Evolutionarily Conserved Transcription Factors Drive the Oxidative Stress Response in *Drosophila*

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Abstract

In order to understand how oxidative stress signal transduction pathways evolve, we analyzed the molecular evolution of the p38 MAPK (p38K) gene family across the genus *Drosophila*. p38K family genes play a vital role in oxidative stress resistance and are also important for organismal development and immunity. We find that the p38Ka and p38Kb genes are highly conserved across the genus and that p38Kc is more recently evolved. We further find that the p38Kb genomic locus includes conserved binding sites for the AP-1 and lola-PT transcription factors. Accordingly, over-expression of either AP-1 or lola-PT in *D. melanogaster* is sufficient to induce transcription of p38Kb under normal conditions, while under oxidative stress only lola-PT over-expression was able to induce p38Kb transcription. In addition, exposure to oxidative stress results in increased p38K protein levels in a number of species. These increased levels are associated with an increased resistance to oxidative stress across species. We also find that the presence of a lola-PT binding site in the p38Kb locus is predictive of the species' survival in response to oxidative stress. Through our comparative genomics approach, we have identified biologically relevant transcription factor binding sites that regulate the expression of p38Kb and are associated with resistance to oxidative stress. These findings reveal a novel mode of regulation for p38 MAP Kinases and suggests that transcription may play as important a role in p38K mediated stress responses as post-translational modifications.

Significance Statement

Organisms encounter a variety of environmental stresses such as oxidative stress throughout their lifetime. Therefore, organisms have evolved a number of mechanisms to combat these stresses. In order to understand how these mechanisms evolved, we have compared the genomes of a diverse set of species across the genus *Drosophila* to examine the p38 MAPK stress response gene family. Our analysis was able to successfully predict transcription factors

that not only regulate our target gene, p38Kb, but do so under different conditions to ensure an appropriate stress response. Therefore, we find that in addition to post-translational regulation, transcriptional regulation of signaling pathways may also play an important role in how organisms are able to adapt to stressful environments or respond to stress conditions as they arise. Furthermore, our comparative genomics approach may be utilized to identify transcriptional regulators of other highly conserved signaling pathways.

Introduction

Organisms encounter oxidative stress both through external environmental exposure and from the internal release of oxygen radicals during ATP synthesis, and have evolved a variety of mechanisms to counteract it. These oxidative stress response mechanisms can include detoxifying enzymes such as catalases as well as signal transduction pathways such as the JNK and p38K MAP Kinases that lead to the induction of downstream antioxidants. In addition to utilizing a variety of different defense mechanisms, the genomes of many organisms have undergone duplication events resulting in families of closely related stress response genes with specific functions. One such gene family that has undergone a considerable expansion is the glutathione transferase (GST) family, which consists of over 70 genes in multiple classes (*1*). Gene expansion is not limited to detoxifying enzymes and has also occurred within signaling pathways. For example, the MAPK family is a highly conserved set of signaling pathways that have diversified and evolved to play a variety of roles in the cell. The MAPK family is divided into the ERK, JNK, and p38K pathways, with an additional newly evolved MAPK family, ERK5, found only in vertebrates. Each pathway consists of a phosphorylation cascade of upstream kinases that leads to the dual phosphorylation of the MAPK protein on a TXY motif. This activated MAPK can phosphorylate target proteins in the cytoplasm and can also translocate to the nucleus where it can phosphorylate downstream transcription factors. A variety of stimuli

such as growth factors and reactive oxygen species can result in the specific activation of a MAPK and its downstream targets, which allows the cell to appropriately respond to that stimulus. In addition, there is a complex web of crosstalk between these pathways, which can allow for the fine tuning of a specific cellular response. Furthermore, the different MAPK cascades have further diverged with multiple ERK, JNK, and p38K genes present in higher organisms (*2*–*5*).

In the fruit fly *Drosophila melanogaster,* there is a single ERK gene, a single JNK gene and three p38K genes: p38Ka, p38Kb, and p38Kc. Loss of any one of these p38K genes is viable, however, p38Ka p38Kb double knockouts are lethal (*6*–*11*), suggesting that these genes are at least partially redundant. Both p38Ka and p38Kb play a role in resistance to heat shock, starvation, and oxidative stress (*6*, *11*, *12*). Furthermore, both of these genes play a role in the immune response with p38Ka regulating the gut DUOX system (*13*), while p38Kb is involved in bacterial phagocytosis (*7*) and antiviral immunity (*14*). In addition, these genes have unique roles in the body. p38Ka plays a role in cardiac function (*15*), while p38Kb is a regulator of lifespan, locomotor functions, and circadian rhythms (*6*, *16*). Interestingly, p38Kc has distinct functions in the cell in regulating innate immunity through the *Ddc* pathway (*17*) and intestinal oxidative stress and lipid homeostasis (*18*). The three p38K genes have been hypothesized to have arisen through gene duplication, and the differences in function suggest that evolutionary pressures may be playing a role in the divergence of these genes. Thus, the *Drosophila* p38K genes provide an excellent system to analyze the molecular and functional evolution of a set of closely related signaling molecules involved in stress responses.

Here, we utilized the sequenced genomes of species within the *Drosophila* genus to analyze the molecular evolution of the p38K family genes. *Drosophila* provide a powerful model for this research, with multiple species with sequenced genomes and known evolutionary

relationships. Additionally, these species have differing environmental ranges spanning cosmopolitan species such as *D. melanogaster* and *D. simulans* and geographically restricted species such as the Hawaiian species *D. grimshawi,* and *D. sechellia* and *D. mauritiana* which are endemic to the Seychelles and Mauritius, respectively. Species across the genus also display differing food ranges including generalists like *D. simulans* and specialists such as *D. mojavensis* (toxic necrotic cacti, (*19*, *20*)) and *D. sechellia* (noni fruit, (*19*, *20*)). Thus, these species also likely face divergent selective pressures for oxidative stress response in their natural environments.

Our molecular evolution analyses suggest that all three p38K genes are under purifying selection and that p38Kc resulted from a recent gene duplication. Interestingly, we find that *D. pseudoobscura* has also experienced a duplication of the p38Kb gene, which corresponds to increased resistance to an oxidative insult. Furthermore, we find that diverse species of *Drosophila* express high levels of p38K protein during oxidative stress, and that there are conserved AP-1 and lola-PT transcription factor binding sites that regulate p38Kb expression under normal and oxidative stress conditions. The levels of p38K expression and the presence of these transcription factor binding sites are also predictors of resistance to oxidative stress. These data suggest that transcriptional regulation of p38Kb may play as important a role in oxidative stress resistance and may boost the effectiveness of the more well-described post-translational regulation of p38Kb in regulating stress responses.

Materials and Methods

Sequences

Sequences corresponding to MAPK family genes were downloaded from FlyBase (flybase.org) and NCBI. Accession numbers are given in Table S1 for DNA sequences and Table S2 for protein sequences.

Phylogenetic trees

Phylogenetic trees for the MAPK family were constructed using amino acid sequences. Sequences were aligned using the ClustalW algorithm with the Gonnet Protein Weight Matrix implemented in MEGA (version 5.2.2, (*21*)), and all positions containing gaps and missing data were eliminated. Initial trees were obtained using the Neighbor-Joining method. The phylogenetic relationships among the sequences were inferred using the maximum likelihood method based on the Jones-Taylor-Thornton model (*22*), and the trees with the highest log likelihood are shown. Each tree was tested with 1000 bootstrap replications (*23*), and the given values are the percentage of trees in which the associated sequences clustered together.

Phylogenetic trees for the *Drosophila* p38K gene family and ITS2 were constructed using nucleic acid sequences. Sequences were aligned using the ClustalW algorithm with the IUB DNA Weight Matrix implemented in MEGA, and all positions containing gaps and missing data were eliminated. Pairwise distances were estimated using the Maximum Composite Likelihood approach and initial trees were obtained using the Neighbor-Joining method. The phylogenetic relationships among the sequences were inferred using the maximum likelihood method based on the Tamura-Nei model (*24*), and the trees with the highest likelihood are shown. Each tree was tested with 1000 bootstrap replications (*23*), and the given values are the percentage of trees in which the associated sequences clustered together.

The output from these analyses were used to draw trees using FigTree v. 1.4.

Chromosome synteny

The regions of interest from the Muller B and E elements across *Drosophila* species were accessed through GBrowse on FlyBase. Synteny was determined manually.

Genetic conservation analysis

Pairwise dN and dS values for p38K family genes across the *Drosophila* species were computed in MEGA (v.5.2.2) using the Nei-Gojobori model. Values were plotted in R.

Drosophila stocks

Drosophila melanogaster Oregon^{R}, y[1] w[*]; P{w[+mC]=UAS-lola.4.7}3, and P{w[+mC]=UAS-lola.4.7}1, w[*] were obtained from the Bloomington Drosophila Stock Center, Indiana University. w^{-};;MHC-GAL4 was used as reported in (*6*) and w^{-} ;UAS-Jun; UAS-Fos is described in (*25*). *Drosophila pseudoobscura* (strain 14011-0121.94), *Drosophila virilis* (strain 15010-1051.87), *Drosophila simulans* (strain 14021-0251.195), *Drosophila yakuba* (strain 14021-0261.00), *Drosophila mauritiana* (strain 14021-0241.151), and *Drosophila ananassae* (strain 14024-0371.13) were obtained from the Drosophila Species Stock Center, University of California San Diego.

Predicting transcription factor binding sites

Transcription factor binding sites were predicted using iMotifs (*26*). The TomTom Motif Comparison Tool v4.9.1 (MEME web server, (*27*)) 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 positions.

qRTPCR

D. melanogaster lines were reared at 25°C in a 12hr:12hr light:dark cycle on standard fly food media. Virgin female flies were collected at eclosion and fed standard fly food media and aged for 1 week. Flies were then transferred to food mixed with 20mM paraquat (Sigma). Food was changed every 3-4 days. Surviving flies were collected when 30% of controls had died. Thoraxes from 6 flies were dissected and pooled. RNA was extracted using Trizol (Thermofisher Scientific) and reverse transcribed using SuperScript IV (Thermofisher Scientific). qRTPCR was

performed using SYBR green (Thermofisher Scientific). Primers designed to amplify p38Kb or the housekeeping gene Arp88 were utilized. To compare p38Kb expression across genotypes, the ΔCt value for each replicate was calculated by subtracting the Ct value of Arp88 from the Ct value of p38Kb. We tested the hypothesis that AP-1 and Lola-PT overexpression would increase the amount of p38Kb transcript using one-tailed Student's t and Wilcoxon signed rank tests (implemented in R).

Lifespan

AP-1 and lola-PT flies and outcrossed controls were reared at 25°C in a 12hr:12hr light:dark cycle on standard fly food media. Flies were collected at eclosion and lifespan was assayed daily. Food was changed every 3-4 days. Differences in survival were assayed using the Log-Rank test. Pairwise comparisons between samples were made using the Pairwise Log-Rank test and p-values were adjusted using the false discovery rate. These tests were performed using the survival package in R.

Paraquat Survival

We find that the efficacy of the paraquat was batch dependent (Table S3). Therefore, all experiments were performed with biological replicates and controls all on the same batch of paraquat. Virgin females from each species were collected at eclosion and transferred to fly food media mixed with 20mM paraquat (Sigma). *D. simulans* and *D. pseudoobscura* were reared at 21°C in a 12hr:12hr light:dark cycle. *D. melanogaster, D. virilis*, *D. yakuba*, *D. ananassae,* and *D. mauritiana* were reared at 25°C in a 12hr:12hr light:dark cycle on standard fly food media. Food was changed every 3-4 days. Survival was scored daily.

AP-1 and lola-PT flies and outcrossed controls were reared at 25°C in a 12hr:12hr light:dark cycle on standard fly food media. Virgin female flies were collected at eclosion and

aged 1 week and then transferred to standard fly food media mixed with either 10mM or 20mM paraquat. Food was changed every 3-4 days. Survival was scored daily. Differences in survival were assayed using the Log-Rank test. Pairwise comparisons between samples were made using the Pairwise Log-Rank test and p-values were adjusted using the false discovery rate. These tests were performed using the survival package in R.

Immunoblots

Three thoraxes from adult female flies where homogenized in 1X Laemmli buffer. Immunoblots were performed as described in (*6*). Membranes were probed with mouse anti-total p38 MAPK (1:1000, Upstate), rabbit phospho p38 MAPK (1:1000, Cell Signaling Technologies), and rabbit anti-alpha tubulin (1:5000) and either goat anti-mouse HRP 1:20,000 (Jackson Labs) or goat anti-rabbit HRP 1:40,000 (Jackson Labs). Membranes were then developed using SuperSignal West Femto kit (ThermoFisher) or Pierce ECL (ThermoFisher) and exposed on autoradiography film. All immunoblots were performed at least in triplicate. Densitometry was performed using Adobe Photoshop. For each replicate, the densitometry value of each p38K band was normalized to its corresponding alpha tubulin control. These normalized values were then fit to linear regression models with each blot treated as a separate blocking factor and compared using ANOVA (implemented in R).

Interaction analysis

To test for a correlation between p38K protein levels and survival on paraquat, we calculated the Pearson's product-moment correlation between the change in protein levels in flies fed paraquat and the median survival following paraquat exposure. To test for a correlation between body size and survival on paraquat, we calculated the Pearson's product-moment correlation between body size of female flies (approximated by thorax length, (*19*)) and the

median survival following paraquat exposure. Analyses were performed in R and correlation data were plotted using ggplot2 in R.

To test the relationship between p38K protein levels, paraquat exposure and the presence of predicted transcription factor binding sites, we used a model in which paraquat exposure and site presence were independent variables and p38K level was the dependent variable. These data were compared using the Analysis of Variance of Aligned Rank Transformed Data method (implemented using the ARTool package in R). Interaction plots were generated using ggplot2 in R.

To test the relationship between the presence of predicted transcription factor binding sites and survival on paraquat, the data were compared using the Analysis of Variance of Aligned Rank Transformed Data method (ARTool package in R). Data were plotted using ggplot2 in R.

Results

Evolution of MAPK family genes across taxa

We first analyzed the relationship between the three MAPK families: ERK, JNK, and p38K across taxa (Tables S4-S6) as well as within a subset of the sequenced *Drosophila* species that are representative of the genus. While humans have multiple ERK and JNK family members, *D. melanogaster* only has one ortholog for each of these genes (*rolled* and *basket*, respectively). In addition, vertebrates have four p38K genes while *D. melanogaster* have three p38K orthologs (*p38Ka*, *p38Kb*, and *p38Kc*). We find that all three MAPK families emerged very early in animal evolution before the split between vertebrates and invertebrates (Figure 1A). In addition, the ERK and JNK families evolved from a shared ancestral gene, whereas the p38K family diverged earlier (Figure 1A). Within the p38K family, the three *D. melanogaste*r p38K genes are not orthologous to the distinct vertebrate p38K genes, suggesting they have evolved since the split between vertebrates and invertebrates (Figure 1A). Analysis of a subset of

Drosophila species reveals that these relationships are also conserved across the genus, with the species having single orthologs of ERK and JNK and multiple members of the p38K family (Figure 1B). This analysis further reveals that p38Kc is more recently diverged from p38Ka and p38Kb (Figure 1B). This is particularly interesting as 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, suggesting that p38Ka and p38Kc may be the result of a tandem gene duplication.

Evolution of p38K genes in Drosophila

To further understand the evolutionary relationship between the p38K genes in *D. melanogaster*, we compared the p38K sequences across 22 sequenced *Drosophila* species. We find that across species each p38K gene forms a distinct cluster (Figure S1). All three of the p38K genes mostly cluster along taxonomic lines as determined by molecular phylogeny (Figure 2A). Interestingly, unlike the other *Drosophila* species, *D. pseudoobscura* has two p38Kb genes (Figure 2C), suggesting a recent duplication of p38Kb in this species. As not all of the 22 sequenced *Drosophila* species have fully annotated genomes, we focused on 12 species that are fully sequenced and annotated to analyze the genomic architecture of the p38K loci. We find that the synteny of the p38Ka and p38Kc genes are highly conserved (Figure 3A), and their proximity suggesting that p38Kc most likely arose from a tandem duplication event of p38Ka*,* after the divergence of the willistoni group (Figures 2D and 3A). Furthermore, in *D. persimilis* p38Kc is truncated as compared to the p38Kc gene in other *Drosophila* species leading to a shortened p38Kc protein. Interestingly, *D. persimilis* also has three additional genes (GL24143, GL24139, GL24140) that have appeared near the p38Kc locus, disrupting its close proximity to p38Ka (Figure 3A). Both *D. sechellia* and *D. grimshawi* have independent duplications of a neighboring gene (CG6178), and *D. grimshawi* also has a new gene (GH19091) inserted into this region.

The synteny of the p38Kb locus is also highly conserved (Figure 3B). The melanogaster subgroup has an additional gene (CG43376) inserted into this region. Furthermore, much like the p38Ka and p38Kc locus, *D. persimilis* has an insertion of four genes (GL21221, GL21222, GL21223, and GL21095) as well as loss of the gene CG31731 in this region (Figure 3B). Though *D. pseudoobscura* has two p38Kb genes (Figure 2C), these genes reside on different chromosomes with p38Kb on the second chromosome (Muller element E) and p38Kb 2 on the fourth chromosome (Muller element B).

The p38K genes are under purifying selection

Since the three p38K genes are highly conserved but with p38Kc more diverged, we investigated if these genes show evidence of selection. We analyzed the dN/dS ratio for each of the p38K genes in 22 different species of *Drosophila* (Figure 4). Both p38Ka and p38Kb are under strong purifying selection with average dN/dS ratios of 0.0741 and 0.0479, respectively (Figure 4). This suggests that amino acid substitutions are not well tolerated in these genes and that they have evolved important functions that when disrupted lead to detrimental effects. Interestingly, the p38Ka and p38Kb proteins are also very similar to each other. For example, in *D. melanogaster* the two proteins are 78.4% identical and 94.5% similar at the amino acid level. Therefore, the limited amino acid differences between the p38Ka and p38Kb proteins may be important for their independent functions.

We also find that p38Kc is under purifying selection but has a higher dN/dS ratio (dN/dS ratio of 0.3346, Figure 4). These data suggest that p38Kc is less constrained than p38Ka and p38Kb, which allowed not only the diversity in amino acid sequence across species, but potentially the evolution of new functions. Furthermore, as p38Kc is under selective pressure, this suggests that these functions are also critical for the well-being of the organism.

Identification of conserved transcription factor binding sites for p38Ka and p38Kb

Though the *D. melanogaster* p38Ka and p38Kb genes are strikingly similar to each other and have some redundant functions (*6*–*8*), these genes have also been shown to have some independent functions (*6*–*11*, *16*). We have also previously reported that the p38Kb protein is highly expressed in muscle and brain, whereas p38Ka is expressed at lower levels in these tissues (*6*), which may contribute to the specific functions of each of these genes. Therefore, we analyzed 1kb upstream of each gene for putative transcription factor binding sites in the 12 *Drosophila* species. We find that p38Ka has four consensus transcription factor binding sites (Figure 5A). Three of these motifs are mostly conserved across *Drosophila* species within the melanogaster group. The first site is a consensus homeobox binding site, which can be bound by members of the Hox family of transcription factors. The other two conserved binding sites are consensus sequences for two isoforms of the transcription factor lola: lola-PO and lola-PK. The final binding site (motif 3) is for an unknown transcription factor and is found only in *D. erecta*.

In comparison, p38Kb has five consensus transcription factor binding sites (Figures 5B and S2) that are conserved across the melanogaster and obscura species groups. Similar to p38Ka, p38Kb has two consensus sites for isoforms of the lola transcription factor, lola-PT and lola-PO. p38Kb also has a widely conserved site which corresponds to the AP-1 transcription factor binding site. The final two motifs represent binding sties for unknown transcription factors. Motif 4 is conserved within the obscura group and also in the more distantly related species *D. takahashii*. Motif 5 may be a binding site that has been repeatedly gained or lost over evolutionary history within the melanogaster species group. Interestingly, Motif 5 has been lost in *D. melanogaster* but retained in its closely related sister species *D. simulans, D. mauritiana* and *D. sechellia*. The lola-PO binding site, on the other hand, is restricted to *D. melanogaster* and its sister species. Furthermore, we identified both AP-1 and lola-PT binding sites in most of the species analyzed.

Both the p38Ka and p38Kb loci include binding sites for transcription factors that play a role in development (the Hox family and AP-1 (*28*–*31*)). Though loss of either p38Ka or p38Kb is viable, double knockout animals are inviable (*6*–*9*, *11*), suggesting that p38K signaling may play an important role in developmental processes much like in mammalian systems (*32*). In addition to its role in development, AP-1, a heterodimer of the transcription factors jun and fos, also plays a role in immune functions (*33*, *34*) and oxidative stress response (*35*). Interestingly, p38Kb has been shown to phosphorylate jun (*36*), suggesting that p38Kb expression may be regulated through a feedback loop.

lola is a key regulator of axon guidance (*37*–*39*). However, lola has also been shown to act in the ovary to regulate programmed cell death in both developmental and starvation conditions (*40*). Furthermore, the ModENCODE project demonstrated that lola is highly expressed in response to adult exposure to the oxidizing agent paraquat (*41*, *42*), though the role of specific isoforms of lola in oxidative stress are not well characterized.

AP-1 and lola-PT regulate the expression of p38Kb in D. melanogaster

Oxidative stress is known to induce the phosphorylation and thus activation of p38K proteins in a variety of systems including *D. melanogaster*. However, the presence of both the AP-1 and lola isoform sites suggests that p38K transcription may also be regulated by oxidative stress. Therefore, we tested if these conserved transcription factor binding sites are important for the regulation of the p38Kb gene. We focused on the AP-1 and lola-PT binding sites since they are exclusive to p38Kb and have links to MAPK signaling pathways and/or oxidative stress. We over-expressed either AP-1 or lola-PT in the muscle using the MHC-GAL4 driver as p38Kb has been shown to act in this tissue to regulate viability and the oxidative stress response (*6*). We find that over-expression of AP-1 is able to induce expression of p38Kb under normal conditions, but not when the flies are exposed to the oxidizing agent paraquat (Figure 6A-B). Over-expression of lola-PT induced p38Kb expression in both normal and oxidative stress

conditions (Figure 6C-D). These data suggest that both AP-1 and lola-PT are capable of mediating p38Kb transcription and that lola-PT may be playing a role in the oxidative stress response by promoting the transcription of p38Kb during stress conditions. As transgenic over-expression of p38Kb in the muscle leads to increased lifespan (*6*), we next tested the effect of AP-1 and lola-PT on lifespan. We find that neither over-expression of AP-1 or lola-PT alters lifespan (Figure S3, Table S7, χ^2=1.8, p-value=0.4 and χ^2= 5.5, p-value=0.06, respectively), suggesting that the levels of increased p38Kb transcription induced by AP-1 or lola-PT are not sufficient to extend lifespan. Over-expression of p38Kb is also protective against oxidative stress exposure (*6*). Therefore, we assessed the effects of AP-1 and lola-PT over-expression on resistance to paraquat. We find that over-expression of AP-1 leads to increased survival in response to both high (20mM) or low (10mM) paraquat exposure (Figure 6E and G, Tables S8 and S9). Interestingly, lola-PT over-expression also leads to increased survival in response to oxidative stress but only in response to low dose paraquat exposure (Figure 6F and H, Tables S8 and S9). These data suggest that AP-1 and lola-PT are able to induce sufficient levels of p38Kb that leads to a readily available pool of p38K that can be activated and induce the oxidative stress response. However, lola-PT mediated protection is limited to the degree of oxidative stress exposure.

Assessing the relationship between p38K and survival across species

To explore the evolution of oxidative stress response across *Drosophila*, we tested the resistance of 7 species to exposure with the oxidizing agent paraquat. We find that there are significant differences in survival between different species (Figure 7A and Table S10). Interestingly, *D. virilis*, the largest species in our study (*19*) did not have the longest survival time on paraquat (Figure 7A and Table S10). Accordingly, we find that body size and paraquat resistance are only weakly correlated (Figure S4, R=0.44, p-value=0.4529), suggesting that body size is not a major determinant of stress resistance.

We find that of the studied species, *D. pseudoobscura* showed the longest survival in response to paraquat (Figure 7A and Table S10). This finding is especially interesting as *D. pseudoobscura* has two copies of the p38Kb gene, suggesting that p38Kb may be important for stress resistance and that both copies may contribute to this response. To examine the potential role of p38K, flies were exposed to either control food or food mixed with 20mM paraquat and collected at an early and late timepoint based on the average survival time for that species. We performed immunoblots to analyze changes in the levels of p38K protein. We observe a decrease in p38K protein levels on control food with time across the species (Figure S5A ($p = 0.026$), and we find no significant differences between species ($p = 0.40$), suggesting that in general, p38K protein levels decline with age in *Drosophila* species.

When flies were subjected to prolonged paraquat exposure, we find an increase in the levels of p38K in *D. melanogaster*, *D. mauritiana*, and *D. pseudoobscura* ($\log_2$ fold change greater than 0.5), a lack of change in *D. simulans* and *D. virilis* ($\log_2$ fold change between -0.5 and 0.5), and decreased levels in *D. yakuba* and *D. ananassae* ($\log_2$ fold change less than -0.5, Figure 7B).

We next tested if the changes in p38K protein levels are associated with species survival when exposed to paraquat. We find that there is a weak positive correlation between the change in p38K levels and paraquat resistance (Figure 7C, $R = 0.52$, $p = 0.23$). When we categorized the species by changes in total p38K as increased, unchanged, or decreased, we find that decreased levels of p38K are associated with poor survival on paraquat when compared to those species with neutral or increased p38K (Figure 7D, p-value=0.00429).

It has been well documented that oxidative stress induces the activation of p38K through its phosphorylation (*43*). We performed Western blots on our samples using an anti-phosphorylated p38K antibody and find that across species and conditions, there is a linear relationship between the changes in levels of total and phosphorylated forms of p38K (Figure S5B-D).

Paraquat resistance and p38K protein levels across Drosophila taxa are associated with AP-1 and lola-PT binding sites

Since over-expression of either AP-1 or lola-PT is able to induce p38Kb transcription in *D. melanogaster*, we next tested if having a conserved AP-1 and/or lola-PT site is important for paraquat resistance. Therefore, we tested 7 species of *Drosophila* with different combinations of these sites (Figure 5B) for oxidative stress resistance. Two of these species, *D. simulans* and *D. mauritiana*, are sister species of *D. melanogaster* that have both the AP-1 and lola-PT binding sites as well as a conserved motif not found in *D. melanogaster*. *D. yakuba* is also a member of the melanogaster subgroup and has the AP-1 binding site but not the lola-PT site. *D. pseudoobscura* is a more distantly related species and has two p38Kb genes both of which have AP-1 and lola-PT binding sites. Finally, we tested two species that do not have the conserved AP-1 or lola-PT sites, *D. ananassae* and *D. virilis*, which is the most distantly related of the species. In order to assess the roles of the AP-1 and lola-PT binding sites, we used ANOVA on Aligned Rank Transformed data to test for interactions between the presence of a transcription factor binding site and p38K protein levels.

Though p38K levels tend to decrease across time (Figure S5), we find that the presence of an AP-1 site is associated with a smaller decrease in p38K levels under standard conditions (Figure 8A, $p=0.037$). In addition, the AP-1 site is also associated with increased levels of p38K on paraquat food (Figure 8A, $p = 0.037$), however, there is no interaction between AP-1 site presence and food treatment (Figure 8A , $p = 0.175$), suggesting that AP-1 regulation of p38K levels is not responsive to paraquat treatment, in line with our qPCR results (Figure 6A-B). The presence of a lola-PT binding site leads to increased levels of p38K on paraquat but not control food (Figure 8B), and significantly interacts with treatment (Figure 8B, $p = 0.013$), suggesting that lola-PT is regulating p38K levels in response to oxidative stress. We find that the presence

of an AP-1 or lola-PT binding site shows a positive trend toward increasing median survival on paraquat food, although these effects fall just short of significance (Figure 8C-D, $p = 0.098$ for AP-1, $p = 0.069$ for lola-PT). These data suggest that AP-1 plays a role in the normal regulation of p38Kb transcription, whereas lola-PT acts in response to oxidative stress to increase p38Kb transcription, and that both modes of regulation can contribute to p38K-mediated oxidative stress resistance. Additionally, we find that across species the presence of lola-PO, motif 4 or motif 5 has no significant interaction with p38K protein levels under any conditions (Table S11).

Discussion

A common theme in biology is the repurposing of signaling pathways to perform new cellular functions. Evolutionary pressures can act on these signaling pathways in various ways leading to sequence divergence, gene duplications or the creation/loss of regulatory elements. In order to understand how these pressures may have influenced an organism's ability to respond to oxidative stress, we utilized the power of the *Drosophila* genome sequencing projects, which have resulted in the sequencing of 22 *Drosophila* species, 12 of which have been fully annotated. We undertook a molecular genomics approach to analyze the genomic architecture of the conserved p38K genes across the genus *Drosophila* which represents 30-40 million years of evolutionary history (*44*). We find a recent duplication event most likely of p38Ka resulted in formation of the p38Kc gene, which has diverged from both p38Ka and p38Kb and has potentially developed new functions as has been observed in *D. melanogaster* (*6–11*, *13–18*, *36*, *45*, *46*). In addition, analysis of the 1kb upstream region revealed loss and gain of transcription factor binding sites for both the p38Ka and p38Kb loci. We find that p38Ka and p38Kb both share lola-PO binding sites, which likely plays a role in regulating the expression of both p38Ka and p38Kb in shared functions. Additionally, each gene has a specific set of

transcription factor binding sites that may play a role in regulating the expression of these genes for specific functions.

To determine if this bioinformatics approach identified biologically relevant regulatory elements, we tested the function of these sites *in vivo* using the genetically amenable species *D. melanogaster*. We find that the p38Kb transcription factor binding sites for both AP-1 and lola-PT are functional as over-expression of AP-1 and lola-PT induce expression of p38Kb under control conditions. In addition, we also found that these sites play different roles in the regulation of p38Kb as only over-expression of lola-PT was able to further induce expression of p38Kb in the presence of the oxidizing agent paraquat. Intriguingly, we find that over-expression of AP-1 in which flies are aged a week before being exposed to paraquat leads to increased resistance compared to lola-PT over-expression. These data suggest that AP-1 may be able to transcribe sufficient amounts of p38Kb pre-exposure that results in a protective effect once the animal encounters an oxidizing environment. Thus, AP-1 and lola-PT are playing unique roles in the regulation of p38Kb expression and this may, in turn, help the organism as it navigates environmental changes. To determine if these sites may play similar roles in other *Drosophila* species, we tested how species with differing combinations of the AP-1 and lola-PT binding sites respond to oxidative stress. Similar to our results with *D. melanogaster*, we find a significant interaction between the presence of an AP-1 site and increased levels of p38K protein regardless of the oxidative stress state, whereas the presence of a lola-PT binding site shows a significant interaction with p38K protein levels only in the presence of oxidative stress. Furthermore, we found a positive trend between the presence of these sites and increased survival. However, these results were not significant, likely due to the limited number of species identified with different combinations of AP-1 and lola-PT binding sites that we could test. Overall our data suggest that AP-1 and lola-PT act in a similar manner across species to regulate p38Kb expression. Much of what is known about how p38K proteins regulate stress responses has focused on the role of post-translational modifications, in particular, the dual

phosphorylation of p38K (*2*, *3*, *5*), and little is known about the role of transcriptional regulation in this process. We find that over-expression of either AP-1 or lola-PT is sufficient to increase p38Kb transcription under non-stress conditions and confer resistance to subsequent oxidative stress. These findings suggest that transcriptional regulation of p38Kb can also play an important role in regulating the oxidative stress response, and the transcription of p38Kb in response to oxidative stress may complement the effects of p38Kb phosphorylation in promoting resistance. However, more research will be necessary to understand the relationship between p38Kb induced transcription and subsequent phosphorylation and how this supports the oxidative stress response.

Our results suggest that we can successfully utilize the genomes of sequenced species to identify biologically relevant regulatory elements that may give insights into how different species have evolved mechanisms for responding to selective pressures. Furthermore, as little is known about the transcriptional regulation of other MAP Kinases, our comparative genomics approach may be utilized to identify conserved transcription factor binding sites that may play important roles in regulating these MAP Kinases and potentially other key signaling genes in response to a variety of cellular or environmental stimuli.

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Figure Legends

Figure 1. Phylogenetic Analysis of the MAPK Family. A) The evolutionary relationship between the ERK1/2 (blue), JNK (black), and p38 (red) MAPK genes from *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans,* and *Saccharomyces cerevisiae*. Note that HOG1 is the *Saccharomyces cerevisiae* homologue of both JNK and p38. B) The evolutionary relationship between the ERK1/2 (blue), JNK (black), and p38 (red) MAPK genes from representative species in the genus *Drosophila*.

Figure 2. Phylogenetic Analysis of each p38K gene in *Drosophila*. The evolutionary relationships of the A) ITS2, B) p38Ka, C) p38Kb, and D) p38Kc genes in 22 sequenced species of *Drosophila*. Red denotes the melanogaster species group, blue denotes the obscura group, black denotes the willistoni group, and grey represents the *Drosophila* subgenus. Note that *D. pseudoobscura* is the only species with 2 copies of the p38Kb gene. p38Kc arose after the split of the obscura group from *D. willistoni*.

Figure 3. Synteny of the p38K genes in *Drosophila*. A) p38Ka and p38Kc lie in close proximity to each other. *D. persimilis* has additional genes present in this locus as well as a truncated p38Kc gene. *D. sechellia* has a duplication of CG6178 as does *D. grimshawi*, which also has an additional gene inserted into the locus. Backslashes indicate additional inserted DNA with no predicted genes. B) p38Kb synteny is also highly conserved. Within the

melanogaster subgroup is the appearance of CG43376. *D. persimilis* has an insertion of 4 genes within this locus and is lacking the CG31731 gene. *D. pseudoobscura* has two p38Kb genes, though the second p38Kb gene is found on a different chromosome.

Figure 4. The p38K genes are under purifying selection. Plot of the dN and dS values for the p38K family genes (p38Ka shown in red circles, p38Kb in blue triangles and p38Kc in black crosses). Each spot represents a pairwise comparison between two species.

Figure 5. Transcription factor binding sites upstream of p38Ka and p38Kb. The 1Kb upstream region for A) p38Ka and B) p38Kb was analyzed for conserved transcription factor binding sites.

Figure 6. AP-1 and Iola-PT mediate p38Kb transcription and oxidative stress resistance. Levels of p38Kb transcription in response to over-expression of AP-1 under A) control conditions and B) 20mM paraquat. Levels of p38Kb transcription in response to over-expression of Iola-PT under C) control conditions and D) 20mM paraquat. Resistance to 20mM paraquat was measured for E) AP-1 (red) or F) Iola-PT (red) over-expression flies. Resistance to 10mM paraquat G) AP-1 (red) or H) Iola-PT (red) over-expression flies. Outcrossed GAL4 and transgene controls are in black and grey, respectively.

Figure 7. Oxidative stress is correlated with p38K protein levels. A) Kaplan-meier plot of species survival during exposure to 20mM paraquat. B) Log_2 fold change of levels of p38K protein during paraquat exposure. C) Scatterplot of the correlation between p38K protein levels and median survival time. D) Median survival of *Drosophila* species with decreased, unchanged or increased levels of p38K during paraquat exposure. a,b indicate significance groups with $p < 0.05$.

Figure 8. The presence of AP-1 and lola-PT binding sites are associated with p38K protein levels. A) The presence of an AP-1 site is associated with increased levels of p38K protein but does not interact with food treatment. B) The presence of a lola-PT site is associated with increased p38K levels during paraquat treatment and interacts with paraquat treatment . Median survival of *Drosophila* species with C) AP-1 and D) lola-PT sites.

Figure S1. Phylogenetic tree of the p38K genes in *Drosophila*. The evolutionary relationships between all three p38K genes across 22 species of *Drosophila*. Black denotes the p38Ka genes, red the p38Kb genes, and grey the p38Kc genes.

Figure S2. Consensus sequences the p38Kb transcription factor binding sites. The consensus sequence of the A) lola-PT, B) AP-1, C) lola-PO, D) Motif 4, and E) Motif 5 transcription factor binding sites. For each binding site, the size of the letter indicates the proportion of confirmed sites that use that base.

Figure S3. Over-expression of AP-1 or lola-PT has no effect on lifespan.
A) Over-expression of AP-1 (red) or B) lola-PT (red) reared on control food has no significant effect on lifespan as compared to outcrossed GAL4 (black) and transgene (grey) controls.

Figure S4. Body size is weakly correlated with paraquat resistance. Scatterplot of correlation between female thorax length and median survival.

Figure S5. Levels of total and phosphorylated p38K have a linear relationship.

A) Log_2 fold change of levels of p38K protein on control food. Scatter plots showing the relationship between changes in total and phosphorylated p38K under B) all conditions, C) control conditions and D) exposure to 20mM paraquat.

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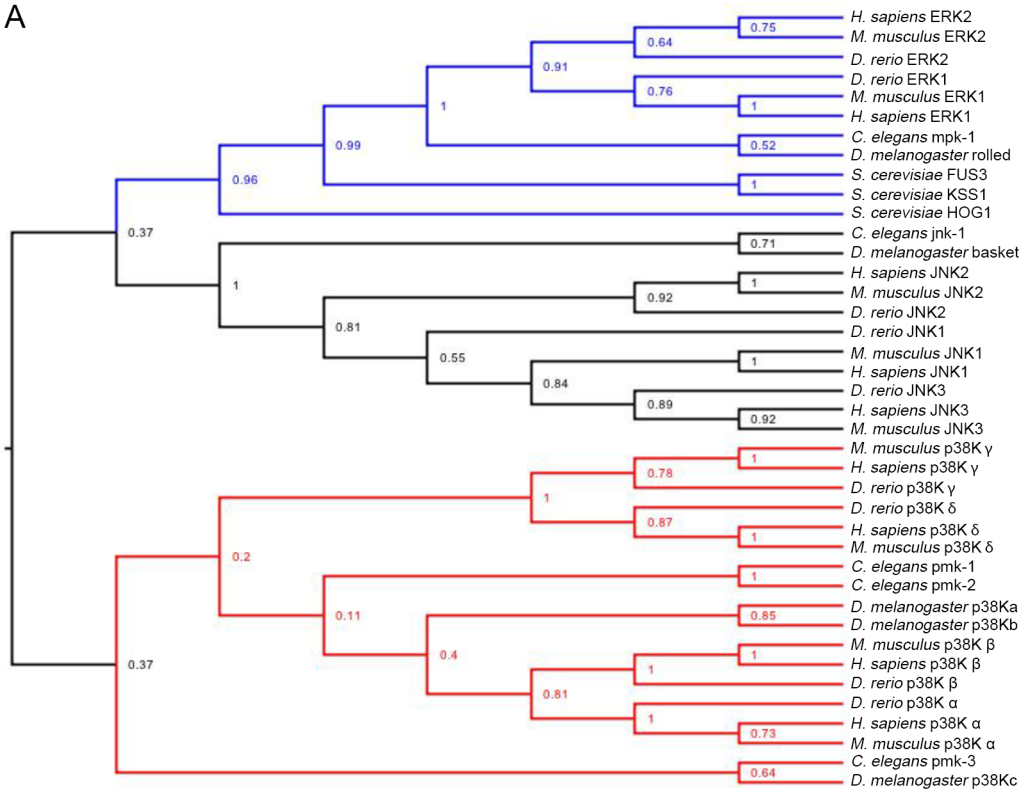
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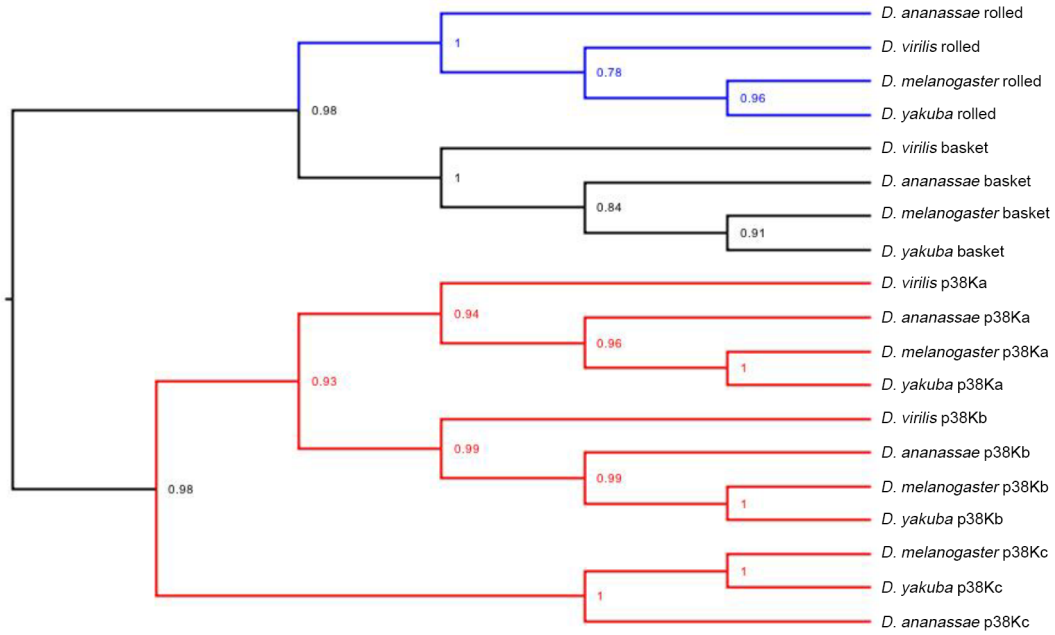
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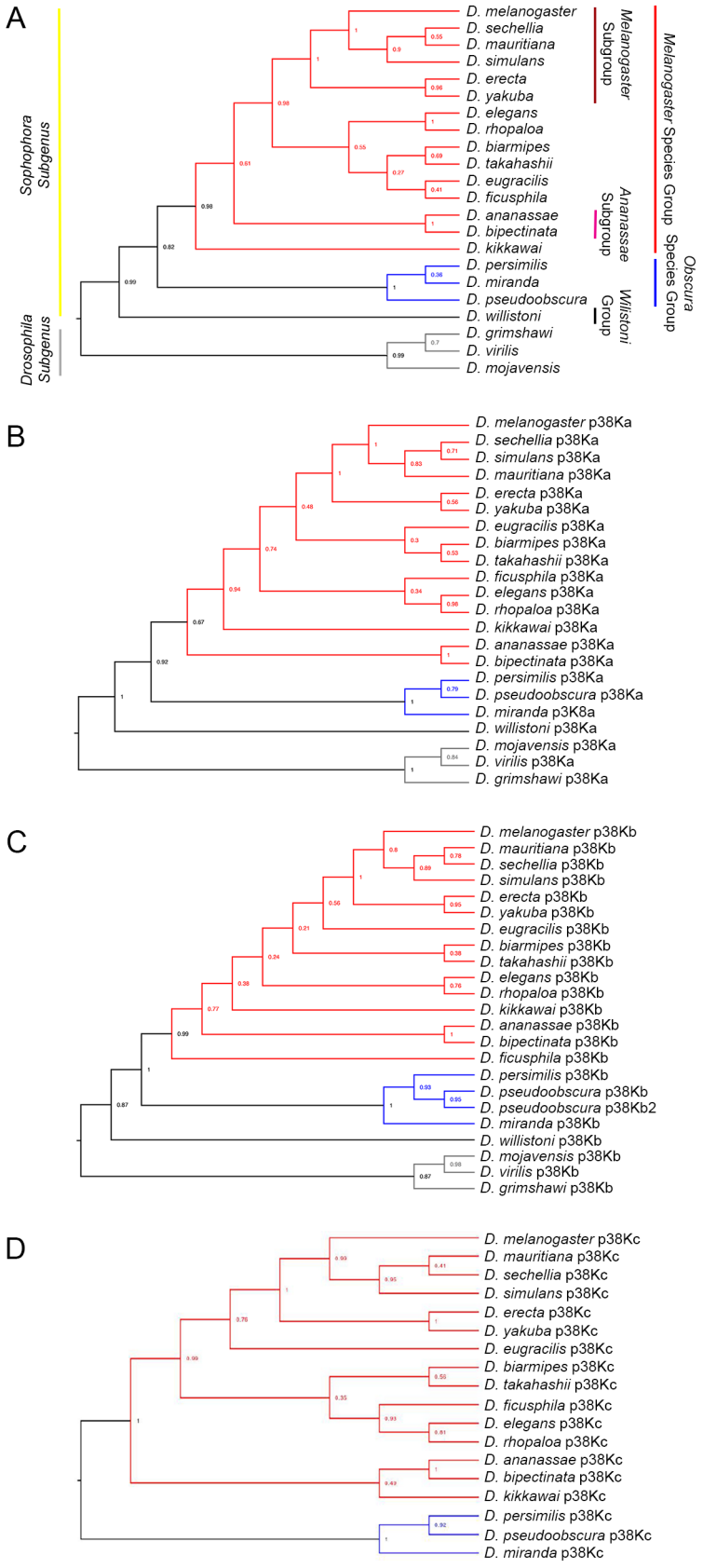
Figure 1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D. ficusphila
D. ananassae
D. bipectinata
D. kikkawai
D. persimilis
D. miranda
D. pseudoobscura
D. willistoni
D. grimshawi
D. virilis
D. mojavensis
Melanogaster Subgroup
Ananassae Subgroup
Willistoni Group
Melanogaster Species Group
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D. kikkawai p38Kc
D. persimilis p38Kc
D. pseudoobscura p38Kc
D. miranda p38Kc

Figure 2

A

Drosophila melanogaster
Drosophila simulans
Drosophila sechellia
Drosophila yakuba
Drosophila erecta
Drosophila ananassae
Drosophila pseudoobscura
Drosophila persimilis
Drosophila willistoni
Drosophila mojavensis
Drosophila virilis
Drosophila grimshawi
Slimp
GL24143
p38c
GL24139
GL24140
p38a
CG6178
GH19091
Myo95E

B

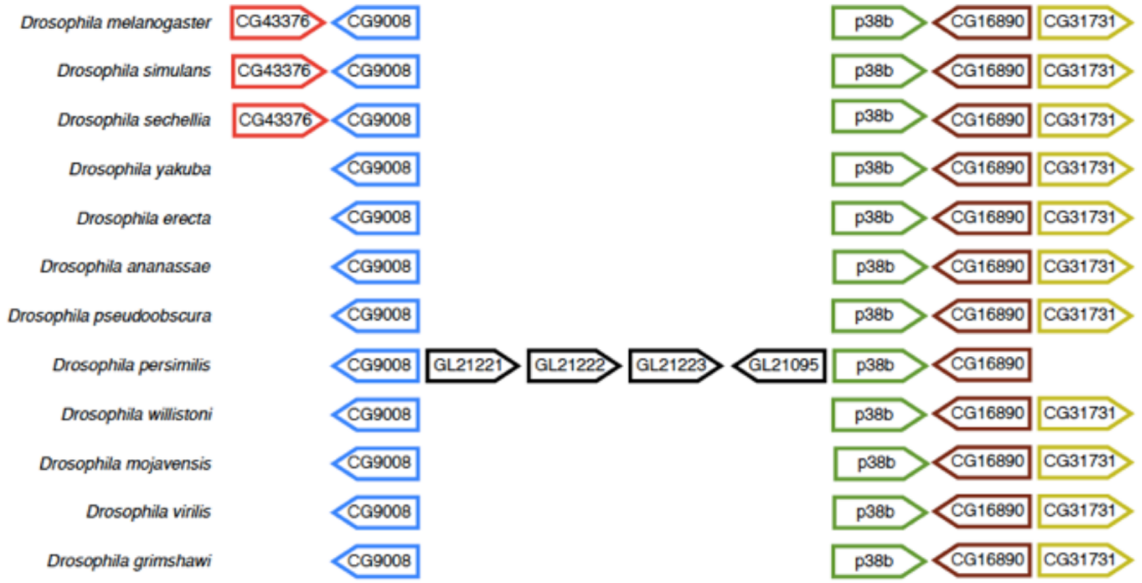
Drosophila melanogaster
Drosophila simulans
Drosophila sechellia
Drosophila yakuba
Drosophila erecta
Drosophila ananassae
Drosophila pseudoobscura
Drosophila persimilis
Drosophila willistoni
Drosophila mojavensis
Drosophila virilis
Drosophila grimshawi
CG43376
CG9008
GL21221
GL21222
GL21223
GL21095
p38b
CG16890
CG31731

Figure 3

A

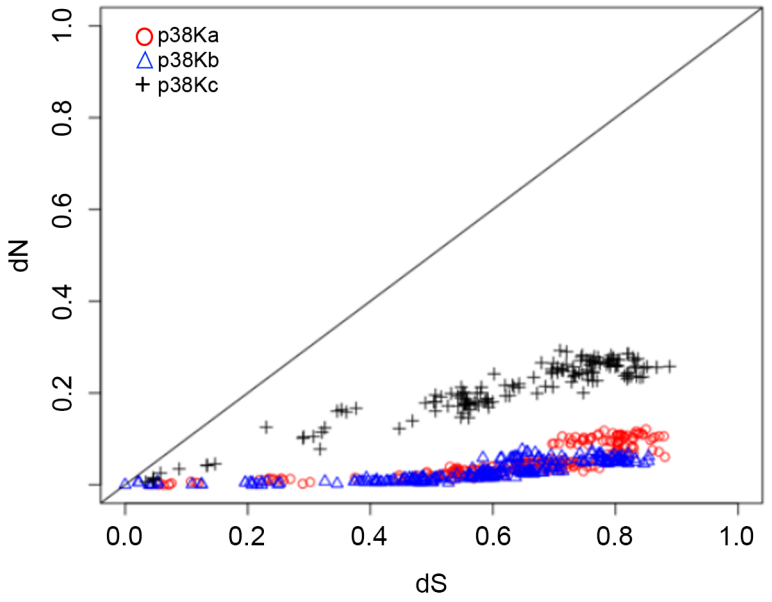

Figure 4

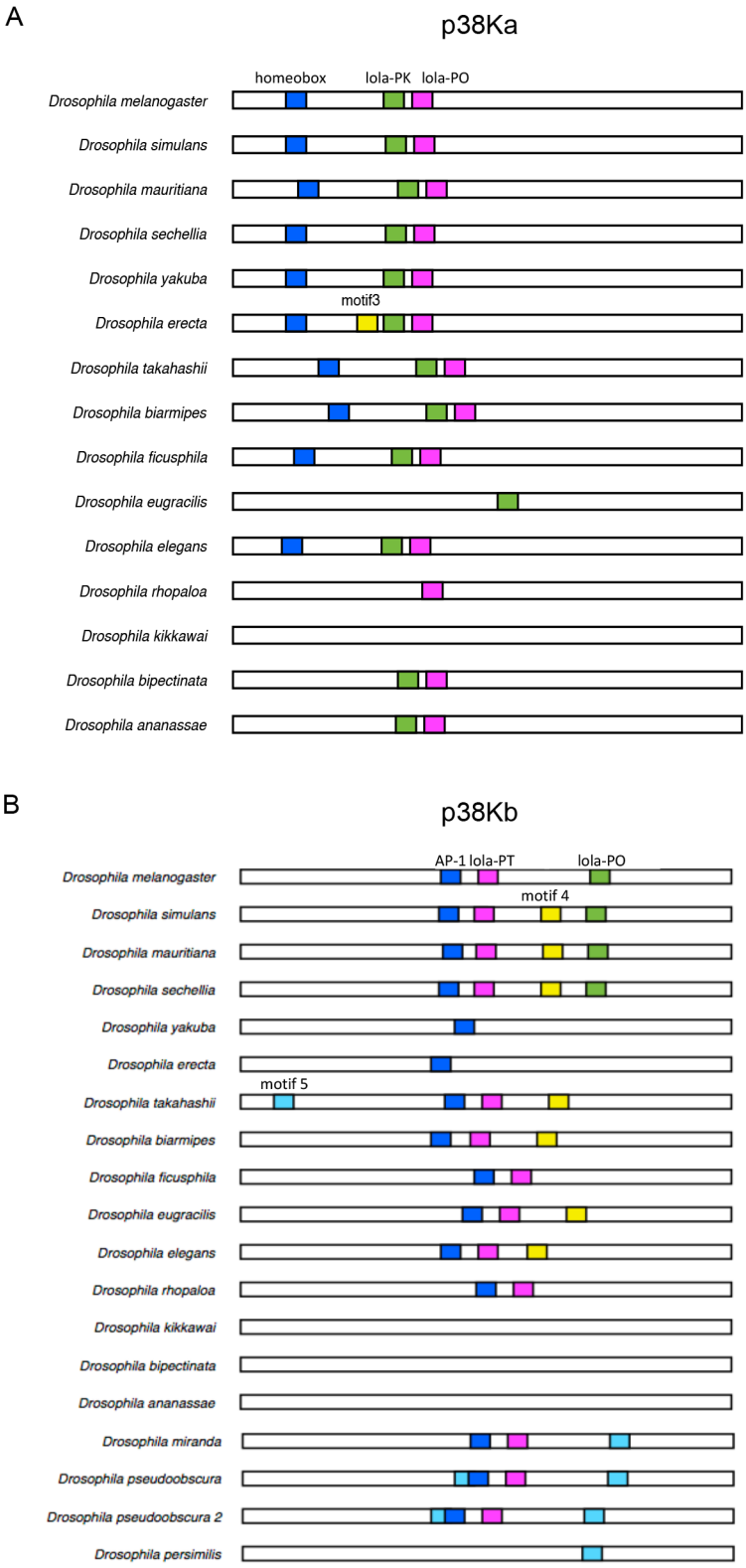
A
p38Ka
homeobox
lola-PK
lola-PO
motif3
Drosophila melanogaster
Drosophila simulans
Drosophila mauritiana
Drosophila sechellia
Drosophila yakuba
Drosophila erecta
Drosophila takahashii
Drosophila biarmipes
Drosophila ficusphila
Drosophila eugracilis
Drosophila elegans
Drosophila rhopaloa
Drosophila kikkawai
Drosophila bipectinata
Drosophila ananassae
B
p38Kb
AP-1
lola-PT
lola-PO
motif 4
motif 5
Drosophila melanogaster
Drosophila simulans
Drosophila mauritiana
Drosophila sechellia
Drosophila yakuba
Drosophila erecta
Drosophila takahashii
Drosophila biarmipes
Drosophila ficusphila
Drosophila eugracilis
Drosophila elegans
Drosophila rhopaloa
Drosophila kikkawai
Drosophila bipectinata
Drosophila ananassae
Drosophila miranda
Drosophila pseudoobscura
Drosophila pseudoobscura 2
Drosophila persimilis

Figure 5

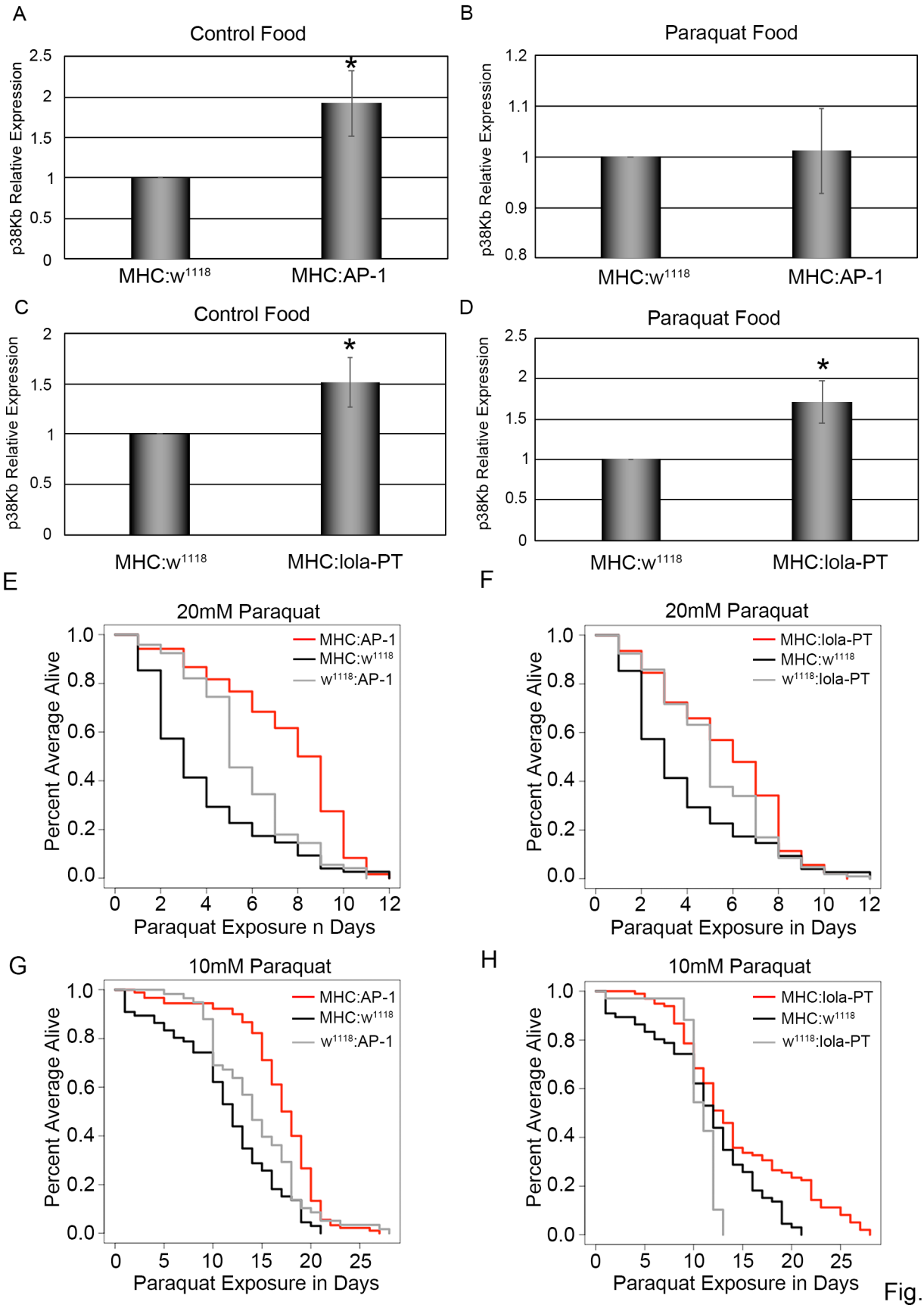
A
Control Food
p38Kb Relative Expression
2.5
2
1.5
1
0.5
0
*
MHC:w1118
MHC:AP-1
B
Paraquat Food
p38Kb Relative Expression
1.2
1.1
1
0.9
0.8
MHC:w1118
MHC:AP-1
C
Control Food
p38Kb Relative Expression
2
1.5
1
0.5
0
*
MHC:w1118
MHC:lola-PT
D
Paraquat Food
p38Kb Relative Expression
2.5
2
1.5
1
0.5
0
*
MHC:w1118
MHC:lola-PT
E
20mM Paraquat
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
MHC:AP-1
MHC:w1118
w1118:AP-1
0
2
4
6
8
10
12
Paraquat Exposure n Days
F
20mM Paraquat
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
MHC:lola-PT
MHC:w1118
w1118:lola-PT
0
2
4
6
8
10
12
Paraquat Exposure in Days
G
10mM Paraquat
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
MHC:AP-1
MHC:w1118
w1118:AP-1
0
5
10
15
20
25
Paraquat Exposure in Days
H
10mM Paraquat
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
MHC:lola-PT
MHC:w1118
w1118:lola-PT
0
5
10
15
20
25
Paraquat Exposure in Days

Fig. 6

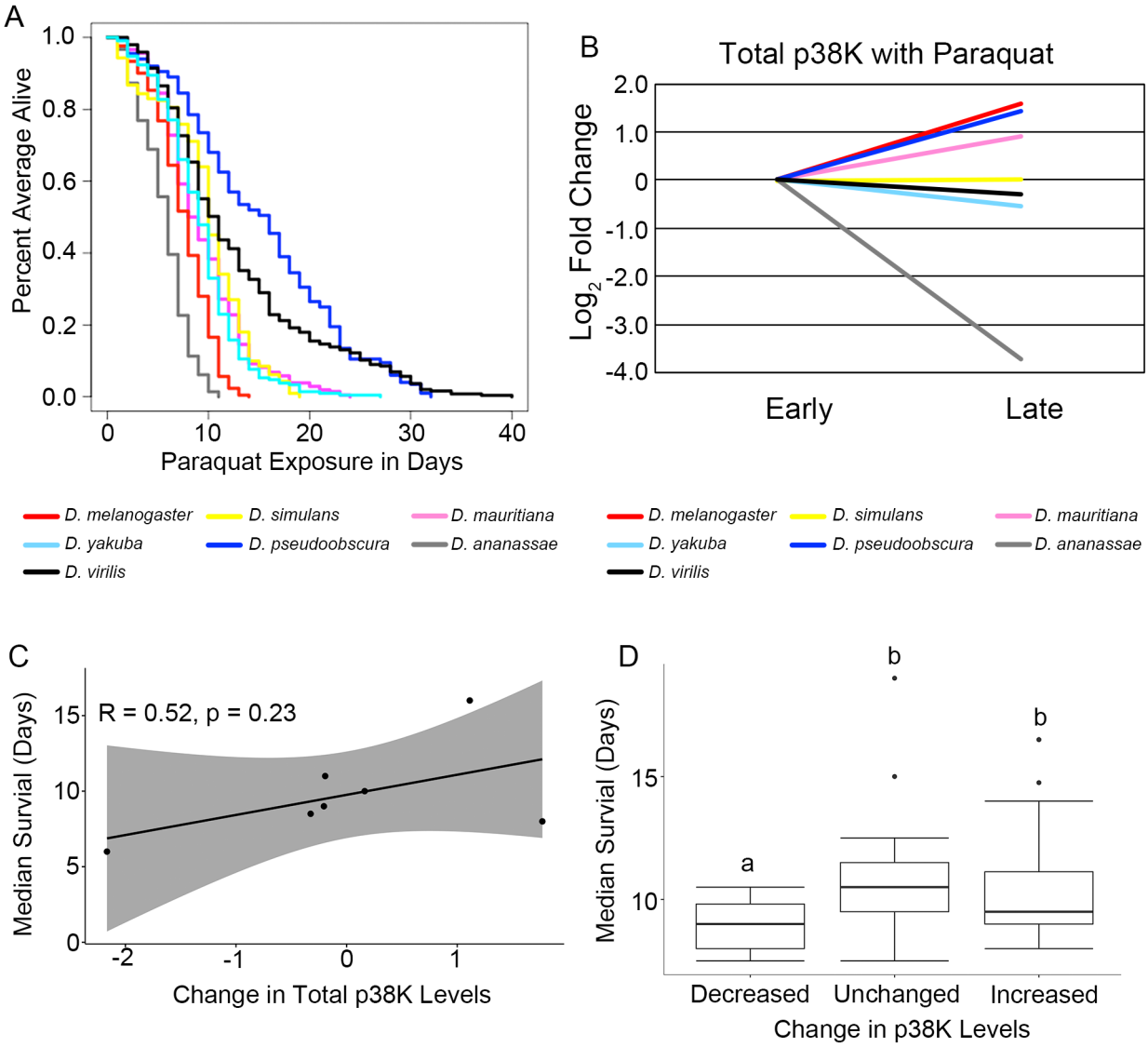
A
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Paraquat Exposure in Days
D. melanogaster
D. simulans
D. mauritiana
D. yakuba
D. pseudoobscura
D. ananassae
D. virilis
B
Total p38K with Paraquat
Log2 Fold Change
2.0
1.0
0
-1.0
-2.0
-3.0
-4.0
Early
Late
D. melanogaster
D. simulans
D. mauritiana
D. yakuba
D. pseudoobscura
D. ananassae
D. virilis
C
R = 0.52, p = 0.23
Median Survial (Days)
15
10
5
0
-2
-1
0
1
Change in Total p38K Levels
D
Median Survial (Days)
15
10
a
b
b
Decreased
Unchanged
Increased
Change in p38K Levels

Figure 7

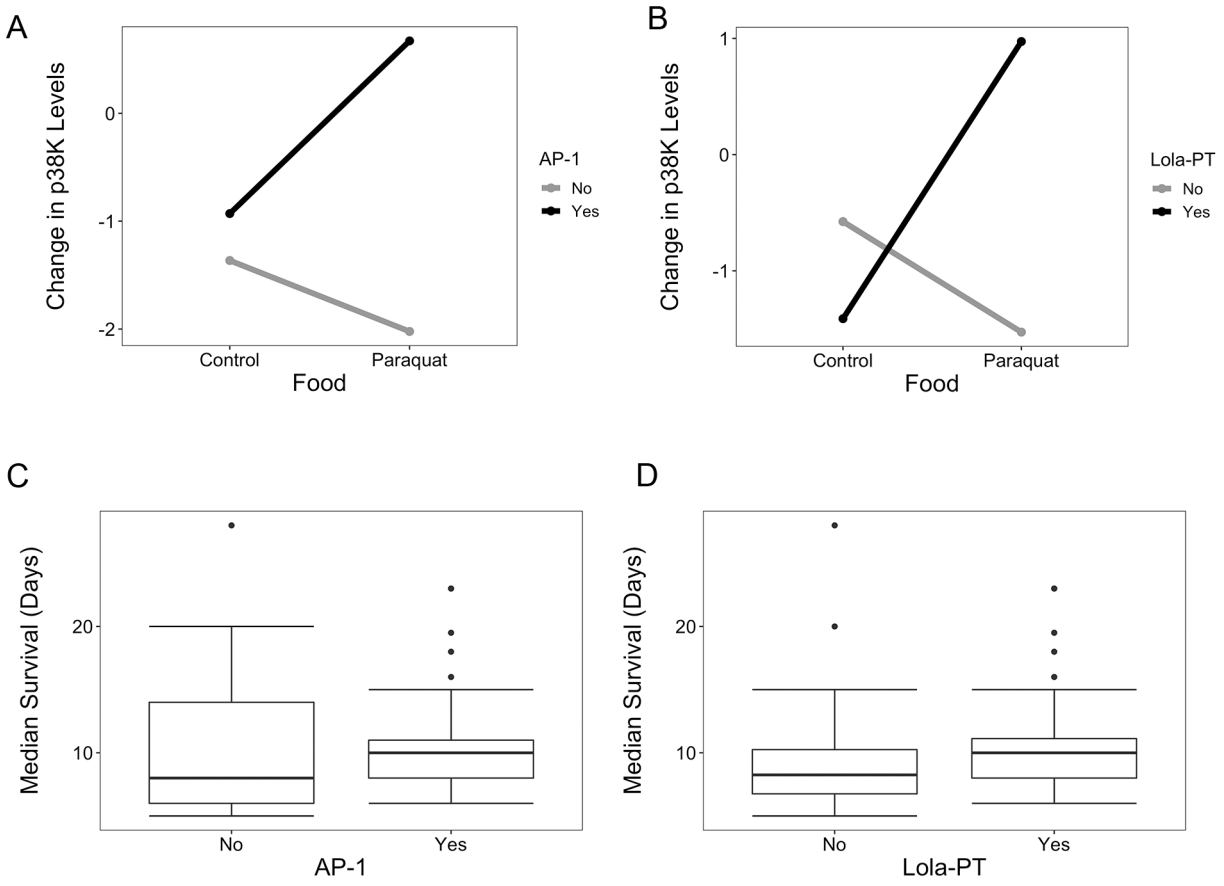
A
Change in p38K Levels
0
-1
-2
Control
Paraquat
Food
AP-1
No
Yes
B
Change in p38K Levels
1
0
-1
Control
Paraquat
Food
Lola-PT
No
Yes
C
Median Survival (Days)
20
10
No
Yes
AP-1
D
Median Survival (Days)
20
10
No
Yes
Lola-PT

Figure 8

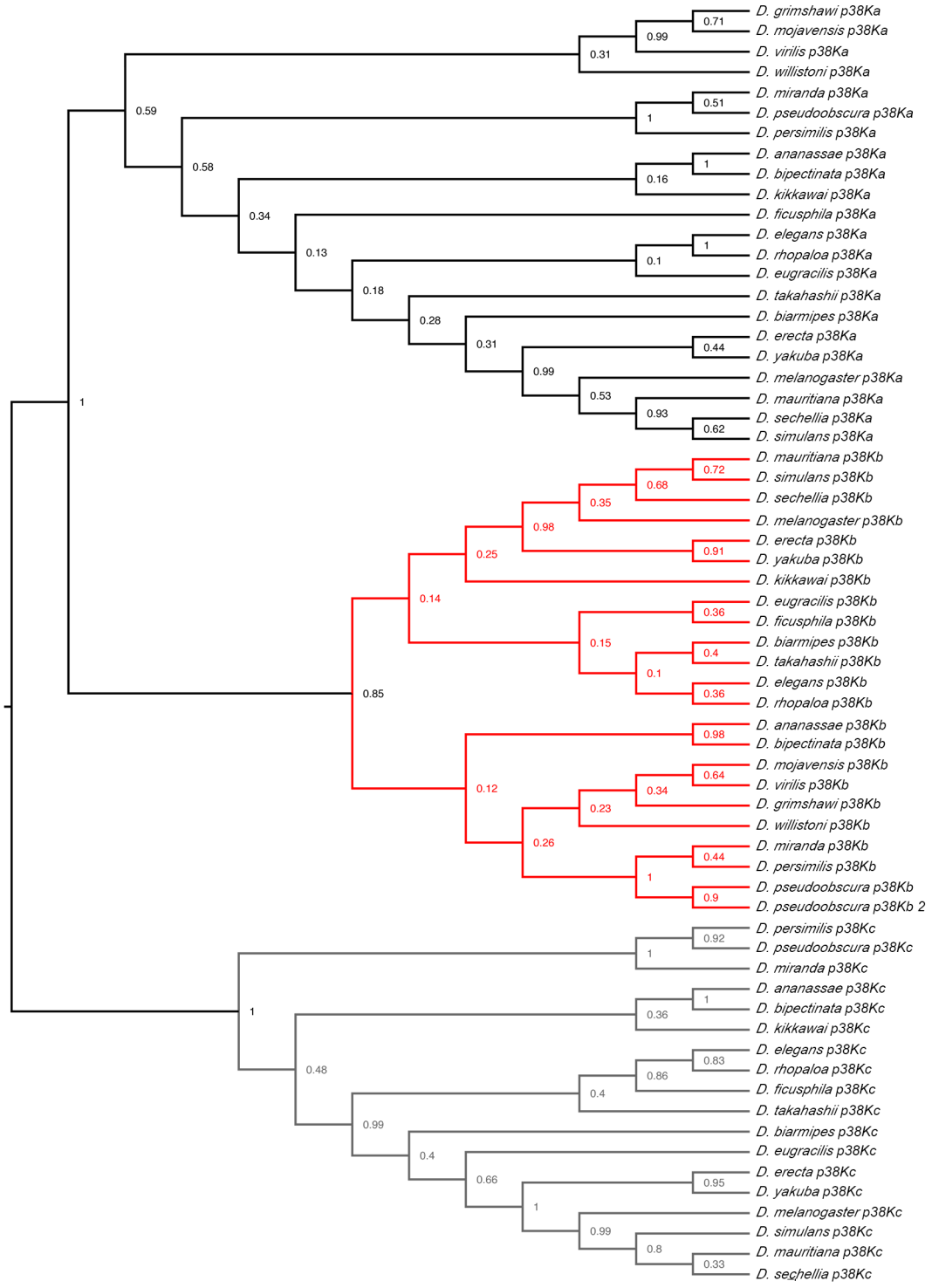
D. grimshawi p38Ka
D. mojavensis p38Ka
D. virilis p38Ka
D. willistoni p38Ka
D. miranda p38Ka
D. pseudoobscura p38Ka
D. persimilis p38Ka
D. ananassae p38Ka
D. bipectinata p38Ka
D. kikkawai p38Ka
D. ficusphila p38Ka
D. elegans p38Ka
D. rhopaloa p38Ka
D. eugracilis p38Ka
D. takahashii p38Ka
D. biarmipes p38Ka
D. erecta p38Ka
D. yakuba p38Ka
D. melanogaster p38Ka
D. mauritiana p38Ka
D. sechellia p38Ka
D. simulans p38Ka
D. mauritiana p38Kb
D. simulans p38Kb
D. sechellia p38Kb
D. melanogaster p38Kb
D. erecta p38Kb
D. yakuba p38Kb
D. kikkawai p38Kb
D. eugracilis p38Kb
D. ficusphila p38Kb
D. biarmipes p38Kb
D. takahashii p38Kb
D. elegans p38Kb
D. rhopaloa p38Kb
D. ananassae p38Kb
D. bipectinata p38Kb
D. mojavensis p38Kb
D. virilis p38Kb
D. grimshawi p38Kb
D. willistoni p38Kb
D. miranda p38Kb
D. persimilis p38Kb
D. pseudoobscura p38Kb
D. pseudoobscura p38Kb 2
D. persimilis p38Kc
D. pseudoobscura p38Kc
D. miranda p38Kc
D. ananassae p38Kc
D. bipectinata p38Kc
D. kikkawai p38Kc
D. elegans p38Kc
D. rhopaloa p38Kc
D. ficusphila p38Kc
D. takahashii p38Kc
D. biarmipes p38Kc
D. eugracilis p38Kc
D. erecta p38Kc
D. yakuba p38Kc
D. melanogaster p38Kc
D. simulans p38Kc
D. mauritiana p38Kc
D. sechellia p38Kc

Figure S1

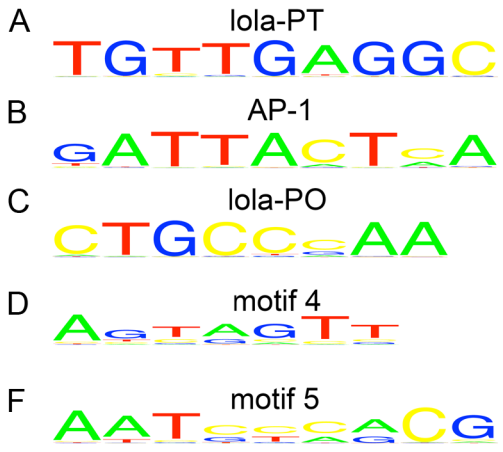

Figure S2

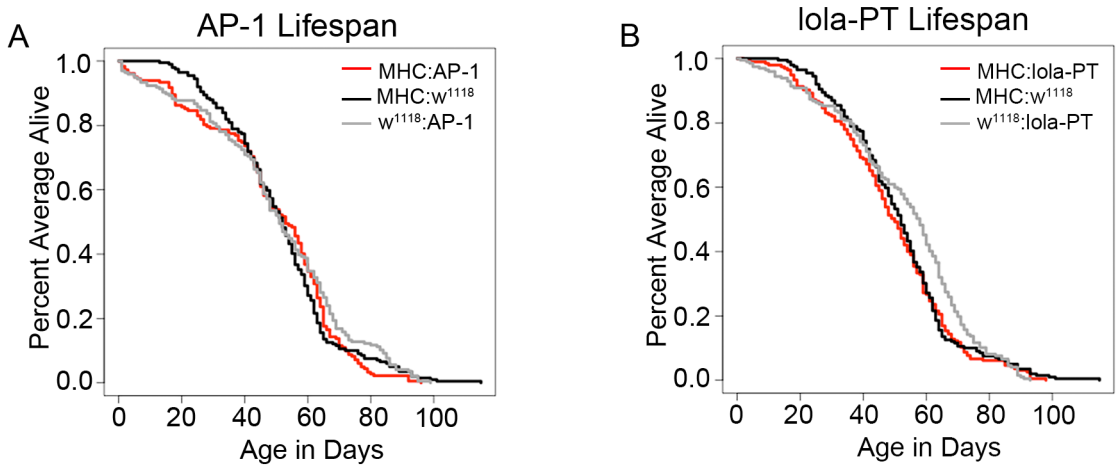
A
AP-1 Lifespan
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Age in Days
MHC:AP-1
MHC:w1118
w1118:AP-1
B
Iola-PT Lifespan
Percent Average Alive
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Age in Days
MHC:Iola-PT
MHC:w1118
w1118:Iola-PT

Figure S3

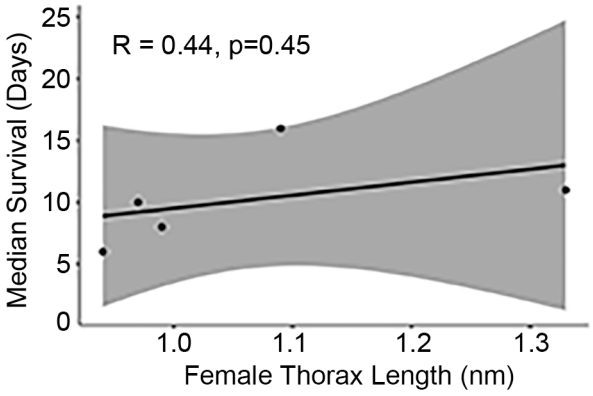
R = 0.44, p=0.45
Median Survival (Days)
25
20
15
10
5
0
1.0
1.1
1.2
1.3
Female Thorax Length (nm)

Figure S4

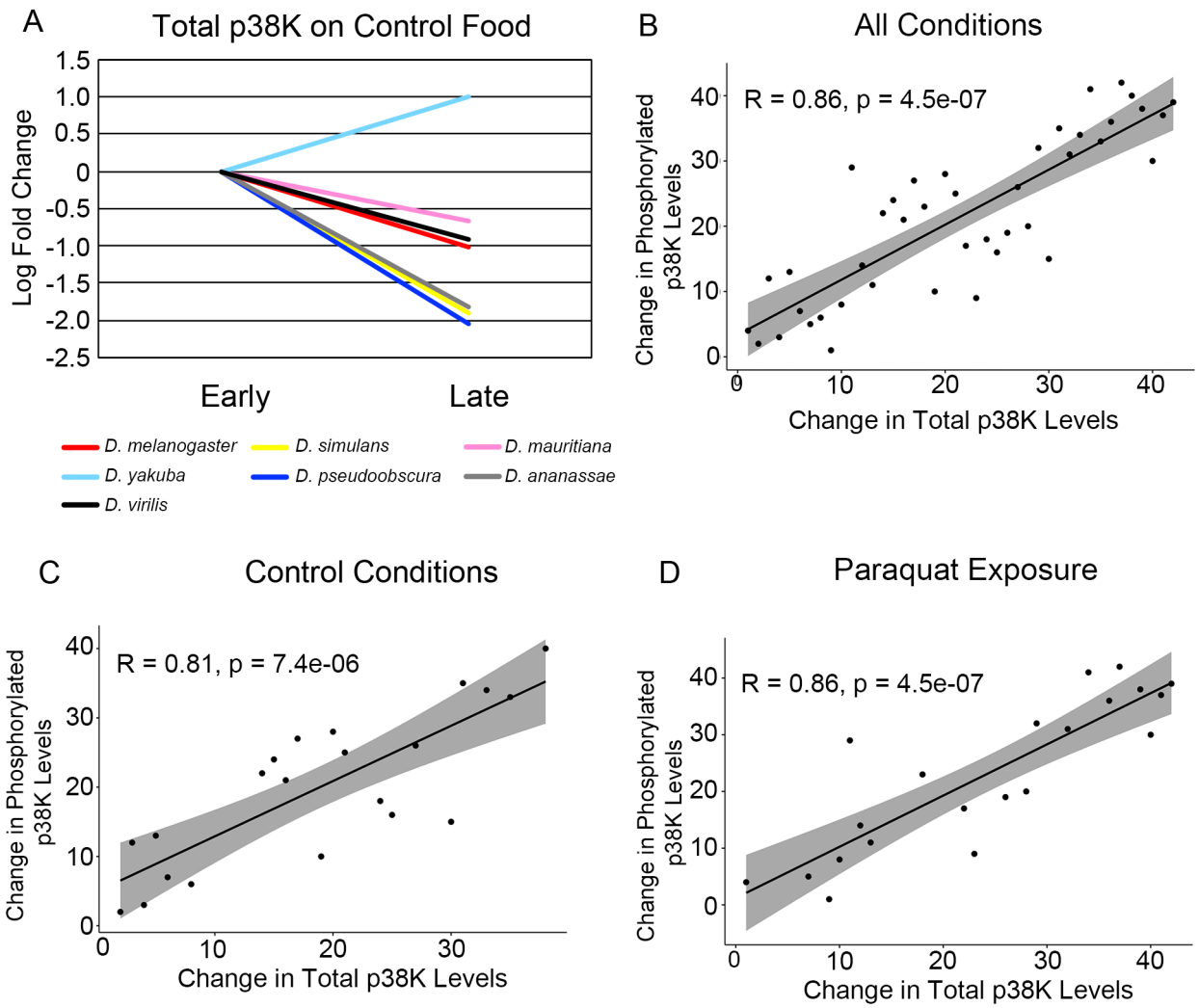
A
Total p38K on Control Food
Log Fold Change
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
Early
Late
D. melanogaster
D. simulans
D. mauritiana
D. yakuba
D. pseudoobscura
D. ananassae
D. virilis
B
All Conditions
R = 0.86, p = 4.5e-07
Change in Phosphorylated p38K Levels
Change in Total p38K Levels
C
Control Conditions
R = 0.81, p = 7.4e-06
Change in Phosphorylated p38K Levels
Change in Total p38K Levels
D
Paraquat Exposure
R = 0.86, p = 4.5e-07
Change in Phosphorylated p38K Levels
Change in Total p38K Levels

Figure S5