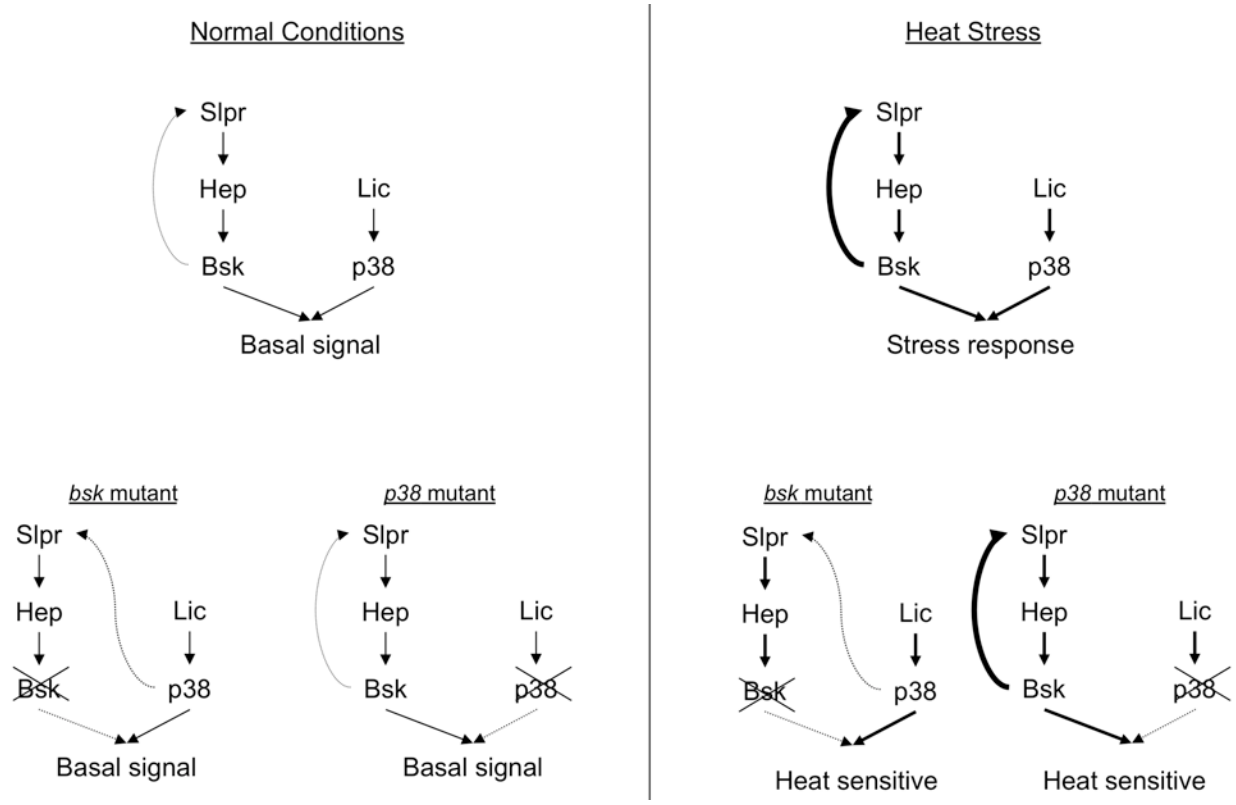

Figure 20: Model of Slpr signaling in normal and heat stress conditions

A model of JNK and p38 signaling during normal and heat stress conditions. Slpr requires Bsk for basal levels of PXSP phosphorylation and p38 can compensate for loss of Bsk activity under normal conditions. With heat stress, both JNK and p38 pathways are required for stress response, such that loss of either pathway renders flies sensitive to heat shock. Bsk increases Slpr PXSP phosphorylation to promote an amplified JNK-dependent heat stress response, and p38 is insufficient to compensate for loss of Bsk activity.

It is interesting to note that in several mammalian tissue culture systems, dual-specificity MAPK phosphatases are heat labile, accounting in part for the accumulation of phosphorylated MAPKs to sustain signaling under stress (Meriin et al., 1999; Palacios et al., 2001; Yaglom et al., 2003). Whether this mechanism is conserved in *Drosophila* has not yet been investigated; however, inactivation of the JNK phosphatase, Puc, could contribute indirectly to the observed increase in phospho-Slpr through inhibition of Bsk dephosphorylation.

While our data reveal a role for PXSP phosphorylation in thermostress signaling, it is still unclear by what mechanism this modification modulates Slpr activity. To probe the effect on Slpr when PXSP phosphorylation is lost, we performed several biochemical experiments. Proteolytic analysis of PXAP and SlprWT revealed similar proteolysis patterns with or without heat shock, indicating analogous protein folding between the two forms, thus ruling out the notion that the PXAP protein is grossly misfolded. Consistent with that observation, the unfolded protein response pathway was not induced in embryos overexpressing PXAP under non-stress conditions. Furthermore, we were unable to detect a biochemical interaction between Slpr and Heat Shock Proteins (HSP70 or HSP90) under normal or stress conditions. It is noteworthy though that there is an abundance of PXAP protein compared to SlprWT by Western immunoblot. While phosphorylation within the PXSP motif may not be required for proper protein folding, it may increase protein turnover to maintain proper spatial distribution or a pool of active Slpr. Perhaps PXAP behaves as a thermosensitive dominant negative by sequestering endogenous Slpr in an inactive pool that is not amenable to signaling. Precedence for this mechanism comes from JNK phosphorylation of MLK3 in which the phosphorylated form is reversibly localized to a triton-soluble fraction of the cell to modulate signaling intensity (Schachter et al., 2006). We are currently examining the localization of Slpr transgenic proteins

under different conditions to explore this possibility. Alternatively, phosphorylation within this motif may affect binding of substrate or upstream effectors. Tests are ongoing to determine the consequences of PXSP phosphorylation at the protein level.

Another question raised by our results is which tissue requires active JNK or p38 signaling during adult heat stress? Accumulating evidence suggests that the nervous system, in particular insulin producing neurosecretory cells, upregulate JNK signaling during oxidative stress response, which counteracts insulin/IGF signaling allowing an adaptive systemic response (Broughton et al., 2005; Karpac et al., 2009; Rulifson et al., 2002; Wang et al., 2005). Additionally, the JNK pathway is normally active in neuronal development, particularly in the mushroom body (Rallis et al., 2010), and in maintaining neuronal homeostasis in both *Drosophila* and mammals (Collins et al., 2006; Stone et al., 2010; Whitmarsh et al., 2001). Notably, we have observed that *arm-Gal4* directs transgenic Slpr protein expression strongly in the larval nervous system, primarily in the mushroom bodies, but to a lesser extent in many other regions, and at relatively low levels in the larval imaginal discs. Whether *arm-Gal4* directs expression in the insulin-producing cells of the adult brain is not known, but would provide an intriguing explanation linking the effects of PXSP phosphorylation to the described adaptive stress response. To directly test whether Slpr is required in the nervous system to mediate heat stress response, we expressed Slpr transgenes in post-mitotic neurons with e*lav-Gal4*. Though overexpression of SlprWT and PXEP evoked mild resistance to heat stress, SlprAAA and PXAP did not supply any resistance. While these transgenes could not protect the adults like SlprWT and PXEP, they did not behave as dominant negative forms of the protein in this context. Therefore, it is likely that response to heat stress in adults requires multiple tissues, including the nervous system.

The results presented in this report demonstrate that the JNK pathway, including Slpr, is required for thermostress signaling. We argue that phosphorylation of the PXSP motif by Bsk, directly or indirectly, provides positive feedback to intensify JNK signaling in an attempt to reestablish homeostasis. Even under nonstress conditions a certain degree of phosphorylation is maintained by the compensatory stress kinases to buffer signaling activity from complete failure in rapidly changing conditions.

5.0 CONCLUSIONS

The work presented here describes how phosphorylation of Slpr affects both its activation to propagate JNK signaling and its regulation in response to heat shock. I have verified that conserved residues within the activation loop are required for Slpr function. Specifically, T295 is essential for Slpr-mediated JNK signaling. Furthermore, I have identified phosphorylation at a conserved MAPK consensus site downstream of the CRIB domain. Loss of this phosphorylation in transgenic overexpression experiments results in sensitivity to heat stress. Active Bsk is required for PXSP phosphorylation, suggesting that this phosphorylation is the result of positive feedback to ensure that homeostasis is maintained under this stress. Together, these data show how modification of Slpr kinase is necessary both in protein activation and in stress response.

5.1 T295 IS REQUIRED FOR SLPR FUNCTION

Upon examination of the sequence of the activation loop of Slpr, it was evident that three conserved residues were putative phosphorylation sites. Some combination of two of these three residues is phosphorylated in MLK homologs (Durkin et al., 2004; Leung and Lassam, 2001), and therefore it is reasonable to predict that two of these residues are phosphorylated for Slpr activation. Transgenic flies expressing SlprAAA, a non-phosphorylatable version of the protein, display defects associated with loss of JNK signaling, indicating that SlprAAA behaves as a

dominant negative. To address which residue is primarily responsible for Slpr activation, double mutants (SlprAAT, SlprASA, and SlprTAA) were generated. Slpr AAT was the only transgene that retained function when overexpressed. Therefore, we conclude that the third residue, T295, is essential for Slpr function. To address how phosphorylation at T295 might affect Slpr function, phospho-mimetic SlprAAE transgenic animals were tested. Preliminary results suggest that SlprAAE cannot rescue *slpr* mutants. One explanation for this is that the phospho-mimetic greatly increases JNK signaling, resulting in puckered embryos, which are embryonic lethal (Martin-Blanco et al., 1998). A second option is that excessive JNK signaling from increased Slpr activity is over the threshold for an apoptotic response, which leads to embryonic lethality (Lennox and Stronach, 2010; Reed et al., 2001). Another possibility is that T295 is not phosphorylated, and this residue is required for another function such as substrate binding. Because T295 lies at the beginning of the P+1 loop, it is likely that the function of this region is disrupted when T295 is mutated to alanine. Further examination of SlprAAE transgenic mutants is underway to determine whether phosphorylation at T295 is required for Slpr activation or if T295 possesses another function that is required by Slpr. We are currently studying if overexpression of SlprAAE upregulates JNK activity, suggesting a role for phosphorylation. If this is not the case, we will pursue experiments that test SlprAAE and SlprAAT binding to Hep, the JNKK that is downstream of Slpr in JNK signaling, using a peptide corresponding to the activation loop of the kinase domain of Hep, which is the substrate phosphorylation site of Slpr.

Kinases commonly require dual phosphorylation within the activation segment to be fully active (Nolen et al., 2004). Phosphorylation at the primary site provides the conformational change that properly positions the activation segment. Either upstream or downstream of the

primary site is a secondary phosphorylation site. This subsequent phosphorylation has varying effects on kinase activity, allowing it to more efficiently bind ATP or its substrate. Secondary phosphorylation can increase kinase kinetics anywhere from 5- to 1000-fold (Dajani et al., 2003; Prowse et al., 2001). Therefore, if Slpr is phosphorylated at T295, SlprAAT and SlprAAE may retain some activity, but full activation of the protein cannot be achieved due to mutation at a putative secondary site. Single mutants, in which one of the three putative phosphorylation sites is changed to alanine, may help tease apart the requirement for multiple phosphorylation sites within the activation loop. If T295 is the primary phosphorylation site, then either SlprAST or SlprTAT should retain full Slpr activity, which can be tested in assays that are sensitive to varying levels of Slpr-mediated JNK signaling (Garlena et al., 2010b). We predict that primary phosphorylation alone, presumably seen in SlprAAT, results in mild JNK pathway activity, while dual phosphorylation would provide full activation and resemble SlprWT overexpression. Indeed, SlprAAT overexpression retains some Slpr function, but the upregulation of JNK signaling is not as strong as overexpressing SlprWT. Additionally, results from double mutant analysis suggests that if T295 is the site of primary phosphorylation, then this phosphorylation is required before a secondary phosphorylation, as loss of function at T295 results in a loss of Slpr function. Together, results from single mutant analysis, in addition to the information provided from the double mutants, would supply further confirmation of a step-wise activation of Slpr.

A major goal in identifying phosphorylation within the activation loop of Slpr is to be able to monitor active Slpr. Phospho-specific antibodies would allow us to identify dynamic Slpr signaling in various contexts, providing more information about the behavior of Slpr to regulate JNK signaling. For instance, we could monitor active Slpr subcellular localization to determine if there is an active pool of Slpr in the cell. Additionally, we could examine temporal

control of Slpr signaling to determine increases or decreases in Slpr activation over time and how this affects particular outcomes throughout development, morphogenesis, or stress response, for example. This information may reveal new insights into the relevant ligands and tissues that contribute to signaling specificity in the JNK pathway.

5.2 PXSP PHOSPHORYLATION IS REQUIRED FOR HEAT SHOCK RESPONSE

Downstream of the CRIB domain of Slpr lies a conserved MAPK consensus site, PXSP. *In vitro* transcribed SKLC runs as a doublet on one dimensional SDS-PAGE, which collapses down to a single band when treated with a phosphatase. A truncated protein lacking the PXSP motif also runs as a single band, suggesting that this is the site of phosphorylation.

Non-phosphorylatable (PXAP) and phospho-mimetic (PXEP) transgenic mutants were made to test the effects of loss or gain of PXSP phosphorylation, respectively. Initial experiments to determine the role of PXSP phosphorylation in the morphological functions of Slpr resembled SlprWT overexpression, suggesting that loss or gain of PXSP phosphorylation did not affect Slpr function in this context. Because PXSP phosphorylation appeared to be dispensable for the known developmental and morphological roles of Slpr, we hypothesized that this regulatory phosphorylation occurs as a result of stress signaling, something to which Slpr had not previously been linked. In fact, little is known about heat stress response in *Drosophila* involving JNK and p38 signaling, but both pathways have been reported to be activated during thermostress in mammalian systems (Adler et al., 1995; Kano et al., 2004; Kyriakis and Avruch, 1996; Murai et al., 2010; Zanke et al., 1996). Furthermore, the Slpr homolog MLK is activated by ceramide (Sathyanarayana et al., 2002), a sphingolipid that accumulates upon heat stress

(Hannun and Luberto, 2000). Additionally, MLK3 interacts with the heat shock protein, HSP90, to mediate stress signaling (Zhang et al., 2004). These studies support further investigation into Slpr-mediated JNK signaling in response to heat stress in *Drosophila*.

We speculated that Slpr could be activated upon heat stress, and PXSP phosphorylation may be required for modulating Slpr-dependent signaling in a system that requires a rapid response. Indeed, adults that express PXAP are sensitive to heat shock, similar to p38 and JNK pathway mutants. Flies expressing SlprWT or PXEP, on the other hand, were resistant to thermostress. Moreover, we found an increase in lethality of embryos expressing PXAP that were heat shocked compared to non-heat shocked PXAP embryos, as well as heat shocked SlprWT and PXEP embryos.

We took a molecular genetic approach to determine which MAPK was responsible for PXSP phosphorylation. SKLC expressed in embryos appears as a doublet on SDS-PAGE in either *p38* or *bsk* mutant backgrounds. However, when the SKLC-expressing embryos are heat shocked, the upper phosphorylated band is enriched in all of the samples except with inhibition of Bsk signaling. This suggests that Bsk is required for stress-induced PXSP phosphorylation. Interestingly, in embryos overexpressing SKLC using the *pnr-Gal4* driver while heterozygous for mutant *puc*, a phosphatase that inactivates Bsk (Martin-Blanco et al., 1998), there was an enrichment of the upper phosphorylated SKLC band (see appendix, Figure 18), providing further support for the requirement of Bsk activity for PXSP phosphorylation.

These results are significant for a number of reasons. First, to our knowledge, this is the first example of JNK signaling being required in response to heat stress in *Drosophila*. Second, we link Slpr to this stress response. Furthermore, Slpr heat stress signaling requires phosphorylation outside of the kinase domain. Third, PXSP phosphorylation relies on Bsk

activity, suggesting positive feedback regulation. Together these data reveal a context-specific mechanism for Slpr regulation, which lends insight to complexities of signal transduction in *Drosophila* such that a regulatory phosphorylation that is not required for activation aids in dynamic stress signaling in response to heat.

There is evidence of individual modifications induced by stress that affect specific protein functions. For example, a well-known tumor suppressor, p53, is activated by upstream kinases, but can also be regulated at distinct phosphorylation sites in response to specific stresses to generate an amplified signal (Saito et al., 2003) thereby ensuring proper signaling levels in response to stressful conditions. It is reasonable to predict that Slpr signal can also be regulated outside of kinase domain phosphorylation to provide a rapid, intensified response to stress. We observe that about 50% of Slpr is phosphorylated at PXSP in non-stress conditions. Perhaps this phosphorylation provides basal Slpr signal during development and morphogenesis, but upon heat stress, Slpr signal is amplified by increasing phosphorylation at PXSP.

5.3 SUMMARY

The work presented here offers insight into previously unknown mechanisms of Slpr activation and regulation. Examination of the activation segment of Slpr reveals a requirement for putative phosphorylation residues. It is possible that T295 is the site of primary phosphorylation to activate Slpr, confirming that Slpr requires phosphorylation similar to its MLK homologs for full activation. This information can serve to monitor the dynamics of Slpr signaling in response to various cues in different contexts. In addition, while involvement of Slpr in JNK signaling during development and morphogenesis has been well established through genetic analysis of

slpr mutants, I have uncovered a novel role of Slpr signaling in heat shock response, linking it to a regulatory phosphorylation site through positive feedback by Bsk activity. By understanding the various mechanisms of Slpr regulation, through both activating phosphorylation within the kinase domain and regulatory phosphorylation at PXSP, we gain a better appreciation of how control of protein activity within a signaling pathway can affect several downstream effects, such as development, morphogenesis, and organism homeostasis.

APPENDIX A

A.1.1 BIOCHEMICAL ANALYSIS OF SLPR

In the course of analyzing Slpr, I took a biochemical approach to elucidate properties of SKLC phosphorylation as well as differences between PXAP and SlprWT. The following data are examples of varying conditions that affected the proteins.

A.2 THE SKLC DOUBLET IS AFFECTED BY VARIOUS DRIVERS AND BUFFER CONDITIONS

SKLC that was overexpressed in embryos by different drivers was extracted in either 2X sample buffer (SB) or RIPA buffer. Using both *arm-Gal4*, which is a ubiquitous driver, and *pnr-Gal4*, which expresses in the dorsal ectoderm, SKLC runs as a doublet when extracted in 2X SB. Interestingly, the soluble portion of the RIPA extraction is enriched for the upper band of SKLC. Phosphatase treatment of this sample results in an electrophoretic shift, indicating that the phosphorylated form of SKLC is extracted using RIPA buffer (Figure 21A). SKLC that was overexpressed using pnr-Gal4 with mutant *puc,* a phosphatase that negatively regulates Bsk, in

the background is also enriched for the upper band (Figure 21B). Although preliminary, this data supports the notion that active Bsk leads to SKLC phosphorylation.

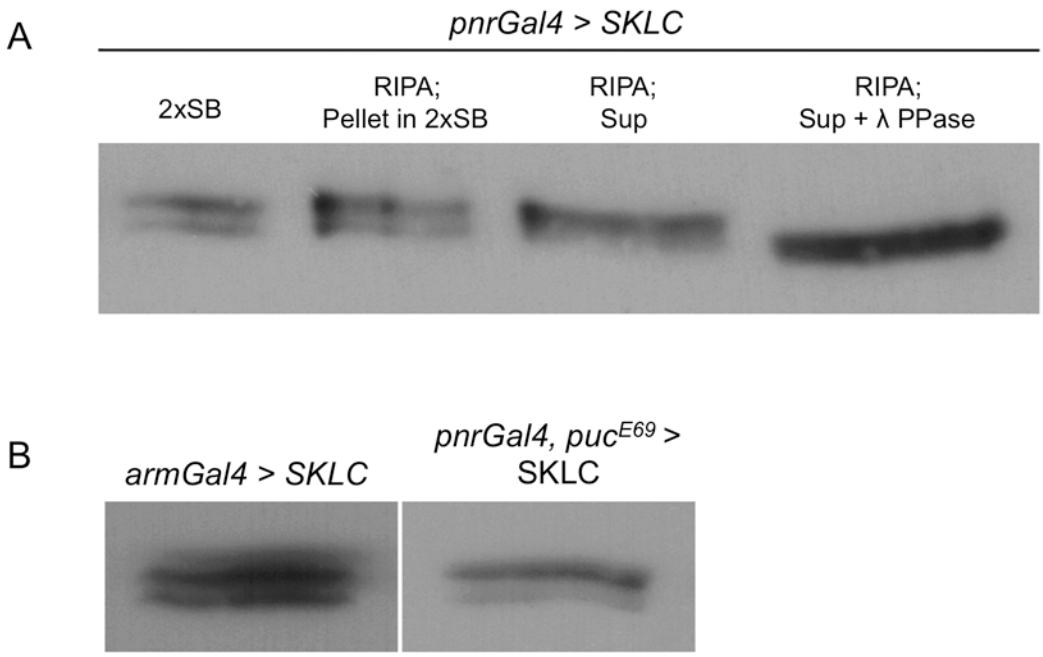

Figure 21: Different Gal-4 drivers and buffer conditions affect SKLC doublets

A. Embryonic lysates made in 2X sample buffer run as a distinct doublet. When using RIPA buffer to make the lysates, the doublet is lost when the supernatant is run on a gel (lane 3), but resuspension of the resulting pellet in 2X SB results in a doublet (lane2). Upon treatment of the RIPA supernatant with λ-PPase, the band shifts down, suggesting that the phosphorylated version is extracted using RIPA buffer. B. The *arm-Gal4* driver produces an even distribution of phosphorylated and non-phosphorylated SKLC. In contrast, the phosphorylated form of SKLC is enriched using the *pnr-Gal4* driver with mutant *puc* in the background. A possible explanation is that there is more active Bsk in this sample, as Puc is a phosphatase that negatively regulates Bsk, and therefore there is increased feedback to phosphorylate SKLC.

A.3 TWO-DIMENSIONAL GEL ANALYSIS OF SKLC

Two-dimensional gel electrophoresis was used to analyze post-translational modifications of Slpr. Embryonic lysates containing overexpressed transgenes were prepared and run in two dimensions: first separating proteins based on isoelectric point, followed by separation by size. $SKLC^{WT}$ and $SKLC^{AAA}$ were visualized by western blot. Both versions of the protein contain numerous isoforms when prepared using PhosphoSafe buffer (Novagen), which contains phosphatase inhibitors (Figure 22, upper two panels). The separation of individual isoforms is inconclusive, so we tested SKLC in a different buffer and treated with a phosphatase to identify which spots are associated with phosphorylated SKLC. Untreated $SKLC^{WT}$ appears as four spots, and one spot is lost upon phosphatase treatment, suggesting that is the only spot corresponding to phosphorylation (Figure 22, lower two panels). Further tests should be performed to identify the modifications that are represented by the other spots on the blot.

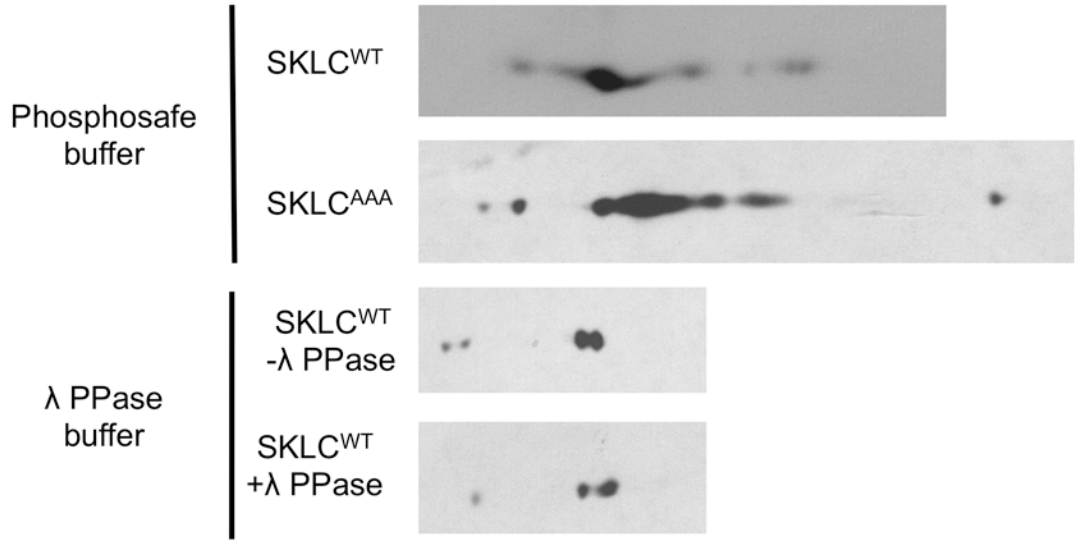

Figure 22: 2D gel analysis of SKLC

Lysates were made from embryos expressing $SKLC^{WT}$ or $SKLC^{AAA}$. Proteins were separated by two dimensional gel electrophoresis and blotted for HA. The top two panels represent lysates prepared in phosphosafe buffer, and the bottom two panels represent lysates made in phosphatase buffer. $SKLC^{WT}$ has four distinct spots in the phosphatase buffer, and one spot is lost upon phosphatase treatment. (Blot: mouse α-HA)

A.4 VARIOUS BUFFER CONDITIONS AFFECT PROTEIN LEVELS

SlprWT and PXAP were extracted from embryos using 2X sample buffer, RIPA buffer, and Phosphosafe buffer (Novagen). Using both 2X SB and RIPA buffers, PXAP appears more abundant than SlprWT by western blot. β-tubulin was used as a loading control. Interestingly, PXAP and SlprWT are extracted at fairly equal levels using PhosphoSafe buffer (Figure 23). Since the detergents present in PhosphoSafe buffer are proprietary, we cannot definitively determine the subcellular localization of these proteins. However, because buffer conditions affect the amount of protein we extract, this suggests that perhaps the proteins are differentially localized in the cell.

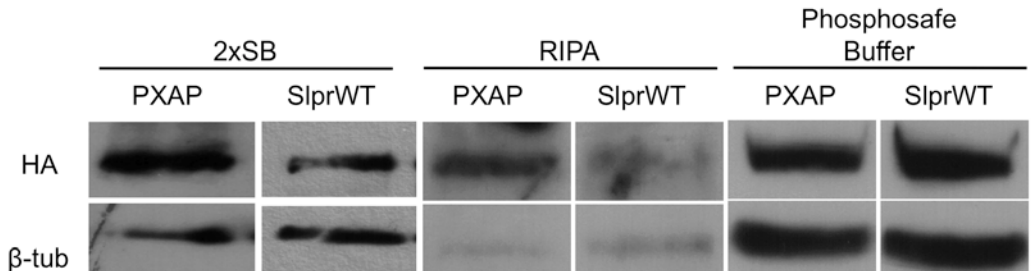

Figure 23: Variable PXAP and SlprWT protein levels are extracted with different buffers

Lysates from embryos overexpressing either PXAP or SlprWT were made in three different buffers. Using 2X SB and RIPA buffer, we notice an increase in the amount of PXAP protein over the amount of SlprWT. However, the Phosphosafe buffer results in roughly equal extraction of PXAP and SlprWT. β-tubulin was used as a loading control.

A.5 ENDOGENOUS SLPR IS STABLE OVER 2 HOURS

Since we had initially observed that overexpressed PXAP was more abundant than the SlprWT transgene, we wanted to test how quickly endogenous Slpr is turned over. S2 cells were treated with cyclohexamide (CHX) to stop nascent protein production. We then extracted endogenous Slpr at timepoints to observe how quickly Slpr is degraded. Over the course of two hours, Slpr is stable. A sample at 24 hours contains less Slpr, but there is still substantial protein present (Figure 24). These data suggest that endogenous Slpr is not turned over rapidly.

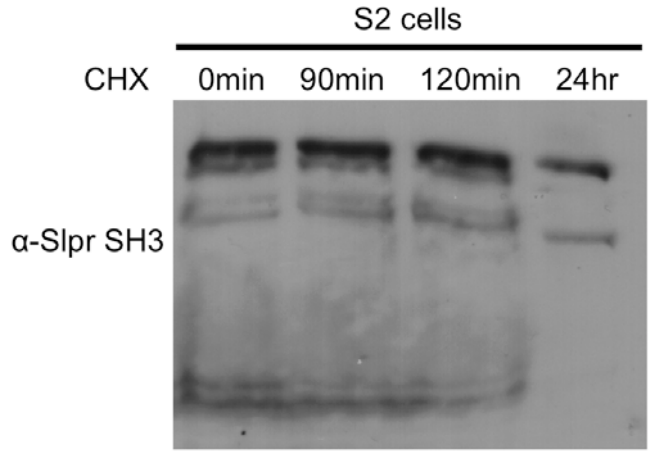

Figure 24: Endogenous Slpr expression in cell lysates from S2 cells treated with CHX

Cyclohexamide was added to S2 cells and incubated at room temperature. Lysates were made in RIPA buffer from samples taken at the given time points to monitor protein turnover. Endogenous Slpr appears stable over 2 hours, and there is still some protein observed after 24 hours. This indicates that Slpr does not turnover rapidly in S2 cell lysates.

A.6 SALIVARY GLAND LOCALIZATION

To test whether protein localization is affected in PXAP mutants, larval salivary glands were utilized due to their large cell size and polytene nature, which leads to abundant protein production. PXAP and SlprWT were overexpressed using HS-Gal4, and protein localization of each during heat shock was monitored. Upon initial heat shock, both SlprWT and PXAP were enriched at the cortex of the cell, as has been previously observed for SlprWT (Figure 25A) (Garlena et al., 2010a), while there was additional cytoplasmic PXAP protein present. As the heat shock continued, PXAP became increasingly cytoplasmic, and by 3 hours, the majority of PXAP was cytoplasmic, while SlprWT remained cortically localized. After four hours at 37°C, PXAP was enriched in the nucleus, and this nuclear localization was dramatically after the animals were released from the heat stress (Figure 25B). However, SlprWT failed to display the same dynamic localization as was observed with PXAP. We conclude that phosphorylation by (MAPK) within Slpr's PXSP motif results in proper sub-cellular localazation, and PXAP mislocalization may lead to faulty signaling in response to environmental stress.

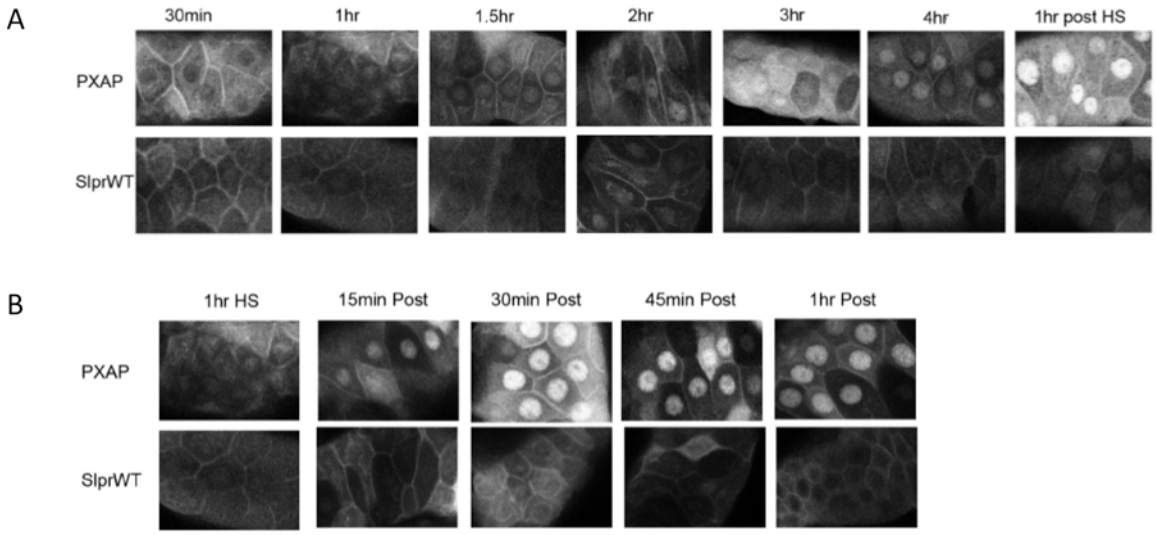

Figure 25: PXAP and SlprWT differentially localize in heat-shocked larval salivary glands

PXAP and SlprWT were overexpressed in larvae using HS-Gal4 and assayed for localization within the salivary glands of third instar larvae. A. Under continuous heat shock, PXAP becomes more cytoplasmic than SlprWT. After four hours of heat shock, PXAP is increasingly nuclear, whereas SlprWT remains cortically localized. B. Protein localization is compared post heat stress. After release from the heat shock, PXAP show strong nuclear localization as early as 15 minutes post heat shock. SlprWT on the other hand, remains excluded from the nucleus and is still enriched at the cortex of the cells. Transgenic protein was visualized using HA staining.

APPENDIX B

B.1.1 REAGENTS GENERATED

B.2 MUTAGENIC PRIMERS

Table 3: Primers used for site-directed mutagenesis

Primer	Sequence
BG2313f	5’-GATCGAATTCCGCCTTGTCGCGTACAGC-3’
BGAAAf	5’-GATGTACAACGCGCAGAGGATGGCTGCGGCGGGCGCGTACGCTTGG-3’
BGAAAr	5’-CCAAGCGTACGCGCCCGCCGCAGCCATCCTCTGCGCGTTGTACATC-3’
BGAAEf	5’-GATGTACAACGCGCAGAGGATGGCTGCGGCGGGCGACGCTTGG-3’
BGAAEr	5’-CCAAGCGTACTCGCCCGCCGCAGCCATCCTCTGCGCGTTGTACATC-3’
BGASAf	5’-GATGTACAACGCGCAGAGGATGAGTGCGGCGGGCGCGTACGCTTGG-3’
BGASAr	5’-CCAAGCGTACGCGCCCGCCGCACTCATCCTCTGCGCGTTGTACATC-3’
BGTAAf	5’-CGCAGAGGATGGCTGCGGCGGGCGCGTACGCTTGG-3’
BGTAAr	5’-CCAAGCGTACGCGCCCGCCGCAGCCATCCTCTGCG-3’
BGAATf	5’-GATGTACAACGCGCAGAGGATGGCTGCGGCGGGC-3’
BGAATr	5’-GCCCGCCGCAGCCATCCTCTGCGCGTTGTACATC-3’
BGPXAPf	5’-CGGAACAGCCGGGAGCACCTTCCTTCTCC-3’

BGPXAPr	5’-GGAGAAGGAAGGTGCTCCCGGCTGTTCCG-3’
BGPXEPf	5’-CGGAACAGCCGGGAGAACCTTCCTTCTCC-3’
BGPXEPr	5’-GGAGAAGGAAGGTTCTCCCGGCTGTTCCG-3’

B.3 RT-PCR PRIMERS

Table 4: Primers used for RT-PCR

Primer	Sequence
XBP intron flank F	5’-CCGAATTCAAGCAGCAACAGCA-3’
XBP intron flank R	5’-TAGTCTAGACAGAGGGCCACAATTTCCAG-3’
HA Forward	5’-GATGGGTATGCTACAAGGACTG-3’
HA Reverse	5’-CCTGCGTAGTCCGGGACGTCATAGGGATA-3’
actin79B forward	5’-CATCCGCAAGGATCTGTATG-3’
actin79B reverse	5’-TTCCTTTTGCATACGGTCAG-3’

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