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The Role of p38 MAPK in Protein Homeostasis and Aging

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Abstract

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ABSTRACT

Aging is characterized by a failure to maintain proper protein homeostasis, potentially leading to tissue dysfunction. Though a variety of genes have been found to regulate lifespan and age-related behaviors how these genetic factors contribute to protein homeostasis has not been fully explored. Here, we report that the evolutionarily conserved aging gene p38 MAPK (p38Kb) regulates age-dependent protein homeostasis. Over-expression of p38Kb results in reduced protein aggregation, while knockout of p38Kb leads to increased protein aggregation. Furthermore, we find that p38Kb regulates protein homeostasis, lifespan, and age-dependent locomotor functions through an interaction with the Chaperone Assisted Selective Autophagy complex; a protein quality control mechanism that selectively degrades misfolded or damaged proteins. We also find that p38Kb regulates the expression of a number of proteins linked to cytoskeletal and neuronal function. Many of these p38Kb-dependent proteins are linked to the human neuropathy Charcot-Marie-Tooth disease and Limb-Girdle Muscular Dystrophy.

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CHAPTER ONE: BACKGROUND AND SIGNIFICANCE

1.1 Aging Processes

Aging is the process of progressive physiological decline leading to impaired cellular and tissue function and ultimately the death of the organism. Age is the single largest risk factor for developing disease including cancer, neurodegenerative diseases, cardiovascular diseases and certain muscular dystrophies (Wagner et al., 2016; Wallace and McNally, 2009). Presently there is an ever-growing aging population who are more susceptible to loss of mobility and independence and a higher chance of developing disease. This older population can have a large financial strain on the economy and present policy challenges for the government (Longo et al., 2015). As a result there is a need for more research dedicated to understanding of the genetic and non-genetic components responsible for aging. For our aging human population, the development of disease interventions and treatment options could increase both our lifespans and healthspans (Olshansky et al., 1990). Throughout the lifespan of an organism, cells and tissues accumulate damage from a variety of sources. Therefore much aging research has been directed at finding the causes of this cellular damage and more recently the identification of compensatory pathways or therapeutic treatments that attempt to restore

homeostasis in damaged cells (Vijg and Campisi, 2008). Only a handful of known pathways, including MTOR, sirtulins, insulin/IGF-1 and MAPK, have been identified that regulate aging processes (Kenyon, 2010). By teasing apart these pathways, we could intervene and slow down the aging processes and the inevitable diseases that come with advanced age.

One of the first aging studies documented calorie restriction in rodents responsible for lifespan extension (McCay et al., 1935). Then in the 1990s, a number of specific genes were identified to effect longevity. For example, daf-2 and daf-16 genes can double lifespan of *Caenorhabditis elegans* (Kenyon et al., 1993). In yeast (*Saccharomyces cerevisiae),* the Sir2 gene and a histone deacetylase RPD3 were identified to effect lifespan (Kaeberlein et al., 1999; Kim et al., 1999). In *Drosophila melanogaster, methuselah,* a putative G-protein coupled receptor, showed lifespan extension and increased resistance to starvation, oxidative stress and heat shock (Lin et al., 1998). Since these initial studies, most of these genes have also been shown to function similarly in mammals to regulate lifespan and aging processes.

It is evident that the processes that control aging appeared to be largely conserved throughout evolution and primarily regulate two central pathways: nutrient sensing/metabolism and stress response (Kenyon, 2005; Vijg and Campisi, 2008). In favorable conditions, both of these systems trigger increased growth and reproduction of the organism. On the other hand, in less favorable conditions these systems work toward cellular protection and organism maintenance. In most cases, environmental interventions (e.g. antioxidants) or longevity- linked genes drive pathways that decrease cellular

damage or increase the activity of compensatory pathways to maintain homeostasis (Kenyon, 2010).

1.2 Oxidative Stress

In the 1950s, Denham Harmon formalized his "free radical theory of aging" where endogenous oxidative radicals build up in the cell, causing cell and tissue damage, thus aging the organism (Harman, 1956). This has been a controversial theory, but numerous studies have supported this hypothesis. For example, *Drosophila* mutants over-expressing the antioxidants SOD1 and catalase show significant lifespan extension (Orr and Sohal, 1994). Also in *C. elegans*, the long-lived age-1 mutant shows an increase in both SOD1 and catalase expression (Friedman and Johnson, 1988; Klass, 1983; Larsen, 1993).

Oxidative stress is defined as the imbalance between the reactive oxygen species (ROS) produced in the cell and the antioxidants and oxidative stress response proteins that work to clear them (Durackova, 2010). Mitochondrial cellular respiration, specifically the electron transport chain and NADPH oxidase, are responsible for the majority of ROS production; *in vivo* about 1-5% of oxygen inhaled is converted into ROS products (Gaki and Papavassiliou, 2014). External sources of oxidative stress include pesticide exposure, ionizing radiation, chemotherapeutics and inflammatory cytokines (Finkel and Holbrook, 2000). Caloric restriction has been linked to lowering respiration levels thus lowering levels of overall ROS (Lin et al., 2000).

At low levels, ROS play positive roles in normal cellular function. For example, growth factors trigger an increase in cytosolic ROS that signal cell proliferation (Finkel and Holbrook, 2000). Also, ROS play roles in activating signal transduction pathways and biochemical processes (e.g. carboxylation or peroxidating reactions) (Durackova, 2010). Nitric oxide (NO·) compounds play critical roles in muscle relaxation and neurotransmission (Martinez et al., 2010). ROS are also upregulated during exercise and promote muscle adaptation (Scheele et al., 2009). Finally, ROS are essential for normal immune function as phagocytic cells use cytosolic ROS to combat infection (Durackova, 2010; Finkel and Holbrook, 2000). Despite these positive roles, if levels of ROS are too high they can severely damage DNA, lipids and proteins. High ROS concentrations can effect the redox state of many signaling molecules, thus interfering with transduction cascades and whole cell signaling. In turn, this cellular damage can result in tissue damage and inflammation, increasing the risk of developing disease (Bokov et al., 2004). There appears to be a critical balance point between the right amount of ROS for optimal cellular function opposed to wide-spread cellular damage (Kenyon, 2010).

To maintain equilibrium there are a number of mechanisms the cell employs to reduce free ROS concentrations. In order to prevent ROS formation, chelating agents (e.g. allopurinol) sequester ions from transition metals therefore preventing radical formation (Durackova, 2010). Once ROS have been formed, antioxidants such as superoxide dismutase - including cytosolic Cu/Zn SOD1, mitochondrial matrix Mn SOD2 and extracellular SOD3 - catalase, peroxiredoxin and gluthanoine reductase act as ROS scavengers (Martinez et al., 2010). If these proteins that manage ROS production fail, organelles damaged by ROS are degraded by lipases or proteases. ROS

overproduction or failure/insufficiency of antioxidant pathways can allow these reactive metabolites to accumulate to a toxic threshold, this process is termed oxidative stress (Gaki and Papavassiliou, 2014; Yacoubian and Standaert, 2009).

Oxidative stress has been linked to the progression of many muscular dystrophies, neuropathies and neurodegenerative diseases including Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis (ALS) (Barnham et al., 2004; Choi et al., 2016; Gaki and Papavassiliou, 2014; Lin and Beal, 2006). Neurons are especially sensitive to oxidative stress because of the large requirement for oxygen by the brain, low endogenous levels of antioxidants, high amounts of lipids that can be readily damaged by ROS and low regenerative capabilities (Barnham et al., 2004; Cui et al., 2004; Gaki and Papavassiliou, 2014). Also, muscle tissue can become oxidatively stressed, partially due to their significant ATP requirement. It is theorized that genetic mutations responsible for many of the muscular dystrophies make the muscle cells more sensitive to oxidative stress and thus induce disease onset and disease progression (Rando, 2002).

1.3 Protein Homeostasis

Along with oxidative stress, protein aggregation is recognized as a key feature of neurodegeneration disorders and muscular dystrophies (Hasegawa, 2016; Verheesen et al., 2006). One of the big questions in the field of aging is the role of a healthy proteome and if it is a requirement for a long lifespan. Especially in adult cells with increased rates of protein misexpression, mutations and cumulative damage from environmental factors, maintaining a healthy proteome would seem to be essential in maintaining proper cell

function (Morimoto and Cuervo, 2009). The lifespan of a protein is typically relatively short and rates for synthesis and degradation are influenced by extracellular stresses and intracellular conditions (Martinez-Vicente et al., 2005). By changing rates of translation and degradation, cells can be finely tuned and adapt to acute or chronic change of cellular conditions (Gidalevitz et al., 2011). Protein turnover happens frequently within the cell and can minimize the number of damaged proteins circulating in the cell. These improperly folded, improperly modified or damaged proteins can self-aggregate, precipitate and form toxic protein inclusions or aggregate structures. Damaged proteins can loose their native conformation thus exposing hydrophobic regions that can negatively interact with other intracellular components or the membrane. Thus, aggregation could potentially be a protective mechanism for the cell to sequester damaged, toxic proteins. "Proteotoxicity" has been linked to neurodegenerative disorders, myopathies, cancer, metabolic disorders, liver disease, retinopathies and diabetes (Koga et al., 2011; Martinez-Vicente et al., 2005). Therefore it is critical that denatured or unfolded proteins are either re-folded or, if too damaged, degraded. In order to maintain protein homeostasis, the cell uses a variety of protein quality control pathways.

While protein degradation is important all throughout development (e.g. embryogenesis and cell differentiation), this process has long been known to naturally decline as the organism ages (Makrides, 1983). It also appears that this decline in proteostasis is one of the key factors causing cellular and organismal aging. In a study of the naked-mole rat, the longest-lived rodent, it was found that they had increased levels of proteasome and autophagy activity (Rodriguez et al., 2016). Also, *C. elegans* studies

show critical links between autophagy and lifespan. The lifespan extension seen in daf-2 mutants was reduced when macroautophagy genes have reduced expression (Hars et al., 2007).

As proper protein homeostasis is essential for cell function, there are number of molecular chaperones and proteolytic systems that oversee the health of the proteome. Highly conserved molecular chaperones such as the heat shock proteins (HSPs) monitor specific organelles or cytosolic proteins for non-native protein conformations (Haslbeck et al., 2005b). In these locations, three families of proteins Hsp70, Hsp90 and HspB are predominantly responsible for protein folding and re-folding, conformational dynamics and can also signal degradation or post-translational modification (Young et al., 2004). Chaperones work with co-chaperones to identify misfolded proteins and attempt re-folding. But if a protein cannot be refolded, then chaperones allow for ligases, E1-E3 ligases, to tag the damaged protein surface with 4-5 ubiquitin molecules. Cellular stress can cause protein unfolding and failure of the cytosolic chaperone protein network has been linked to many diseases (Macario and Conway de Macario, 2005). Proper chaperone activity also is important for aging processes. Studies in worms and flies, show that the overexpression of HSPs leads to lifespan extension (Morrow et al., 2004; Walker et al., 2001). Also, a studies of centenarians show increased expression of chaperones, importantly some HSPs (Marini et al., 2004).

After chaperons and the E3 ligases identify and tag damaged proteins, these proteins are sorted into different degradation pathways. BAG proteins target the damaged protein for to the proteasome or lysosome for degradation (Connell et al., 2001). BAG-1 has an ubiquitin-like mediating domain, which allows for the shutting of damaged

proteins toward the proteasome (Demand et al., 2001). The classical method of protein degradation is the ubiquitin/proteasome system (UPS) that is responsible for destruction of improperly folded, unfolded or damaged (e.g. oxidized) proteins. The proteasome consists of a catalytic core (20S) which degrades the proteins and the peripheral regulatory unit (19S) which recognizes specific target proteins and can adjust the rate of turnover (Koga et al., 2011). Many studies suggest that the UPS is also linked to aging. With age, ubiquitin levels decrease and the ubiquitin molecules can become mutated resulting in decreased degradation of damaged proteins (Shang et al., 1997). Also, the levels of the 20S proteasome decrease with age and this is accompanied by reduced efficiency of assembly of the complete 26S proteasome (Tonoki et al., 2009; Vernace et al., 2007). Aging is also associated with a decrease in ATP, in turn reducing the efficiency of the UPS (Vernace et al., 2007). Furthermore, cells cultured from centenarians have very efficient proteasome systems (Chondrogianni et al., 2000).

In addition to the UPS, damaged proteins can be degraded through a parallel process called autophagy that can degrade both cytosolic components and whole organelles through the lysosome. Another member of the BAG family, BAG-3, competes with BAG-1, and shuttles damaged proteins to the autophagosome (Fuchs et al., 2010; Gamerdinger et al., 2009; Rosati et al., 2011). Multiple types of autophagy have been characterized including macroautophagy, microautophagy, Chaperone Mediated Autophagy and Chaperone Assisted Selective Autophagy. Compared to other types of autophagy where chaperones individually select and target damaged proteins, macroautophagy is a relatively non-selective process. An autophagosome is formed as a portion of the ER membrane called the phagophore surrounds and sequesters entire

portions of the cytosol or organelles. Then this structure fuses with the lysosome to degrade the engulfed contents. The abilities of macroautophagy appear to decline with age, in part due to incomplete lysosome/autophagosome fusion (Terman, 1995) . Since entire organelles can be removed via macroautophagy, this process serves a powerful regulator of ROS production by removing damaged mitochondria (Terman et al., 2004). Microautophagy functions similarly to macroautophagy without the requirement of the intermediate autophagsome formation. This pathway plays a role in maintain basal levels of protein turnover (Mortimore et al., 1988).

Unlike macroautophagy and microautophagy, autophagy mediated by chaperone complexes show high specificity for degradation targets. It was thought that autophagy did not rely on polyubitiqitin tags, but recent work on Chaperone Mediated Autophagy (CMA) and Chaperone Assisted Selective Autophagy (CASA) show a requirement for ubiquitin molecules tagging damaged proteins (Kraft et al., 2010). CMA relies on specific chaperones identifying a specific subset of proteins with a pentapeptide KFERQ motif (Behl, 2011; Cuervo and Dice, 1998; Kiffin et al., 2004). CMA target proteins are directly translocated to the lysosome through an interaction with the surface receptor protein - lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo and Dice, 1996; Kaushik and Cuervo, 2009). The CMA mediated protein turnover is increased by oxidative stress (Kiffin et al., 2004; Massey et al., 2004). During oxidative insult, there is a transcriptional upregulation of LAMP-2A that increases the degradation of oxidatively damaged proteins, and LAMP-2A shows a preference for oxidatively damaged proteins (Kiffin et al., 2004). Like other proteostasis systems, the CMA has also been shown to have age-dependent functional decline. LAMP-2A has been shown to less efficiently

translocate cargo to the lysosomal lumen in older rodents and mammalian cell culture, reducing the overall efficiency of the system (Cuervo and Dice, 2000). The decline of CMA function allows for the build up of pathogenic protein targets such as mutant forms of huntingtin and α-synuclein (Dice, 2007; Massey et al., 2004; Thompson et al., 2009). Even when pathogenic proteins are being cleared from the cell, these mutated protein can monopolize these system and put a strain on overall cell proteostasis (Kettern et al., 2010).

Another type of chaperoned autophagy is the Chaperone Assisted Selective Autophagy (CASA) complex. CASA is comprised of a core set of chaperones and co-chaperones. HspB8 and Hsc70 recognize misfolded proteins and attempt refolding (Arndt et al., 2010). If too damaged, Hsc70 can recruit the co-chaperone, BAG-3 (starvin in flies) (Rosati et al., 2011). BAG-3 interacts with the substrate by a proline-rich domain and will target the protein for autophagy (Carra et al., 2008). BAG-3's involvement is one of the key differentiating factors for this type of autophagy. Interestingly, there is shift in the levels of BAG-1 to BAG-3, as autophagy pathways are upregulated with age and levels of oxidative stress (Gamerdinger et al., 2009). This results in a switch from proteasomal to lysosomal degradation of oxidatively damaged and ubiqutinated proteins, which could serve as a cellular adaptation in times of oxidative stress or high protein aggregation (Kettern et al., 2010).

The core CASA chaperones, Hsc70-HspB8-BAG-3, associate with CHIP, an E3 ubiqutin ligase, that covalently links a ubiquitin tag to the protein destined for degradation (Arndt et al., 2010). p62 (ref(2)p in flies) recognizes this ubiquitinated protein and facilitates transport to the autophagosome/lysosome for protein destruction.

Interestingly, in mammalian cells, p62 concentrations also increase with age, which is thought to increase the potential number of oxidized and ubiquitinated proteins moved to the lysosome (Gamerdinger et al., 2009). In *Drosophila* muscle, the CASA complex has been shown to have a role maintaining protein homeostasis at the z-disk, a site of mechanical stress and high protein turnover (Arndt et al., 2010). On of the identified CASA substrates is damaged filamin, which normally functions to crosslink actin filaments (Arndt et al., 2010). Currently, we are investigating the role of p38 MAPK in regulating protein homeostasis through the CASA complex.

1.4 Mitogen Activated Protein Kinases (MAPKs)

Extracellular stress signals are known to activate a group of Mitogen Activated Protein Kinases (MAPK) in all eukaryotic cells. MAPKs are a group of Ser/Thr kinases that function in many biological processes. Conventional MAPKs from mammalian systems are ERK1/2 (Extracellular Signal Regulated Kinase), JNK 1/2/3 (c-Jun amino (N)- terminal Kinase) and p38 α/β/γ/δ kinase. ERKs generally mediate growth and differentiation response while JNK and p38K respond to cellular stress including oxidative stress, osmotic shock, cytokines and UV radiation and can induce inflammatory response, apoptosis and survival responses. A key structural similarity of all these kinases is a Thr-X-Tyr motif on the activation loop that is dually phosphorylated by an upstream kinase. MAPKs respond to a host of extracellular and intracellular stimuli such as growth factors, cytokines, hormones and oxidative stress cues. MAPK malfunction also appear to play a role in many types of disease (Cargnello and Roux, 2011).

There is an evolutionarily conserved upstream activation sequence responsible for the activity of MAPKs. Receptor activation, by external stimuli, leads to direct or indirect phosphorylation of the initial kinase, MAPKKK. In turn, this kinase phosphorylates and activates MAPKK. Lastly, the MAPKK will dual phosphorylate the conserved threonine and tyrosine residues on the activation loop of the MAPK. Phosphorylation allows for a conformational change of the MAPK, allowing for ATP and substrate binding. Once activated, the MAPK can interact with downstream targets by phosphorylating a serine or threonine residue followed by a proline residue. Because this series of residues is fairly common, p38K exhibits a higher degree of substrate specificity through docking and binding motifs, activation loop length and specific interactions with scaffolding proteins (Roux and Blenis, 2004; Sharrocks et al., 2000). Furthermore, there are a few identified domains adjacent to the substrate's phosphorylation site that further help with substrate specificity and efficiency of phosphorylation (Kallunki et al., 1996). For each of the MAPKs there are specific upstream factors that are responsible for their activation and unique downstream targets. MAPK substrates include a wide variety of transcription factors, other protein kinases, cell-surface receptors, phospholipases and cytoskeletal proteins. But a full network map of the downstream targets of each MAPK is still unknown.

One of the MAPKs, p38K plays critical roles in managing cellular response to stresses such as DNA damage, UV radiation, inflammatory cytokines and oxidative stress. Once activated, p38K can determine the cell's stress response: from preventing growth and differentiation or in extreme stress, triggering apoptosis. As such, p38K regulates many pathways linked to protein homeostasis and can increase recycling

pathways for damaged proteins (Sui et al., 2014). To control autophagy pathways, p38K interacts with Atg9, a key component of the autophagy machinery (Webber and Tooze, 2010b). Additionally, we now find that p38K can interact with the CASA complex to mediate protein degradation (Figure 1.1).

In *Drosophila,* there are only two p38K genes: p38Ka and p38Kb, with p38Kb playing a role in a wider variety of functions in flies. Loss of either gene results in viable animals, but loss of both genes is lethal (Craig et al., 2004; Vrailas-Mortimer et al., 2011). Tissue specificity of upstream activators and various stimuli can lead to specific functions of p38K within a tissue. In the muscle, p38K has a critical role in in muscle differentiation, normal muscle activity and locomotor behaviors (Cuadrado and Nebreda, 2010; Vrailas-Mortimer et al., 2011). Also during exercise, p38K promotes expression of Pgc-1α, a regulator of mitochondrial biogenesis and muscle adaptation (Akimoto et al., 2005). Muscle-specific over-expression of p38Kb also provides increased resistance to starvation, heat shock and oxidative stress (Vrailas-Mortimer et al., 2011). Finally, levels of p38Kb in muscle play a critical role in lifespan. While p38Kb mutants have a significantly reduced lifespan, over-expression leads to a 37% lifespan extension in *Drosophila* (Vrailas-Mortimer et al., 2011). Similar effects are not seen with p38Ka mutants.

1.5 Muscular Dystrophies

The integrity of muscle tissue is influenced by both age and oxidative stress; in turn these become risk factors for developing or worsening symptoms of muscular dystrophies. *Drosophila* provide an excellent model to study genes and mechanisms that

underlie many muscular dystrophies. The muscle tissue in flies show same structural and functional features to humans (Baylies et al., 1998). Additionally flies mutant for genes linked to muscular dystrophy like dystrophin (Kucherenko et al., 2011; Wairkar et al., 2008), the dystroglycan/dystrophin complex (Shcherbata et al., 2007) and lamin (Munoz-Alarcon et al., 2007) show age-dependent muscle degeneration and mobility defects similar to human symptoms.

Muscular dystrophies are a group of genetically inherited disorders that effect predominantly voluntary skeletal muscle causing muscle weakness, and in more extreme diseases or with advanced ages, muscle atrophy. Muscular dystrophies include a wide range of diseases including congenital (present at birth) and age-dependent. While many genetic mutations responsible for muscular dystrophies have been identified, the underlying mechanism for many types is still unknown. Muscular dystrophies progress as mutant proteins accumulate thus impairing and overwhelming proteasome and autophagy systems (De Palma et al., 2012). In mouse models of muscular dystrophies, abnormally high numbers of chaperone proteins associated with the UPS and CASA complex are seen in effected tissue (Janue et al., 2007; Kley et al., 2013). Members of the CASA complex have been shown to localize to the muscle z-disk in the flight muscle of flies (Arndt et al., 2010). Mutations of CASA complex members, BAG-3 and HspB8, have been linked to muscular dystrophies and neuropathies (Ghaoui et al., 2016; Rosati et al., 2011; Selcen et al., 2009). As age and oxidative stress are also risk factors involved in many of these diseases (Choi et al., 2016), we wanted to explore the relationship of p38K and members of the CASA complex to three types of muscular dystrophies: Limb-Girdle Muscular Dystrophy, Charcot-Marie-Tooth disease and myofibrillar myopathy.

Limb-girdle muscular dystrophy

Limb-Girdle Muscular Dystrophy (LGMD) are a genetically diverse group of disorders that effect the voluntary proximal muscles of the shoulder and pelvic girdles. LGMD can start in childhood or adulthood and will cause muscle weakness and eventually muscle atrophy. Other than loss of mobility, some patients will also experience cardiopulmonary complications. There are currently 30 subtypes of LGMD caused by a number of mutations affecting proteins of the sarcolemma, sarcomere, cytoplasm or nucleus (Sarparanta et al., 2012; Thompson and Straub, 2016). It appears that myocytes attempt to degrade these mutated proteins, sometimes via the CASA complex. For example, Hsc70 interacting co-chaperones DNAJB6 and BAG-3 have been shown to cause LGMD (type 1D) (Sarparanta et al., 2012). In LGMD patients, DNAJB6 was found in cytoplasmic inclusions of muscle tissue colocalizing with HspB8 and BAG-3 (Sato et al., 2013).

Oxidative stress and inflammatory cytokines, which are activators of p38K, are linked to LGMD (Haslbeck et al., 2005a). Calpain 3, another LGMD causing gene (type 2A) normally functions as a calcium-dependent, non-lysosomal cysteine protease (Richard et al., 1995). In Calpin 3 knockout mice, p38K activation was decreased downstream of CaMKII (Kramerova et al., 2016). As a result, there is a decrease in downstream targets of p38K such as PGC1α and Mef2 that play a role in protein damage control, protein turnover and proper mitochondrial function (Kramerova et al., 2016). Dysferlin, causative in LGMD type 2B, is also shown to interact with p38K in dysferlin deficient mice (Suzuki et al., 2012). Dysferlin plays a role in muscle membrane repair

with cavelolin-3, mutations of either protein are linked to levels of p38K expression (Capanni et al., 2003).

Charcot-Marie-Tooth Disease

Charcot-Marie-Tooth disease (CMT) is an inherited peripheral neuropathy, affecting both neurons and muscles. According to the NIH it is one of the most common inherited neurological disorders, seen in 1 out of every 2,500 people in the United States (NIH, 2007). Both sensory and motor nerves are affected leading to muscle weakness, changes in gait, loss of sensation and, later in the disease progression, muscle atrophy. Symptoms normally develop from adolescence through mid-adulthood. There are a variety of mutations responsible for CMT and affect the integrity of the peripheral axon or the myelin sheath.

Protein aggregation and mislocalization are linked to CMT. For example, a mutation in small integral membrane protein of lysosome/late endosome (SIMPLE) is responsible for CMT type 1C. Mutations in this gene also cause an increase in protein aggregation and increase in the activity of autophagy pathways (Lee et al., 2011). Mutations in PMP22, linked to the most common type of CMT (type 1A), show increase in oxidative stress markers from patient biopsies defective in PMP22 (Seco-Cervera et al., 2014). In addition, studies in *Drosophila* of the CMT type 4A gene, found that mutations in Gdap1 causes a change in mitochondrial size, distribution and morphology (Lopez Del Amo et al., 2015). This mitochondrial change could underlie the increase in oxidative stress. Finally, loss of sensory nerves is another CMT symptom. Interestingly,

we have observed in *Drosophila* that p38Kb mutant flies show an increase in grooming behavior; which is indicative of loss of sensation in flies.

Myofibrillar Myopathy

The presentation of exclusively LGMD is relatively rare; most patients also have concomitant distal myopathy or myofibrillar myopathy (Straub and Bushby, 2008). Myofibrillar myopathy (MFM) is a muscular dystrophy encompassing a wide range of skeletal muscle disease symptoms. Patients show muscle weakness originating in the distal limbs but the disease can spread, affecting proximal, respiratory and cardiac muscles. Although there are early-onset forms, the majority of MFM cases appear later in adulthood. Severity of the disease is variable: from mild muscle weakness to severe, lethal forms where the respiratory and cardiac functions are critically affected.

Characteristic MFM biopsies show disintegration of myofibrils originating at the Z-disk leading to protein aggregation, which culminates in myofibrillar degeneration and muscle weakness (Ruparelia et al., 2014). While sporadic cases have been documented, most documented MFM cases are due to mutations transmitted in an autosomal dominant manner. All MFM associated genes play a role in proper Z-disk function, including proteins like BAG-3, filamin and cofilin (Ferrer and Olive, 2008; Kley et al., 2013; Selcen et al., 2009). Increased oxidative stress and protein aggregation are two hallmark cellular features of this disease (Selcen et al., 2004). Interestingly, MFM patients also showed increased immunoreactivity to HDAC6, an upstream autophagy marker, in affected tissues (Kley et al., 2012; Kley et al., 2013). HDAC6 serves as a vital integrator for protein homeostasis by upregulating compensatory lysosome-autophagy pathways

when UPS is impaired (Pandey et al., 2007). HDAC6 has been shown to trigger an increase heat shock proteins, including Hsc70, and work via p38K (Kastle et al., 2012).

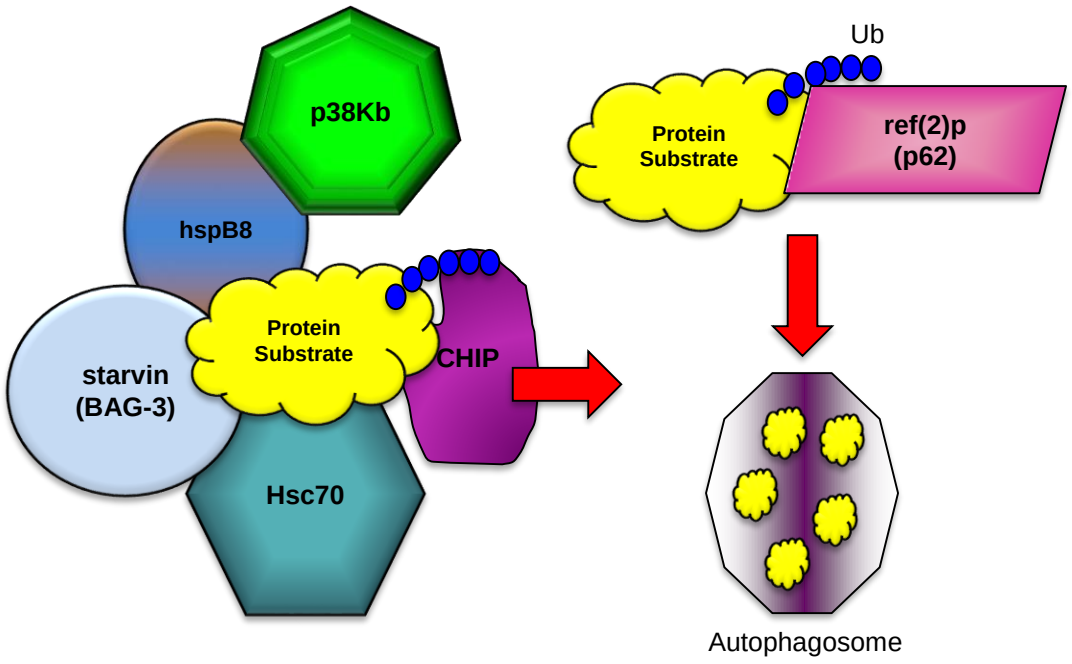


Figure 1.1: **Model of chaperone assisted selective autophagy (CASA) complex.** The CASA complex works in protein homeostasis through the identification and targeting of misfolded or damaged proteins for lysosomal degradation. The complex is comprised of a few core members including chaperones Hsc70 and HspB8 that identify damaged or misfolded proteins. BAG-3 (starvin in flies) is a co-chaperone that targets protein degradation via autophagy pathways. CHIP, an E3 Ubiquitin ligase, will covalently link 4-5 Ubiquitin molecules on the surface of the damaged proteins. p62 (ref(2)p in flies) identifies this ubiquitin tags on the surface of the damaged protein. The protein is moved it to the autophagosome and subsequently, the lysosome, where protein degradation occurs. We hypothesized that p38Kb interacts with the CASA complex to regulate protein aggregation within the adult muscle of *Drosophila*.

CHAPTER TWO: P38 MAPK INTERACTS WITH CHAPERONE ASSISTED SELECTIVE AUTOPHAGY TO REGULATE AGE-DEPENDENT PROTEIN HOMEOSTASIS

2.1 Introduction

Protein turnover is critical for maintaining tissue health as many proteins become damaged or misfolded during normal tissue functions. Therefore, the cell utilizes a variety of protein quality control mechanisms to refold or degrade these damaged proteins, including the ubiquitin proteasome system and macroautophagy. During aging, protein quality control mechanisms become less efficient leading to the accumulation of damaged or misfolded proteins that begin to form protein aggregates (Taylor and Dillin, 2011). It has been hypothesized that these damaged proteins form toxic aggregates that may lead to the negative phenotypes associated with normal aging, such as impaired tissue function (Taylor and Dillin, 2011). Furthermore, decreased protein aggregation has been associated with longevity. For example, over-expression of Foxo leads to an increased lifespan but also a concordant decrease in protein aggregation in *C. elegans*, *Drosophila*, and mice (Ben-Zvi et al., 2009; Cohen et al., 2006; Demontis and Perrimon, 2010; Morley et al., 2002; Palazzolo et al., 2009), suggesting that lifespan and protein aggregation are tightly linked processes. However, the molecular mechanisms that

underlie the relationship between aging and protein homeostasis have not been fully characterized.

One pathway that has been linked to both aging and protein homeostasis is the stress response p38 MAPK (p38K) pathway. In mammalian systems, there are four p38K genes (α, β, γ, and δ), and p38Kα has been linked to both the inhibition (Schnoder et al., 2016) (Webber and Tooze, 2010a) and induction (Liu et al., 2009; Younce and Kolattukudy, 2010) of macroautophagy, in particular in response to oxidative stress (Zhuang et al., 2016) (Duan et al., 2011). In addition, p38Kα has been linked to regulating macroautophagy in cellular senescence (Henson et al., 2014; Luo et al., 2011; Rudolf et al., 2014). However, how p38K signaling may contribute to protein homeostasis in response to natural aging is not well understood. The fruit fly *Drosophila melanogaster* has two p38K genes (p38Ka and b), and we have previously reported that p38Kb acts in the adult musculature to regulate aging. We found that over-expression of p38Kb leads to increased lifespan while loss of p38Kb results in a short lifespan and age-dependent locomotor behavior defects (Vrailas-Mortimer et al., 2011). In addition, p38Kb has been implicated in regulating muscle protein homeostasis, with loss of p38Kb leading to increased polyubiquitination of insoluble proteins and alterations in oxidative stress dependent translation (Belozerov et al., 2014). Our *Drosophila* p38Kb aging model is an ideal system for testing how the p38K signaling pathway regulates age-dependent protein homeostasis and how this impacts lifespan and age-dependent locomotor behaviors.

As *Drosophila* p38Kb acts in the muscle to regulate aging, we have tested for interactions with the Chaperone Assisted Selective Autophagy (CASA) complex, a

protein quality control mechanism that has been shown to regulate protein turnover in the muscle in both flies and mice (Arndt et al., 2010; Ulbricht et al., 2013). The CASA complex consists of three core proteins: the molecular chaperones HspB8 and Hsc70 (Hsc70-4 in flies) and the co-chaperone BAG-3 (starvin in flies). The CASA complex binds to specific protein substrates that are damaged or misfolded. Those substrates that cannot be refolded are polyubiquitinated by the E3 ubiquitin ligase CHIP, targeted to the autophagosome by p62 (ref(2)p in flies) and subsequently degraded through the autophagosome-lysosome system (Behl, 2011; Gamerdinger et al., 2009; Kettern et al., 2010; Min et al., 2008; Terman et al., 2007). Here, we report that p38Kb regulates age-dependent muscle protein homeostasis through an interaction with the CASA complex.

2.2 Results

p38Kb regulates age-dependent protein homeostasis.

p38Kb null mutant animals have a short lifespan of ~4-5 weeks and age-dependent locomotor behavior defects as compared to genetic background controls (Vrailas-Mortimer et al., 2011). In addition, p38Kb mutants have increased levels of insoluble polyubiquitinated proteins by biochemical analysis (Belozerov et al., 2014). However, protein aggregate formation had yet to be visualized in the p38Kb mutants. Therefore, we analyzed how protein aggregation is altered in both the p38Kb mutants as well as p38Kb over-expression animals. We find that loss of p38Kb leads to an increased number of protein aggregates in the adult indirect flight muscle at 1 week and 3 weeks of age (Figure 2.1A-B) and increased aggregate size with age (Figure 2.1C-D). We find that over-expression of p38Kb in the adult muscle, which extends lifespan, leads to decreased

protein aggregate number and size throughout the lifespan (Figure 2.1E-H). These results suggest that decreased protein aggregate number and size may be linked to longevity and that protein aggregate accumulation and increased size may be toxic, leading to decreased lifespan and potentially the impaired locomotor function of the p38Kb mutants.

p38Kb colocalizes with the CASA complex in the adult flight muscle.

As p38Kb acts in the muscle to regulate aging and protein homeostasis, we tested for an interaction between p38Kb and the CASA complex, which has been previously reported to localize to the muscle Z-disc (Arndt et al., 2010), an area of high protein turnover. We find that a FLAG-tagged p38Kb colocalizes with the Z-disc marker alpha-actinin and is also present at the M-line (Figure 2.2A). Furthermore, p38Kb colocalizes with HspB8, Hsc70-4 and starvin (stv) at the Z-disc and M-line (Figure 2.2B-D), and with the E3 ubiquitin ligase CHIP only at the Z-disc (Figure 2.2E). These data suggest that p38Kb may directly interact with the CASA complex in the muscle.

p38Kb physically interacts with the CASA complex in the adult flight muscle.

To determine if p38Kb interacts with the CASA complex in the adult muscle, we performed co-immunoprecipitation experiments using endogenously GFP tagged CASA complex proteins. In order to capture transient interactions, we expressed a FLAG-tagged p38Kb kinase dead construct that is able to be activated and bind to a target but cannot phosphorylate it, leading to a delayed release of the target (Hattori et al., 2013). We immunoprecipated each endogenously GFP tagged CASA complex protein and probed for the FLAG tagged p38Kb and found that p38Kb co-immunoprecipitates with the core

members of the CASA complex (Figure 2.2F-H) with stronger binding at younger ages than older ages. To further verify this interaction, we tested if the CASA complex can co-immunoprecipitate with endogenous p38Kb and find that HspB8, Hsc70-4 and stv co-immunoprecipitate with phosphorylated p38K (Figure 2.2I), suggesting that p38Kb activation may be important for its interaction with the CASA complex.

p38Kb genetically interacts with the CASA complex to regulate lifespan.

Muscle specific over-expression of p38Kb results in lifespan extension (Vrailas-Mortimer et al., 2011), which may be associated with decreased protein aggregate number and size (Figure 2.1E-H). Therefore, we tested if the p38Kb mediated lifespan extension requires the CASA complex. We find that expression of a dominant negative Hsc70-4 alone results in early lethality and reduces the p38Kb mediated lifespan extension by 19.4% as compared to p38Kb over-expression alone (Figure 2.3A), completely blocking the lifespan extension. In addition, expression of HspB8 RNAi in the muscle does not alter lifespan compared to its respective controls (Figure 2.3B). Although HspB8 inhibition reduces the p38Kb lifespan extension by 10.7%, this still leads to an overall lifespan extension as compared to controls (Figure 2.3B).

Inhibition of stv with a strong muscle driver (Mef2-GAL4) results in a severely reduced lifespan (on average 4 days) and completely abrogates the p38Kb mediated lifespan extension, resulting in an 81.2% reduction as compared to p38Kb over-expression (Figure 2.3C). Interestingly, over-expression of p38Kb is able to rescue a subset of the stv RNAi animals, allowing them to live relatively normal lifespans (Figure 3C). In order to further analyze the relationship between p38Kb and stv, we utilized the

MHC-GAL4, which drives weaker expression of UAS transgenes (Vrailas-Mortimer et al., 2011). Weaker inhibition of stv also reduces lifespan as compared to controls and partially blocks the p38Kb mediated lifespan extension by 22.5% (Figure 2.3D). These data suggest that p38Kb requires the CASA complex and intact protein quality control in order to regulate lifespan but also that over-expression of p38Kb can rescue the lifespan defects caused by inhibition of the CASA complex (Table 2.1 – 2.7).

p38Kb over-expression requires the CASA complex for improved muscle function.

We have previously reported that p38Kb plays an important role in muscle function, with loss of p38Kb resulting in age dependent locomotor dysfunction (Vrailas-Mortimer et al., 2011). Therefore, we tested if p38Kb over-expression results in improved muscle function. We analyzed flight behavior in which good fliers will collect in the top portion of the chamber and poor fliers will fall to the bottom of the chamber. We find that over-expression of p38Kb results in better fliers, with more flies distributed in the top and middle portions of the chamber as compared to background controls at one week of age (Figure 2.3E-F), however, this benefit is lost by 5 weeks of age (data not shown). Though inhibition of either HspB8 or stv results in normal flight behaviors at both young (Figure 2.3E-F) and old ages (data not shown), inhibition of the CASA complex prevented the improved flight behavior observed in the p38Kb over-expression animals (Figure 2.3E-F), suggesting that p38Kb requires the CASA complex for both lifespan and muscle function.

p38Kb genetically interacts with the CASA complex to regulate age-dependent protein homeostasis.

As we find that p38Kb genetically interacts with the CASA complex to regulate lifespan and muscle function, we next tested how p38Kb and the CASA complex interact to regulate protein homeostasis. We find that expression of dominant negative Hsc70-4 in the muscle results in a wing posture defect in which the wings are either held in an upright position or drooped down by the sides (Figure 2.4A). Over-expression of p38Kb has no effect on the Hsc70-4 mediated wing posture defect (Figure 2.4A). This type of wing posture defect is linked to muscle degeneration (Greene et al., 2003), suggesting that Hsc70-4 plays an important role in muscle maintenance. Furthermore, when we dissected the thoraxes of the Hsc70-4 dominant negative flies, the muscles were thin and wasted, resulting in a "hollow" phenotype (Figure 2.4D), suggesting muscle degeneration. Over-expression of p38Kb in the Hsc70-4 dominant negative background is unable to rescue the muscle loss (Figure 2.4E), similar to what we observe with lifespan and wing posture. Due to this muscle wasting phenotype, analysis of protein aggregation in the Hsc70-4 dominant negative background was not reliable (data not shown).

We find that inhibiting HspB8, which doesn't affect lifespan (Figure 2.3B), leads to increased protein aggregate number at both young and old ages (Figure 2.4F-G) but does not affect aggregate size (Figure 2.4H-I). Additionally, inhibition of HspB8 is not sufficient to prevent the reduced number and size of protein aggregates observed in the p38Kb over-expression animals (Figure 2.4F-I), which was surprising given that

inhibiting HspB8 in a p38Kb over-expression background partially blocks the lifespan extension mediated by p38Kb (Figure 2.3B).

Next, we examined the effects of stv inhibition on p38K mediated protein aggregation. For stv inhibition using the stronger Mef2-GAL4 driver, we analyzed protein aggregate number and size at 2 days of age (Figure 2.5A and C) as these animals live on average for 4 days (Figure 2.3C). At this very early time point, we find that both aggregate number and size are highly variable in the Mef2-GAL4 controls. Despite this variability, we find that inhibiting stv results in a trend towards more aggregates and a significant increase in aggregate size (Figure 2.5A and C). Over-expression of p38Kb results in a trend towards fewer and smaller aggregates at two days (Figure 2.5A and C), which becomes statistically significant by 1 week of age (Figure 2.5B and D). In addition, inhibition of stv in the p38Kb over-expression background doesn't lead to a statistically significant increase in aggregation or aggregate size, though there is a trend towards an increase in number at both 2 days and 1 week (Figure 2.5A and B). This is particularly interesting as the p38Kb lifespan extension is severely reduced by inhibition of stv. However, it may be that the portion of these flies that go on to have more normal lifespans (Figure 2.3C) do so due to having reduced protein aggregation.

Using the weaker MHC-GAL4 driver to induce stv inhibition also results in increased protein aggregate number and size at 5 weeks (Figure2. 5E-H). Inhibition of stv in the p38Kb over-expression background at young ages did not result in a further increase in aggregate number or size as compared to p38Kb over-expression alone. However, in old flies, inhibition of stv prevents the p38Kb mediated reduced protein aggregation (Figure 2.5F). These data suggest that p38Kb genetically interacts with the

CASA complex to regulate protein homeostasis and that the extent of protein aggregation is sensitive to the levels of p38Kb and the CASA complex members.

p38Kb regulates the activity of the CASA complex.

To further explore the link between protein homeostasis and aging, we tested if over-expression of the CASA complex is able to rescue the reduced lifespan observed in p38Kb null mutants. We find that over-expression of either Hsc70-4 or HspB8 had no effect on the p38Kb short lifespan defect (data not shown). However, over-expression of stv, which has no effect on lifespan alone (Figure 2.6B), results in a further shortening of the p38Kb mutant lifespan by an additional 36% as compared to p38Kb mutant controls (Figure 2.6A), suggesting that p38Kb may be limiting for stv function. If stv requires p38Kb activity, then co-over-expression of p38Kb and stv may lead to a further extension of the lifespan. Indeed, we find that co-over-expression of stv and p38Kb leads to an additional 5% increase in lifespan relative to p38Kb over-expression alone (Figure 2.6B). These data suggest that p38Kb may act upstream of the CASA complex and that p38Kb is required for regulating the activity or efficiency of the complex through an interaction with stv.

To further explore how p38Kb may be interacting with the CASA complex, we examined if loss of p38Kb alters the localization of the CASA complex in the muscle. We find that while stv can still localize to the Z-disc and M-line in p38Kb null mutants, stv expression is also more diffuse (Figure 2.6C-E). As p38Kb mutants have elevated levels of protein aggregates, these data indicate that in the absence of p38Kb, stv

localization is impaired as is subsequent CASA complex mediated clearance of damaged and/or misfolded proteins.

Since co-over-expression of p38Kb and stv leads to a further increase in lifespan (Figure 2.6B), this suggests that these animals may also have a further decrease in protein aggregate number and size. We find that over-expression of stv alone results in fewer aggregates at young and old ages (Figure 2.6F-G). We also find that co-over-expression of p38Kb and stv leads to reduced aggregate number at young ages, which is not significantly different from over-expression of p38Kb or stv alone (Figure 2.6F). By 5 weeks of age, co-over-expression of p38Kb and stv flies have comparable aggregate number to controls (Figure 2.6G). These data suggest several possibilities. The first is that co-over-expression of p38Kb and stv provides beneficial effects in early adulthood that continue throughout adulthood leading to increased lifespan despite the presence of protein aggregates. Another possibility is that aggregate size and/or content may play a more important role in determining lifespan as compared to overall aggregate number. When we examine aggregate size, we find that stv over-expression results in reduced aggregate size only at older ages (Figure 2.6H-I). Co-over-expression of p38Kb and stv leads to smaller aggregates at both young and old ages similar to p38Kb over-expression animals alone (Figure 2.6H and I). These data suggest that aggregate size may play a greater role in predicting longevity. Though we don't see a significant difference in aggregate number or size between p38Kb alone and p38Kb and stv co-over-expression, there is a trend towards smaller and fewer aggregates in the co-overexpression animals. In addition, it may be that over-expression of p38Kb and stv together promotes the rapid

clearance of particularly toxic protein species that allows for increased longevity without significant changes in protein aggregate size or number.

These data suggest that p38Kb may be regulating the activity/efficiency of the CASA complex so that when p38Kb is over-expressed, this leads to improved recognition of damaged/misfolded CASA targets and/or improved refolding or targeting of substrates for degradation as required. If p38Kb does regulate the CASA complex in this manner, then inhibiting the degradation step should result in an accumulation of poly-ubiquitinated protein aggregates. Therefore, we removed a single copy of ref(2)p, the lysosomal adaptor protein that mediates the degradation of CASA targets, in the p38Kb over-expression background. Interestingly, loss of a single copy of ref(2)p alone results in fewer aggregates at old age (Figure 2.7B), which may reflect compensation by other protein clearance mechanisms. We also find that loss of a single copy of ref(2)p prevents the reduced protein aggregation observed in the p38Kb over-expression animals at both young and old ages (Figure 2.7A-B). In addition, we find that loss of ref(2)p is also sufficient to block the p38Kb mediated lifespan extension (Figure 2.7C) and improved flight behavior at 1 week of age (Figure 2.7D). These data suggest that p38Kb does indeed regulate the activity of the CASA complex by promoting the lysosomal clearance of damaged CASA complex targets.

2.3 Discussion

How protein aggregation contributes to aging has been an area of great interest. One outstanding question is if protein aggregation is a consequence or cause of aging. It has been hypothesized that protein aggregates accumulate with age as the amount of

damaged or misfolded proteins increase. However it is not clear whether or not these aggregating proteins are toxic leading to tissue dysfunction and further drive the aging process. In order to further understand the link between aging and protein homeostasis, we have utilized the p38Kb aging model. We find that p38Kb regulates age-dependent protein homeostasis through an interaction with the CASA complex. Our data suggest that activated p38Kb is associated with the CASA complex and plays a role in promoting the proper localization of stv within the muscle. Stv has a conserved MAPK docking site as well several potential p38K phosphorylation sites that might be the targets of p38Kb mediated regulation of CASA complex function. One possibility is that p38Kb mediated phosphorylation of stv is required for the localization of a functional CASA complex to the Z-disc, where damaged proteins are rapidly turned over. Another possibility is that p38Kb is required for the stability of the CASA complex and that over-expression of p38Kb leads to more stable complexes that can efficiently refold damaged proteins or target them for degradation as needed. Additionally, in the ubiquitin proteasome system, protein targets often need to be phosphorylated before they can be ubiquitinated and then targeted for degradation (Pines and Lindon, 2005), therefore, another possibility is that p38Kb serves a similar purpose for the CASA complex and phosphorylates the proteins that can't be refolded as a signal for these proteins to be polyubiquitinated and then targeted for degradation through the lysosome.

Previous studies have found that long-lived fly strains such as over-expression of Foxo or parkin result in reduced protein aggregate formation (Demontis and Perrimon, 2010; Rana et al., 2013). Therefore, we expected to find a similar link between lifespan and protein aggregation using the p38Kb model. As expected, we find that the short-lived

p38Kb mutants, which exhibit premature locomotor behavior defects (Vrailas-Mortimer et al., 2011), have large and numerous protein aggregates. We also find that over-expression of p38Kb, which extends lifespan, leads to improved flight behavior and results in fewer and smaller aggregates. In addition, we find that inhibition of the CASA complex member stv results in a reduced lifespan as well as increased aggregate number and size, much like inhibition of p38Kb. These data suggest that aging and protein homeostasis are tightly linked processes and that reduced aggregation is beneficial while increased aggregation is detrimental to the health of the animal.

However, this relationship doesn't necessarily hold true. Over-expression of stv, which does not extend lifespan, results in fewer aggregates throughout adulthood but has no effect on aggregate size at young ages. In addition, inhibition of HspB8, which doesn't negatively affect lifespan or flight behavior, results in a significant increase in protein aggregation but without a concordant increase in aggregate size. Furthermore, co-over-expression of p38Kb and stv results in an additional lifespan extension as compared to p38Kb over-expression alone. However, this increased lifespan does not correspond to an additional reduction in protein aggregate number, though it does have a trend towards a further decrease in aggregate size. These data suggest that aggregate size may be a more influential characteristic of the aggregates to determine either the beneficial or toxic effects of these aggregates. Another possibility is that neither aggregate number or size is the critical factor, but rather it is the content of the aggregates that plays the greatest role in longevity and tissue health. As aggregate protein content is currently unknown, further research will be needed to understand the contribution of specific aggregating protein species to health and lifespan.

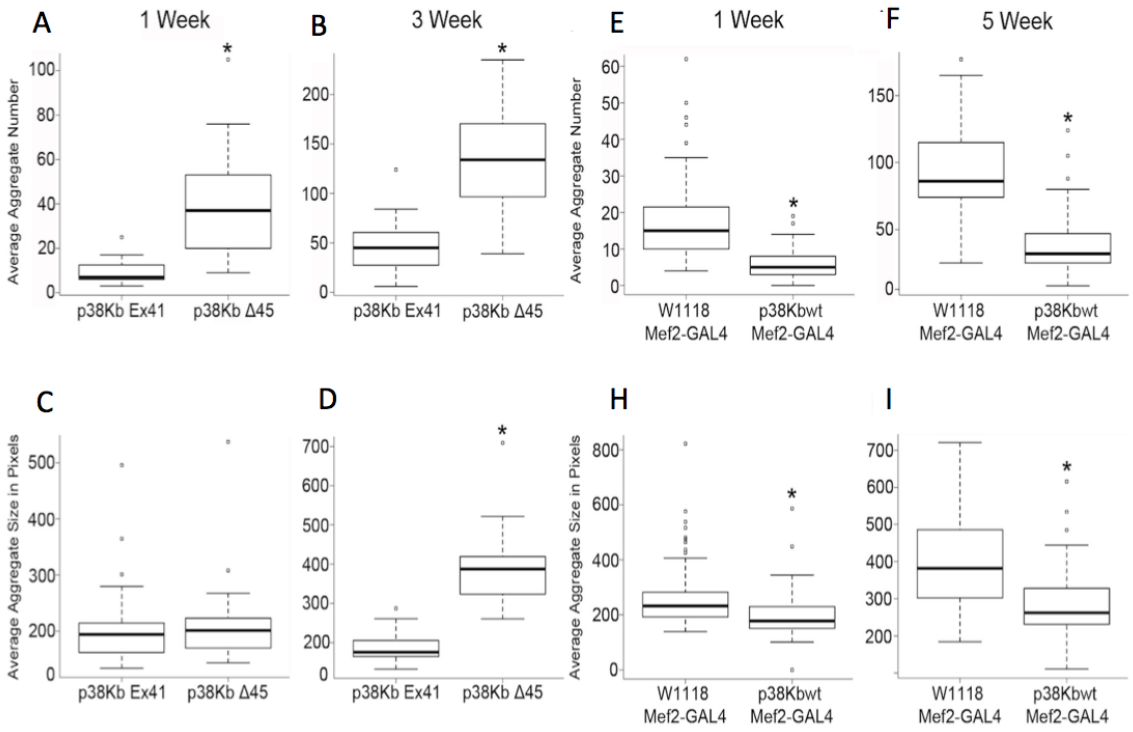


Figure 2.1. p38Kb regulates age-dependent protein homeostasis.
Box-Whisker Blots of aggregate number in p38Kb mutants at **A)** 1 week and **B)** 3 weeks of age and aggregates size at **C)** 1 week and **D)** 3 weeks of age. Aggregated number in p38Kb over-expression animals at **E)** 1 week and **F)** 5 weeks of age and aggregate size at **G)** 1 week and **H)** 5 weeks of age. Asterisks denote an adjusted p-value of <0.001.

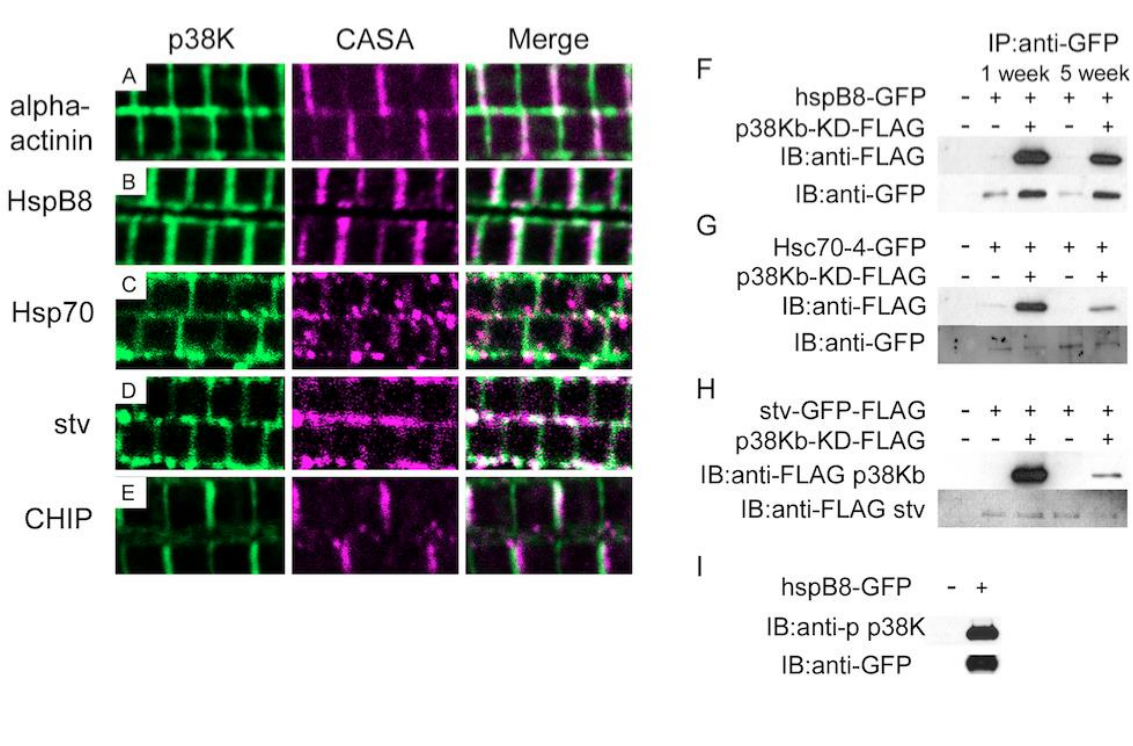


Figure 2.2. p38Kb colocalizes and co-immunoprecipitates with the CASA complex. Localization of a FLAG-tagged p38Kb (green in A-E and A''-E'') in the adult indirect flight muscle. **A)** FLAG-tagged p38Kb localizes to the Z-disc (arrows) as exhibited by colocalization with the Z-disc protein alpha-actinin (magenta, **A'** and **A''**), as well as the M-line (arrowheads). **B-E)** p38Kb colocalizes with each CASA complex member (magenta, **B'-E'**) at the Z-disc. Over-expression of a FLAG tagged p38Kb in the muscle in an **F**) endogenous HspB8-GFP fusion protein background, **G**) endogenous Hsc70-GFP fusion protein background, and **H)** endogenous stv-GFP-FLAG fusion protein background. Muscle lysates were immunoprecipated using anti-GFP coated beads. Immunoblots were probed with anti-FLAG to detect the presence of p38Kb in the IP lysates. Immunoblots were performed using anti-GFP to demonstrate successful pull down of **F)** HspB8 and **G)** Hsc70 and anti-FLAG to demonstrate successful pull down of **H)** stv. Note: stv is tagged with both GFP and FLAG. **I)** Endogenous phospho-p38 co-immunoprecipitates with HspB8-GFP, Hsc70-4-GFP, and stv-GFP. Muscle lysates were immunoprecipitated with anti-GFP beads, and immunoblots were probed with anti-phospho-p38K.

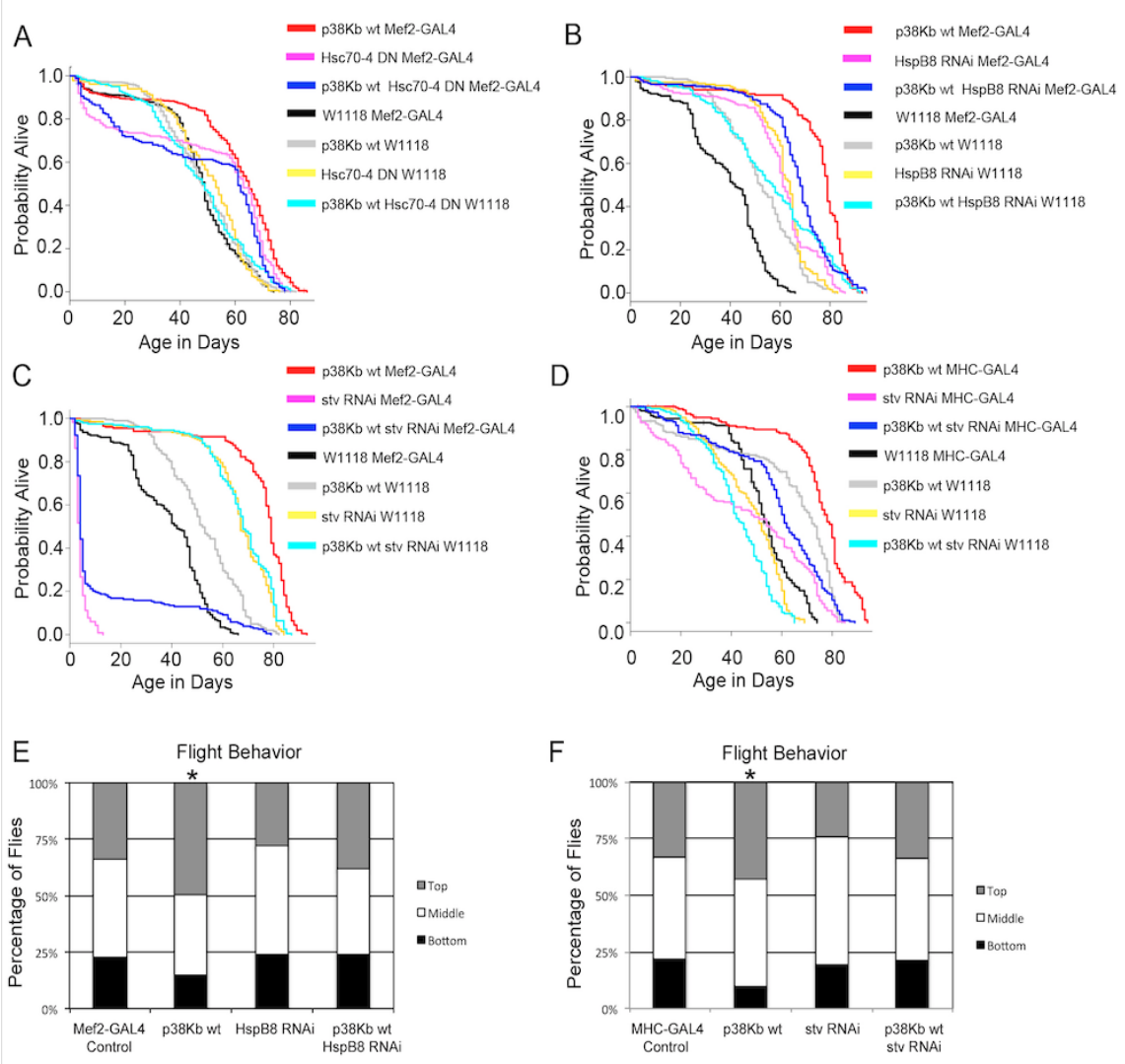


Figure 2.3. p38Kb requires the CASA complex for lifespan extension and flight behavior. A-D) Over-expression of p38Kb (red line) results in an increased lifespan as compared to the Mef2-GAL4 control and p38Kb transgene control (black line and gray lines, respectively). **A)** Inhibition of Hsc70-4 using a dominant negative construct results in a decreased lifespan as compared to the Hsc70-4 dominant negative transgene and GAL4 controls (compare pink line to yellow and black lines) and also prevents the p38Kb mediated lifespan extension (compare red line to blue line). **B)** Knockdown of HspB8 has no effect on lifespan as compared to the HspB8 RNAi transgene control (pink line and yellow line). Inhibition of HspB8 blocks the p38Kb lifespan extension (compare red line to blue line). **C)** Knockdown of stv using the Mef2-GAL4 results in a decreased lifespan (pink line compared to yellow and black lines) and is sufficient to prevent the p38Kb mediated lifespan extension (compare red line to blue line). **D)** Knockdown of stv using the MHC-GAL4 results in a decreased lifespan (pink line compared to yellow and

black lines) and prevents the p38Kb mediated lifespan extension (compare red line to blue line). **E** and **F)** Distribution of flies throughout the flight chamber. Poor fliers are at the bottom, while good fliers are collected at the top of the chamber. Over-expression of p38Kb leads to better flight performance at 1 week of age, which is blocked by inhibition of **E)** HspB8 or **F)** stv. Asterisks denote an adjusted p-value of <0.01.

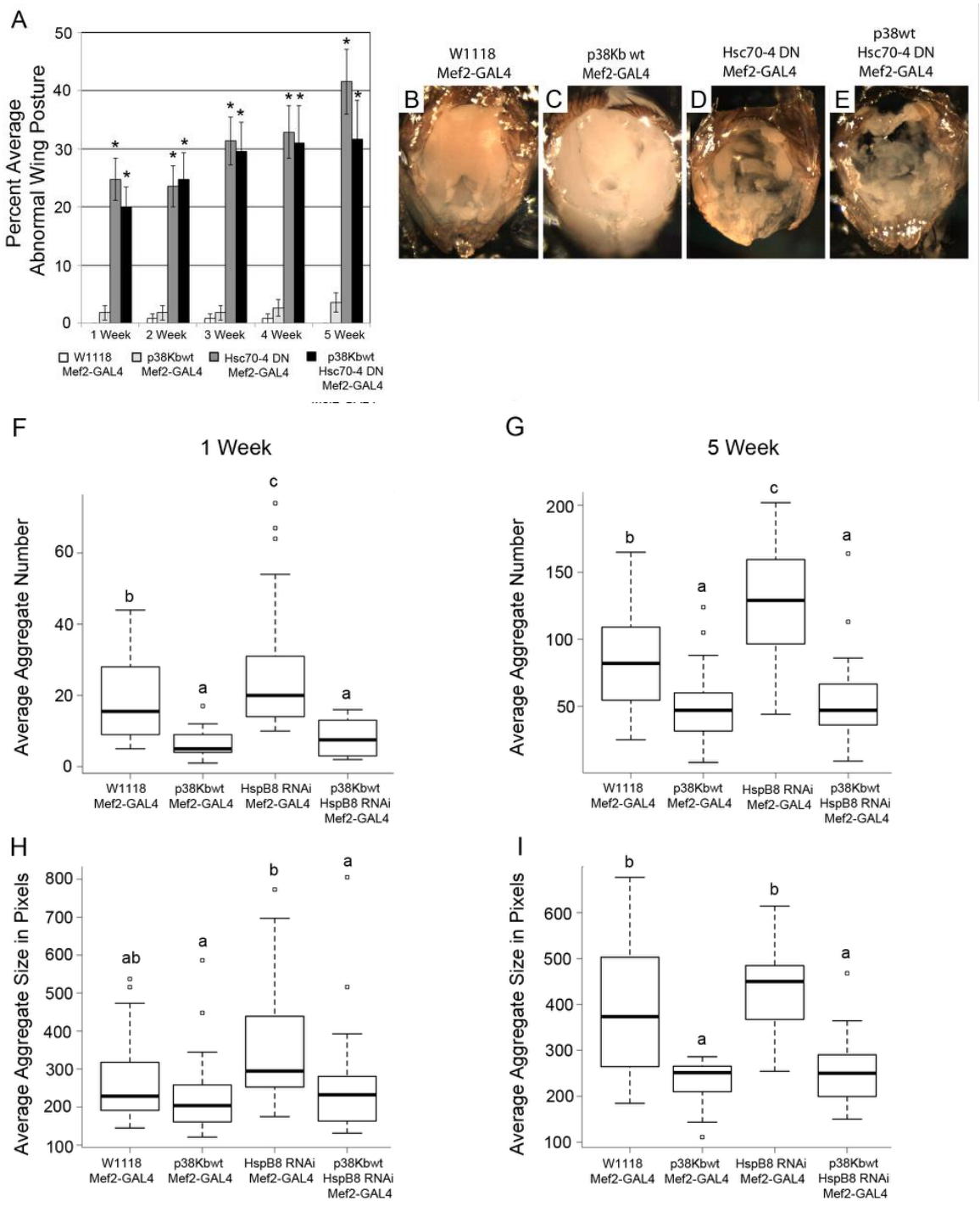
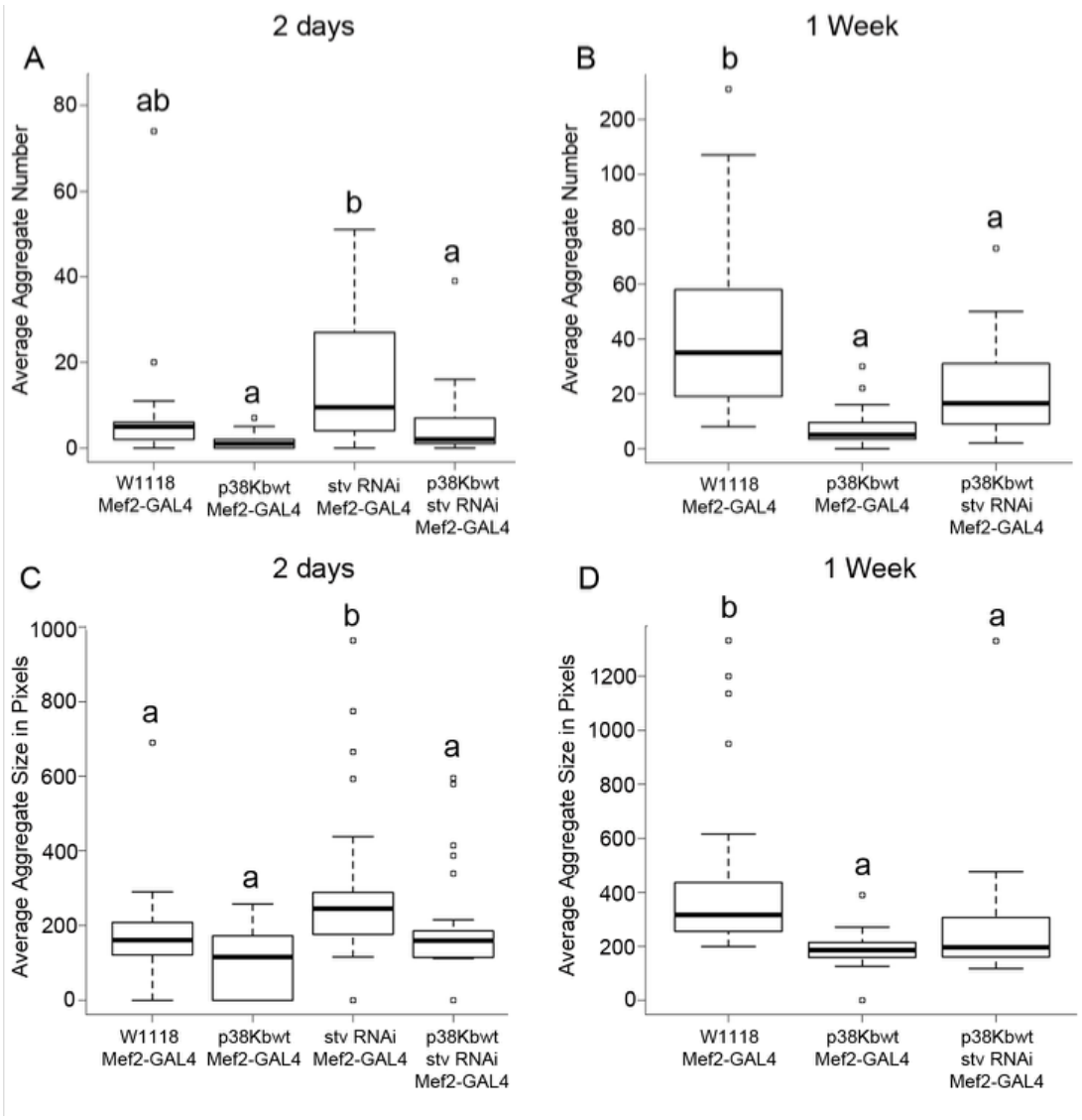


Figure 2.4. p38Kb genetically interacts with HspB8 to regulate protein homeostasis. **A)** Inhibition of Hsc70-4 results in an abnormal wing posture that is not rescued by p38Kb over-expression. **B-E)** Thorax dissections at 1 week of age. **D)** Inhibition of Hsc70-4 results in muscle wasting, which **E)** is not rescued by over-expression of p38Kb. **F-I**) Knockdown of HspB8 leads to increased protein aggregate number **F-G)** but not size **H-I)**. Over-expression of p38Kb prevents the HspB8 mediated increase in aggregate size

and number at all time points. Asterisks denote a p-value of <0.01. a, b, and c are confidence groups with a p value of <0.05


A
2 days
Average Aggregate Number
ab
a
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B
1 Week
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C
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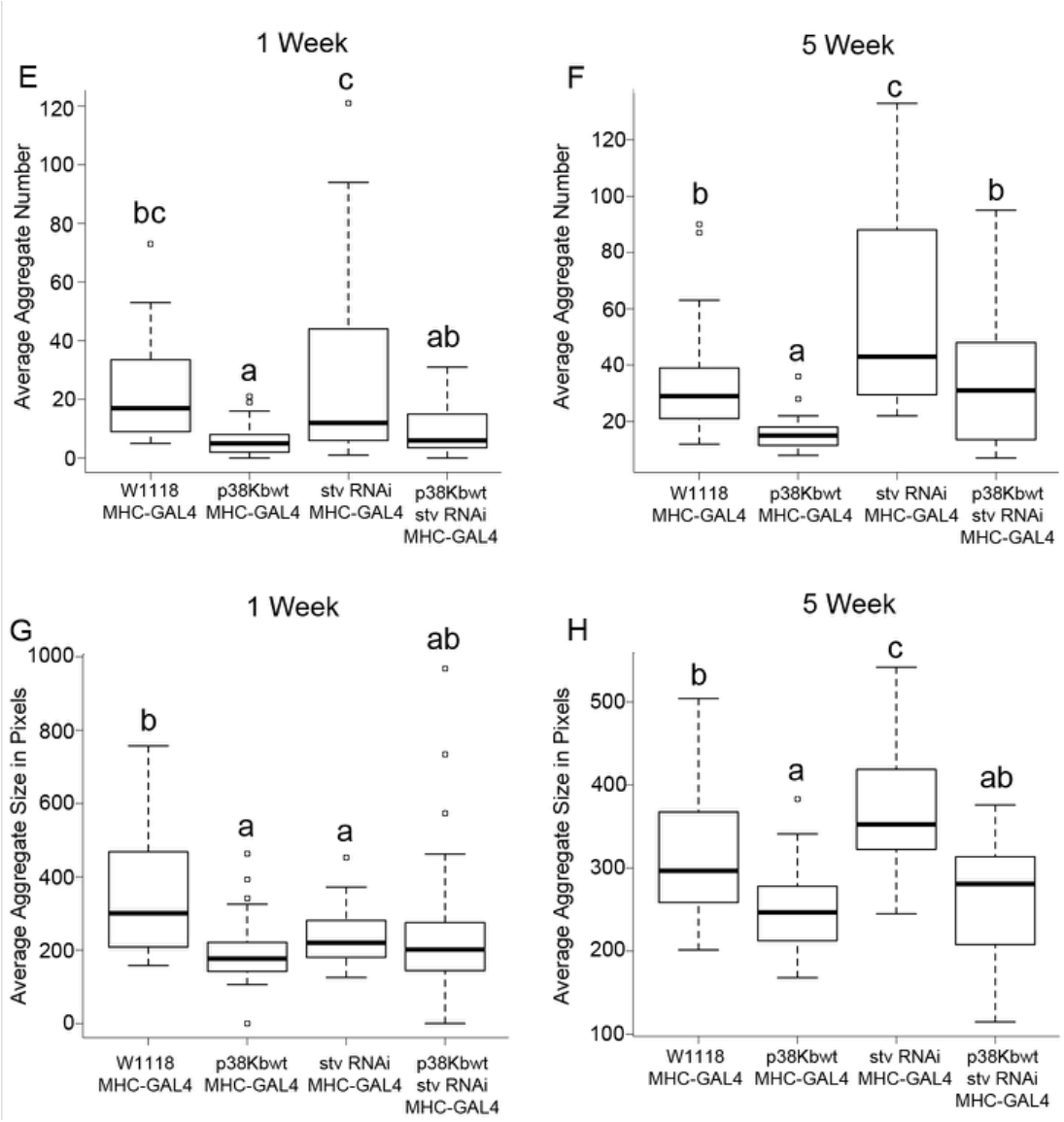
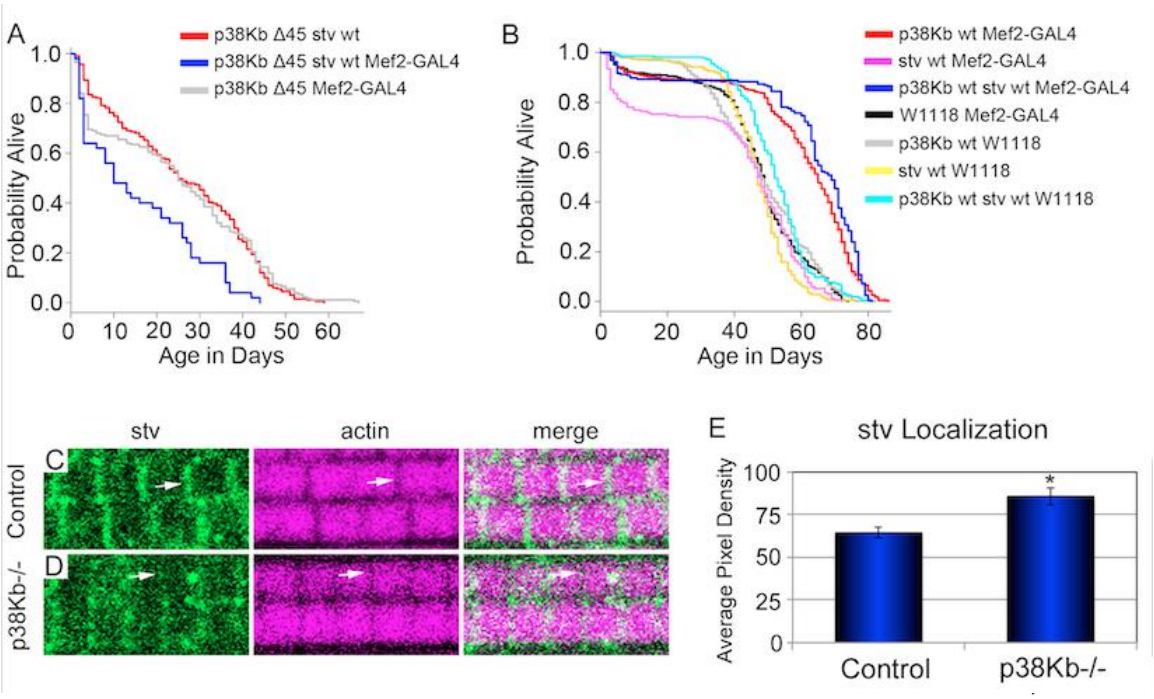


Figure 2.5. p38Kb genetically interacts with stv to regulate protein homeostasis. **A-D)** Protein aggregation in the stv knockdown background using the Mef2-GAL4. Protein aggregate number at **A)** 2 days and **B)** 1 week and protein aggregate size at **C)** 2 days and **D)** 1 week. Inhibition of stv using the Mef2-GAL4 results in a trend toward increased protein aggregate number and a significant increase in size at 2 days of age. Knockdown of stv is not sufficient to prevent the p38Kb mediated reduction in protein aggregation number and size at both 2 days and 1 week. **E-H)** Protein aggregation in the stv knockdown background using the MHC-GAL4. Protein aggregate number at **E)** 1 week and **F)** 5 weeks and protein aggregate size at **G)** 1 week and **H)** 5 weeks. Inhibition of stv using the MHC-GAL4 leads to an increase in protein aggregate number and size at 5 weeks age and partially suppresses the p38Kb mediated reduction in both aggregate number and size at 5 weeks of age. a, b, and c are confidence groups with a p value of >0.05.


A
p38Kb Δ45 stv wt
p38Kb Δ45 stv wt Mef2-GAL4
p38Kb Δ45 Mef2-GAL4
Probability Alive
Age in Days
B
p38Kb wt Mef2-GAL4
stv wt Mef2-GAL4
p38Kb wt stv wt Mef2-GAL4
W1118 Mef2-GAL4
p38Kb wt W1118
stv wt W1118
p38Kb wt stv wt W1118
Probability Alive
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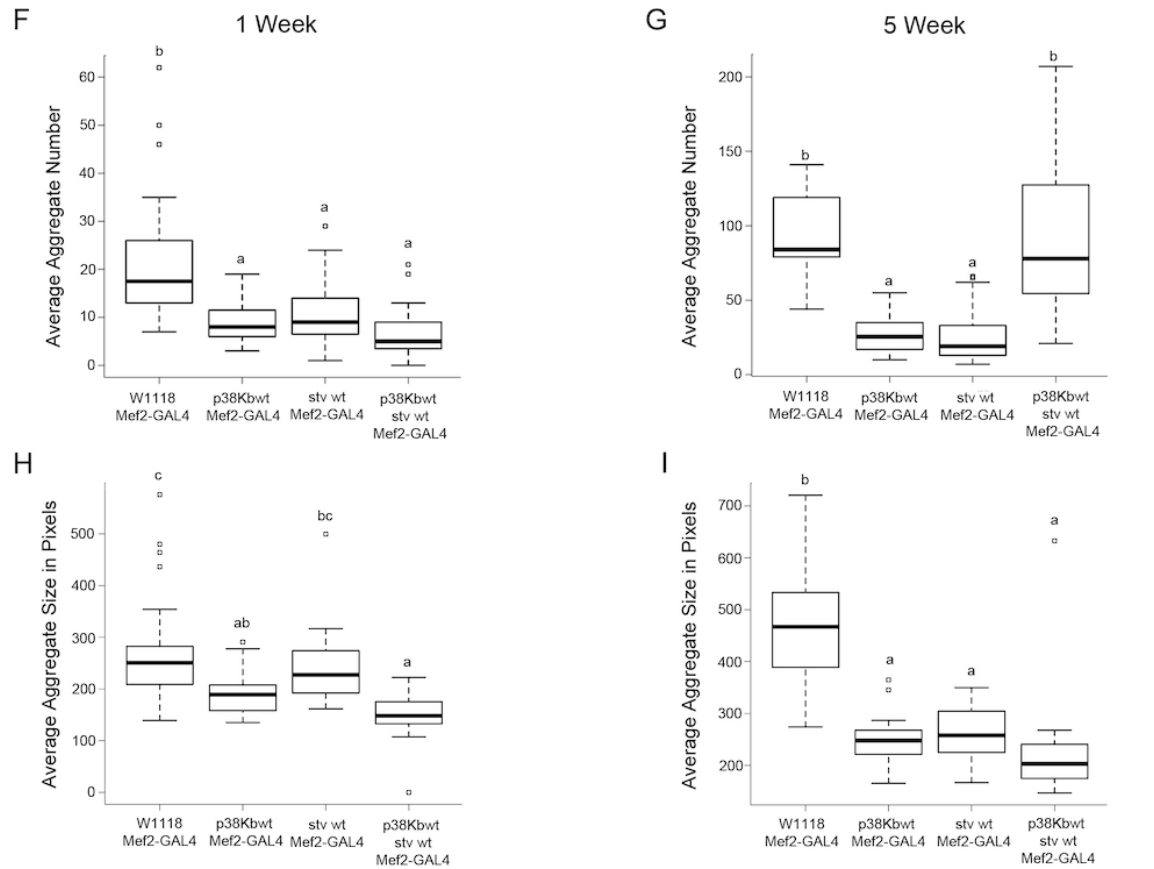
actin
merge
C
Control
D
p38Kb-/-
E
stv Localization
Average Pixel Density
Control
p38Kb-/-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stv wt Mef2-GAL4
p38Kbwt stv wt Mef2-GAL4
G
5 Week
Average Aggregate Number
H
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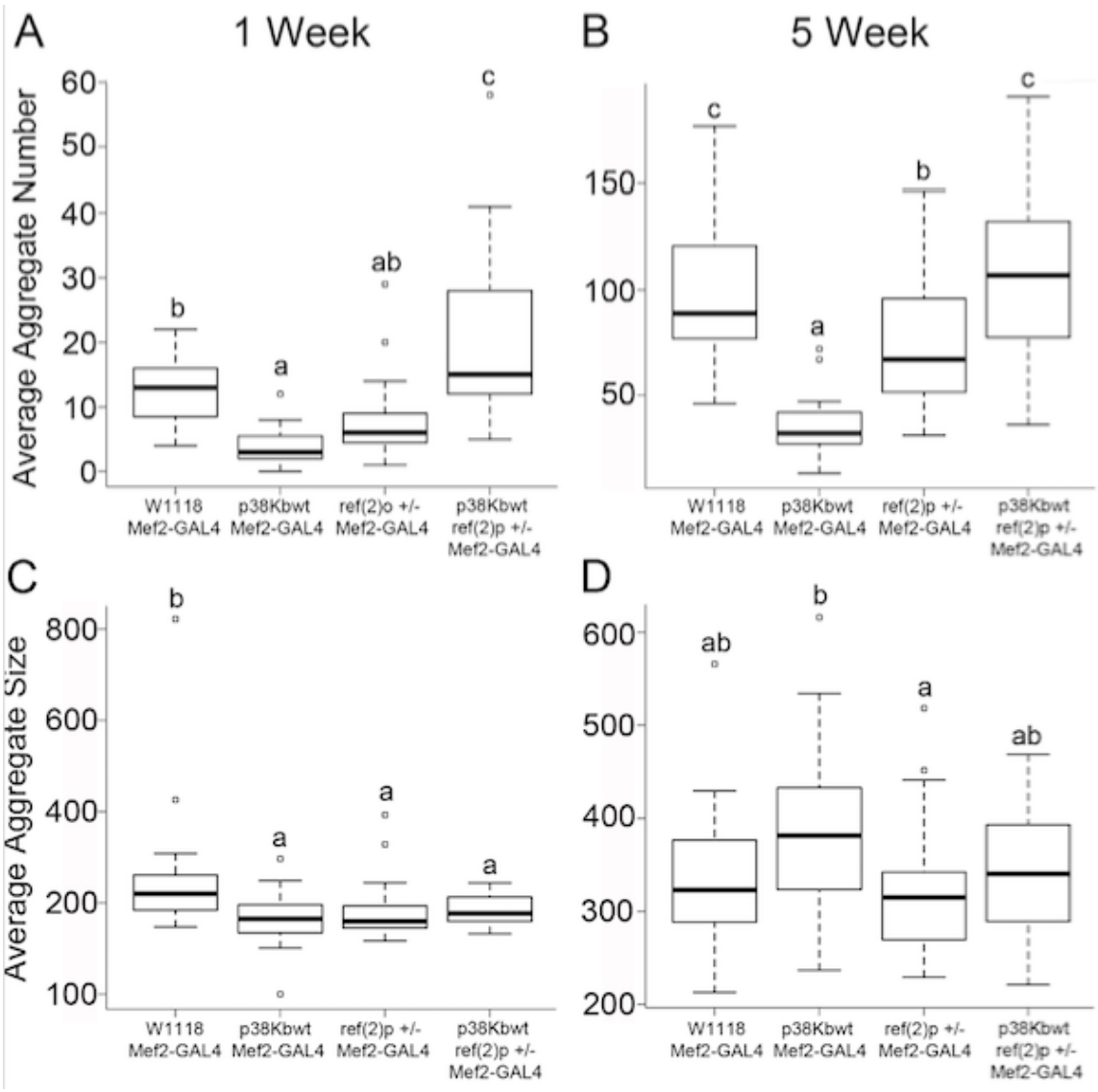
Average Aggregate Size in Pixels

Figure 2.6. p38Kb and stv co-overexpression in protein homeostasis.
A) Over-expression of stv in the p38Kb mutant background results in a further reduction of lifespan as compared to p38Kb mutant controls (compare blue line to red and grey lines). **B)** Over-expression of stv alone has minor effects on lifespan (pink line as compared to yellow and black lines), however, co-over-expression of stv and p38Kb results in a further increase in lifespan (compare red line to blue line). **C)** stv localizes to the adult muscle Z-disc and M-line in control animals (white arrows). **D)** stv localization is disrupted in p38Kb mutants. **E)** Quantification of average pixel density. Protein aggregate number at **F)** 1 week and **G)** 5 weeks and protein aggregate size at **H)** 1 week and **I)** 5 weeks measured in stv over-expression backgrounds. Over-expression of stv leads to reduced aggregate number at 1 and 5 weeks and aggregate size at 5 weeks. Co-over-expression of stv and p38Kb does not result in a further decrease in protein aggregate number but trends towards decreased aggregate size at both 1 and 5 weeks of age. a, b, and c are confidence groups with a p value of ≤ 0.05.


A
1 Week
Average Aggregate Number
60
50
40
30
20
10
0
b
a
ab
c
W1118 Mef2-GAL4
p38Kbwt Mef2-GAL4
ref(2)o +/- Mef2-GAL4
p38Kbwt ref(2)p +/- Mef2-GAL4
B
5 Week
150
100
50
c
a
b
c
W1118 Mef2-GAL4
p38Kbwt Mef2-GAL4
ref(2)p +/- Mef2-GAL4
p38Kbwt ref(2)p +/- Mef2-GAL4
C
Average Aggregate Size
800
600
400
200
100
b
a
a
a
W1118 Mef2-GAL4
p38Kbwt Mef2-GAL4
ref(2)p +/- Mef2-GAL4
p38Kbwt ref(2)p +/- Mef2-GAL4
D
600
500
400
300
200
ab
b
a
ab
W1118 Mef2-GAL4
p38Kbwt Mef2-GAL4
ref(2)p +/- Mef2-GAL4
p38Kbwt ref(2)p +/- Mef2-GAL4

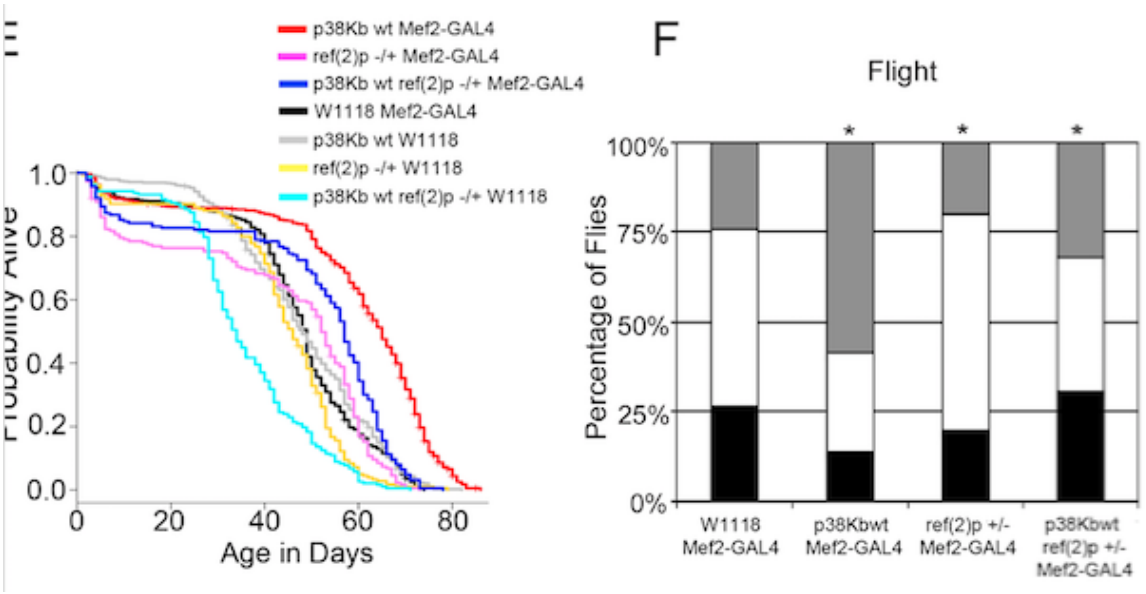


Figure 2.7. p38Kb regulates CASA complex activity
A-B) Protein aggregate number in ref(2)p heterozygous mutant backgrounds at **A)** 1 week and **B)** 5 weeks. Loss of a single copy of ref(2)p prevents the p38Kb mediated reduced protein aggregation at 1 week and 5 weeks of age. **E)** Loss of a single copy of ref(2)p results in a normal lifespan (pink line compared to black and yellow lines) and is sufficient to prevent the p38Kb mediated lifespan extension (compare blue line to red line). **F)** Distribution of flies throughout the flight chamber. Poor fliers are at the bottom, while good fliers are collected at the top of the chamber. Heterozygosity for ref(2)p results in poorer fliers, with more flies accumulating in the middle portion of the chamber. Over-expression of p38Kb results in better fliers, which is blocked by loss of a single copy of ref(2)p. a, b, and c are confidence groups with a p value of >0.05.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control	p value vs UAS-p38Kb wt Mef2-GAL4
W1118 Mef2-GAL4	38 days	42 days	206	-	0
UAS-p38Kb wt Mef2-GAL4	74.2 days	79 days	203	0	-
UAS-HspB8 RNAi Mef2-GAL4	59 days	61 days	232	0	0
UAS-p38Kb wt UAS-HspB8 RNAi Mef2-GAL4	66.3 days	68 days	208	0	7.16E-09
UAS-p38wt W1118	52.2 days	52 days	203	0	0
UAS-HspB8 RNAi W1118	60.7 days	64 days	201	0	0
UAS-p38Kb wt UAS-HspB8 RNAi W1118	56.3 days	57 days	211	0	1.33E-12

Table 2.1. Inhibition of HspB8 prevents p38Kb lifespan extension.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control	p value vs UAS-p38Kb wt Mef2-GAL4
W1118 Mef2-GAL4	46.3 days	49 days	515	-	0
UAS-p38Kb wt Mef2-GAL4	58.2 days	64 days	182	0	-
UAS-Hsc70-4 DN Mef2-GAL4	56.9 days	66 days	208	0	4.99E-05
UAS-p38Kb wt UAS-Hsc70-4 DN Mef2-GAL4	46.9 days	61 days	192	3.03E-10	2.54E-09
UAS-p38wt W1118	47.4 days	48 days	730	0.2006667	0
UAS-Hsc70-4 DN W1118	49 days	53 days	182	0.1093313	0
UAS-p38Kb wt UAS-Hsc70-4 DN W1118	46.6 days	48 days	181	0.06216	0

Table 2.2. Inhibition of Hsc70-4 prevents p38Kb lifespan extension.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control	p value vs UAS-p38Kb wt Mef2-GAL4
W1118 Mef2-GAL4	38 days	42 days	206	-	0
UAS-p38Kb wt Mef2-GAL4	74.2 days	79 days	203	0	-
UAS-stv RNAi Mef2-GAL4	4.2 days	4 days	116	0	0
UAS-p38Kb wt UAS-stv RNAi Mef2-GAL4	66.3 days	68 days	208	1.33E-10	0
UAS-p38wt W1118	52.2 days	52 days	203	0	0
UAS-stv RNAi W1118	65.8 days	67 days	206	0	0
UAS-p38Kb wt UAS-stv RNAi W1118	13.4 days	4 days	203	0	6.48E-16

Table 2.3. Strong inhibition of stv prevents p38Kb lifespan extension.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control	p value vs UAS-p38Kb wt Mef2-GAL4
W1118 MHC-GAL4	51 days	52 days	212	-	0
UAS-p38Kb wt MHC-GAL4	73.5 days	77 days	175	0	-
UAS-stv RNAi MHC-GAL4	44.3 days	48 days	217	7.98E-03	0
UAS-p38Kb wt UAS-stv RNAi MHC-GAL4	56.9 days	60.5 days	182	1.31E-11	0
UAS-p38wt W1118	59.4 days	70 days	178	0	4.43E-13
UAS-stv RNAi W1118	46.2 days	50.5 days	194	1.47E-05	0
UAS-p38Kb wt UAS-stv RNAi W1118	41.3 days	41 days	197	4.24E-15	0

Table 2.4. Intermediate inhibition of stv prevents p38Kb lifespan extension.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control	p value vs UAS-p38Kb wt Mef2-GAL4
W1118 Mef2-GAL4	46.3 days	49 days	515	-	0
UAS-p38Kb wt Mef2-GAL4	58.2 days	64 days	730	0	-
stv EP Mef2-GAL4	39.7 days	47 days	182	0.03458824	0
UAS-p38Kb wt stv EP Mef2-GAL4	61.3 days	69 days	205	0	0.03651667
UAS-p38wt W1118	47.4 days	48 days	598	0.1806	0
stv EP W1118	46.1 days	47 days	189	3.47E-03	0
UAS-p38Kb wt stv EP W1118	51.9 days	52 days	206	2.00E-03	0

Table 2.5. p38Kb and stv co-over-expression further extends lifespan.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control
p38Kb-/- Mef2-GAL4	24.1 days	25 days	200	-
p38Kb-/- stv EP	25.6 days	25 days	207	0.936
p38Kb-/- stv EP Mef2-GAL4	15.5 days	10 days	50	1.03E-04

Table 2.6. Over-expression of stv fails to rescue p38Kb mutant short lifespan.

Genotype	Average Age	Median Age	n	p value vs GAL4 Control	p value vs UAS-p38Kb wt Mef2-GAL4
W1118 Mef2-GAL4	46.3 days	49 days	515	-	0
UAS-p38Kb wt Mef2-GAL4	58.2 days	64 days	730	0	-
UAS-ref(2)p -/+ Mef2-GAL4	42.3 days	52 days	187	0.829	0
UAS-p38Kb wt UAS-ref(2)p -/+ Mef2-GAL4	48.7 days	57 days	190	2.97E-05	0
UAS-p38wt W1118	47.4 days	48 days	598	0.1901053	0
UAS-ref(2)p -/+ W1118	43 days	46 days	185	6.03E-04	0
UAS-p38Kb wt UAS-ref(2)p -/+ W1118	35.5 days	34 days	193	0	0

Table 2.7. Inhibition of ref(2)p prevents p38Kb lifespan extension.

CHAPTER THREE: P38 MAPK DEPENDENT GLOBAL PROTEOMIC CHANGES WITH AGING IN BRAIN AND MUSCLE

3.1 Introduction

In humans, there are a number of age-dependent changes in the structure and function of specific tissues. The integrity of muscle and nervous tissue are critical for proper function of the organism, but both show an age-dependent decline (Nair, 2005; Pakkenberg and Gundersen, 1997). Reduction in muscle mass with age leads to muscle weakness, muscle wasting, fatigue and sometimes loss of mobility (Short et al., 2004). In turn, this contributes to the development of many metabolic disorders including type II diabetes, cardiovascular disease and obesity and the exacerbation of musculoskeletal diseases (Nair, 2005; Wallace and McNally, 2009). Also, muscle protein synthesis is effected by age with an age-dependent decrease in the translation of many muscle proteins, importantly myosin heavy chain (Balagopal et al., 2001) . There is also a significant ATP requirement in healthy muscle. But with age, there is a decrease in mitochondrial protein synthesis resulting in reduced mitochondrial biogenesis and a decrease in overall ATP production (Barazzoni et al., 2000).

Similarly, in the brain there is well-recognized phenomenon of age-dependent cognitive functional decline starting in mid-life and worsening with age (Beason-Held et al., 2016; Deeg et al., 1990; Villeda et al., 2011). Additionally, cognitive and memory

impairments are exacerbated by age-related diseases like Alzheimer's disease, Parkinson's disease and other dementias. The brain's white matter atrophies with age (Double et al., 1996) and dendritic branching and spine density also show age-dependent reduction (de Brabander et al., 1998). Nervous tissue also show an age-related decline in mitochondrial function and production of mitochondrial proteins in the brain (Corral-Debrinski et al., 1992; Pollard et al., 2016).

Studies from model organisms such as *Drosophila* and *C. elegans* highlight how genes linked to lifespan extension also inhibit age-related changes in specific tissues (Arey and Murphy, 2016). For example, changes in expression of FOXO or insulin signaling members reduce normal age-related changes in muscle tissue, in part due to decreasing protein aggregation (Demontis and Perrimon, 2010; Wessells et al., 2004). Also, some tissues appear to have larger roles in coordinating aging processes of the whole organism. Localized expression of age-related genes (e.g. DAF-16) in specific tissues results in lifespan extension for the animal (Biteau et al., 2010; Libina et al., 2003) We also find that p38 MAPK (p38Kb) over-expression in the muscle, mediates both the maintaining muscle tissue function and lifespan extension (Vrailas-Mortimer et al., 2011).

The four mammalian p38K genes are differently expressed in human tissues but have over 60% sequence homology to one another and over 90% homology in their kinase domains (Coulthard et al., 2009). In mammalian systems, p38K-α is the predominant p38K and both complete and conditional p38K-α knockout mice are embryonic lethal (Adams et al., 2000; del Barco Barrantes et al., 2011; Hui et al., 2007; Perdiguero et al., 2007). But single, double and triple knockouts of p38K-β, p38K-γ and

p38K-δ all results in viable mice (Aouadi et al., 2006). Each of the four mammalian p38Ks have unique sets of upstream activators and downstream targets. This creates a complex network that has only been partially identified, making studies of p38K and its interacting partners very difficult in mammalian systems. The p38K genes of *Drosophila melanogaster* are highly conserved to mammalian forms. In *Drosophila* there are only two p38K genes: p38Ka and p38Kb. p38Ka and p38Kb mutants result in viable animals, but loss of both genes is lethal (Craig et al., 2004; Vrailas-Mortimer et al., 2011). The p38Kb mutants show a reduction in lifespan; living for 20 days as compared to the 48 days in control animals (Vrailas-Mortimer et al., 2011). Unlike p38Ka mutants, the loss of p38Kb also increases their sensitivity to oxidative stress and age- dependent locomotor defects (Vrailas-Mortimer et al., 2011).

We find that in *Drosophila* p38Kb plays a critical role in aging when over-expressed in muscle tissue, and to a lesser extent in neuronal tissue; over-expression of p38Kb in muscle results in a 37% lifespan extension and increased resistance to oxidative stress (Vrailas-Mortimer et al., 2011). Additionally in the muscle tissues of flies the levels of phosphorylated active p38K increase with age (Belozerov et al., 2014). p38K appears to play a critical role in mediating aging processes and maintaining tissue function therefore we wanted to explore levels of proteins that are affected by p38K expression. The evolutionary conservation, shorter lifespan and genetic tractability of *Drosophila* make it an ideal system to explore the age-dependent protein network affected by p38Kb.

We now find that p38Kb decreases age-dependent protein aggregation through an interaction with the Chaperone Assisted Selective Autophagy (CASA) complex, a

mediator of lysosomal autophagy (Chapter 2). We also find that p38Kb regulates the activity of the CASA complex. Cell dysfunction can arise as a result of insufficient protein degradation by the proteasomal or lysosomal systems. Proteostasis dysfunction, resulting in protein aggregation, is seen in many age-dependent neurodegeneration and musculoskeletal diseases (Robertson and Bottomley, 2010; Wallace and McNally, 2009). Through inhibition of members of the CASA complex or p38Kb, we see an increase in age-dependent protein aggregation. However, there are only a couple of identified targets of the CASA complex. . In addition, little is known about the downstream target of p38Kb that contribute to the aging process. In this study, we performed a quantitative proteomic screen to identify age-dependent changes relating to p38Kb expression in heads and thoraxes of *Drosophila*. We find that p38Kb regulates the expression of a number of proteins linked to cytoskeletal and neuronal function in both these tissues. Also many of the identified proteins are linked to human neuromuscular and neurodegenerative disease including Charcot-Marie-Tooth disease and Limb-Girdle Muscular Dystrophy.

3.2 Results

In order to understand the tissue specific effect of p38Kb on the *Drosophila* proteome, we analyzed both heads (predominantly brain tissue in the fly) and thoraxes (predominantly muscle tissue) at weekly time points. Short-lived p38Kb mutants were aged 1-5 weeks, long- lived p38Kb over-expression animals were aged 1-10 weeks and wild type controls were aged 1-8 weeks. Heads and thorax lysates were analyzed through LC-ESI-MS/MS. This type of mass spectroscopy allows for quantitative proteomics of

heterogeneous samples, with proteins at low expression. From this analysis, proteins identified were compared to a database of 25,000 proteins, and we find 2,247 proteins of detectable expression levels were identified in the heads or thoraxes at one or multiple time points in one or more genotypes.

Proteome Signature as Predictor of Age

In order to determine if a specific proteomic signature is a predictor of age, we used MLSeq's machine learning and trained on protein expression from 2/3 of the wild type and the p38Kb w1118 and Mef-2-GAL 4 control samples from heads and thoraxes, respectively. These control samples that were binned into the correct chronological age (young, 1-3 weeks, middle, 4-6 weeks, and old, 7-8 weeks). This data set trained the program on protein expression levels with different ages. The remaining 1/3 of the control samples were then tested to determine with what accuracy the program assigned the proper age (young, middle or old) to the samples. We found that the program predicted with 93% accuracy on both heads and thoraxes. Interestingly, we find that the head can be divided into young, middle, and old aged samples, whereas the thorax is divided into young and old ages. We then tested how over-expression of p38Kb affects these predicted proteomic ages in the muscle and brain. The p38K over-expression in the head leads to a prolonged middle age classification, with proteomes of young (1-3 weeks) and old p38Kb over-expression flies (7-10 weeks) being assigned into the middle age category. Conversely, the head proteome of the p38K mutants entered middle and old age earlier than controls.

We then analyzed which proteins were the best predictors of age in the heads and thoraxes, respectively. The top 20 proteins in the head of control animals (Table 3.1) include proteins involved in cytoskeletal processes and the oxidative stress response with an unknown gene, CG1561, as the number one predictor of aging in the heads. CG1661 has only been identified in a couple of other studies. In a study of *parkin*, whose mutation causes familial early-onset Parkinson's disease, CG1561 was identified as being downregulated in *parkin Drosophila* mutants (Greene et al., 2005). In another study of Fat/hippo/atrophin signaling in neurodegeneration, CG1561 transcripts were found to be upregulated in atrophin mutants (Napoletano et al., 2011). Further characterization of this gene might yield interesting results regulating key aging pathways. Other age-predictor proteins are linked to regulation of stress, energy processing, aging and protein homeostasis. Additionally, the human homologues of many of these proteins are linked to neurodegeneration and neuromuscular disease.

In the thoraces, the top 20 proteins identified as key age predictors in control animals include proteins involved in apoptosis, immune response, oxidative stress response and cytoskeleton, with Hsc70-5 rating as the best predictor of age (Table 3.2). Interestingly, over-expression of p38Kb in the head affects the expression of 18 of the 20 age-predictor proteins in heads. When comparing the predictor genes for the thoraxes 11 of 20 proteins were matched to the p38Kb over-expression in the heads. Surprisingly, in thoraces with p38Kb over-expression, only 3 proteins matched the thorax age-predictor proteins.

p38K Expression Affects the Proteome in Drosophila Head and Thorax

We investigated the impact of p38Kb expression on the proteome using Linear Models for Microarray Data (limma) in R. Both the p38Kb over-expression and p38Kb mutants were compared to their respective controls over each week through the entire experimental timecourse. We find that 358 proteins in the head and 1,373 in the thorax were changed in the p38Kb over-expression animals. Furthermore, 113 proteins were changed in the heads and 307 in the thoraxes of p38Kb mutants. In both the over-expression and mutant p38Kb affected proteins were linked to cytoskeleton dynamics, nerve and brain function and gene expression processes (i.e. transcription/translation) (Figure 3.1).

It was also seen that p38Kb regulates a number of proteins that are linked to Limb Girdle Muscular Dystrophy (LGMD) and Charcot-Marie-Tooth (CMT) disease. One of these proteins was lamin, an intermediate filament and part of the nuclear envelope. Mutations of lamin are responsible for the accelerated aging disorder of progeria. Affected children will begin aging within the first two years of life with characteristic aging phenotypes such as stiff joints, hip dislocation, atherosclerosis and stroke. Mutations in lamin are also associated with CMT disease. CMT is a progressive hereditary sensory and motor neuropathy and is the most common inherited form of a neuromuscular disorder, affecting 1 in 2,500 people (Reilly et al., 2011). Mutations linked to this disease effect the integrity of the lower motor neuron axon or myelin sheath. Patients exhibit muscle weakness, difficulty walking, changes in gait, loss of sensation and muscle atrophy. Lastly, expression of titin was effected. Titin is an abundant protein in skeletal muscle and functions as a molecular spring by connecting

sacromeric Z and M lines. There are a number of diseases associated with mutations of titin including LGMD, myofibrillar myopathy, tibial muscular dystrophy and cardiomyopathies.

We also noticed that overall protein expression of p38Kb mutant thoraxes were very similar to the p38Kb over-expression in heads. There appears to be coordinated communication between tissues and suggests that a subset of tissues might be driving aging processes for the whole animal. From lifespan studies, p38Kb plays a larger role in the muscle tissue as opposed to neuronal tissue. Therefore, p38Kb could be exerting its activity at different rates in specific tissues over the lifespan of the organism. Interestingly, 68 proteins were changed in both p38Kb over-expression and mutant animals in the head (Table 4.3) and 69 in the thorax (Table 4.4). In the head, the majority proteins identified play roles in gene expression, cytoskeleton dynamics and nervous system function. Whereas in the thorax, many proteins were involved in signaling pathways, as well as gene expression and cytoskeletal processes. In both the thorax and head, we find that the expressions of many proteins that are changed by p38K expression also are linked to the human disease, CMT. Three identified proteins – DCTN1-p150, Dap160 and CG9279 - are part of the dynein-dynactin complex; mutations in this pathways cause axonal CMT type 2. Dynein plays a role in cargo transport along microtubules. Dynein further associates with dynactin, together this complex specifies and transports cargo, namely signaling and trophic factors, along the length of the axon. Mice deficient in dynein or dynactin show inhibited retrograde transport causing degeneration and loss of motor neurons (Hafezparast et al., 2003; LaMonte et al., 2002). Also, the anterograde motor kinesin protein, KIFIB, has also been linked to causing

CMT2. The perturbation of normal axonal transport through the dynein-dynactin-kinesin complex and interactors are linked to other diseases of muscle and motor neuron degeneration (Hurd and Saxton, 1996; Peeters et al., 2013; Puls et al., 2003; Puls et al., 2005). Our study identified two kinesin family members, unc-104 and Klp3A, whose expression changed based on levels of p38Kb.

Finally, we identified two lamin proteins, lamin and the lamin B receptor, with altered expression; mutations of human lamin (LMNA) cause axonal CMT2. LMNA null mice show presence of nonmyelinated axons, reduced axon density and axonal enlargement, consistent with CMT2 patients (De Sandre-Giovannoli et al., 2002). Mutations in LMNA have also been linked to limb-girdle muscular dystrophy (type 1B) (Muchir et al., 2000) and Emery-Dreifuss muscular dystrophy (Bonne et al., 1999).

In order to further highlight p38Kb's interaction network, we catalogued proteins that change in their expression levels in both tissues in p38Kb mutants and over-expression (Table 4.5). There were only 4 proteins whose expression changed in these conditions; surprisingly two of these are unclassified proteins, CG6701 and CG1561. The significance of CG1561 being identified in these stringent conditions is especially interesting, as we have previously identified it to be the number one predictor of aging in heads.

3.3 Discussion

We have found that p38Kb regulates the expression of a number of proteins in both heads and thoraxes. These data demonstrate the role specific tissues have in aging processes. Interestingly the best predictors of aging were different in heads and thoraces,

suggesting that different pathways manage aging pathologies differently in these tissues. Potentially, localized tissue specific mediators, such as antioxidants, can provide rejuvenating effects for the whole organism. The integrity of muscle and neuronal tissue are essential to the organism's healthspan and lifespan. Failure of these tissues occurs synonymously with a proteostatic failure, but the mechanism is unclear of this "chicken or the egg" scenario. As we have shown that p38Kb regulates protein homeostasis through the CASA complex (Chapter 2), proteins identified in this screen can be tested as potential degradation targets for this autophagy pathway.

Many of the p38K-dependent proteins are also linked to human diseases, including a number of muscular dystrophies and neurodegenerative diseases (Figure 3.2). Interestingly of these proteins, we identified 8 fly homologs of genes linked to Limb-Girdle Muscular Dystrophy and 27 homologs linked to Charcot-Marie-Tooth disease. p38K could potentially be an important node for therapeutics through which to treat CMT or LGMD. High levels of activated p38K might trigger apoptotic events, which are seen in many neuropathies, but treatments inhibiting or regulating activity of p38K could encourage cell survival. p38Kb expression appears to be an important regulator of proteins involved with axonal health and function. In light of the genetic links to CMT, p38Kb might be playing a particularly important role at the level of the motor neuron. Previous studies have also linked proper p38K signaling to axonal health. In a study of the progeroid neurodegenerative disorder, Ataxia Telangiectasia, Barascu et al. hypothesized a link between p38K and axonal demylination via lamin in mammalian cell culture. They hypothesize that p38K activation through chronic oxidative stress increases laminB1 expression to a threshold that it negatively interacts with the myelin protein

synthesis, resulting in neurological defects (Barascu et al., 2012). In another study, a yeast two-hybrid screen, the dynein-dynactin complex was found to interact with MKK6/3, upstream activators of p38K (Cheung et al., 2004). This connection of p38K as an important regulator of axonal health opens up an exciting new mechanism to explore in the pathology and treatment options of CMT and other neuropathies.

Gene	Machine Learning Score	Name	Function	Human Ortholog	Human Disease
CG1561	100		linked to neruodegeneration and parkin	ACAD11: acyl-CoA dehydrogenase family member 11	
Arr1	41.881	Arrestin 1	photoreceptor cell deactivation	ARRB2: beta arrestin	
eys	35.278	eyes shut	eye development, extracellular matrix component	EYS: eyes shut homolog	Retinitis Pigmentosa 25
GlyP	17.073	Glycogen phosphorylase	glycogen phosphorylatse activity, lifespan, muscle function, interacts with foxo and parkin	PYGM: phosphorylase, glycogen, muscle	Glycogen Storage Disease V
se	16.977	sepiapterin GSTO4	gluathione dehydrogenase activty	GSTO1: glutathione S-transferase omega 1	Charcot-Marie-Tooth disease
PPO3	16.74	Prophenoloxidase 3	pigment production, conversion of dopamine, immune response		
PPO2	16.74	Prophenoloxidase 2	pigment production, conversion of dopamine, immune response		
CG34417	14.812		predicted actin binding	SMTNL1: smoothelin like 1	
CG16935	12.251		zinc-containing alcohol dehydrogenase family, predicted NADPH activity	MECR: mitochondrial trans-2-enoyl-CoA reductase	
cathD	11.686	cathepsin D	aspartic peptidase, autophagic cell death, immune clotting	CTSD: cathepsin D	Neuronal Ceroid Lipofuscinosis
rtp	9.447	retinophilin	apoptotic cell engulfment	MORN4: MORN repeat containing 4	
Fon	9.395	fondue	hemolymph coagulation, metamorphosis		
ATPsyngamma	7.504	ATP synthase, γ subunit	phagocytosis, immunity, ATP synthase activity	ATP5C1: ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	

Rab32	7.235	Rab32	regulation of autophagy, linked to aging and neurodegeneration	RAB32: RAB32, member RAS oncogene family	
Gene	**Machine Learning Score**	**Name**	**Function**	**Human Ortholog**	**Gene**
Jar	6.478	jaguar, myoVI	actin, cytoskeleton binding	MYO6: myosin VI	Deafness, autosomal recessive 37/autosomal dominant 22
c11.1	6.405	c11.1	predicted role in heart function	MROH1: maestro heat like repeat family member 1	
PPO1	5.686	Prophenoloxidase 1	conversion of dopamine, catechol oxidase activity, immune response		
Lam	5.603	Lamin	cytoskeleton	LMNA: lamin A/C	Cardiomyopathy 1A, Emery-Dreifuss Muscular Dystrophy 2/3, Congenital Muscular Dystrophy, Charcot-Marie-Tooth disease 2B, Hutchinson-Gilford progeria
Pdh	5.166	Photoreceptor dehydrogenase	oxidative stress response, linked to neurodegeneration, lifespan	HPGD: hydroxyprostaglandin dehydrogenase 15-(NAD)	Hypertrophic Osteoarthropathy, Isolated Congenital Digital Clubbing
Few	4.573	flower	apoptosis, linked to aging	CACFD1: calcium channel flower domain containing 1	
Tsf1	4.552	Transferrin 1	Iron binding, immunity, linked to aging	MELTF; melanotransferrin	

Table 3.1: Machine Learning Results from Heads. The top 20 proteins identified to be best predictors of aging in the head of control flies. The top predictor of aging is CG1561, a protein with links to neurodegeneration and *parkin*.

Gene	Machine Learning Score	Name	Function	Human Ortholog	Human Disease
Hsc70-5	100	Heat shock protein cognate 5	protein chaperone	HSPA9: heat shock protein family A (Hsp70) member 9	Anemia, Sideroblastic, Even-Plus Syndrome (Epiphyseal and Vertebral Dysplasia)
GstD1	35.535	Glutathione S transferase D1	lifespan, neurodegeneration, oxidative stress		
Cpr64Aa	30.118	Cuticular protein 64Aa	structural constituent of chitin-based larval cuticle	zinc finger protein 160	
PPO1	18.058	Prophenoloxidase 1	conversion of dopamine, catechol oxidase activity, immune response		
PHGPx	13.526	PHGPx	peroxidase activity, oxidative stress response	GPX4; glutathione peroxidase 4	Spondylometaphyseal Dysplasia
PPO2	8.533	Prophenoloxidase 2	pigment production, conversion of dopamine, immune response		
PPO3	8.533	Prophenoloxidase 3	pigment production, conversion of dopamine, immune response		
Strn-Mlck	6.201	Stretchin-Mlck	Calcium/Calmodulin-dependent protein kinases, myosin light chain kinase activity, oxidative stress, lifespan, muscle	MYLK: myosin light chain kinase	Aortic Aneurysm, familial
Idgf2	4.065	Imaginal disc growth factor 2	imaginal disc growth factor receptor binding, immuntiy, clotting	CHIT1: chitinase 1	Chitotriosidase Deficiency
Esyt2	3.172	Extended synaptotagmin-like protein 2 ortholog	membrane trafficking	ESYT2: extended synaptotagmin 2	
Zasp66	2.777	Z band alternatively spliced PDZ-motif	alpha-actinin binding, muscle function	PDZ and LIM domain 3	Myofibrillar Myopathy 4 and Cardiomyopathy 1C

		protein 66			
Act57B	2.664	Actin 57B	cytoskeleton, muscle function, cell trafficking, synapse organization	ACTB, ACTC1 and ACTA1: actin, beta, cardiac 1 and skeletal alpha 1	Juvenile-Onset Dystonia, Nemaline Myopathy 3, Cardiomyopathy 1R
Gene	**Machine Learning Score**	**Name**	**Function**	**Human Ortholog**	**Human Disease**
tgo	2.484	tango Hif-1 beta	myosin binding, muscle and nervous system development	ARNT2; aryl hydrocarbon receptor nuclear translocator 2	Webb-Dattani Syndrome (Hypothalamo-Pituitary-Frontotemporal Hypoplasia)
CG10211	2.204		Haem peroxidase, oxidative stress response, myotube formation?	myeloperoxidase	Alzheimer's Disease, Myeloperoxidase Deficiency
Est-6	1.685	Esterase 6	type-B carboxylesterase/lipase family		
ND-B14.7	1.67	NADH dehydrogenase (ubiquinone) B14.7 subunit	nervous system function	NADH:ubiquinone oxidoreductase subunit A11	Mitochondrial Complex I Deficiency
RpL18	1.622	Ribosomal protein L18	ribosome	ribosomal protein L18	
RpL3	1.489	Ribosomal protein L3	ribosome	ribosomal protein L3	
ND-42	1.488	NADH dehydrogenase (ubiquinone) 42 kDa subunit	NADH dehydrogenase activity, glucose metabolism, mitophagy PINK1, lifespan, oxidative stress response	NADH:ubiquinone oxidoreductase subunit A10	Leigh Syndrome (Necrotizing Encephalopathy), oculopharyngeal muscular dystrophy
AdSS	1.454	Adenylosuccinate Synthetase	lifespan, inteaction with SOD2, immunity, purine biosythesis?,	adenylosuccinate synthase like 1	

Plp	1.36	Pericentrin-like protein	cytoskeleton	pericentrin	Microcephalic Osteodysplastic Primodial Dwarfism, Type II

Table 3.2: Machine Learning Results from Thoraces. The top 20 proteins identified to be best predictors of aging in the thorax of control flies. The number one predictor of aging is Hsc70-5. Other important proteins like GstD1, PPO1/2/3 and PHPDx are all involved in oxidative stress response.

Gene	Full Name	Function/Feature	Human Ortholog	Human Disease
Cyt-b5-r	Cytochrome b5-related	mitochondrial	FADS3; fatty acid desaturase 3	
Fas2	Fasciclin 2	neurons	NCAM2; neural cell adhesion molecule 2 and NCAM1	
if	inflated	integrin, neurons and muscle	ITGA8; integrin subunit alpha 8	Renal Hypodysplasia/Aplasia 1; Rhda1
wor	worniu	neurons, RNA	SNAI3; snail family zinc finger 3, 2 and 1	Piebald Trait; Pbt And Waardenburg Syndrome, Type 2D; Ws2D
AspRS	Aspartyl-tRNA synthetase	neurons	DARS; aspartyl-tRNA synthetase	Hypomelination with brainsteam and spinal cord involvemnt and leg spasticity: HBSL
nec	necrotic	serpin family, immune and proteolysis	SERPINC1; serpin family C member 1	Antithrombin III Deficiency; AT3D
pros	prospero	neurons, protein localization	PROX1; prospero homeobox 1	
trpl	transient receptor potential-like	calcium signaling	TRPC5; transient receptor potential cation channel subfamily C member 5	
Cct5	T-complex Chaperonin 5	chaperonin, protein folding	CCT5; chaperonin containing TCP1 subunit 5	Neuropathy, Hereditary Sensory with Spastic Paraplegia, Autosomal Recessive
Klp3A	Kinesin-like protein at 3A	kinesin	KIF4A; kinesin family member 4A	Mental Retardation, X-linked 100: MRX100
Gp150	Gp150	leucine rich repeat	TLR6; toll like receptor 6/2	Mycobacterium Tuberculosis
TART-element \gag	TART-open reading frame 1	transposable_element _gene --> GAG protein		
glu	gluon	condensin chromosome maintenance	SMC4; structural maintenance of chromosomes 4	
Prosbeta2	Proteasome β2 subunit	20S proteasome subunit	PSMB7; proteasome subunit beta 7	

Gene	Full Name	Function/Feature	Human Ortholog	Human Disease
sns	sticks and stones	adhesion, actin cytoskeleton organization	NPHS1; NPHS1 nephrin	Nephrotic Syndrom, Type 1: NPHS1
Ugt35b	UDP-glycosyltransferase 35b	UDP-glycosyltransferase	UGT2B15; UDP glucuronosyltransferase family 2 member B15	
CG8677	CG8677	chromatin silencing	RSF1; remodeling and spacing factor 1	
ThrRS	Threonyl-tRNA synthetase		TARS; threonyl-tRNA synthetase	
AlaRS	Alanyl-tRNA synthetase		AARS; alanyl-tRNA synthetase	Charcot-Marie-Tooth Disease, Axonal Type 2N: CMT2N, Epileptic Encephalopathy, Early Infantile 29: EIEE29
CG12182	CG12182		???	
CG33090	CG33090	non-lysosomal glucosylceramidase family.	GBA2; glucosylceramidase beta 2	Spastic Paraplegia 46, Autosomal Recessive: SPG46
Spn88Ea	Serpin 88Ea	serpin family, immune and proteolysis	SERPINI1; serpin family I member 1	Encephalopathy, Familial with Neuroserpin Inclusion Bodies; FENIB
CG7766	CG7766	phosphorylase b kinase regulatory chain family	PHKA2; phosphorylase kinase, alpha 2 (liver) and 1	Glycogen Storage Disease IXa1; GSD9A1
CG1561			acyl-CoA dehydrogenase family member 11	
SWIP	SWIP	WASHS7 family interacts with WASP	KIAA1033; KIAA1033	Mental Retardation, Autosomal Recessive 43; MRT43
CG9981	CG9981		ATP11C; ATPase phospholipid transporting 11C, 11B and 11A	
CG5080	CG5080	???	???	
ND-B14.5B	NADH dehydrogenase (ubiquinone) B14.5 B subunit	Mitochondrial Complex 1-NADH: Ubiquinone Oxidreductase Complex Subunits	NDUFC2; NADH:ubiquinone oxidoreductase subunit C2	

Gene	Machine Learning Score	Name	Function	Human Ortholog
IM33	Immune induced molecule 33		WFIKKN1; WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1	
CG11321	CG11321	ring finger domain, protein polyubiquitination	RNF31; ring finger protein 31	
w-cup	world cup	meiosis	???	
CG5122	CG5122	carnitine O-acetyltransferase activity	CRAT; carnitine O-acetyltransferase	
CG16885	CG16885		???	
CG17549	CG17549		???	
CG10188	CG10188	Rho GEF	ARHGEF18; Rho/Rac guanine nucleotide exchange factor 18	
Dscam1	Down syndrome cell adhesion molecule 1	axon guidance	DSCAML1; Down syndrome cell adhesion molecule like 1	
CG7639	CG7639		SAMM50; SAMM50 sorting and assembly machinery component	
Gale	UDP-galactose 4'-epimerase	NAD(P)-dependent epimerase/dehydratase family	GALE; UDP-galactose-4-epimerase	Galactose Epimerase Deficiency
CG13900	CG13900	neurons, splicing	SF3B3; splicing factor 3b subunit 3	
Cpr62Bb	Cuticular protein 62Bb	cuticle protein	ZNF160; zinc finger protein 160	
CG10077	CG10077	RNA helicase?	DDX5; DEAD-box helicase 5 and 17	
CG4461	CG4461	Hsp20 like chaperone	HSPB3; heat shock protein family B (small) member 3	
CG9279	CG9279	DYNACTIN COMPLEX, dynein binding	DCTN1; dynactin subunit 1	Distal Hereditary Neuropathy
CG14609			RPAP2; RNA polymerase II associated protein 2	
CG11334	CG11334	eIF-2B alpha/beta/delta subunits family	MRI1; methylthioribose-1-phosphate isomerase 1	
Hexo2	Hexosaminidase 2	glycosidase	HEXB; hexosaminidase subunit beta and alpha	Sandhoff Disease

Gene	Machine Learning Score	Name	Function	Human Ortholog
sals	sarcomere length short	positive regulator of actin, muscle	SCAF1; SR-related CTD associated factor 1	
CG32201	CG32201	Prolyl 4-hydroxylase, alpha subunit		
CG32437	CG32437		???	
Sec16	Sec16 ortholog		SEC16A; SEC16 homolog A, endoplasmic reticulum export factor	
CG34202	CG34202		???	
su(r)	suppressor of rudimentary		DPYD; dihydropyrimidine dehydrogenase	Dihydropyrimidine Dehydrogenase Deficiency
eIF-2gamma	Eukaryotic initiation factor 2γ	translation factor GTPase family.	EIF2S3; eukaryotic translation initiation factor 2 subunit gamma	
DNApol-epsilon255	DNA polymerase ε 255kD subunit	DNA repair	POLE; polymerase (DNA) epsilon, catalytic subunit	Colorectal cancer 12; CRCS12 Facial dysmorphism, Immunodeficiency, Livedo and short stature: FILS
CG45067	CG45067		PROM1; prominin 1	Stargardt Disease 4; STGD4, Retinitis Pigmentosa 41: RP41, Cone-Rod Dystrophy 12: CORD12, Retinal Macular Dystrophy 2: MCDR2
unc-104	unc-104 ortholog (C. elegans)	kinesin	KIF1A; kinesin family member 1A and B	Spastic Paraplegia 30, Autosomal Recessive; SPG30 , Neuropathy, Hereditary Sensory, Type IIC: HSN2C, Mental Retardation, Autosomal dominant 9: MRD9
CG31548	CG31548	Short-chain dehydrogenase/reductase	HSD17B14; hydroxysteroid (17-beta) dehydrogenase 14	
CG12276	Activator of SUMO 1	sumolyation, neurons	SAE1; SUMO1 activating enzyme subunit 1	
CG12909		zn finger	LYAR; Ly1 antibody reactive	
Galpha49B	G protein α q subunit	axon guidance, locomotion	GNAQ; G protein subunit alpha q	Sturge-Weber Syndrome, SWS, Capillary

				Malformations, Congenital; CMC
Gene	**Machine Learning Score**	**Name**	**Function**	**Human Ortholog**
CG15267	down and out	behavioral mutant	SMYD4; SET and MYND domain containing 4	
CG6701	???	dendrite morphogenesis	MOV10; Mov10 RISC complex RNA helicase	
CG1600	Death resistor Adh domain containing target	circadian, alcohol		
PPO2	Phenoloxidase 2	ox stress		
sec10	Sec10 ortholog (S. cerevisiae)	synaptic vesicles	EXOC5; exocyst complex component 5	
fbp	Fructose-1,6-bisphosphatase	metabolism	FBP2; fructose-bisphosphatase 2	
Evi5	Ecotropic viral integration site 5	GTPase activator activity	EVI5; ecotropic viral integration site 5	

Table 3.3: p38K Mutants and Over-Expression Effected the Expression of 68 Proteins in the Head. Identified proteins mediated three main cellular processes: cytoskeletal, gene expression and neuronal health.

Gene	Full Name	Function/Feature	Human Ortholog	Human Disease
Adgf-A	Adenosine deaminase-related growth factor A	cell proliferation	CECR1; cat eye syndrome chromosome region, candidate 1	Polyarteritis Nodosa, Childhood-Onset, Pan Syndrome
CG7028		Dual Specificity Tyrosine-Phosphorylation Regulated Kinases	PRPF4B; pre-mRNA processing factor 4B	
CG2918		hsp70 domain	HYOU1; hypoxia up-regulated 1	
CG1561		acyl-CoA dehydrogenase family member 11		
CG8209		ubiquitin related domain	UBXN1; UBX domain protein 1 and 4	
CG3326		AAA ATPase family	FIGNL1 and spastin	Familial Spastic Paraplegia 2 FSP2
Nup154	Nucleoporin 154kD		NUP155; nucleoporin 155kDa	Familial Atrial Fibrillation 15; ATFB15
Prx2540-1	Peroxiredoxin 2540-1	oxidative stress	PRDX6; peroxiredoxin 6	
CG8858		armadillo repeats	KIAA0368; KIAA0368	
Akap200	A kinase anchor protein 200		MARCKSL1; MARCKS-like 1	
slgA	sluggish A		PRODH; proline dehydrogenase 1	Hyperprolinemia, Type 1:HYRPRO1 and Schizophrenia 4; SCZD4
stwl	stonewall	myb transcription factor		
fon	fondue			
CG10700			AIFM3; apoptosis inducing factor, mitochondria associated 3	
SF1	Splicing factor 1		SF1; splicing factor 1	

Gene	Full Name	Function/Feature	Human Ortholog	Human Disease
shf	shifted	regulates hh signaling. wing development	WIF1; WNT inhibitory factor 1	
Inx2	Innexin 2	gap junctions		
ash1	absent, small, or homeotic discs 1	Histone-lysine methyltransferase family	ASH1L; ash1 (absent, small, or homeotic)-like (Drosophila)	
CG14556		??		
glu	gluon	mitosis	SMC4; structural maintenance of chromosomes 4	
Mekk1	Mekk1	STE 11 kinases	MAP3K4; mitogen-activated protein kinase kinase kinase 4	
Sec16	Sec16 ortholog	ER stress	SEC16A; SEC16 homolog A, endoplasmic reticulum export factor	
Best1	Bestrophin 1	chloride channels	BEST1-4; bestrophin 1-4	Bestrophinopathy, Autosomal Recessive; Arb, And Vitreoretinochoroidopathy; Vrcp And Retinitis Pigmentosa 50; Rp50 Macular Dystrophy, Vitelliform, 2; Vmd2
CG43347		Zn finger binding	PRDM4; PR domain 4 and PRDM14	
Fim	Fimbrin	actin binding and ca binding	PLS3; plastin 3 and PLS1 and LCP1; lymphocyte cytosolic protein 1	Bone Mineral Density Quantitative Trait Locus 18: BMND18
vir-1	virus-induced RNA 1			
Tollo	Tollo		TLR3; toll like receptor 3	susceptibility to Human Immunodeficiency Virus type 1
Dap160	Dynamin associated protein 160		ITSN1; intersectin 1 and ITSN2	ITSN1; intersectin 1 and ITSN2

Gene	Machine Learning Score	Name	Function	Human Ortholog
Traf6	TNF-receptor-associated factor 6	zinc ring finger domain	TRAF6; TNF receptor associated factor 6	
CG11883			NT5E; 5'-nucleotidase ecto and GOLM1; golgi membrane protein 1	Calcificaiton of Joints and Arteries
CG4049		helicase???	ERCC6; excision repair cross-complementation group 6 and AD54L2; RAD54-like 2 (S. cerevisiae)	Macular Degeneration, Age-Related 5: ARMD5, Lung Cancer, De Sanctis-Cacchione Syndrome Cerebrooculofacioskeletal syndrome1: COFS1
Adar	Adenosine deaminase acting on RNA	RNA editing	ADARB1; adenosine deaminase, RNA specific B1	
ValRS	Valyl-tRNA synthetase	Cytoplasmic Aminoacyl-Trna Synthetases	VARS; valyl-tRNA synthetase	Combined Oxidative Phosphorylation Deficiency 20; COXPD20
Mdr65	Multi drug resistance 65	Belongs to the ABC transporter superfamily.	ABCB4; ATP binding cassette subfamily B member 4	
CG1979	???	sleep ?	???	
CG5482			FKBP8; FK506 binding protein 8	
CG31797	???		???	
CG46281	???		???	
ck	crinkled	myosin family	MYO7A; myosin VIIA	
CG3107	???	Belongs to the peptidase M16 family	PITRM1; pitrilysin metallopeptidase 1	
Tsf2	Transferrin 2	iron binding	MELTF; melanotransferrin	
ProtB	Protamine B		???	
CG8086	???		ODF3; outer dense fiber of sperm tails 3	
CG42540	???		STOM; stomatin	
wit	wishful thinking	dpp receptor	BMPR2; bone morphogenetic protein receptor type 2	

CG1924	???	calcium binding	CANX; calnexin	
Gene	**Machine Learning Score**	**Name**	**Function**	**Human Ortholog**
DNApol-delta	DNA-polymerase-δ	DNA synthesis	POLD1; polymerase (DNA) delta 1, catalytic subunit	
CG5521	???	RAP GTPase family	RALGAPA1; Ral GTPase activating protein catalytic alpha subunit 1	
CG42271	???	Phosphatidylinositol Phosphate And Inositol Phosphate Phosphatases	INPP4A; inositol polyphosphate-4-phosphatase type I A	
DCTN1-p150	Dynactin 1, p150 subunit	dynein binding	DCTN1; dynactin subunit 1	Neuronopathy, Distal Hereditary Motor VIIB; HMN7B, Amyotrophic Lateral Sclerosis 1; ALS1. Perry Syndrome
LBR	Lamin B receptor	Anchors lamina and heterochromatin to inner nuclear membrane	LBR; lamin B receptor	Reynolds Syndrome Greenberg Dysplasia; GR8GD, Pelger- Huet Anomaly; PHA
CG7741	???	Belongs to the CWF19 family	CWF19L1; CWF19-like 1, cell cycle control (S. pombe)	Spinocerebellar Ataxia, Autosomal Recessive 17: SCAR17
CG34459	???	helicase		
CG8915	???		YTHDC2; YTH domain containing 2	
CG17233	???		CCDC82; coiled-coil domain containing 82	
CdGAPr	Cd GTPase activating protein-related	Rho GTPase Activating Proteins	ARHGAP33; Rho GTPase activating protein 33 and 32	
sle	slender lobes	mushroom body development	???	
CG6209	???		???	
Sec31	Sec31 ortholog	ER	SEC31A; SEC31 homolog A, COPII coat complex component	

Oat	Ornithine aminotransferase precursor	class-III pyridoxal-phosphate-dependent aminotransferase family	OAT; ornithine aminotransferase	Gyrate Atrophy of Choroid and Retina: GACR
Gene	**Machine Learning Score**	**Name**	**Function**	**Human Ortholog**
CG6701	???	dendrite morphogenesis	MOV10; Mov10 RISC complex RNA helicase	
CG8768	???		SDR39U1; short chain dehydrogenase/reductase family 39U member 1	
Pi3K92E	PI3K		PIK3CD; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	
clu	clueless	mitochondrial function/localization	CLUH; clustered mitochondria (cluA/CLU1) homolog	
HDC19897	mystery gene			
CG31784	?????		????	
CG7669	hemingway		CFAP97; cilia and flagella associated protein 97	

Table 3.4: p38K Mutants and Over-Expression Effected the Expression of 69 Proteins in the Thoraxes. Identified proteins were involved in cytoskeletal processes, gene expression, neuronal health and stress response.

Gene	Full Name	Function/Feature	Human Ortholog	Human Disease
Sec16	Sec16 ortholog	Ras GTPase binding, ER stress	SEC16A; SEC16 homolog A, endoplasmic reticulum export factor	
CG1561		acyl-CoA dehydrogenase family member 11		linked to neruodegeneration and parkin
glu	gluon	condensin chromosome maintenance	SMC4; structural maintenance of chromosomes 4	
CG6701	???	dendrite morphogenesis	MOV10; Mov10 RISC complex RNA helicase	

Table 3.5: p38K Mutants and Over-Expression Effected the Expression of 4 Proteins in both Heads and Thoraxes. One of these proteins, CG1561, is also a key regulator of aging in the head as predicted by the machine learning.

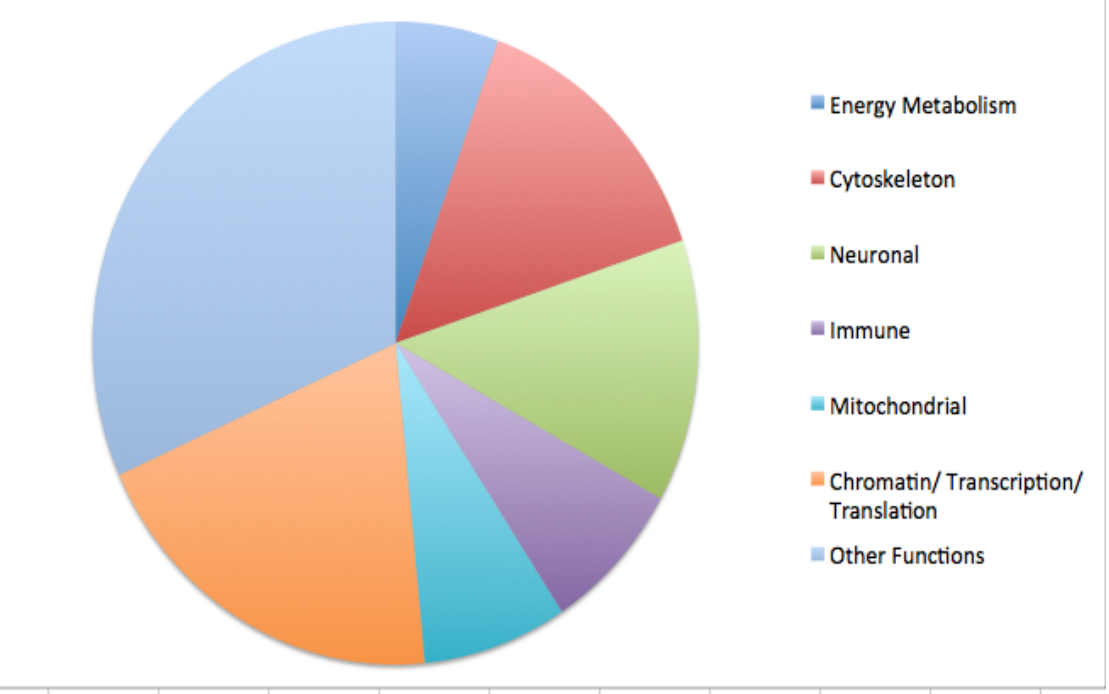


Figure 3.1: The Cellular Functions of Protein Expression Effected by p38Kb Expression. Functional categories for protein expression levels that were effected by p38K expression in in both heads and thoraces.

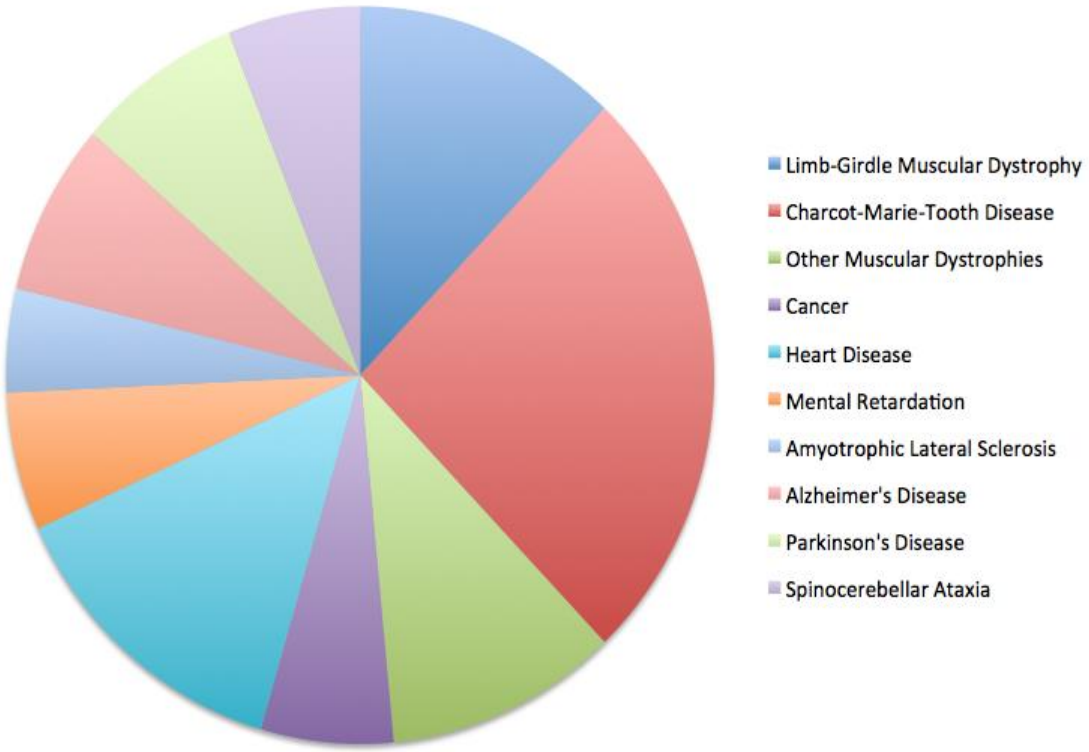


Figure 3.2: Genes linked to Human Diseases Effected by p38Kb Expression.
Proteins that were affected by p38K expression in heads and thoraces are linked to many muscular dystrophies, neurodegeneration diseases, heart disease and cancers.

CHAPTER FOUR: THE EVOLUTION OF P38 MAPK IN *DROSOPHILA* SPECIES

4.1 Introduction

Genetic mutations are a driving force for natural selection and the evolution of species. Mutations can affect a single gene resulting in the altered expression of the gene, or the production of a modified protein product. Alternatively, mutations can occur at the chromosomal level (e.g. the deletion, duplication, inversion or translocation of chromosomal regions) and can have an effect on a larger number of genes and their associated protein products. Mutations that affect the function or expression pattern of a gene (i.e. when and where it is expressed) may prove beneficial to an organism with changing environmental conditions, and thus become fixed within a population. Interestingly, when there is a chromosomal duplication, a duplicated gene is not normally under the selective pressures of the original gene, and as such can provide the raw genetic material for the evolution of new genes. In turn, the accumulation of new and different genes allows for divergent evolution as populations separate and eventually forms different species (Wunderlich et al., 2012).

Signal transduction pathways are highly specialized to allow organisms to respond to a range of inputs, and understanding the evolution of these pathways can provide insight into the responses to developmental signals and environmental stress. Mitogen-activated protein kinases (MAPK) are serine-threonine protein kinases that

respond to a wide variety of extracellular stimuli and control a number of cellular processes including cell growth, proliferation, stress response and apoptosis. MAPKs are evolutionarily conserved in all eukaryotic cells and respond to extracellular factors like growth signals, cytokines, hormones and cellular stress (Widmann et al., 1999). MAPK signaling cascades are characterized by extracellular signals which activate an intracellular response via a receptor tyrosine kinase on the plasma membrane surface (Shilo, 2014). The receptors work with groups of evolutionarily conserved upstream kinases, or small GTP binding proteins of the Rho/Rac family to control the activity of the MAPKs via a phosphorylation cascade (Cargnello and Roux, 2011). First, phosphorylation of a MAPKKK leads to a subsequent phosphorylation of a MAPKK which can dual phosphorylate the conserved Thr-X-Tyr motif to activate the MAPK (Figure 4.1). This phosphorylation step causes a conformational change that allows the MAPK to bind ATP as well as specific substrates. Once activated, MAPKs phosphorylate either a Ser or Thr followed by a Pro residue on downstream targets. MAPKs are responsible for activating large sets of transcription factors (many in the Activating Transcription Factor (ATF) family), other protein kinases, cytoskeleton associated proteins and phospholipases (Chakrabarti et al., 2014; Widmann et al., 1999).

Conventional MAPKs fall into three categories: extracellular signal-regulated kinases (ERK), c-Jun amino (N)-terminal kinases (JNK) and p38 kinases (p38K). These three classes of MAPKs have undergone a variety of gene duplications and possibly gene loss, resulting in the generation of subclasses of MAPKs with specialized functions. For example in mice there are four p38K genes (p38K-α, β, γ, and δ). p38K-α plays a primary role and a complete or conditional knockout of this gene is embryonic lethal

(Adams et al., 2000; Tamura et al., 2000). p38K-α mediates the stress and inflammation responses, but also play critical roles in the cell cycle and proliferation (Coulthard et al., 2009). The other p38K genes appear to be less critical, as mutations of p38K-β or p38K-δ or double knockout of p38K-γ and p38K-δ all produce viable fertile mice (Aouadi et al., 2006). p38K-α and p38K- β are expressed widely in most murine tissues and mediate similar growth and stress responses (Coulthard et al., 2009). The expression pattern of p38K-γ and p38K-δ is more restricted; these two regulate aspects of cellular growth and proliferation (Coulthard et al., 2009).

Whereas in the fruit fly, *Drosophila melanogaster,* there are two conventional p38K genes (p38Ka and p38Kb) and a third gene (p38Kc) that has lost the canonical Thr-Gly-Tyr phosphorylation site. p38Ka plays a role in stress response and immunity and a p38Ka null allele is still viable but these flies shows increased susceptibility to heat shock, starvation and oxidative stress (Craig et al., 2004). p38Kb regulates aging, the oxidative stress response and immunity. p38Kb null mutants are also viable but showed a greatly reduced lifespan, age-depended locomotor effects and increased sensitivity to heat shock, oxidative stress and starvation (Vrailas-Mortimer et al., 2011). Finally, p38Kc null mutants are also viable but show increased sensitivity to oxidative stress and decreased ability to produce melanin, which has a role in immune response (Chakrabarti et al., 2014; Davis et al., 2008). It is possible that other pathways compensate in these single null mutants because double knockouts of p38Ka and p38Kb are lethal (Vrailas-Mortimer et al., 2011). But p38Kc appears to play less of a conventional MAPK role because double knockouts of p38Ka and p38Kc are still viable.

In order to better understand the patterns of p38K evolution within the *Drosophila* genus, we have utilized the sequenced genomes of the 12 *Drosophila* species (Figure 4.2). From our analysis, we find that p38Ka and p38Kb are highly conserved, and p38Kc has diverged most recently within the *Drosophila* species and does not contain the canonical MAPK motifs. Additionally, we find conserved transcription factor binding sites for Homeobox, AP-1 and lola that could control the activity of p38Ka and p38Kb genes across *Drosophila* species.

4.2 Results

Analysis of MAPK genes in humans, mice, zebrafish, *Drosophila melanogaster*, *Aedes aegypti* (mosquito), *Apis mellifera* (honeybee), and *C. elegans* finds that the emergence into the 3 MAPK families (ERK, JNK and p38K) happened very early in animal evolution before the split between vertebrates and invertebrates. Furthermore, we find that the three p38K genes in *D. melanogaste*r are not orthologous to the p38K genes found in other species, suggesting that the three p38K genes arose from a single ancestral p38K gene (Figure 4.3).

In *D. melanogaste*r, the p38Kb gene resides on the second chromosome; while both p38Ka and p38Kc reside in close proximity on the third chromosome. Analysis of the p38Ka and p38Kc genes across the 12 species of *Drosophila* revealed a high level of evolutionary conservation of genes between the species (Figure 4.4A). p38Kc appears to have been a later addition to *Drosophila,* appearing between *D. willistoni* and *D. persimilis.* Furthermore, in *D. persimilis* p38Kc is truncated as compared to the p38Kc gene in other *Drosophila* species. Interestingly, *D. persimilis* also has additional genes

that have appeared around the p38Kc locus, disrupting its close proximity to p38Ka (Figure 4.4A). These data suggest that p38Kc most likely arose from a gene duplication event of p38Ka. Similar analysis of the p38Kb locus finds that p38Kb is highly conserved within the *Drosophila* genus. However, much like with p38Ka and p38Kc, *D. persimilis* has additional genes inserted in the p38Kb locus (figure 4.4B). Additionally, *D. pseudoobscura* is unique as there are two p38b genes (figure 4.5), which are located 8555kb apart on the second chromosome.

In order to determine if the p38K genes are under selective pressures, we performed a sequence alignment and created a phylogenetic tree of p38K across 22 *Drosophila* species with available genome sequence data. We find that across species, the p38Ka and p38Kb genes clustered according to species relatedness (Figure 4.5). But p38Kc has diverged more across the different species. We next determined the dN/dS ratio for each of the p38K genes to determine which type of selection each gene is under. Non-synonymous changes (dN) measure the degree to which amino acid changes are made in two homologous sequences. Synonymous changes (dS) measure how two homologous sequences differ with respect to silent mutations and can be used as a molecular clock for evolution of a gene between closely related species (Nei and Gojobori, 1986). Comparing the ratio of the dN/dS for two homologous sequences can determine what type, if any, selection a protein is under. When the ratio of dN/dS equals one there is no selection occurring and mutations affecting the genetic code are largely neutral. When the ratio is below one purifying selection (or negative selection) is occurring. In this scenario there is selection against any deleterious non-synonymous mutations. Conversely, if the ratio is above one, diversifying selection (or positive

selection) is occurring. Diversifying selection is driven by amino acid changes and, if passed onto offspring, can result in a new species.

We looked at the dN/dS ratio for p38Ka, p38Kb and p38Kc genes in 22 different species of *Drosophila* using Molecular Evolutionary Genetics Analysis (MEGA) (Figure 4.6). In the case of p38Ka and p38Kb dN/dS <<1 therefore purifying selection is operating. In this scenario, an occasional amino acid substation may have been caused by selection, but not to an extent to overcome the overall effects of purifying selection. As such, this type of selection prevents deleterious mutations from accumulating within a population. But p38Kc shows a higher ratio of non-synonymous to synonymous change, suggesting that p38Kc is more amenable to changes that could lead to new functions for this gene. But as the slope is still below one, this indicates that p38Kc is still under selective pressures to maintain certain functions within the cell or tissue (Figure 4.6).

We then analyzed the genes of p38Ka, p38Kb and p38Kc. When comparing only the *D. melanogaster* alignment of p38Ka and p38Kb, these two sequences are very highly conserved and only differ in 19 residues between the two proteins (figure 4.7A). But interestingly, p38Kc differs in a few key regions from p38Ka and p38Kb. First, the conserved TXY motif characteristic to all MAPKs (specifically p38Ks have a TGY motif) is different in p38Kc. This motif serves as the activation loop in which the Thr and Tyr residues serve as dual phosphorylation sites to activate the MAPK. But p38Kc lacks both the glycine and the tyrosine of the TGY motif (TGY to TDH) and therefore can't be phosphorylated in the canonical MAPK manner. Secondly, p38Kc differs in its interaction domain where canonical MAPKs phosphorylate a Ser or Thr on targets. In the case of p38Kc there are extra residues present at this site that could suggest that p38Kc

binds or interacts differently to other targets than p38Ka or p38Kb (figure 4.7A). Next, we compared the sequences for p38Ka, p38Kb and p38Kc among all 12 species (figure 4.7B-4.7D). There is a very high degree of sequence homology for p38Ka and p38Kb among all 12 species, but p38Kc shows a large amount of sequence variability.

We have previously reported that p38Kb protein is highly expressed in muscle and brain, whereas p38Ka is expressed in lower levels in these tissues (Vrailas-Mortimer et al., 2011), suggesting that p38Ka and p38Kb may have different roles in these tissues. Since these expression levels differ for p38Ka and p38Kb, we tested if there were differences in which transcription factors might be controlling the expression of these genes. We analyzed 1kb upstream of each gene for transcription factor consensus regions in the 12 *Drosophila* species (p38Ka motifs shown in figure 8A and p38Kb in figure 4.8C). There are a few homologous motifs that were conserved among most or all of the species (colored highlights). These identified motifs were then matched to known transcription factor binding sites (p38Ka in figure 8B and p38Kb in figure 4.8D).

From this analysis of p38Ka, we find conserved binding sites for a Homeobox transcription factor and two different isoforms of lola (lola-PO and lola-PK). Homeobox is responsible for Hox patterning and segmentation during *Drosophila* development (Lawrence and Morata, 1994). Additionally p38K has been found to interact with Homeobox CDX2/3 in humans to drive differentiation of intestinal cells (Houde et al., 2001). Lola (*longitudinals lacking*) plays critical roles in axon guidance, synapse development and neural circuitry from development through adult stages and the oxidative stress response (Gates et al., 2011; Goeke et al., 2003). There are more than 20 lola isoforms, each with similar BTB dimerization domains but unique DNA binding

domains, allowing for each isoform to target specific genes (Gates et al., 2011; Goeke et al., 2003; Horiuchi et al., 2003). With p38Kb, we find two lola isoforms (lola-PO and lola-PT) and the transcription factor AP-1, which is a heterodimer of Jun and Fos (figure 4.8D). AP-1 plays a role in cell differentiation, cell growth and apoptosis and is known to interact with MAPKs (Karin, 1995; Shaulian and Karin, 2002).

Finally on an organism level, since p38K is known to play a role in oxidative stress, we investigated resistance to oxidative stress in 7 different species of *Drosophila*. Populations of different species were tested for their survival to the oxidizing agent, paraquat (Figure 4.9.). *D. pseudoobscura* preformed the best on this treatment, while *D. ananassae* died within a few days of exposure. These findings are especially interesting as *D. psudoobscura* has two copies of the p38Kb gene, suggesting that both copies are expressed and help manage this stress response.

4.3 Discussion

The MAPK family is highly conserved in homologous amino acid sequences, targets of activation and overall function. Even though MAPKs are activated by a number of upstream MAPKKK and MAPKK, each of the MAPKs (ERK, JNK and p38K) show high specificity for downstream targets and affect the cell function in unique ways. But these three MAPKs work in concert to coordinate cellular response to extracellular cues. As the branching of MAPKs into three families occurred very early in animal evolution, it is evident that these kinases play an integral role in cellular function.

On of the large roles of p38K is mediating oxidative stress and immune system response. As *Drosophila* live in fermenting and decaying food matter it is imperative that

they have sufficient defense systems to combat their environment. *D. ananassae* is native to tropical regions of the South Pacific (Makino and Kawata, 2012). In these tropical regions, abundant fruits like guava, papaya and star fruit are much higher in antioxidant properties than fruits from other parts of the world (Lim et al., 2007). Because their nutrient medium is enriched in antioxidants, *D. ananassae* might not have as robust p38K signaling. In contrast, *D. pseudoobscura* (native to western parts of North America) and *D. virilis* (native to eastern coast of Asia) (Makino and Kawata, 2012) performed the best on the paraquat treatment. Based on these environments, p38K signaling may be more robust in these species. Additionally *D. pseudoobscura* has two p38Kb genes that could lead to increased signaling in response to oxidative stress.

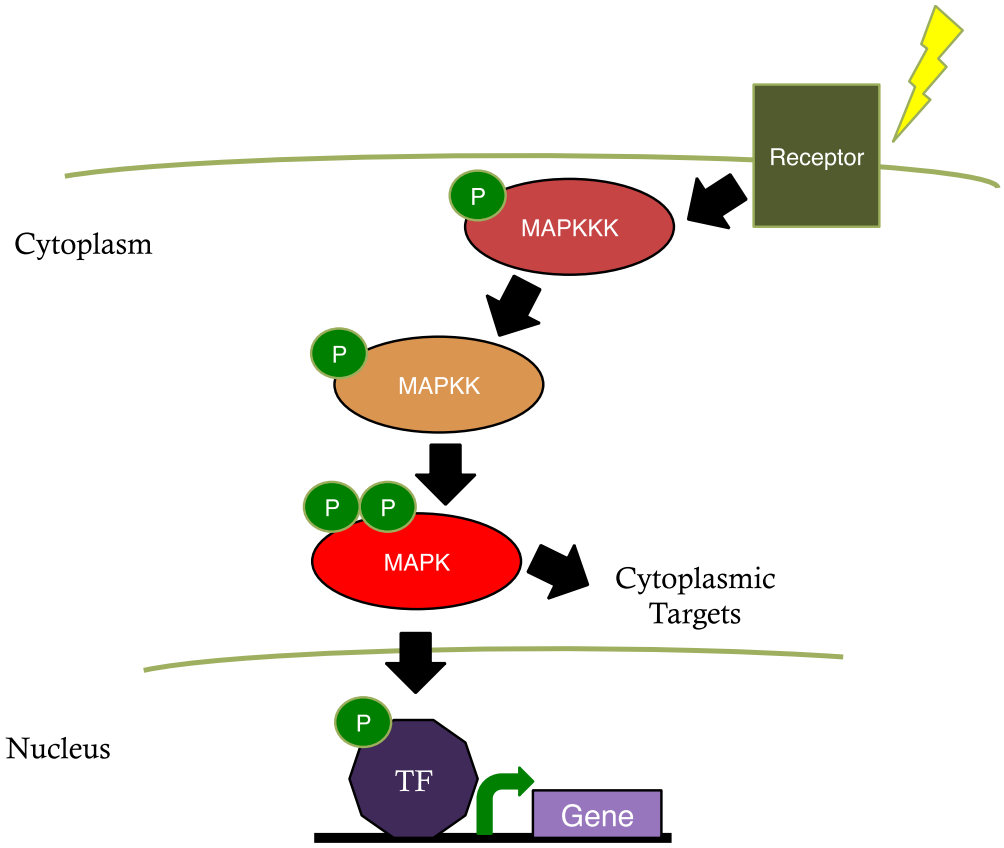


Figure 4.1: **MAPK activation cascade**. Mitogen Activated Protein Kinases (MAPKs) are activated through a series of phosphorylation steps on upstream kinases. Upon receiving an extracellular signal, such as oxidative stress, heat shock or osmotic stress, a receptor, frequently receptor tyrosine kinase integrates the signal and initiates the phosphorylation of MAPKKK. Upon activation the MAPKKK will phosphorylate the MAPKK. The MAPKK can dually phosphorylate the Thr-X-Tyr motif on the MAPK. Once activated MAPK can phosphorylate a number of targets including transcription factors or cytoplasmic proteins.

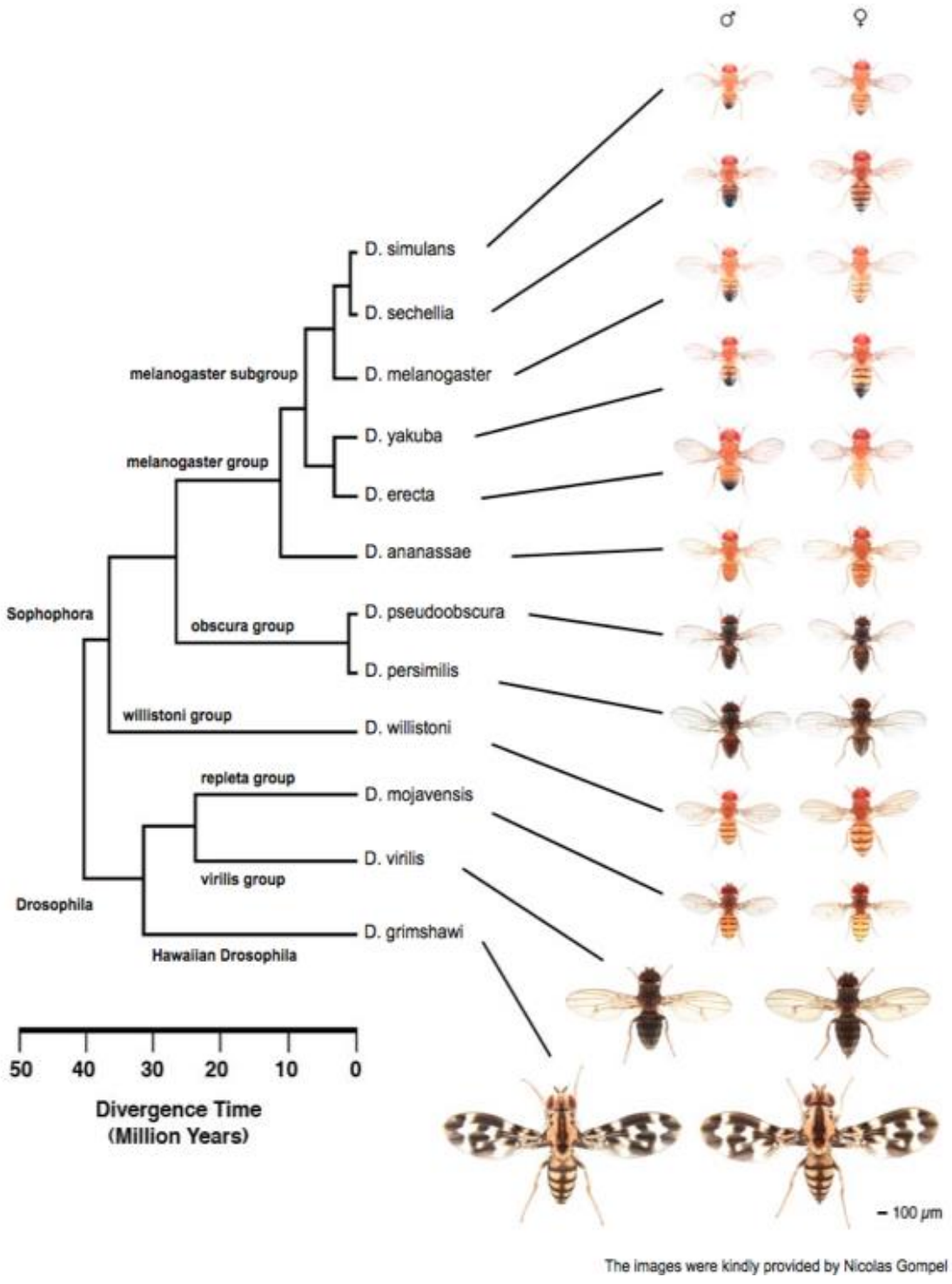


Figure 4.2: Partial phylogenetic tree of *Drosophila* genus. Phylogenic tree showing evolutionary relatedness of the 12 fully sequenced *Drosophila* species. Image from FlyBase (http://flybase.org/static_pages/species/sequenced_species.html).

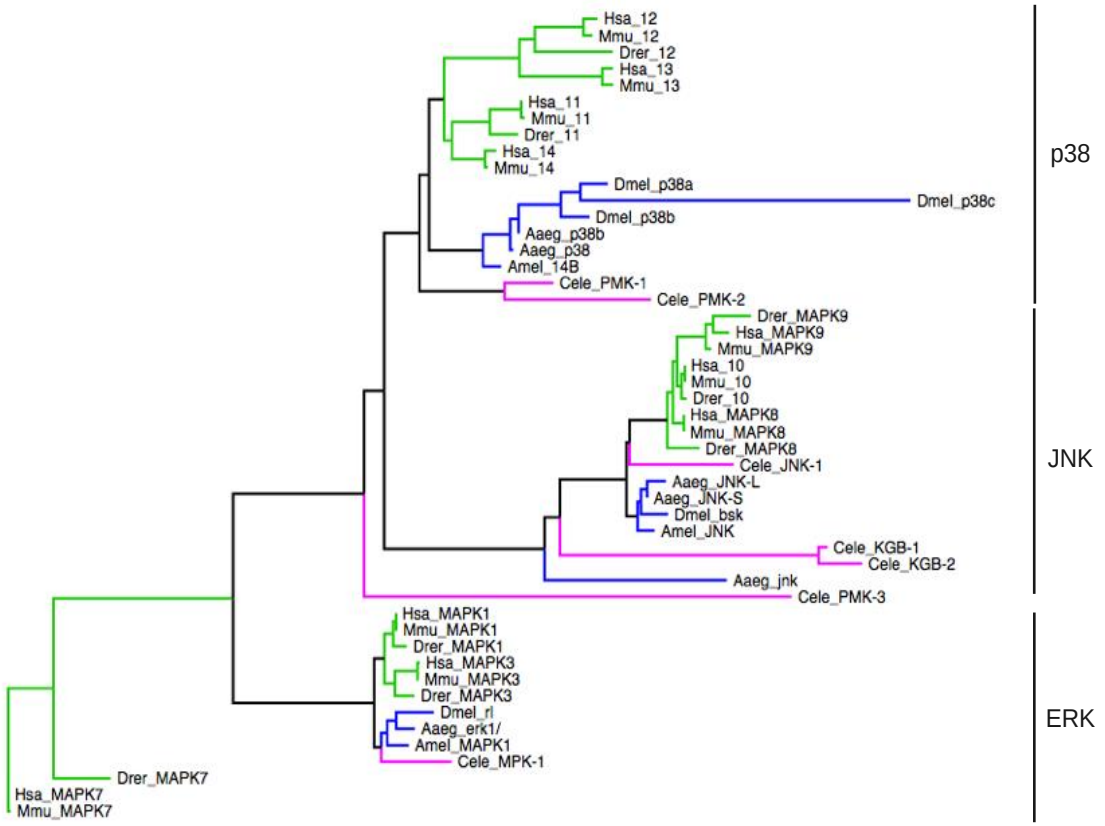
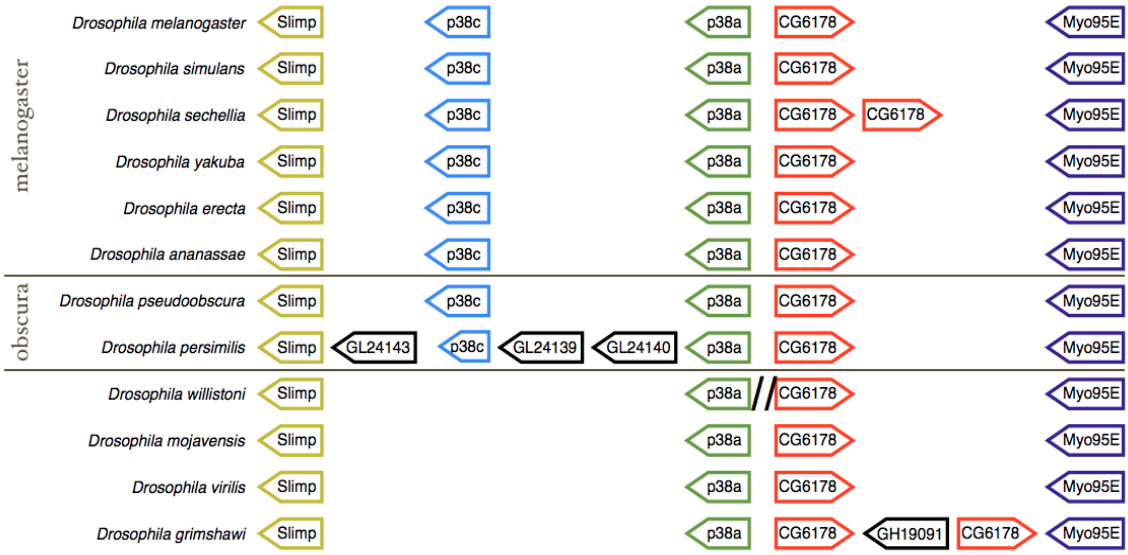


Figure 4.3: Phylogenetic tree of MAPK sequences in 7 key model organisms. The branching of MAPKs into the 3 conserved families of ERK, JNK and p38K happened early in animal evolution and show conserved homology.

Species listed as: Aaeg: Aedes aegypti (mosquito), Amel: Apis mellifera (honeybee), Cele: Caenorhabditis elegans (roundworm), Dmel: Drosophila melanogaster (fruit fly), Drer: Danio rerio (zebrafish), Hsa: Homo sapiens (human), Mmu: Mus musculus (mouse).

4.4A:



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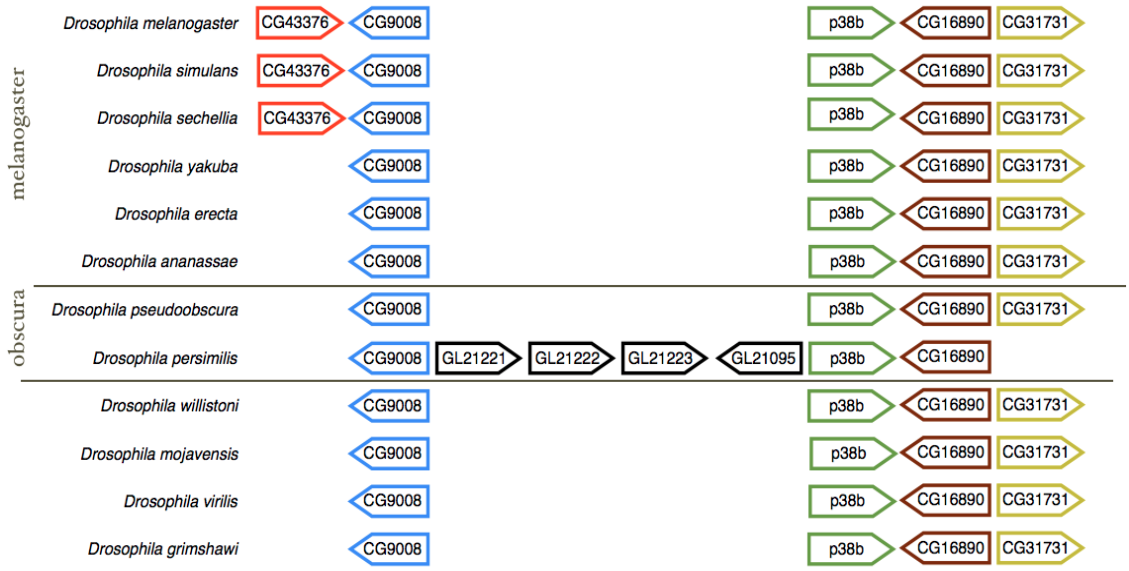


Figure 4.4: **Chromosome mapping of p38Ka, p38Kb and p38Kc**. Location of p38K genes in 12 representative species of *Drosophila.* (A) The locations of p38Ka and p38Kc on chromosome III. p38Kc is a newer gene emerging between *D. willistoni* and *D. persimillis*. (B) Chromosome II shows the conserved p38Kb gene in all species.

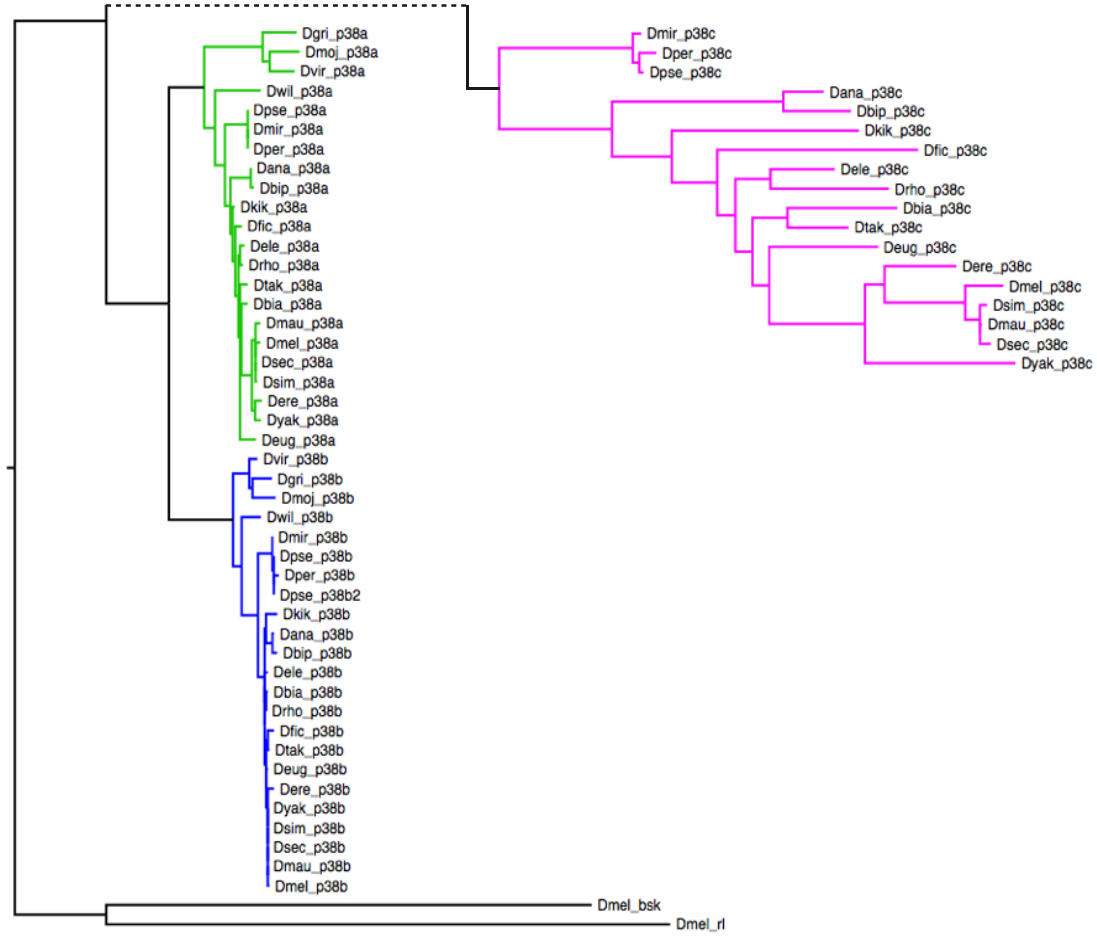


Figure 4.5: **Phylogenetic tree of the three p38 MAPK sequences in genus *Drosophila.*** The three p38K genes are compared to JNK (*Drosophila* homolog *basket*) and ERK (*Drosophila* homolog *rolled*). p38Ka is represented in green, p38Kb in blue and p38Kc in pink. p38Kc evolved more recently and is more closely related to p38Ka than p38Kb. *D. psuedoobscura* has two p38Kb genes, with a small amount of divergence between them.

Species abbreviated as: Dana: Drosophila ananassae, Dbia: Drosophila biamipes, Dbip: Drosophila bipectianta, Dele: Drosophila elegans, Dere: Drosophila erecta, Deug: Drosophila eugracilis, Dfic: Drosophila ficusphila, Dgri: Drosophila grimshawi, Dkik: Drosophila kikkawai, Dmau: Drosophila mauritiana, Dmel: Drosophila melanogaster, Dmir: Drosophila miranda, Dmoj: Drosophila mojavensis, Dper: Drosophila persimilis,, Dpse: Drosophila pseudoobscura, Drho: Drosophila rhopaloa, Dsec: Drosophila sechellia, Dtak: Drosophila takahashii, Dsim: Drosophila simulans, Dvir: Drosophila virilis, Dwil: Drosophila willistoni, Dyak: Drosophila yakuba. Genes abbreviated are bsk: basket (Drosophila homolog of JNK) and rl: rolled (Drosophila homolog of ERK).

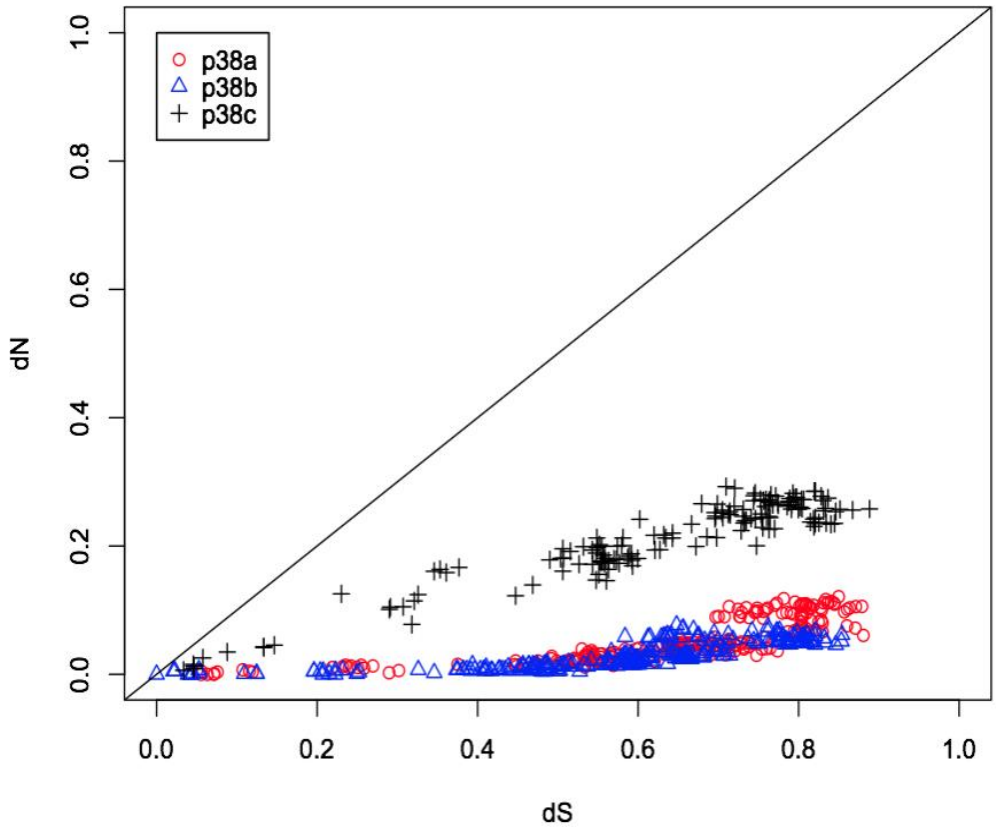
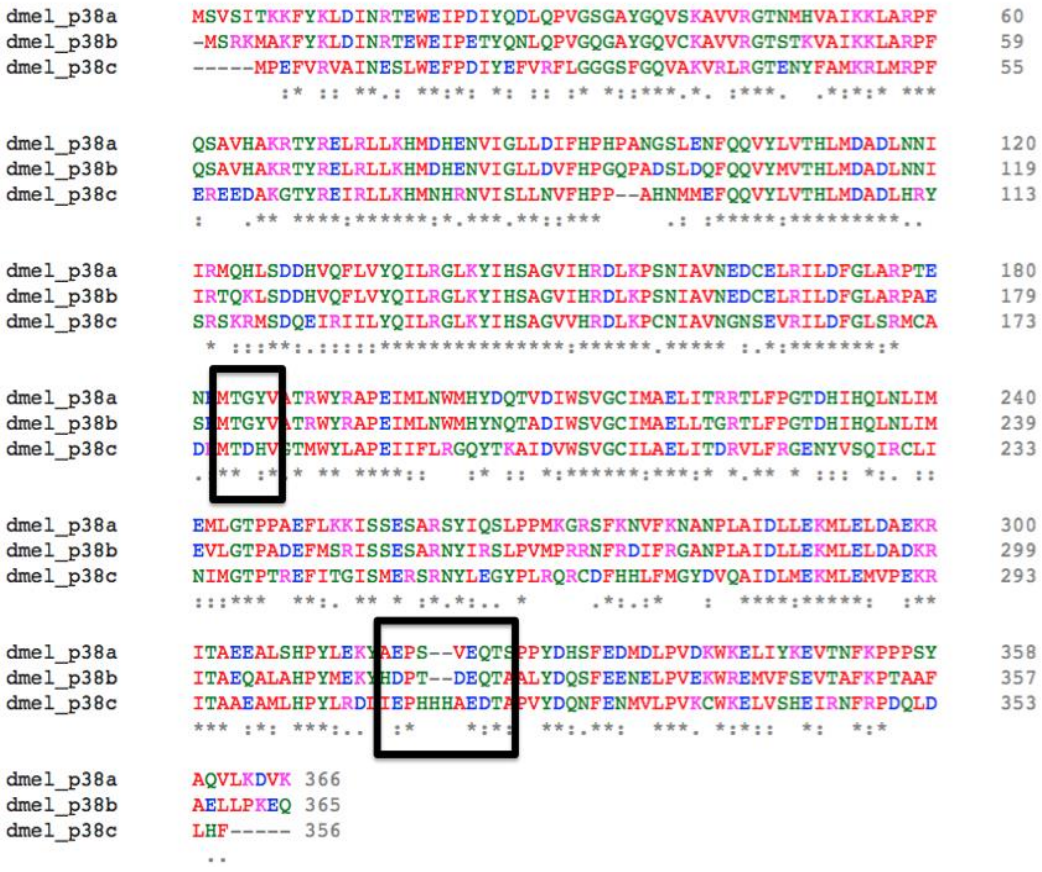


Figure 4.6: **dN/dS ratio of p38Ka, p38Kb and p38Kc**. A comparison of the non-synonymous changes (dN) to synonymous changes (dS) in different species of *Drosophila*. p38Ka is represented in red circles, p38Kb in blue triangles and p38Kc in black hash marks. Neutral selection is represented as a slope equal to one. All of the p38K genes fall below a slope of one indicating that there is selection against deleterious mutations. However, there is more change being tolerated in p38Kc, possibly due to its more recent appearance within the *Drosophila* genus.

4.7A: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dmel_p38c -----MPEFVRVAINESLWEFPDIYEFVRFLGGGSFGQVAKVRLRGTENYFAMKRLMRPF 55
dmel_p38a QSAVHAKRTYRELRLLKHMDHENVIGLLDIFHPHPANGSLENFQQVYLVTHLMDADLNNI 120
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dmel_p38a IRMQHLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPTE 180
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dmel_p38b SIMTGYVATRWYRAPEIMLNWMHYNQTADIWSVGCIMAELLTGRTLFPGTDHIHQLNLIM 239
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dmel_p38c LHF----- 356

4.7B:

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dsec_p38a      ----------------------------------------------MSVSITKKFYKL     12
dyak_p38a      ----------------------------------------------MSASVTKKFHKL     12
dere_p38a      ----------------------------------------------MSASITKKFHKL     12
dana_p38a      ----------------------------------------------MSASKAQKFYKV     12
dpse_p38a      ----------------------------------------------MSASKTQKFYKL     12
dper_p38a      ----------------------------------------------MSASKTQKFYKL     12
dwil_p38a      ---------------------------------------------MAATSTRKAQKFYKL     15
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dgri_p38a      ----------------------------------------------MSACKIRKFYKL     12
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dsec_p38a      DINRTEWEIPDIYQDLQPVGSGAYGQVSKALVRGT--NMHVAIKKLARPFQSAVHAKRTY     70
dyak_p38a      DINRTEWEIPDIYQDLQPVGSGAYGQVSKALVRGT--TMHVAIKKLARPFQSAVHAKRTY     70
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dgri_p38a      DINRTEWEVPEIYEQLQPVGSGAYGQVCKAKVRGT--NTDVAIKKLSRPFQSTVHAKRTY     70
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dyak_p38a      RELRLLKHMDHENVIGLLDIFHPHAANASLENFQQVYLVTHLMDADLNNIIRMQHLSDDH    130
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4.7C:

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dsec_p38b      MSRKMAKFYKLDINRTEWEIPETYQNLQPVGQGAYGQVCKAVVRGTSTKVAIKKLARPFQ     60
dyak_p38b      MSRKMAKFYKLDINRTEWEIPETYQNLQPVGQGAYGQVCKAVVRGTSTKVAIKKLARPFQ     60
dere_p38b      MSRKMAKFYKLDINRTEWEIPETYQNLQPVGQGAYGQVCKAVVRGTSTKVAIKKLARPFQ     60
dana_p38b      MSRQMAKFYKLDINRTEWEIPETYQNLQPVGQGAYGQVCKAVVRGTNTKVAIKKLARPFQ     60
dpse_p38b      MSRKMPKFYKLDINRTEWEIPETYQSLQPVGQGAYGQVCKAVVRGTSTKVAIKKLARPFQ     60
dper_p38b      ----MPKFYKLDINRTEWEIPETYQSLQPVGQGAYGQVCKAVVRGTSTKVAIKKLARPFQ     56
dwil_p38b      MSRKMPKFYKLDINRTEWEIPETYQNLQAVGQGAYGQVCKALVQNTNTKVAIKKLARPFQ     60
dmoj_p38b      ----MPKFYKIEINRTEWEIPETYQNLQAVGQGAYGQVCKAVVRGTSTKVAIKKLARPFQ     56
dvir_p38b      ----MPKFYKLDINRTEWEIPETYQNLQSVGQGAYGQVCKALVRGTTTKVAIKKLARPFQ     56
dgri_p38b      ----MPKFYKLDINRTEWEIPETYQNLQPVGQGAYGQVCKALVRGTTTKVAIKKLARPFQ     56
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dsim_p38b      SAVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     120
dsec_p38b      SAVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     120
dyak_p38b      SAVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     120
dere_p38b      SSVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     120
dana_p38b      SAVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLEQFQQVYMVTHLMDADLNNII     120
dpse_p38b      SAVHAKRTYRELRLLKHMEHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     120
dper_p38b      SAVHAKRTYRELRLLKHMEHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     116
dwil_p38b      SAVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLEQFQQVYMVTHLMDADLNNII     120
dmoj_p38b      SAVHAKRTYRELCLLKHMDHENVIGLLDVFHPGQPADSLDQFQQVYMVTHLMDADLNNII     116
dvir_p38b      SAVHAKRTYRELRLLKHMDHENVIGLLDVFHPGQPADSLEQFQQVYMVTHLMDADLNNII     116
dgri_p38b      SAVHAKRTYRELCLLKHMDHENVIGLLDVFHPGQPADSLEQFQQVYMVTHLMDADLNNII     116
               *:********** *****:********************:********************

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dsim_p38b      RTQKLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dsec_p38b      RTQKLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dyak_p38b      RTQKLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dere_p38b      RTQKLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dana_p38b      RTQKLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dpse_p38b      RTQKLSDDHVQFLIYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dper_p38b      RTQKLSDDHVQFLIYQILRGLKYIHSAGVIHRDLKPSQ----------------HRES     158
dwil_p38b      RTQKLSDDHVQFLVYQILRGLKYIHSAGVIHRDLKPSNIAVNEDCELRILDFGLARPAES     180
dmoj_p38b      RTQKLSDEHVQFLVYQILRGLKYIHSAGVIHRDLKPSNLAVNEDCELRILDFGLARPAES     176
dvir_p38b      RTQKLSDEHVQFLVYQILRGLKYIHSAGVIHRDLKPSNLAVNEDCELRILDFGLARPAES     176
dgri_p38b      RTQKLSDEHVQFLVYQILRGLKYIHSAGVIHRDLKPSNLAVNEDCELRILDFGLARPTES     176
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dsec_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     240
dyak_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     240
dere_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     240
dana_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     240
dpse_p38b      EMTGYVATRWYRAPEIMLNWMHYNKTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     240
dper_p38b      EMTGYVATRWYRAPEIMLNWMHYNKTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     218
dwil_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     240
dmoj_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     236
dvir_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     236
dgri_p38b      EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIHQLNLIME     236
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dsec_p38b       VLGTPADEFMSRISSESARNYIRSLPVMPRRNFRDIFRGANPLAIDLLEKMLELDADKRI     300
dyak_p38b       VLGTPADEFMSRISSESARNYIRSLPVMPRRNFRDIFRGANPLAIDLLEKMLELDADKRI     300
dere_p38b       VLGTPADEFMSRISSESARNYIRSLPVMPRRNFRDIFRGANPLAIDLLEKMLELDSDKRI     300
dana_p38b       VLGTPADEFMSRISSESARNYIRSLPVMPRRNFRDVFRGANPLAIDLLEKMLELDAEKRI     300
dpse_p38b       VLGTPAEEFMSRISSESARNYIRSLPVMPRRNFRDIFRGANPLAIDLLEKMLELDADQRI     300
dper_p38b       VLGTPAEEFMSRISSESARNYIRSLPVMPRRNFRDIFRGANPLAIDLLEKMLELDADQRI     278
dwil_p38b       VLGTPAEEFMNRISSDSARSYIRSLPVMPRRHFRDVFRGANPLAIDLLEKMLELDADRRI     300
dmoj_p38b       VLGTPNDEFMNKISSESARTYIRSLPVMPRRNFRDVFRGANPLAIELLEKMLELDAEKRI     296
dvir_p38b       ILGTPNDEFMSKISSESARTYIRSLPVMPRRNFRDIFRGANPLAIDLLEKMLELDAEKRI     296
dgri_p38b       VLGTPNDEFMNKISSDSARTYIRSLPVMPRRSFRDVFRGANPLAIDLLEKMLELDAEKRI     296
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dsim_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVEKWREMVFSEVTAFKPTAAFAEL     360
dsec_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVEKWREMVFSEVTAFKPTAAFAEL     360
dyak_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVEKWREMVFSEVTAFKPTAAFAEL     360
dere_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVEKWREMVFSEVTAFKPSAAFAEL     360
dana_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVERWREMVFTEVQAFKPTAAFAEL     360
dpse_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVEKWREMVFTEVQAFRPTQAFAEL     360
dper_p38b       TAEQALAHPYMEKYHDPTDEQTAALYDQSFEENELPVEKWREMVFTEVQAFRPTQAFAEL     338
dwil_p38b       TAEQALAHPYMEKYHDPTDEQTSALYDQSFEENELPVEKWKDLVFTEVQSFKPPQAFAEL     360
dmoj_p38b       TAEQALEHPYMEKWHDPSDEATSTLYDQSFEEDENTVEKWKEMVFTEVRRFKPPQAFAEL     356
dvir_p38b       TAEQALAHPYMEKWHDPSDEATSTLYDQSFEENELPVEKWKELVFTEVRDFKPPQAFADL     356
dgri_p38b       TAEQALAHPYMEKWHDPSDEATSTLYDQSFEETELPVEKWKESVFKAVREFKPPPTFAEL     356
                ****** ******:***:** *::********* *  **:*:: **. *  *:*  :**:*

dmel_p38b       LPKEQ--- 365
dsim_p38b       LPKEQ--- 365
dsec_p38b       LPKEQ--- 365
dyak_p38b       LPKEQ--- 365
dere_p38b       LSKEQ--- 365
dana_p38b       LPKEQ--- 365
dpse_p38b       LPKDL--- 365
dper_p38b       LPKDL--- 343
dwil_p38b       LQQDAQQK 368
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dvir_p38b       LPQAQ--- 361
dgri_p38b       LSQAK--- 361
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4.7D:

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dsec_p38c      --MSKFVKVAINERLWEFPDIYEFVCFLGGGSFGQVAKVRLRGTENYVAMKRLMHPFERE     58
dyak_p38c      --MEGFTRVEVDKIVWEFPDSYQLVSFLGGGSFGRVAKVRIRGTKEYFALK--MRPLESE     56
dere_p38c      MALPRFVRMQIGEGVWEFLDTYEFVSFLGGGSFGQVAKMRVKGTEKHVAMKKLLRPFERE     60
dana_p38c      --MPGFHSVEVNNRNWVIPDIYEVLEPLGRGSFGQVAKVQLRNTNIQVAMKKLLTPFESE     58
dpse_p38c      --MSKFTKFVMDEKAWEVPDVYEIERLLGAGSFGQVSKAKLRGGDVNVAIKKLLQPFETA     58
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dsim_p38c      EDAKGAYREIRLLKHMNHRNVISLLNVFHPPA----HNMMDFQQVYLVTHLMDADLHRYS     114
dsec_p38c      EDAKGAYREIRLLKHMNHRNVISLLNVFHPPG----HNMMDFKQVYLVTHLMDADLHRYS     114
dyak_p38c      EDAKGAYREIRLLKHMDHRNITCLLNVFHPPA----V-GMAFRQVYLVTHLMDEDLQVYS     111
dere_p38c      EDAKSAYREIRLLKHMNHPNVISLLNVFHPPA----R-IMAFVQIYLVTHLMDEDLHRYS     115
dana_p38c      EDAKRVYREIKLLKHMNHRNVISLLDVFHGPSPNPNPTLDDFQEVYLVTDLMYKDLHRVT     118
dpse_p38c      EHAKRVYREIRLLKHMDHPNVISLLDVFHPSSPS--PTLENFQQVYLVTHLMDADLHKTI     116
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dsim_p38c      RSKKMSDHEIRIILYQILRGLKYIHSAGVVHRDLKPCNIAVNGNSEVRILDFGLSRMCAD     174
dsec_p38c      RSKRMSDNEIRIILYQILRGLKYIHSAGVVHRDLKPCNIAVNGNSEVRILDFGLSRMCAD     174
dyak_p38c      RSRRMREHEIRPILYQILRGLKCMHSAGVVHRDLMPCNIAINANNEVRILDFGLSRRYAE     171
dere_p38c      RSQRMSEHEIRPIIYQILRGLKYIHSAGVVHRDLKPCNIAVNGNNEVRILDFGLSRLCAD     175
dana_p38c      REVRLSERQVKFILFQILKGLKHIHSAGVLHRDLKPGNIAVNENCELRILDFGMARLLSM     178
dpse_p38c      RSQKLSDNQIRVILYQILRALKYIHSAGVLHRDLKPGNIAVNKDCELRILDFGMARLNSK     176
               *. :: :.::: *::***:.** :*****:**** * ***:* : *:*******::*  :

dmel_p38c      --KMTDHVGTMWYLAPEIIFLRGQYTKAIDVWSVGCILAELITDRVLFRGENYVSQIRCL     232
dsim_p38c      --KMTDYVGTLWYRAPEIIFLRGQYTKAIDVWSVGCILAELITGRVLFPGENYPNQIRCL     232
dsec_p38c      --NMTDYVGTLWYRAPEIIFLRGQYTKAIDVWSVGCILAELITGRVLFPGENYPSQIRCL     232
dyak_p38c      ITRPKDFVGTLWYWAPELLFLRGQNTKAIDMWSVGCILAELISGRVLFPGEHYFHQLECL     231
dere_p38c      --NMTDFVGTMWYRAPEQLFLRGQYTKAIDMWAVGCILAELISGRVLFPGQDYFDQLRRL     233
dana_p38c      --DMTDRVCTLWYRAPEILFGWGQYTKAIDMWSVGCILAELISGRPLFPGTR-RDQIMVV     235
dpse_p38c      --DMTTYVTTRWYRAPEILFCWRNYSKAIDMWSVGCIFAEMITGRPLFPGRDYTNQLDCI     234
                   .  * * ** *** :*   : :*****:*:****:**:*: * ** *     *:  :

dmel_p38c      INIMGTPTREFITGISMERSRNYLEGYPLRQRCDFHHLFMGYDVQAIDLMEKMLEMVPEK     292
dsim_p38c      IDIIGTPTREFITGISMEKSRSYLERYPLRQRCDFHHLFMGTDVQAVDLMEKMLEIVPEK     292
dsec_p38c      IDIIGTPTREFITGISMEKSRSFLERYPLRQRCDFHHLFMGTDVQAVDLMEKMLEMVPEK     292
dyak_p38c      LDVMGTPTEEFVSGIGLERSRKYVKKCPSRERCDFHHLFPGANIQAVDLMEKMLEMKPER     291
dere_p38c      LDVMGTPTREFVSGIDSQYSRNYVERYPLRQRCDFHHLFLGADIQAVDLMEKMLEMVPER     293
dana_p38c      IEVMGKPSEEFVSGISDPYARQYLDRIPPRERRNFREIFPDANPNALDLLEKMLDMIPQN     295
dpse_p38c      IDIMGTPSDEFKSKIDLESARTYVESLPRRTKSDFMELFGMGNYQAVDLIEKMLVMDPDN     294
               :::::*.*: ** : *    :* ::.  * * : :* .:*   : :*:**:**** : *:.

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dsim_p38c      RITAAEAMLHPYLRDLIEPHHHAEDTAPVYDQNFENLV-LPVKCWKELVANEIRNF----     347
dsec_p38c      RITAAEAMLHPYLRDLIEPHHHAEDTAPVYDQNFENLV-LPVKCWKELVANEIRNF----     347
dyak_p38c      RITASDAMRHPYLRDFIQPHHIYEDVAPTYDQNFENLI-LPVNGWKELIHNEIQNFKRK-     349
dere_p38c      RITAADAMRHPYLRDLIEPHHHDEDIAPVYDQNFENLV-LPVNFWKELISNEIQNFQPNX     352
dana_p38c      RITVEEALNHSYFKGFSDPYFLEDDTAQPYDQNFENMN-LPINCWKELILTEIRNFIPPP     354
dpse_p38c      RITADEALRHPFLKNLVQPQHNDEDTAPLYDQNFENMDWLSVKCWKELVLNEIMNFRPPP     354
               ***. :*: * ::: : :* .  :* *  *******:  * :: ****:  ** **
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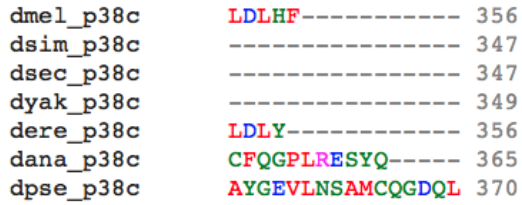

Figure 4.7: Clustal Omega alignments of p38K protein sequences. (A) Comparison of *D. melanogaster* p38Ka, p38Kb and p38Kc. The box that appears around residue 185 highlights the canonical p38K T-G-Y motif that is absent in p38Kc. The second box at residue ~315 shows the MAPK interaction domain. There are extra residues on p38Kc suggesting different target interactions than p38Ka and p38Kb. Comparison of 12 sequenced *Drosophila* species sequences for (B) p38Ka (C) p38Kb and (D) p38Kc. Red colors represent small, hydrophobic residues, blue are acidic residues, magenta are basic residues, green are hydroxyl, sulfhydryl and amine residues and grey are unusual amino acids. Asterisks represent single, fully conserved residues. A colon represents strong conservation between residues and a period represents weak conservation among residues.

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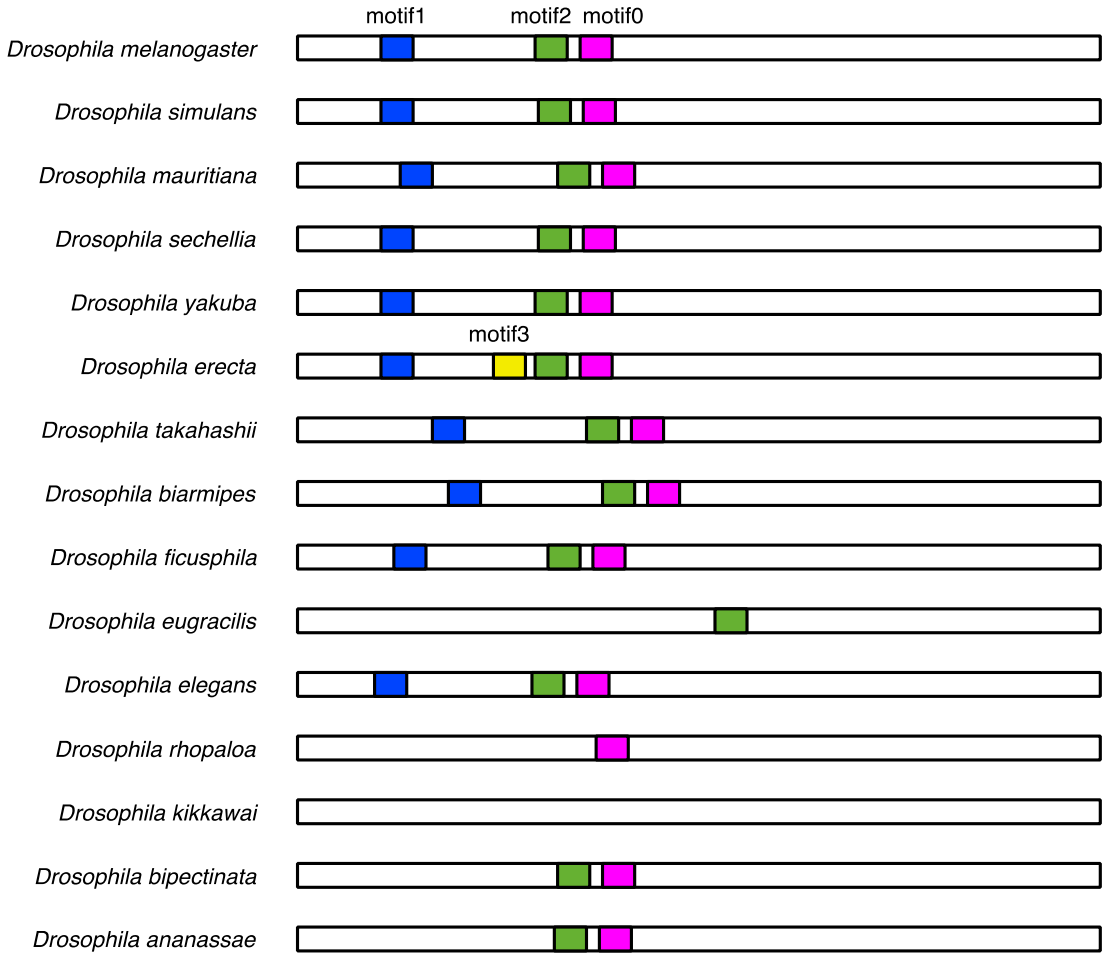
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motif2
motif0
motif3
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Drosophila simulans
Drosophila mauritiana
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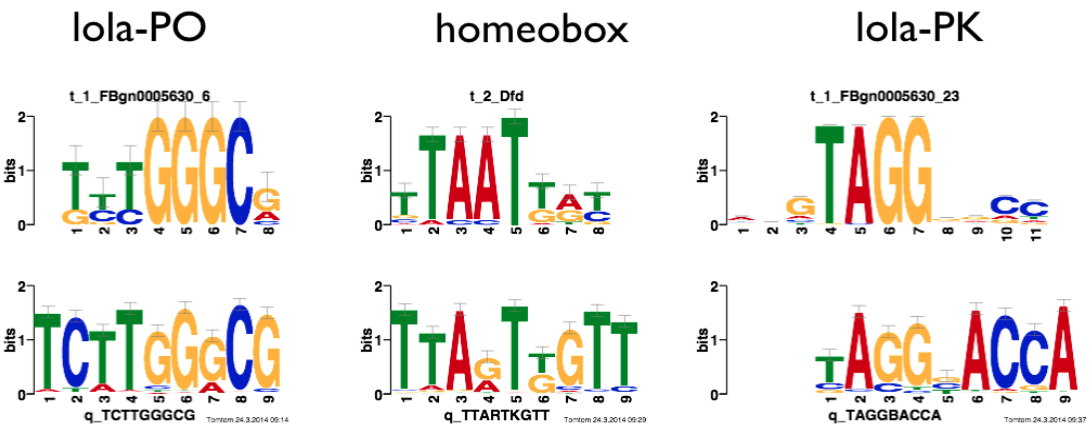
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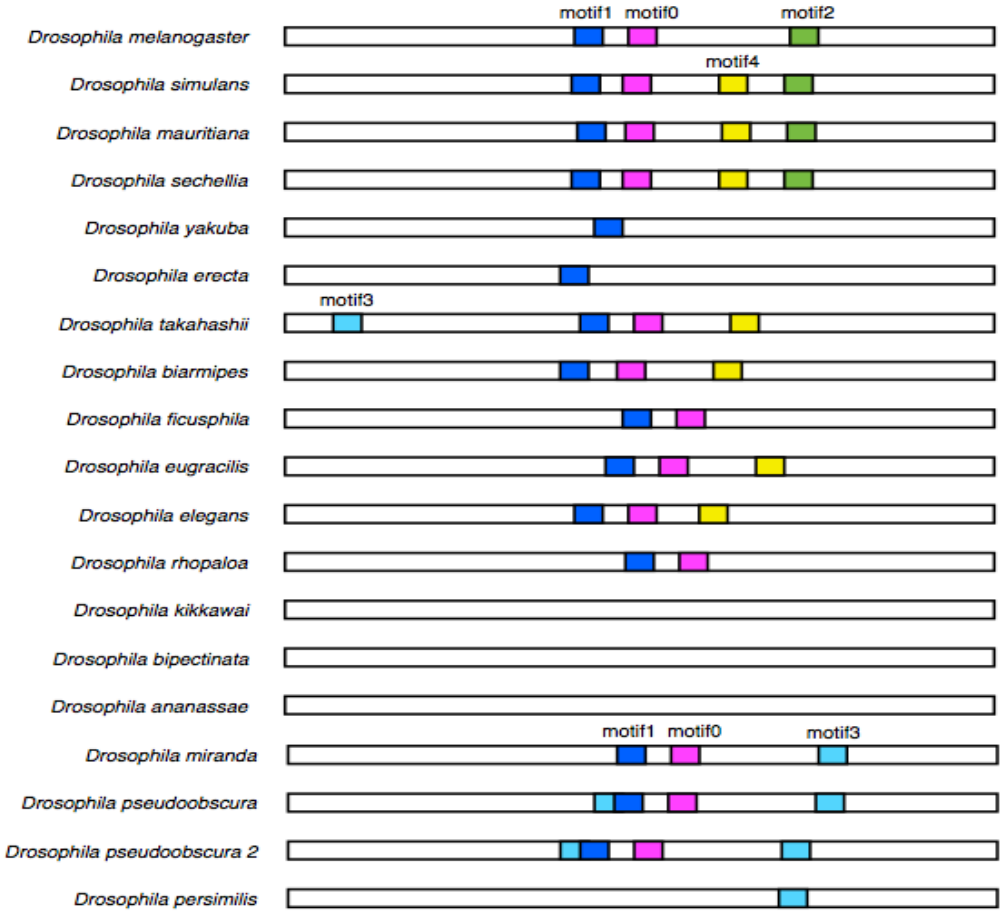

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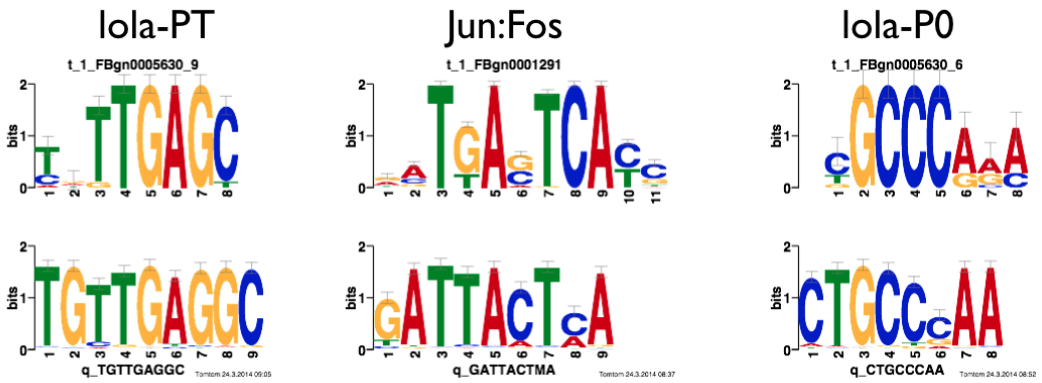

Figure 4.8: Transcription factor binding sites for p38Ka and p38Kb. (A and C) iMotif analysis of 1kb upstream of p38Ka (A) and p38Kb (C) genes for consensus

regions. The motifs that matched in all or most of the *Drosophila* species are highlighted as colored bars. (B and D) These consensus motifs were matched to known transcription factor binding sites using TomTom Motif Comparison. The size of the letter corresponds to the program's confidence at specific position. Previously classified binding sites in database (top row) are shown compared to species consensus motif sequence from iMotif (bottom row). (B) There are 3 transcription factor binding sites that are identified upstream of p38Ka: two lola isoforms (lola-PO and lola-PK) and Homeobox. (D) Two lola isoforms (lola-PO and lola-PT) and Jun:Fos are identified upstream of p38Kb.

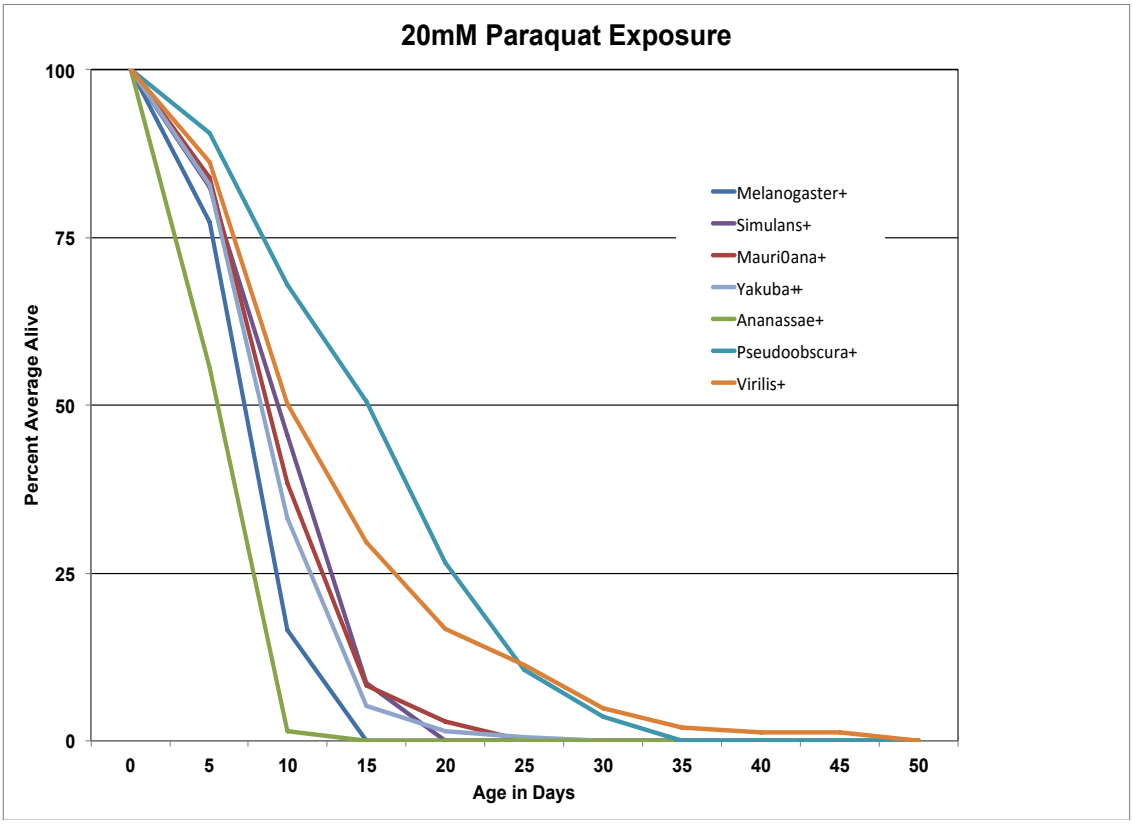

Figure 4.9: **Oxidative stress response in *Drosophila***. Female flies (n=200) from 7 representative *Drosophila* species were placed on standard media with paraquat, a free radical producer. From these survival curves, *D. ananassae* performed the worst but large populations of *D. virilis* and *D. pseudoobscura* survived for longer.

CHAPTER FIVE: DISCUSSION

The MAPK family plays a crucial role in integrating extracellular signals that can be amplified, targeted and regulated by the intercellular and extracellular environments to maintain homeostasis. Because of this critical physiological role, MAPKs play an fundamental role in a variety of organisms including yeasts, plants and animals (Mizoguchi et al., 1997; Widmann et al., 1999). In this study, we find that one of the MAPKs, p38 MAPK, plays an essential, evolutionary conserved role in maintaining protein homeostasis and regulates aging processes in *Drosophila*. The data herein provides a look at p38K on different levels: molecular, tissue, organismal and in an evolutionary context within the *Drosophila* genus.

5.1 – The Role of p38Kb in Protein Homeostasis and Aging

p38K has been previously linked to oxidative stress, locomotor function and lifespan (Kurata, 2000; Vrailas-Mortimer et al., 2011). Here we find that p38Kb regulates protein aggregation in the *Drosophila* adult muscle through the Chaperone Assisted Selective Autophagy (CASA) complex. The CASA complex recognizes damaged and misfolded proteins and degrades these via the autophagosome/lysosome pathway. The complex is comprised of a group of chaperones and co-chaperones, HspB8,

Hsc70-4 and BAG-3 (starvin in flies) that work with CHIP, an E3 ubiquitin ligase, to identify and attach a ubiquitin tag to proteins destined for degradation (Arndt et al., 2010). p62 (ref(2)p in flies) recognizes this ubiquitinated damaged protein and facilitates transport to the lysosome for protein destruction. We find that p38Kb colocalizes with the CASA complex members at the z-disk, a region of high protein turnover in the muscle, and physically binds to these CASA members (Figure 2.2). Additionally we find that p38Kb activity in the muscle affects the number of protein aggregates with age (Figure 2.1). Furthermore, p38Kb acts through the CASA complex to regulate protein homeostasis, age-dependent locomotor functions and the lifespan of *Drosophila* (Figure 2.3-2.5). p38Kb might be interacting with HspB8 and/or starvin to control activity of the CASA complex. We find that p38Kb expression may be limiting for starvin localization and function (Figure 2.6), and this could be the mechanism through which p38Kb regulates CASA activity.

Future Directions

As p38K is a known regulator of oxidative stress, it would be of interest to explore the relationship of p38Kb and the CASA complex in regulating protein aggregation with oxidative stress exposure. As both p38K (Kurata, 2000) and BAG-3 (Gamerdinger et al., 2009) are upregulated in oxidative stress conditions, the CASA complex might become more active under oxidative stress conditions to try to clear oxidatively-damaged proteins. To identify how p38Kb and CASA complex interact to effect levels of aggregation with oxidative stress, we can test decreased expression (RNAi or dominant negatives) and over-expresssion of starvin, HspB8, Hsc70-4, ref(2)p

and p38Kb. These flies would be treated with paraquat or hydrogen peroxide and, subsequently, levels of protein aggregation quantified. Also, p38K might mediate oxidative stress responses outside of the CASA complex, working with other autophagy related proteins, such as Atg9. We could also perform a very similar experiment with p38Kb and Atg9 mutants/ over-expression with oxidative stress.

Next, it would be interesting to determine the specific targets of the CASA complex and contents of the aggregates. Most likely there will be a general set of proteins in every aggregate targeted by the CASA complex. But in oxidative stress conditions or with age, there might be specific proteins that appear in the aggregates that are linked to human diseases. In order to test this hypothesis, we could isolate aggregates from muscle tissue, (modified from (Demontis and Perrimon, 2010; Nezis et al., 2008), at different ages or oxidative stress conditions, and then identify aggregate contents through mass spectroscopy.

In order to understand how p38Kb interacts and controls the activity of the CASA complex, we want to explore if p38Kb is directly regulating activity of one or more of the CASA complex members. Both HspB8 and starvin have evolutionarily conserved p38K phosphorylation sites and docking sites. By mutating these phosophorylation sites and/or docking sites in HspB8 and starvin, we could perform studies of protein aggregation, lifespan and motor function. Alternatively, p38Kb could be directly phosophorylating targets that are in turn identified by CASA complex members.

5.2 - The Role of p38Kb in Proteomic Changes with Age

We have studied changes in the proteomic signature from heads (predominantly neuronal tissue) and thoraxes (muscle tissue) in *Drosophila* at different ages and different

levels of p38Kb expression. From the machine learning, we have identified proteins that are the best indicators of aging in heads and muscle. Interestingly, one of these proteins, CG1561, has yet to be characterized but could play a crucial role in aging processes. Also in this this study we identify a number of proteins whose expression levels are directly affected by p38Kb expression. A number of these identified proteins are linked to human disorders including muscular dystrophies, neurodegeneration diseases, cancer, cardiovascular disease and stroke.

Future Directions

Complex signaling pathways are responsible for orchestrating all basic cellular activities and responding to extracellular cues. Integration of all of these demands requires a high degree crosstalk and feedback between cell pathways. The duration of MAPK phosphorylation is controlled by phosophatases and provide an additional level of negative feedback through which to control MAPK activity (Owens and Keyse, 2007). p38K phosphorylation kinetics and crosstalk to other pathways are critical in determining groups of downstream targets (Coulthard et al., 2009). While sustained phosophorylation triggers apoptotic processes, short, transient phosphorylation is linked to gene expression promoting growth and survival (Murphy and Blenis, 2006).

Our future studies will investigate p38K activity with different oxidative stressors – the combination of age and level of oxidative stress is predicted to effect phosphorylation kinetics of p38K and potentially change the proteome network. The combination of these two studies will provide a better interaction map of p38K under basal and stressed conditions.

Currently, we are analyzing samples from oxidatively stressed flies with and without normal p38K activity (p38Kb kinase-dead). We are using different oxidative stressors, hydrogen peroxide and paraquat. In the case of the weaker oxidative stress, hydrogen peroxide, stress-relieving gene expression might be seen, while the stronger oxidative stressor, paraquat, could show gene expression or activation for an apoptotic response.

Even though we performed a quantitative proteomic screen (Chapter 3), we could verify protein hits using western blots or age-dependent expression patterns in specific tissues with immunofluorescence. Many of the identified proteins match transcripts identified in studies of *Drosophila* transcriptome with age by (Kim et al., 2005; Landis et al., 2004; Zou et al., 2000). Since p38K regulates the activity of a number of transcription factors, we could perform qRT-PCR to find at what level p38K affects the proteome. Additionally, it would be interesting to focus on the subset of identified proteins that are linked to disease pathways. Protein aggregation is a large risk factor for neurodegeneration and muscular dystrophies. By affecting levels of p38Kb/CASA complex members (RNAi or over-expression), we could look for colocalization with poly-ubiquitin protein aggregates (Chapter 2) and disease-linked proteins. Additional compounding risk factors for disease, such as age and level of oxidative stress, could be explored as well in this study.

We have identified a number of genes that relate to many muscular dystrophies. In order to screen for potential therapeutics that affect locomotor function, we could use the *Drosophila* Activity Motor (DAM) system. Using different fly mutants for muscular dystrophy associated genes, we can screen a large number of compounds and possibly

identify compounds that restore locomotor function. By working with short-lived, genetically amenable *Drosophila* in simultaneously running, 32-chambered DAM monitors offers an opportunity to screen large numbers of compounds and potentially identify a handful of effective treatments for further study in mammalian systems.

5.3 – The Evolution of p38K in *Drosophila* Species

We find that p38Ka and p38Kb are highly conserved across species in the *Drosophila* genus (Figure 4.5). The third p38K gene, p38Kc, evolved more recently; most likely from a gene duplication event of p38Ka (Figure 4.5). p38Kc shows the highest amount of nucleotide variability and is not a true p38 MAPK as it lacks the conserved TGY motif for phosphorylation and the canonical substrate binding domain is not conserved (Figure 4.7). By identifying consensus regions among species upstream of the p38Ka and p38Kb genes, we identified known transcription factor binding domains (Figure 4.8). In the case of p38Ka, we find that lola-PO, lola-PK and homeobox are all potential transcription factors. And for p38Kb, we have identified lola-PO, lola-PT and AP-1 transcription factors. Finally, as p38K regulates oxidative stress, we looked at the survival of different species with paraquat exposure (Figure 4.9). We find that species that performed the best was *Drosophila pseudoobscura,* which is especially interesting because *D. pseudoobscura* has two p38Kb genes so both could be working to mediate the cellular stress response.

Future Directions

Based on the findings of the paraquat survival study, we are interested to know if activation of p38K is different among the species in response to oxidative stress. Paraquat-treated and control flies from different time points are being analyzed by western blots to look at the levels of phosphorylated p38K (the active form) and total levels of p38K. It will be interesting to see if species that lived the longest on paraquat – *D. psudoobscur*a and *D.virilis* – show increased phosphorylated p38K or total amounts of p38K because of paraquat exposure. Whereas, *D. ananassae*, a species native to environments rich with antioxidant foods, might show less p38K activation and thus died the quickest on paraquat.

Also, it would be interesting to confirm transcription factors hypothesized through species consensus sequences analysis. While Homeobox and AP-1 have been previously linked to modulating p38K activity, lola has not. Through western blot analysis, we can explore the levels of p38K when levels of these transcription factors are lowered (e.g. RNAi or over-expression lines for lola, Jun and Fos). As p38K is a well-known mediator of oxidative stress, these same genotypes can also be tested under different oxidative stress conditions (paraquat or hydrogen peroxide) to assess levels of p38K by western blot and densitometry.

CHAPTER SIX: MATERIALS AND METHODS

Methods: Chapter Two

Genotypes

UAS-p38Kb wt, UAS-p38Kb Kinase Dead, p38Kb $^{\Delta 45}$, w^{1118}, Mef2-GAL4 and MHC-GAL4 were as described in (Vrailas-Mortimer et al., 2011).

UAS- HspB8 RNAi (w^{1118}; P{GD7609}v44831), UAS-stv RNAi 34408 (w^{1118}; P{GD10796}v34408), UAS-stv RNAi 34409 (w^{1118}; P{GD10796}v34409/TM3) are described in (Dietzl et al., 2007) and are from the Vienna Drosophila Resource Center.

stv-GFP trap (w^{11118};; Pbac{754.P.F3v30} $stv^{+CPTI\ 002824}$), HspB8-GFP trap (w^{1118} PBac{810.P.FSVS-2}CG14207^{CPTI004445}), Hsc70-4 GFP trap (*w^{1118} PBac{544.SVS-1}N^{CPTI002347}*) are from the Kyoto Stock Center.

y^1 w^{67c23}; P{y[+mDint2] w[BR.E.BR]=SUPor-P}ref(2)P^{KG00926}, UAS-hsc70-4 K71S (w^{126}; P{w[+mC]=UAS-Hsc70-4.K71S}G), UAS-hsc70-4 wt (w^{126}; P{w[+mC]=UAS-Hsc70-4.WT}B), y^1 w^{67c23}; P{w[+mC] y[+mDint2]=EPgy2}EY04969 are from the Bloomington Drosophila Stock Center.

Full Genotype	Referenced Genotype
UAS-p38Kb wt	p38Kb wt OE
UAS-p38Kb Kinase Dead	p38Kb kinase dead
p38Kb Δ45	p38Kb mutant
w1118	Control
UAS- HspB8 RNAi (w1118; P{GD7609}v44831)	HspB8 RNAi 44831
UAS-stv RNAi 34408 (w1118; P{GD10796}v34408)	stv RNAi 34408
UAS-stv RNAi 34409 (w1118; P{GD10796}v34409/TM3)	stv RNAi 34409
stv-GFP trap (w11118;; Pbac{754.P.F3v30} stv+CPTI 002824)	stv- GFP trap
HspB8-GFP trap (w1118 PBac{810.P.FSVS-2}CG14207CPTI004445)	HspB8 GFP trap
Hsc70-4 GFP trap (w1118 PBac{544.SVS-1}NCPTI002347)	Hsc70-4 GFP trap
y1 w67c23; P{y[+mDint2] w[BR.E.BR]=SUPor-P}ref(2)PKG00926	ref(2)p lof
UAS-hsc70-4 K71S (w126; P{w[+mC]=UAS-Hsc70-4.K71S}G)	Hsc70-4 DN
UAS-hsc70-4 wt (w126; P{w[+mC]=UAS-Hsc70-4.WT}B)	Hsc70-4 OE
y1 w67c23; P{w[+mC] y[+mDint2]=EPgy2}EY04969	stvEP
Drivers	
Mef2- GAL4	strong muscle driver
MHC-GAL4	weaker muscle driver

Immunofluorescence and protein aggregate analysis

Adult flies were fixed in 4% paraformaldehyde for 48hrs at 4°C. Indirect flight muscles were then dissected in 1X PBS, permeablized in 1X PBS 0.15% Triton-X 100, and blocked in NGS + 0.15% Triton-X 100. Samples were incubated in primary antibody at 4°C overnight, washed in 1X PBS 0.15% Triton-X 100, and incubated in secondary antibody at room temperature for 2hours. Samples were mounted in Vectasheild mounting medium (Vectorlabs) and visualized using a laser scanning confocal microscope. Antibodies: rabbit anti-GFP 1:400 (Invitrogen), mouse anti-FLAG M2

1:1000 (Sigma), rabbit anti-stv 1:1000 (gift of Jrög Höhfeld), rat anti-α actinin 1:100 (Abcam) rabbit anti-CHIP 1:500 (EMD4Biosciences Calbiochem), anti-mouse IgG-Alexa Fluor 488 1:200 (Life Technologies), anti-mouse IgG- Alexa Fluor 568 1:500 (Life Technologies) anti-rabbit IgG- Alexa Fluor 488 1:500 (Life Technologies) and Rhodamine Phalloidin 1:2000.

Indirect flight muscles were prepared as described above from 9 individual flies per genotype. Protein aggregates were identified using mouse anti- polyubiquitin 1:1000 (Enzo Life Sciences). Three muscles from each individual fly were imaged as z-series and flattened into a single image using confocal microscopy for a total of 27 muscles per genotype. Images were analyzed using Image J "Analyze Particles" function with a100 $pixels^2$ set for the minimum aggregate size. (100 $pixels^2$ equals 1.5 μm^2). Aggregate number and size were analyzed using ANOVA followed by Tukey's HSD using the R package "multcomp." Within genotype and across time point analyses were performed using the Welch two sample t-test in R.

Lifespan

For lifespan experiments using UAS-Hsc70-4 DN, ref(2)p -/+, $p38Kb^{\Delta 45}$, and the stv EP lines, female animals were kept on standard molasses *Drosophila* media. Due to a change in lab food, the HspB8 RNAi, stv RNAi 34408, and stv RNAi 34409 lifespan experiments were performed on the standard Bloomington *Drosophila* media. Virgin flies were collected and reared at 25°C in a 12hour:12hour light:dark cycle. Flies were put on new food twice a week. The number of dead animals were scored daily. Lifespan was analyzed using a log rank test to compare genotypes with censored data on all genotypes

and then on all pairwise comparisons using the R package “survival” with Benjamini and Hochberg correction (false discovery rate < 0.05).

Co-immunoprecipitation

Flies expressing an endogeonously GFP tagged HspB8, Hsc70-4 or stv and/or a FLAG tagged p38Kb KD construct were aged 1 week or 5 weeks. Forty thoraxes per genotype per condition were homogenized in high salt buffer (0.5 M KCl, 35% glycerol,10 mM HEPES pH 7.0, 5 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, 0.1% NP40 25 mM NaF, 1 mM Na_2VO_4, 1 mM DTT, Complete protease inhibitor). The lysate was flash frozen in liquid nitrogen and quickly thawed at 37°C. Then lysates were rocked at 4°C for 30 minutes and centrifuged at 14,200 x g for 30 minutes at 4°C. The supernatant was transferred to equilibrated beads anti-Flag (M2) agarose (Sigma) or anti-GFP agarose (Chromotek) and rocked for 2 hours at 4°C. Beads were collected using a magnetic bar and washed four times with IP buffer (50 mM HEPES pH 7.0, 100 mM KCl, 0.4% NP40 1.5 mM $MgCl_2$, 5% glycerol, 25 mM Na, 1 mM Na_2VO_4, 1 mM EDTA, 1 mM DTT, Complete protease inhibitor). Lyastes were then analyzed by immunoblotting using rabbit anti- GFP 1:1000 (Invitrogen), mouse anti-FLAG M2 1:1000 (Sigma), or rabbit anti-phospho-p38 1:1000 (Cell Signaling Technologies).

Immunoblotting

Wild type flies were aged 3, 15, 30, and 45 days. Three thoraxes were dissected and homogenized in 1x Laemmli buffer. Immunoblots were performed as described in (Vrailas-Mortimer et al., 2011). Membranes were developed using SuperSignal West

Femto kit (ThermoFisher) or Pierce ECL (ThermoFisher) and exposed on autoradiography film. Antibodies used were: rabbit anti- GFP 1:1000 (Invitrogen), rabbit anti-starvin 1:10,000 (gift of Jrög Höhfeld), mouse anti-actin 1:5,000,000 (Sigma), mouse anti- HRP 1:20,000 (Jackson Labs), rabbit anti-HRP 1:40,000 (Jackson Labs).

Starvin localization

Indirect flight muscles were prepared as described above. Indirect flight muscles for five individual flies were analyzed for average pixel density using ImageJ in three different non-overlapping locations on each muscle for a total of 15 measurements per genotype. Average pixel density was analyzed by Student's t-test using R.

Flight behavior

Female animals were collected and aged 1 or 5 weeks. Animals were tested in groups of 20-25 in 10-15 trials. Animals were introduced into a 500mL graduated cylinder that is lightly coated in paraffin oil. Poor fliers land in the bottom third of the cylinder while the best fliers land at the top third of the cylinder. Flight behavior was analyzed using a Fisher's Exact test to compare distribution of flies in the bottom, middle, and top thirds of the flight chamber. All pairwise comparisons were made with Benjamini and Hochberg correction (false discovery rate < 0.05) using R.

Methods: Chapter Three

Genotypes

UAS-p38Kb wt, p38Kb Δ45, w1118, Mef2-GAL4 were as described in (Vrailas-Mortimer et al., 2011).

Tissue Preparation

Virgin female flies were reared on standard *Drosophila* media at 25°C in a 12hour:12hour light:dark cycle. Flies were put on new food twice a week. Flies were aged 1-5 weeks (p38K mutant: p38Kb Δ45,) 1-8 weeks (controls) and 1-10 weeks (p38K over-expression: UAS-p38Kb wt). Tissues from heads and thoraxes were dissected, collected in triplicate at each time point and stored at -80°C.

Timecourse Analysis

Using the Linear Models for Microarray Data (limma) package in R data from p38K mutants and p38K over-expression were compared pairwise to controls. Three replicates from each tissue were compared at all time points. (df= 4 for p38Kb Δ45 and p38Kb Excision 41 at 1-5 weeks and df= 7 for UAS-p38Kb wt; Mef2-GAL4, w1118 UAS-p38Kb wt and w1118 Mef2-Gal4 at 1-8 weeks).

Machine Learning

MLSeq in R was trained on randomized two-thirds of the data from control tissues. Then it tested the remaining one-third of data. Patterns based on these training sets binned data from the heads of control flies into young (1-3 weeks), middle (4-6

weeks) and old (7 -8 weeks). Data from the thoraces of control flies was sorted into young (1-4 weeks) and old (5 -8 weeks for controls). Once trained on controls, the p38Kb mutants or p38Kb over-expression were tested and categorized the proteomes as young, middle or old aged. Predicted data was 93% accurate as compared to raw data sets for heads and thoraxes.

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Methods: Chapter Four

Phylogenetic trees

Sequences aligned with ClustalW or ClustalOmega and non-aligned regions were trimmed with BioEdit. The trees were drawn using Phyml 3.1 and FigTree v. 1.4.

Chromosome alignment

Chromosome II and III regions of interest from GBrowse data available on FlyBase.

Genetic conservation analysis – dN/dS ratio

dN and dS values for different NUMBER species were computed in MEGA (Molecular Evolutionary Genetics Analysis) v.5.2.2 using the Nei-Gojobori model. Values were plotted in R.

Predictive transcription factor binding

Transcription factor binding sites were predicted using iMotifs. The TomTom Motif Comparison Tool v4.9.1 (MEME web server) compared the resulting predicted sites to all known *Drosophila* transcription factor binding sites. The size of the letter corresponds to the program's confidence at specific position.

Paraquat Survival Curves

Virgin female flies (n=200) were placed on standard *Drosophila* media with 20mM paraquat (Sigma). Flies were placed on new food twice a week. *D. simulans* and *D. pseudoobscura* were kept at 21°C on a 12hour:12hour light:dark cycle. *D. melanogaster*, *D. virili*s, *D. yakuba*, *D. ananassae* and *D. mauritiana* were kept at 25°C on a 12hour:12hour light:dark cycle. Flies were scored daily.

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