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Muscle-specific roles for p38K MAP kinase in the regulation of locomotor activity in *Drosophila*

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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p38K kinase (p38K) is a member of the well-studied MAP Kinase family of protein kinases that are involved in a variety of cellular signaling pathways and are capable of regulating gene expression. The p38K kinase in particular, belongs to a subfamily of MAP kinases known as the SAPKs (stress activated protein kinases) which are involved in the stress response. p38K is activated by a number of extracellular stressors, such as the reactive oxygen species (ROS) released by oxidative stress. This type of stress leads to mitochondrial dysfunction and apoptotic death in many cell types (e.g. Parkinson's Disease patients are particularly vulnerable to its effects). Although p38K is a *bona fide* stress responsive signaling protein, cellular targets of p38K signaling in the context of stress and *in vivo* roles for p38K in stress regulation remain unknown.

Recently, the Sanyal lab has studied the function of p38K in *Drosophila* using a double knockout model consisting of null mutations in both p38K *Drosophila* homologs: p38a and p38b. The p38K double knockouts exhibit a reduced lifespan, increased sensitivity to oxidative stress, and locomotor dysfunction. While these findings show how a general lack of p38K can impair locomotor ability, they do not specify what cell types require p38K for normal locomotor function. The aim of the current study was to identify these p38K-dependent cell types by using the GAL4-UAS system to selectively inhibit p38K in specific tissues. Using this system, we were able to test the effects of inhibiting p38K (using a dominant-negative p38K transgenic protein) in distinct cell types on locomotor function. Here, we not only find that locomotor deficits result from overexpressing the dominant-negative form of p38b in all cell types, but also that these deficits are observed when p38K is solely inhibited in muscle cells. We predict that this locomotor impairment may be caused by an increase in oxidative stress-related damage in the muscles resulting from the disruption of the p38K signaling pathway.

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INTRODUCTION

MAP Kinases

Mitogen-activated protein kinases (MAPKs) form part of highly conserved intra-cellular signaling cascades which are crucial for normal development and physiology of all eukaryotic organisms (Boutros et al. 2008). In humans, the duration of MAPK phosphorylation controls cell differentiation and proliferation, and any abnormalities in this process could lead to disease pathologies (Kim & Choi 2010). In *Drosophila*, MAPK signaling has also proven to be a key element in the cell differentiation and development of various tissue types. For example, in developing *Drosophila* wing cells, the location of MAPK within the cell dictates cell fate and proliferation (Marenda et al. 2006), while *Drosophila* eye development is regulated by MAPK pathways which either activate or down-regulate key transcription factors determining the precise pattern and identity of photoreceptors in the compound eye (Kumar et al. 2003).

Despite the fact that MAPKs have various functions and signal transduction through these cascades often impinges on a wide array of downstream targets, MAPK pathways follow a general pattern. In one common form, MAPK signaling is usually initiated through ligand binding and activation of a membrane bound receptor such as a receptor tyrosine kinase (RTK). Upon ligand binding, the activated form of the RTK autophosphorylates on its tyrosine residue. The phosphate group attached to the tyrosine residues then serves to phosphorylate a sequence of other proteins which creates a signaling cascade. Towards the end of this sequence, RAS bound GDP is converted to RAS-GTP which activates the RAS protein, allowing it to bind to MAPKKK (MAP

kinase kinase kinase). In short, activated RTKs lead to the activation of a signaling molecule such as Ras, followed by the activation of the first of three closely linked components of the core MAPK pathway, MAPK kinase kinase, (Orton et al. 2005). MAPKKK, its downstream kinase target MAPKK , MAP kinase kinase, and its final target MAPK, are bound together in a complex. Through a sequential chain of phosphorylation events, phosphorylated MAPK is released from this complex and then either phosphorylates its targets in the cytoplasm, or translocates into the nucleus to phosphorylate nuclear targets such as transcription factors (Figure 1). This conserved phosphorylation relay, i.e. activated MAPKKKs phosphorylate MAPKKs which ultimately phosphorylate and activate the MAPKs, is present in every single MAP kinase cascade known in living organisms.

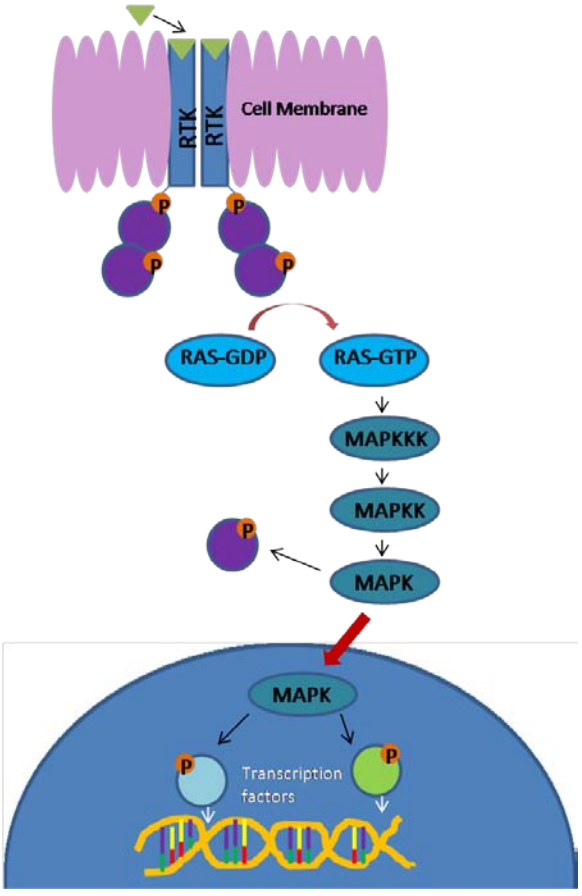


Figure 1. RTK Signaling Pathway

Signal transduction is initiated by the binding of an extracellular ligand to the RTK located on the cell membrane. The activated RTK then proceeds to phosphorylate various proteins, inducing a signaling pathway within the cell. Farther down the pathway, the RAS protein is activated which ultimately activates MAPK after further signaling. The activated MAPK will either phosphorylate proteins in the cytoplasm or translocate into the nucleus to phosphorylate transcription factors.

p38K and the Oxidative Stress Response

The MAPK family consists of three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38K mitogen-activated protein kinases (p38K). Of these, the JNK and p38K MAPKs are also known as stress-sensitive protein kinases (SAPKs) due to their activation by extracellular stressors (de Nadal et al. 2010). The SAPKs use stressors as environmental cues to initiate intracellular signaling and create the appropriate cell response. In the marine bivalve M. galloprovincialis, for example, the p38K pathway is activated in response to different forms of environmental stress including change in water salinity and aerial exposure (Gaitanaki et al. 2004). Other extracellular stressors which increase p38K phosphorylation include heat shock, ultraviolet rays and oxidative stress (Nebreda & Porras 2000).

Reactive oxygen species (ROS) are a widely prevalent source of oxidative stress which can be released through exposure to commonly used toxic chemicals such as the herbicide Paraquat and insecticides such as Rotenone (Ji et al. 2010). ROS are found naturally within the cell as byproducts of metabolic processes in the mitochondria; however, when the cell is exposed to oxidative stress this causes an increase in ROS levels which can damage proteins, lipids, and DNA (Liu et al. 2009).

Since ROS have been found to induce p38K activation in cells (Ouyang & Shen 2006), the p38K signaling pathway may help to attenuate these deleterious effects by eliciting the appropriate cellular response. In a cell culture study, for example, Proteasome inhibitors caused an increase in p38K and prevented some of the cytotoxicity caused by oxidative stress. However, this protection did not occur if p38K inhibitors were

added in the presence of the proteasome inhibitors, suggesting that protection was conferred through selective activation of p38K (Yamamoto et al., 2007).

Although we know p38K is involved in the oxidative stress response, not much is known about the function of this signaling pathway functions or how it affects the organism as a whole. However, because ROS triggers p38K signaling, it may be linked to diseases related to oxidative stress damage such as Parkinson's Disease.

Parkinson's Disease and ROS

One major aspect of the pathology of Parkinson's Disease (PD) is a loss of dopaminergic neurons in the substantia nigra (Maetzler et al. 2009). ROS produced by oxidative stress can ultimately lead to apoptosis and have been found to contribute to this neurodegeneration. (McCubrey et al. 2006). Since the mitochondria is responsible for maintaining low levels of ROS within the cell, another major factor contributing to this disease is mitochondrial dysfunction which may be caused by mitochondrial complex 1 inhibitors such as 1-methyl-4-phenylpyridinium (MPP+) (Arduíno et al. 2009). These toxins and the resultant ROS ultimately lead to cell death in dopaminergic neurons. Thus, a major cause for sporadic instances of PD seems to result from chronic exposure to agents that increase ROS damage.

In addition to the vast majority of sporadic PD cases, several instances of juvenile onset PD, along with other forms of PD, have been reported and linked to mutations in a handful of genes. This has allowed the development of genetic models of PD that closely approximate human PD. For instance, to study Parkinson's disease *in vivo*, genetic

models often use animals with mutations in the *parkin* gene, a gene encoding a ubiquitin-protein ligase which is one predominant cause of autosomal recessive parkinsonism, where 18% of cases are *parkin* related. In many model organisms such as mice and *Drosophila*, Parkin knockouts have succeeded in mimicking symptoms of Parkinson's Disease in humans by demonstrating increased neurodegeneration and dysfunction in dopaminergic neurons (Terzioglu et al. 2008).

These *parkin* models have shown increased sensitivity to oxidative stress and ROS in a number of cases. The degree of Parkin expression has been directly correlated with the death rate of cells exposed to ROS, with a higher death rate in cells with low levels of Parkin expression (Kuroda et al. 2006). In Parkin knockdown zebrafish which initially show a loss of dopaminergic neurons in a brain area homologous to the substantia nigra in humans, MPP+ exposure enhances this effect (Flinn et al. 2009). Parkin knockout mice also exhibit increased mitochondrial damage when exposed to rotenone. This evidence indicates that Parkin plays a key neuroprotective role against mitochondrial toxins in certain cells. Interestingly, further impairment to the mitochondria is observed when the mice are exposed to both rotenone and a p38 inhibitor than when exposed to rotenone alone, suggesting p38K plays a similar role to Parkin in attenuating the damage caused by ROS (Casarejos et al. 2006).

Studies indicate that interaction between Parkin and p38K signaling pathways helps regulate apoptosis and neuroprotection, but little is known about this interplay. Some studies suggest Parkin negatively regulates p38K pathways to reduce apoptosis (Hasegawa 2008). In addition cross-talk with other signaling pathways involved in PD could also modulate MAPK signaling.

Parkin and p38K in *Drosophila*

Given the general lack of consensus on the precise mechanisms by which p38K MAP kinases might regulate oxidative stress and PD onset and incidence, we chose to explore cellular functions of p38K in the genetic model organism *Drosophila melanogaster*. The presence of conserved signaling pathways, excellent tools for genetic analysis and a wide range of cellular, electrophysiological and behavioral assays which pertain to *Drosophila* render it an excellent model organism for studying cellular functions of p38K and its relationship to PD, through genetic manipulation.

Two p38K homologs have been identified in *Drosophila*: p38Ka and p38Kb. *Drosophila* p38Ka mutants show increased vulnerability to stresses such as oxidative stress, heat shock, and starvation (Craig et al. 2004). Initial work in the Sanyal lab has also shown that p38K double knockout animals (that lack both p38Ka and p38Kb) exhibit locomotor dysfunction, oxidative stress sensitivity, dopaminergic neuron loss, and decreased lifespan. In p38Kb knockout animals a similar phenotype is displayed to a lesser extent than the double knockouts, but to a much greater extent than the p38Ka knockouts, suggesting that p38Kb is the more prominent form of p38K in these animals.

Significantly, flies with a knockout for the *parkin* gene express the same phenotypes as the p38K mutants. Increased mitochondrial dysfunction has already been observed in *Drosophila parkin* mutants, along with other characteristics such as reduced lifespan, locomotor deficits, and male sterility (Greene et al. 2003). Furthermore, while both *parkin* null mutants and p38Kb null mutants are viable, a combination of these mutations results in synthetic lethality (Unpublished data Sanyal lab), leading us to

hypothesize that these genes may work in parallel pathways to regulate behaviors and phenotypes characteristic of Parkinson's disease. Taken together, these observations strongly support the idea that p38K MAP kinases regulate oxidative stress and interact with other genetic determinants of PD to contribute to both sporadic and inherited forms of PD.

Targeted Overexpression of p38bKD

This study aims to further analyze the role of p38K using two p38bKinase-Dead transgenes (p38bKD3 and p38bKD10) that can be used to target expression of a dominant-negative form of p38Kb to tissues of choice. The design of this new transgene is as follows. In wild type animals p38Kb initiates signaling by binding to and phosphorylating target proteins by transferring a phosphate from one molecule of ATP (Tong et al. 1997). The dominant-negative form of p38Kb (engineered by mutating the highly conserved ATP binding loop in the kinase domain of p38K) is expected to bind to target proteins without phosphorylating them, antagonizing the endogenous p38K protein and attenuating further signaling downstream of p38K. Since previous research with a different dominant-negative form of p38Kb (in which a phosphorylation site in p38Kb is altered to an Alanine residue) in *Drosophila* has produced a *decapentaplegic*-like developmental phenotype in the wings (Adachi et al. 1999), this allows us to test the efficacy of our transgene, albeit in a context different from cell types that are used to model PD (i.e. muscles and neurons, see later).

A remarkable advantage of using *Drosophila* for these analyses is the ability to direct expression of a transgenic protein, such as p38KD, to tissues of choice using the GAL4-UAS system. In the GAL4-UAS system, a specific genomic enhancer is chosen to drive the expression of GAL4 on one chromosome, and once the GAL4 is synthesized, it binds to UAS sites located upstream of the p38KD gene on a separate chromosome and drives its transcription. This process produces overexpression of the p38KD gene in a certain cell type determined by the chosen genomic enhancer (Figure 2) (Brand et al. 1993). This system then allows us to select a variety of genomic enhancers to target overexpression of p38KD in different tissues, so by creating different GAL4-UAS-p38KD animals we can screen for abnormal phenotypes modified by a lack of p38K signaling in various tissues.

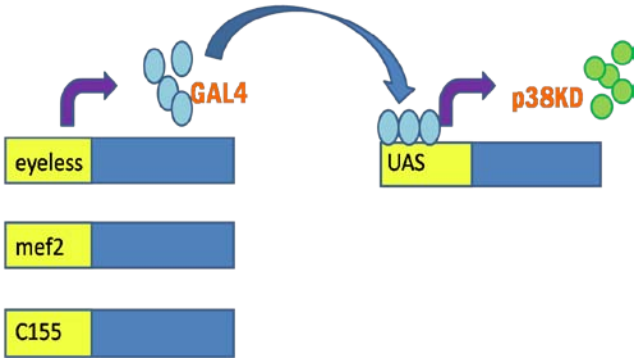


Figure 2 The GAL4-UAS System

On the chromosomes located on the left, a genomic enhancer (eyeless, mef2, C155) located upstream of the GAL4 gene is used to drive its expression. Once GAL4 is produced, it binds to UAS sites on the second chromosome, driving expression of p38KD. The specific genomic enhancers determine which cell types will experience p38KD overexpression.

p38K mutants display a range of motor deficits that worsen with age (Figure 3). However, the specific requirement for p38K signaling in different tissues, i.e. the cellular locus for these phenotypes, is not clear from these experiments. A major reason for developing the p38Kb-KD transgene was to address this issue. In this report, I have

analyzed the outcome of expressing p38Kb-KD, and thereby inhibiting p38K signaling, in different target tissues on motor phenotypes in adult *Drosophila*. Different behavioral assays were implemented to investigate the effects of the p38KD transgene overexpressions on several aspects of fly locomotion. My results suggest that while inhibition of p38K in neurons does not impair locomotion, inhibition of p38K in all tissues produces locomotor phenotypes that are very reminiscent of those seen in p38K mutants. Targeted p38K inhibition in muscle tissue also phenocopies p38K mutants and is consistent with our earlier observation that p38K mutant phenotypes can be rescued through selective add back of wild type p38Kb in muscles.

RESULTS

A. Unpublished results from the Sanyal Laboratory

Prior to analyzing the effect of the p38Kb-KD transgenes, I worked in collaboration with members of the Sanyal laboratory (Alysia Vrailas Mortimer, PhD) to characterize the role of p38K MAP kinase using newly generated mutations in p38Kb, combined with existing mutations in p38Ka. As mentioned previously, *Drosophila* have two p38K genes: p38Ka and p38Kb. When the study was initiated there were no known mutations in p38Kb, hence transposon induced deletion alleles of p38Kb were first generated and verified in the Sanyal laboratory. These were then combined with reported deletions of p38Ka, to derive double mutants that lack both p38K genes (double knockouts). p38K double mutants were found to have a severely reduced lifespan and not unexpectedly, exhibit heightened sensitivity to both general stressors (such as heat shock and starvation) and specific oxidative stress induced by exposure to Hydrogen Peroxide and the herbicide Paraquat (Figure 3). Although the single and the double mutants showed deficits compared to the wild type animals, the double knockouts demonstrated the most drastic impairment followed by the p38Kb mutants. Consistent with the idea that oxidative stress damages dopaminergic neurons, p38K double knockout animals (p38K-DKO) also showed a significant loss of dopaminergic neurons in three specific dopaminergic neuron clusters in the fly brain.

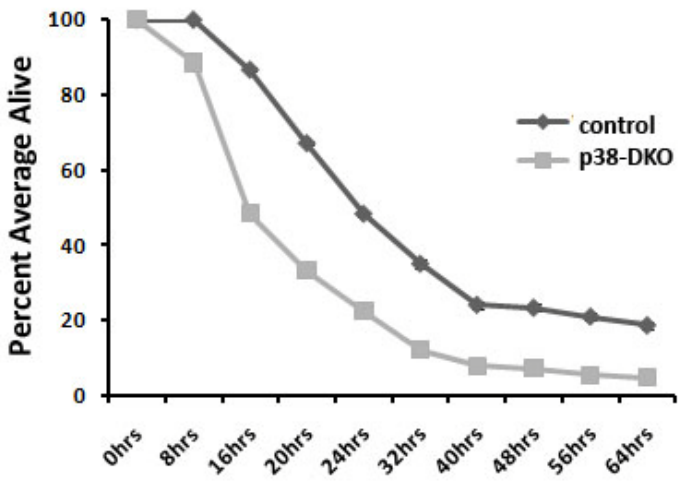


Figure 3. Loss of p38K leads to increased sensitivity to oxidative stress. p38K double mutants show a higher mortality upon exposure to 5Mm Paraquat, while a greater percentage of the wild type population survives for a longer period of time.

Motor deterioration is a hallmark of Parkinson's disease. When tested, p38K mutants were found to show alterations in motor phenotypes as well. Specifically, these animals exhibited drastic locomotor dysfunction in three different assays. In the climbing and countercurrent assays, where animals were allowed a certain time frame to climb up either a vertical graduated cylinder or a set of plastic tubes respectively, the p38K-DKO (double knockout) mutants climbed at a significantly slower pace with a smaller percentage of animals reaching the top at most time points. (Figures 4 and 5). Thus, these assays demonstrate that the mutants exhibit a poor negative geotaxis response. This evidence suggests that a lack of p38K impairs fly movement. However, these experiments do not reveal the identity of the tissue(s) in which p38K function is required to prevent motor deficits. Rescue experiments in which a wild type p38K protein is added back to different tissues in a background that is mutant for p38K suggests that the primary site of action of p38K is the muscle tissue (Figure 6). Interestingly, *parkin* mutant phenotypes in flies also reside in the muscle and we speculate that this is because

in flying insects muscle tissue is metabolically one of the most active tissue types. To directly test the idea that p38K perturbation in muscle is sufficient to cause motor deficits that are reminiscent of those found in *parkin* mutants it is essential to first demonstrate that muscle-specific knock down of p38K leads to motor deficits. Therefore, I hypothesized that expression of a newly generated dominant-negative p38 MAP kinase transgene in muscles will phenocopy or replicate motor phenotypes observed in p38K mutant animals. In the process of testing this hypothesis, I will confirm that the dominant-negative transgene, generated by mutating the kinase domain of *Drosophila* p38K, performs as expected and inhibits endogenous p38K. As a corollary to testing this hypothesis, I also aimed to test the effects of reducing p38 signaling in different cell types other than muscle tissue. All these experiments made use of the GAL4-UAS system described previously and results from this project are outlined below.

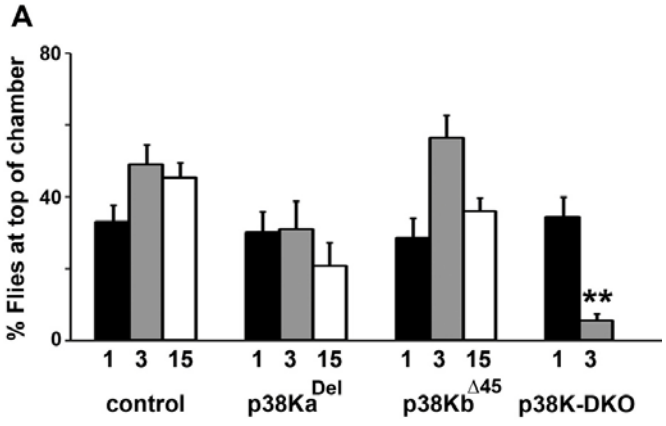
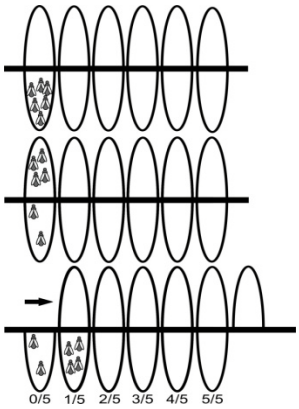


Figure 4. p38K double knockouts perform poorly in the climbing assay Flies from each genotype were tested at different ages (1, 3, and 15 days old) for climbing. Flies were tapped to the bottom of a graduated cylinder, and the number of flies that had reached the top was recorded at 10 sec. At 3 days old, p38K double knockouts had a significantly smaller percentage of flies at the top.

Figure 5a. Countercurrent Apparatus. Flies are placed in tube 1. They are tapped down to the bottom and allowed 15 sec to climb up, and then the top half of the tubes is shifted to the right. The flies which were able to climb this far are transferred to the next tube. This process is repeated 5 times. The good climbers with the best negative geotaxis response will be transferred farther along the set, with the best climbers reaching tube 6.



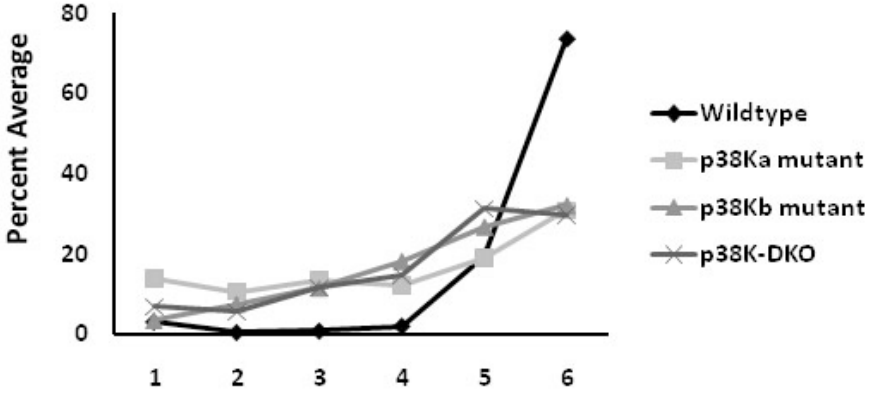


Figure 5b. p38K Mutants Show Climbing Impairment and a Poor Negative Geotaxis Response in the Countercurrent Assay A greater percentage of the p38 mutants tend to remain in the first set of tubes while most wild type flies easily reach the 6th tube.

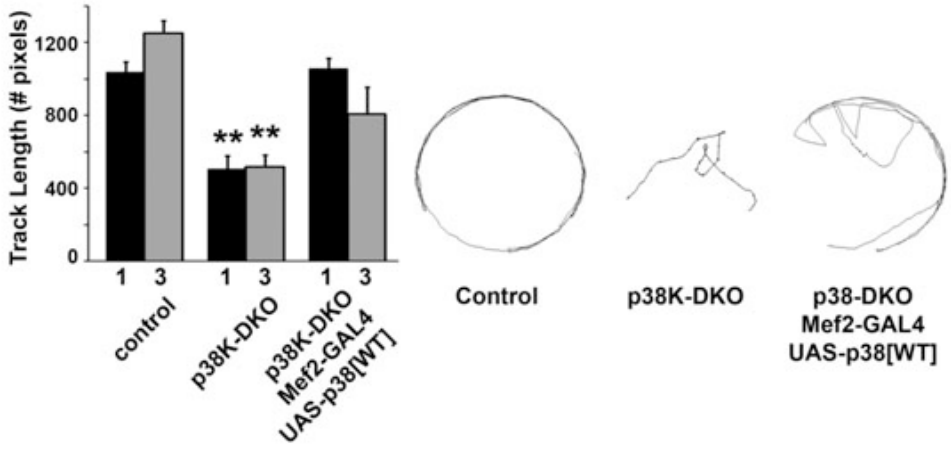


Figure 6. Overexpression of p38K in muscles shows a rescue phenotype for locomotor function. In the walking assay, p38K double knockouts travel a much smaller distance than the control group. When animals with a p38-DKO background have an overexpression of wild type p38K in muscle tissue (p38K-DKO Mef2-GAL4 UAS-p38[WT]) they tend to walk more than the p38K-DKO mutants, and the length of their track resembles that of the control group.

METHODS

Targeted p38bKD Overexpression

To produce GAL4-UAS-p38KD animals, 10 male flies with the genomic enhancers driving GAL4 expression were mated to 10 female flies with the UAS site driving p38KD. The following genomic enhancers were used to express p38KD in the respective cell types: Tubulin-GAL4:all cells, C155-GAL4:neurons, and Mef2-GAL4:muscles. The crosses gave rise to progeny with the following genotypes:

Tub-GAL4/UAS-p38bKD3	Tub-GAL4/UAS-p38bKD10
C155-GAL4/UAS-p38bKD3	C155-GAL4/UAS-p38bKD10
Mef2-GAL4/UAS-p38bKD3	Mef2-GAL4/UAS-p38bKD10

These groups of flies were tested alongside with a control group which consisted of flies with the same genomic enhancer but no p38bKD transgene. These animals were produced by collecting 10 males with the genomic enhancers driving GAL4 expression and mating them to 10 wildtype (OR) females. The progenies had the following genotypes:

Tub-GAL4/OR

C155-GAL4/OR

Mef2GAL4/OR

Locomotor Behavior Tests

Climbing Assay Female flies were collected at 0 days old and tested at 3 days and 15 days of age in groups of 8-10. Each group of flies was placed in a 25ml graduated cylinder and tapped down to the bottom. Flies were then allowed 30 seconds to climb up the cylinder while being videotaped, and the number of flies above the 25ml mark was recorded at 5sec, 10 sec, and 30 sec time points. This procedure was repeated for a total of 10 trials for each genotype. The following genotypes were tested: Tub-GAL4/UAS-p38bKD3 (Tub/KD3), Tub-GAL4/UAS-p38bKD10 (Tub/KD10), Tub-GAL4/OR (Tub/OR), C155-GAL4/UAS-p38bKD3 (C155/KD3), C155-GAL4/UAS-p38bKD10 (C155/KD10), C155-GAL4/OR (C155/OR).

DAM Assay Female flies were collected at 0 days old and tested at 1-3 days of age. Individual flies from each genotype were placed in separate tubes and their locomotor activity was measured using infrared beam breaks to detect movement. During the first six days the flies were exposed to 12 hour light/dark cycles. On day 7 the flies were maintained in total darkness for a period of 6 days. Activity was measured based on the amount of infrared beam breaks. The following genotypes were tested: C155-GAL4/UAS-p38bKD3 (C155/KD3), C155-GAL4/UAS-p38bKD10 (C155/KD10), and C155-GAL4/OR (C155/OR).

Walking Assay At day 0 female flies were collected had their wings removed by using a small blade to slice them off. Flies were then tested at 3 and 15 days of age. Individual flies were placed on a hollow glass surface in the shape of a circle which was covered with a clear plastic slide to enclose the area. Fly walking was videotaped for

1min and the videos were then analyzed using Image J software. 10 flies from each of the following genotypes were tested: Tub-GAL4/UAS-p38bKD3 (Tub/KD3), Tub-GAL4/UAS-p38bKD10 (Tub/KD10), Tub-GAL4/OR (Tub/OR), Mef2-GAL4/UAS-p38bKD3 (Mef2/KD3), Mef2-GAL4/UAS-p38bKD10 (Mef2/KD10), Mef2-GAL4/OR (Mef2/OR)

Statistical Methods

To analyze the statistical differences in my data, t-tests were applied for 2 samples assuming unequal variances. When using the standard t-test the data must follow three conditions: a large enough sample size (n), simple random samples, and normal distribution. Here we assume these conditions are met. However, under certain circumstances when the data does not fit these criteria, one should use nonparametric tests, such as the Mann-Whitney U Test to analyze the significance.

RESULTS

B. Results obtained during the course of the current study

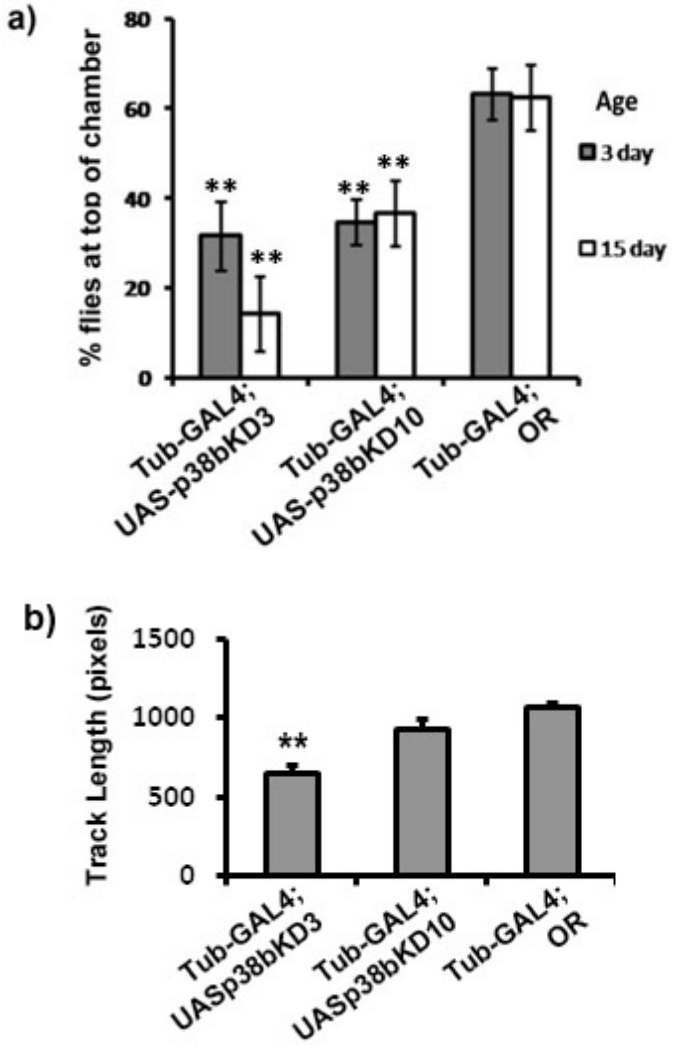


Figure 7. p38K Inhibition in all cell types leads to locomotor deficits in climbing and walking assays. a) In the climbing assay, flies with p38K inhibition demonstrate a poor climbing ability and negative geotaxis response at the 10 sec point compared to wildtype flies b) In the walking assay, these animals travel a shorter distance during a one minute time span (left) and show walking impairment in their path traces (right).

Overall p38K Inhibition Leads to Locomotor Deficits

The tubulin GAL4 driver is used to overexpress a gene of interest in all cell types, therefore this genomic enhancer was used to produce an overexpression of p38bKD throughout the animal by exposing this GAL4 driver and its respective chromosome to a

UAS site on a separate chromosome located upstream of the p38bKD gene in the Tub/KD3 and Tub/KD10 groups. In the Tub animals, the control groups outperformed the KD groups in all the locomotor tests (Figure 7), indicating that a general overexpression of p38bKD impairs locomotor function.

In the climbing assay (Figure 7a), the Tub/OR animals were the fastest climbers with a significantly greater percentage of animals reaching the top of the cylinder at the 10 sec time point than both Tub/KD groups in both 3 days old and 15 day old animals (63% at 3 days of age, 62% at 15 days of age). Although most animals from all groups managed to reach the top by the end of the trial, the difference observed in the percentage of animals at the top at the 10 sec time point indicates the Tub/KD animals moved at a slower pace. The Tub/KD3 group had the smallest percentage of animals at the top (32% at 3 days of age,14% at 15 days of age) followed by the Tub/KD10 group (35% at 3 days of age, 37% at 15 days of age),illustrating these animals exhibit a poor climbing ability and negative geotaxis response.

Similar movement deficits were observed in the Tub/KD groups in the walking assay (Figure 7b and c), where the Tub/OR group traveled the greatest distance on average out of all three groups (1065 pixels). Although the Tub/KD10 animals did not show a significant difference in distance travelled compared to the control (928 pixels, p-value < 0 .106), the average distance travelled by the Tub/KD3 animals was almost half of that of the control group (645 pixels, p-value< 0.01). The Tub/KD3 animals also spent an unusual amount of time grooming or standing still instead of walking. This suggests that an overall decrease of p38K signaling in the Tub/KD animals diminished locomotor activity in multiple paradigms.

Differences between the two transgenes tested in this study are often observed with multiple UAS insertion lines and often arise from differences in transgene expression levels (see discussion). Identifying one particular line that would be uniformly useful in future experiments was also a part of this study. In this case, our results suggest the KD3 transgene would be more useful for testing locomotor function since greater impairment was observed in these animals.

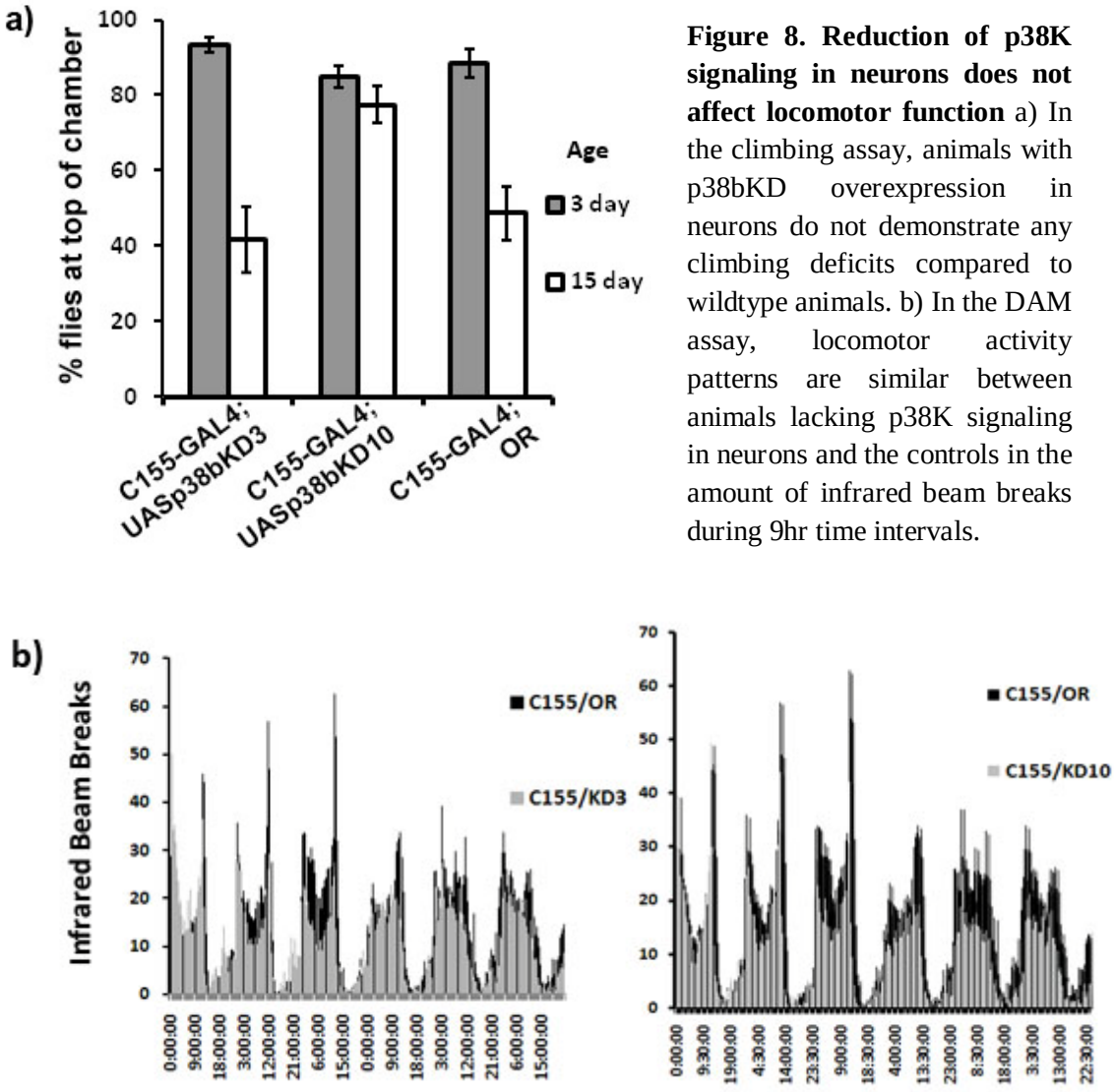


Figure 8. Reduction of p38K signaling in neurons does not affect locomotor function a) In the climbing assay, animals with p38bKD overexpression in neurons do not demonstrate any climbing deficits compared to wildtype animals. b) In the DAM assay, locomotor activity patterns are similar between animals lacking p38K signaling in neurons and the controls in the amount of infrared beam breaks during 9hr time intervals.

p38K Inhibition in Neurons Does Not Impair Locomotor Activity

Since our previous findings revealed that a lack of p38K either in genetic mutants or through inhibition of p38K throughout the animal (previous section) leads to locomotor dysfunction, we hypothesized p38K signaling would have the greatest impact on either neurons or muscle cells due to their involvement in locomotion. We have also shown previously that p38Kb is expressed in dopaminergic neurons and p38-DKO mutants show dopaminergic neuron loss. An observed a lack of p38K results in both locomotor dysfunction and loss of dopaminergic neurons, this suggests that p38K might play a role in neurons to affect locomotion. However, rescue data presented in Figure 6 and elsewhere shows clearly that a specific overexpression of wild type p38K in muscles is both necessary and sufficient to revert p38K-dependent locomotor phenotypes.

To unambiguously test whether p38K is required in Drosophila neurons for normal locomotion, the pan-neuronally expressing C155 GAL4 driver was used to generate an overexpression of p38bKD in neurons in C155/KD3 and C155/KD10 animals. Consistent with our rescue data, these two groups did not exhibit consistent or significant deficits in locomotor function compared to the C155/OR control group (Figure 8a). In the climbing assay, all three groups moved at approximately the same pace and had about the same percentage of animals at the top of the cylinder at the 10 sec time point in 3 day old animals (C155/KD3-93%, C155/kD10-85%, C155/OR-89%). In 15 day old animals the C155/KD3 and C155/OR groups also showed the same percentage of animals at the top (42% and 49%), but the C155/KD10 group a higher percentage (77%). Currently, we do not have an explanation for why expression of KD10 leads to

apparently better climbing in 3 day old animals, but we speculate that this might be due to strain differences that need closer inspection.

There was also no difference noted between the overall locomotion of KD groups and control groups in the DAM assay (Figure 8b and c). In this test, individual flies are monitored for horizontal locomotion in a sealed glass tube under 12:12 light-dark conditions. In addition to providing an estimate of overall locomotion, this is also the standard method for estimating differences in circadian rhythm in flies. In our experiment, all C155 groups seemed to have a robust circadian rhythm with normal peaks of activity during the day and showed similar cumulative movement over a 24 hour period. These data indicate that knock down of p38K signaling in neurons using two of our transgenes is not sufficient in disrupting locomotor behavior in flies. Together, these results suggest that either locomotor activity in adult flies is not dramatically controlled by p38K activity in neurons (consistent with our rescue data), or more trivially, the degree of p38K inhibition achieved using our transgenes is not sufficiently high to elicit neuronally derived locomotor phenotypes (see discussion).

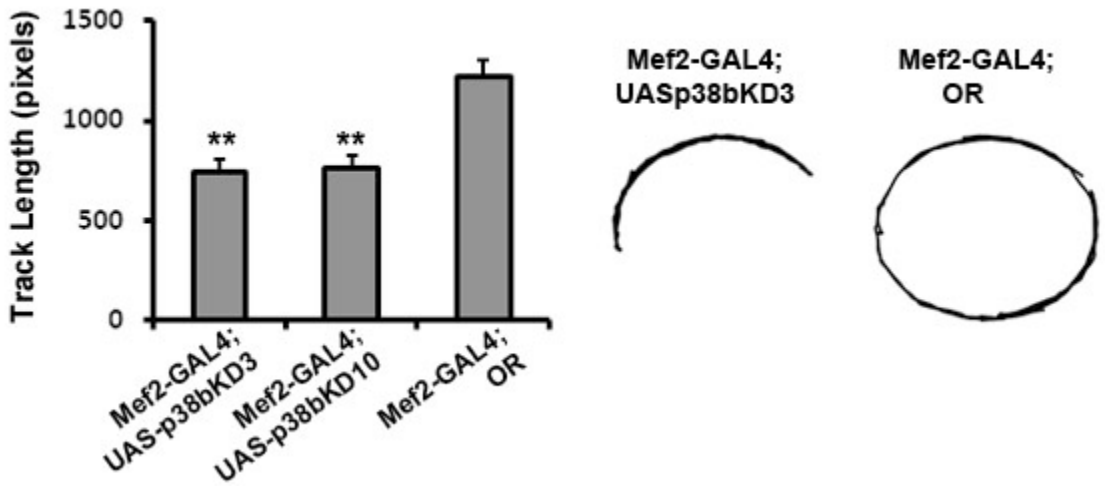


Figure 9. Muscle inhibition of p38K impairs walking. Expression of either p38-KD transgene results in slower walking as compared to age matched wild type genetic controls. This is consistent with observed behaviors in p38K double mutant animals.

p38K Inhibition in Muscle Tissue Impairs Locomotor Function

After finding that the locomotor deficits resulting from overexpressing p38bKD in the Tub animals were not due to the effects of p38bKD in neurons, we investigated whether or not these deficits were related to the expression of this gene in muscle cells. Our previous experiments demonstrated how a targeted overexpression of wild type p38K in muscle cells rescues locomotor function in p38K deficient animals (Figure 6). We hypothesized that overexpressing a gene which decreases p38K signaling in muscle cells of animals with a wild type background will therefore produce the opposite results and impair locomotor function.

To overexpress p38bKD in muscle cells, the Mef2 GAL4 driver was used to generate Mef2/KD3 and Mef2/KD10 animals. These animals performed poorly on the walking assay compared to the Mef2/OR control group, demonstrating that p38K

inhibition in muscle cells hinder locomotor ability (Figure 9). The average distances travelled by the Mef2/KD3 and Mef2/KD10 animals were 738.4 pixels and 759.9 pixels respectively, while that of the control animals was 1215.3 pixels. In addition, behaviors resembling those of the Tub/KD groups were observed as these animals also had the tendency to pause for short periods of time and groom while the control animals were in constant movement.

DISCUSSION

Previously, we have shown how a lack of p38K signaling leads to impaired locomotor activity by testing p38-DKO mutants in the climbing and walking assays. While this study used p38-DKO mutants to characterize the function of this MAPK, our current experiments focused on studying the effects of reducing p38K signaling by overexpressing p38bKD in certain tissues. To investigate the effect of inhibiting p38K mediated signaling throughout the animal (and thereby attempt to phenocopy the genetic mutations in p38K), the tubulin genomic enhancer was used to drive expression of the kinase dead dominant negative p38K in all cell types in Tub/KD animals. Consistent with our predictions, these animals showed a phenotype closely resembling that seen in p38-DKO mutants in both the climbing and walking assays. This evidence further suggests that the locomotor dysfunction observed in both of these groups is directly caused by a lack of p38K signaling and discredits other possibilities (such as the presence of other unmapped mutations in the p38K-DKO genetic background). Together with rescue of locomotor phenotypes in p38K-DKO animals through transgenic reexpression of wild type p38Kb, these results firmly establish a role for p38K signaling in the regulation of motor activity in *Drosophila*.

When comparing the phenotypes of the two different kinase-dead transgenes, we found the KD3 transgene to be more effective in generating locomotor deficits in the Tub animals in both the climbing and walking assays and in the walking assay for the Mef2 animals. In the climbing assay there was a 3% difference in 3 day old animals and a 23% difference in 15 day old animals that reached the top between the KD3 and KD10 groups. In the walking assay there was a 283 pixel difference in distance travelled in the Tub

animals and a 22 pixel difference in the Mef2 animals. This difference in behavior could be related to the levels of expression of each KD transgene. Since the transgenes were inserted at presumably different loci in the Drosophila genome, their levels of expression could vary due to their position relative to the local genomic landscape proximal to the site of insertion. For instance, locally active enhancers are likely to influence expression of the transgene both under basal conditions and in the presence of GAL4. Such variations are commonly observed for UAS driven transgenes in *Drosophila*; hence our experiments are carried out with two different transgenic insertions. In this case it seems the KD3 transgene had higher levels of expression which produced a greater difference in phenotype in these animals compared to the KD10 groups. This assumption could later be tested by performing a Western blot to quantify the amount of p38bKD in KD3 and KD10 animals. Since the transgenic p38KD protein is epitope tagged at the C-terminus with the FLAG epitope, a simple Western blot using anti-FLAG antibodies can be used for this experiment.

In addition to analyzing the effects of general p38bKD overexpression, we chose to study localized p38bKD overexpression in different tissue types to determine the regions where p38K signaling has the greatest impact. Previous experiments in the lab have shown that p38K is expressed throughout the animal, for example in cholinergic neurons in the brain, glutamatergic motor neurons, dopaminergic neurons, and in muscle. Since we observe motor phenotypes following inhibition of p38K signaling, directly investigating the function of p38K in neurons is essential. Furthermore, CHMP gene mutations which lead to muscle atrophy demonstrate downregulation of the p38K

signaling pathway in motor neurons (Cox et al. 2010), suggesting p38K signaling in neurons may be required for maintaining normal muscle function.

When p38bKD was overexpressed in the neurons, however, no locomotor impairment was observed in either the climbing assay or the DAM assay. The C155/KD groups seemed to perform just as well as the control group in both tests, exhibiting similar locomotor patterns. These data indicate that p38K may play a different role in neurons independent of locomotor regulation. An alternative explanation may be that the level of p38bKD expression in neurons was not sufficient to display a phenotype in the C155/KD groups. Although overexpression of p38bKD presumably knocks down a certain amount of p38K signaling, wild type p38K is still being expressed and not all signaling is blocked. p38bKD is able to prevent p38K from signaling by binding to its target proteins, but a higher concentration of wild type p38K than p38bKD would render this method ineffective by facilitating wild type p38K binding. Therefore, neurons may express a surplus of p38K which allows this signaling pathway to remain intact and function normally even in the presence of p38bKD. Future experiments should test the effects of higher levels of p38bKD expression in neurons using different KD transgenes or stronger GAL4 driver lines. An independent assay to test the efficacy of p38K inhibition should also prove useful in this respect.

Finally, p38bKD overexpression was also targeted in muscle tissue, since this cell type, along with motor neurons, forms part of the neuromuscular junction responsible for locomotion. Here, we find that p38K expression in muscles plays a critical role in maintaining proper locomotor activity, since animals with p38bKD overexpression exhibit significant walking impairment. One explanation for these results is that the

locomotor deficits arise from an increase in stress in the muscle cells. p38K is activated by stress, so an inhibition of the p38K pathway in muscle cells would prevent them from producing a normal stress response. This would intensify the deleterious effects of extracellular stressors and may ultimately result in metabolism changes and cell degeneration.

Oxidative stress in particular, has been found to damage muscle tissue and lead to atrophy. (Powers et al. 2005) In skeletal muscle cells, indirect activation of the p38K MAPK pathway was able to attenuate the effects of oxidative stress and offer cell protection (Yang et al. 2010). This evidence implies muscle cells with reduced activity in the p38K pathway (such as those of the Mef2/KD animals) would be particularly vulnerable to the effects of oxidative stress and demonstrate accelerated aging. Oxidative stress is also thought to induce muscle aging, since the amount mitochondrial ROS in skeletal muscle increases with age (Chabi et al. 2008). Our previous research suggests p38K protects cells from oxidative stress-related aging since animals lacking p38K die at a faster rate upon exposure to oxidative stress (Figure 3) and have a shorter lifespan. Prior studies have also shown aging is related to muscle loss (Hughes et al. 2002) and activates the p38K pathway in muscle cells (Moylan et al. 2007), indicating the p38K pathway in muscles may serve as a mechanism to prevent aging. In the current study we found locomotor impairment caused by lack of functioning p38K may be heightened with age. In the climbing assay, the Tub/KD3 animals performed significantly worse at 15 days of age than at 3 days of age. In this group the percentage of animals that reached the top decreased by 18% within the 12 days of aging, while the control group only showed a 1% difference in the age groups. To explore whether this age-dependent performance is due

to lack of p38K signaling in muscles, future experiments should test the locomotor function of Mef2/KD animals at different ages and note if there is a decline in performance

Since PD has been linked to oxidative stress and patients exhibit muscle impairment and have a shorter lifespan, one possibility is that these symptoms may be caused by a malfunction in the p38K pathway in muscle cells. The other alternative stems from the observation that in *Drosophila* models, the most severe phenotypes in genetic models of PD are seen in muscles. Thus, muscle degeneration and mitochondrial phenotypes are observed in *parkin* mutants. Since p38K mutants interact genetically with *parkin* mutants, we consider it likely that both Parkin and p38K affect muscle physiology. Reasons for this might be due to the fact that muscles are one of the more metabolically active tissues in flying insects and thus are more prone to generating (and suffering damage from) byproducts of oxidative metabolism. In general, it seems likely that there is a confluence of cellular mechanisms that underlie stress, aging and age-related degenerative disorders such as PD. Signaling mediated by the p38K MAP kinase appears to be at the center of these pathways since *a)* p38K inhibition either through mutations or transgenic inhibition (this study) results in age-dependent deterioration in locomotion, *b)* p38K mutants are more sensitive to oxidative stress and show increased protein carbonylation and activation of stress pathways, *c)* increased p38K signaling increases resistance to such stress and improves lifespan, and finally *d)* in addition to genetic interactions between *parkin* and p38K, there are close parallels between the types of phenotypes that are exhibited by mutations in these genes. Future studies will be aimed at trying to derive mechanistic connections between p38K and Parkin, and more

generally at trying to identify tissue specific targets of p38K that might be involved in mediating specific phenotypes observed in p38K mutations. One such candidate, the detoxifying enzyme SOD2 (super oxide dismutase), is already being studied as a potential downstream target of p38K in muscles.

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