4-27-2017

The Mechanism of Life Span Extension in rpd3-mutant Drosophila

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5-8-2017

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University of Connecticut, 2017

Abstract

It was previously discovered that mutations in *rpd3*, a gene coding for the mammalian histone deacetylase 1 (HDAC1) homologue, led to an increased lifespan in *Drosophila* (Rogina et al., 2002). From changes in *dSir2* gene expression observed in this initial study and the fact that flies on a calorically restricted diet did not live longer, it was hypothesized that the mechanism of longevity extension overlapped with that of caloric restriction (CR). CR is one of the most effective ways to extend life span in a variety of species ranging from yeast to mammals. CR affects many aspects of metabolism including mitochondrial function, the insulin signaling pathway, and the TOR signaling pathway. Because of this, we investigated these pathways in *rpd3*-mutant *Drosophila*. Our results indicated that there are no changes in mitochondrial biogenesis, but small changes in mitochondrial function may exist. Interestingly, we saw a decrease in the insulin signaling pathway that suggests insulin signaling may mediate some of the effects observed in *rpd3* mutants. In addition we performed mRNA sequencing and determined that there may be changes in the innate immune system of *rpd3* mutants. We also provide a protocol for the measurement of spontaneous locomotor activity in *Drosophila* (Woods et al., 2014). Taken together, these results provide insight into the pathways involved in life span extension in *rpd3*-mutant *Drosophila*.

The Mechanism of Life Span Extension in *rpd3*-mutant *Drosophila*

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B.S., Biology, University of Louisville, 2008

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of Doctor of Philosophy

at the

University of Connecticut

2017

APPROVAL PAGE

Doctor of Philosophy Dissertation

The Mechanism of Life Span Extension in *rpd3*–mutant *Drosophila*

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2017

Acknowledgements

During my time in the MD/PhD program at UConn, there have been many people who have helped me make it through this journey. Dr. Rogina has been a great mentor who taught me more about how to do good science, and she was always willing to help when there was a problem in the lab. Ryan Rogers, PhD is a good friend who made being in the lab even more enjoyable. The help and support of everyone else in the Rogina lab also made this a great experience. My committee consisting of Drs. James Li, Gordon Carmichael, William Mohler, and Arthur Gunzl provided tons of support in moving my project forward and making sure I stayed on track. Their suggestions, whether it be for alternative techniques or how to analyze data, helped me develop as a scientist. The rest of the Genetics department including students, faculty, and staff have all been extremely help and have made being in this program an enjoyable time. Drs. Kream and Pilbeam have been essential to my progress in the MD/PhD program and have provided guidance and mentoring in many steps along the way. Tracy has always made sure to keep our program organized as well. Many other faculty and staff at UCHC have helped throughout my time here, and there are too many to list in one page. Thank you everyone!

Last but not least, I have to thank my family and friends for their support throughout this process. It's been a long road, and I could not have done it without my parents, my sister, and Ivy.

The NIH (AG023088) funded this work. I am honored to submit this dissertation to the Department of Genetics and Developmental Biology at the University of Connecticut in partial fulfillment of the requirement of the degree of Doctor of Philosophy in the field of Biomedical Science.

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Chapter 1: Aging and Rpd3

1.1 Introduction

As the population continues to live longer, research on the biology of aging will be necessary in order to extend the number healthy years that a person lives. In the earlier days of molecular biology, many researchers considered the aging process to be simply a passive process that occurred without regulation (Kenyon, 2010). We now know that aging is a complex biological process that involves changes in an organism ranging from the length of DNA to the size of an individual organ (Blasco, 20007; Courchesne et al., 2000). A recent review defined nine key hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin et al., 2013). In addition, almost of all of these processes are related through various cellular responses and signaling pathways. Importantly, these processes are regulated by many signaling and genetic pathways that can be targeted chemically or genetically to increase the life span of an organism.

The earliest theory on the mechanism of aging suggested that free radicals cause damage to cellular components, leading to a gradual decline in cellular function (Harman, 1956). In this paper it was also proposed that chemical agents could combat either the free radicals or the cellular decline, leading to healthier aging. Since then much work has been focused on describing the mechanism of the aging process, with one goal being an increased life span for humans. Recently the focus of the aging research community has not only been on extending life span, but

also health span, or the number of healthy, disease-free years of life. One group has tried to define health span as the period when an organism has 50% or more of the maximal functional capacity of a wild type animal (Bansal et al., 2015).

Drosophila melanogaster, commonly known as the fruit fly, has been used as a model organism to study aging for almost 100 years now, and it remains a relevant model organism for multiple reasons (Loeb and Northrop, 1916). *Drosophila* has the advantage of a short life span compared to multiple other model organisms, with some of the longest-lived fruit flies surviving almost 120 days (Lin et al., 1998). Fruit flies are also relatively cheap and easy maintenance of *Drosophila* colonies. *Drosophila* have also been a useful model organism because of the fact that many human diseases and conditions can be modeled in the fruit fly (Bier, 2005). 75% of human disease genes have a related sequence in the *Drosophila* genome. Fruit flies can be used to model diseases such as Huntington's, Fragile X Syndrome, or colorectal cancer. In addition, conditions such as aging have been extensively studied in fruit flies by our lab and many others.

The main advantage of using *Drosophila* in any research is their genetics, and the ability to modify their genes relatively easily. The genome of *Drosophila melanogaster* was completely sequenced in 2000 using whole-genome shotgun sequencing (Adams et al., 2000). 120 Mb of euchromatin are spread out on 2 autosomes and the X chromosome while a small fourth chromosome only contains about 1 Mb of euchromatin. The Y chromosome is almost entirely heterochromatin. Throughout the history of *Drosophila* research, many techniques have been developed to modify gene expression. Some of the earlier techniques include the

use of *Drosophila*'s transposable element, known as the P-element, to transform germ line cells (Rubin and Spradling, 1982). Although this method allowed high-frequency transfer of an exact sequence of DNA into embryos, the insertion into chromosomes happened at random positions. A later discovery that unlocked the true power of *Drosophila* genetics is the GAL4-UAS system (Brand and Perrimon, 1993). This initial discovery allowed for targeted gene expression in a tissue or cell-specific manner. To accomplish this, the *GAL4* gene was attached to a tissue-specific enhancer and inserted randomly into the genome, which drove GAL4 expression from different genomic enhancers. Then the target gene would be cloned behind GAL4 binding sites, called the Upstream Activating Sequence (UAS). Flies carrying these two constructs are crossed, and the tissue-specific expression of GAL4 causes the transcription of the target gene to be activated. This system was enhanced further with the discovery of a method to allow temporal specificity as well (Osterwalder et al., 2001). In this study, flies were generated with a conditional RU-486-dependent GAL4 protein. When flies are fed food containing RU-486, a steroid compound also known as mifepristone, the GAL4 fusion is able to dimerize and bind to the UAS, causing the activation of the target gene as in the initial GAL4-UAS system. Flies can be switched off or on food with RU-486 at any time to study the effects of temporal overexpression of a gene of interest. Following the discovery of RNA-mediated gene interference (RNAi) to knock down gene expression, a library of fruit flies have been developed to allow the conditional inactivation of a gene in a specific tissue (Dietzl et al., 2007). It is now possible to overexpress or knock down a gene of interest in both a temporal and tissue-specific manner in *Drosophila*.

Drosophila is not only easily genetically manipulated, but it is relatively simple to alter environmental conditions and study their effect on aging as well. For example, it has been shown that both male and female fruit flies have extended longevity with reduced sexual activity (Partridge and Farquhar, 1981; Smith, 1958). Like many poikilotherms, the life span of fruit flies has been shown to vary with temperature. These results in which decreased fecundity and body temperature extend life span may apply to mammals as well, which could lead to therapeutic potentials for increasing the health span in humans (Partridge and Farquhar, 1981; Conti et al., 2006).

Although fecundity and external temperature can affect the life span greatly, a major determinant of life span is caloric intake. Caloric restriction (CR) is normally a 20-40% reduction in the amount of calories consumed, without malnourishment (Ruetenik and Barrientos, 2015). CR was first discovered to extend the life span of male rats (McKay et al., 1935). In this seminal work, the components of rat chow were lowered so that the rats in the CR groups had lower quantities of nutrients, but the different components such as proteins, carbohydrates, and vitamins were all present. The first analysis of CR in *Drosophila* was performed by lowering the yeast concentrations of fly food, and this group found that the lowered caloric content of food resulted in a longer life span (Chippindale et al., 1993). This group also found that flies with decreased yeast in their diet had decreased fecundity and an increased resistance to starvation. A more recent report suggested that the beneficial effects of CR on reduced mortality in *Drosophila* can be seen as quick as two days following a switch from a normal to a

CR diet (Mair et al., 2003). In this report, flies were switched from a normal diet to CR or vice versa at either day 14 or 22 of life, and it was found that their mortality rate was equivalent to flies who had been raised permanently on the new diet. Currently, CR remains as the most consistent way of extending longevity in model organisms ranging from yeast to possibly non-human primates (Tatar, 2011; Colman et al., 2009).

1.2 Histone deacetylases and their relation to aging

Rpd3 (Reduced Potassium Dependency-3) is a zinc-dependent lysine deacetylase (KDAC) in *Drosophila* whose mammalian homologue is HDAC1. KDACs were initially named histone deacetylases (HDACs) because the first target of deacetylation was the ε-amino group of lysine residues in histones (Inoue and Fujimoto, 1969). Rpd3 as well as other zinc-dependent HDACs were placed in the classical family of HDACs to distinguish them from Sir2 and sirtuins, which are NAD^+-dependent histone deacetylases (Yang and Seto, 2008). The classical family consists of Class I, II, and IV HDACs, while sirtuins are in Class III. *rpd3* was first cloned in *Saccharomyces cerevisiae*, and its gene product was determined to encode a 433-amino acid protein with a molecular mass of 48 kDa (Vidal and Gaber, 1991). In the same report, Vidal and Gaber showed that while Rpd3 can both activate and repress specific transcription of different genes, overall it seems to be more of a transcriptional repressor. However, a study in both yeast and *Drosophila* showed that *rpd3* mutation results in enhanced gene silencing at specific regions of the chromosome (De Rubertis et al., 1996). The discovery that Rpd3 is an orthologue of

a mammalian HDAC came in 1996 when the HDAC1 protein was purified from nuclear fractions of bovine thymus (Taunton et al., 1996). The first mutational analysis of *rpd3* in *Drosophila* was published in 2000, in which Mottus et al. showed that *rpd3*-mutant strains had decreased levels of *rpd3* RNA expression. They also showed that homozygous *rpd3* mutations were embryonic lethal in *Drosophila* (Mottus et al., 2000).

Previously our lab has shown that *rpd3* mutations extend the life span of *Drosophila* through a mechanism that may overlap with CR. Our lab found that 2 different strains of heterozygous *rpd3*-mutant fruit flies had an extended life span compared to their genetic controls (Rogina et al., 2002). Interestingly, the life span of these mutants was not further increased by placing them on food with reduced caloric content, suggesting that the mechanism of life span extension in *rpd3*-mutant flies overlaps with the mechanism of extension seen in CR. Consistent with these data, our lab found that the levels of *dSir2* expression were increased similarly in *rpd3* mutants and control flies on CR. As discussed below, dSir2 is an important effector protein in the CR response in many organisms (Guarente, 2008). Our lab expanded on these results with further analysis of the interactions in *rpd3* and *dSir2* in aging *Drosophila* (Rogina and Helfand, 2004). Flies who were generated with mutations in *rpd3* and *dSir2* had a median life span the same as control flies or a *dSir2* mutant, while *rpd3* mutants alone lived longer. These results suggested that *dSir2* was a downstream mediator of *rpd3*'s effects on longevity.

Many other groups have researched the effects of HDACs and their relation to aging. Previous to our lab's study in flies, it was published that deletion of *rpd3* in *S.*

cerevisiae resulted in an increased life span (Kim et al., 1999). This study suggested that in yeast, *rpd3* mutation functions through its modifications of histones and changes in gene expression. More recently, a study in yeast has shown results suggesting that Rpd3 acts independently of CR through its ability to post-translationally modify proteins in the AMPK signaling pathway (Lu et al., 2011). In this study, yeast Rpd3 was shown to deacetylate Sip2, a β-regulatory unit of Snf2, which is the yeast orthologue of mammalian AMPK. The increased acetylation of Sip2 by generation of lysine-to-glutamine mutants, which mimics hyperacetylation, or by deletion of *rpd3* resulted in an increased replicative life span. They also showed that *rpd3* deletion led to increased trehalose levels compared to wild-type yeast, and that hyperacetylated Sip2 mutants were more resistant to hydrogen peroxide, a form of oxidative stress. A previous study in yeast showed similar results in which *rpd3* deletion extended the replicative life span, but there was not further increase in life span when these cells were placed on low-glucose medium (Jiang et al., 2002). Both of these studies hypothesize that Rpd3 and CR may have a common downstream effector(s), but Lu et al. provide evidence that these longevity-extending alterations are in two different pathways (2011). They propose that both the nutrient-sensing Target of Rapamycin (TOR) pathway and AMPK converge on Sch9, the yeast homologue of mammalian Akt/S6K. Currently it is still unknown whether Rpd3 and CR are acting independently of each other, or if there are overlapping mechanisms mediating longevity extension.

Rpd3 has been shown to function as part of a complex to exert its regulatory effects (Kasten et al., 1997). This study showed that Rpd3 copurifies with Sin3, and

that transcriptional repression by Sin3 was dependent on Rpd3. Interestingly, Sin3 is also known as Rpd1, because mutations of this gene in yeast also conferred a reduced dependency for potassium in yeast (Vidal et al., 1991). This overlap in phenotype provides further evidence for a complex containing Rpd3 and Sin3. Now it is known that two different Rpd3/Sin3 complexes form in yeast, known as Rpd3L and Rpd3S for large and a small, respectively (Yang and Seto, 2008). The Rpd3L complex is generally found to deacetylate histones at promoters, yet the Rpd3S complex targets transcribed regions of genes. The Rpd3/Sin3 complex does not bind DNA directly, but instead is targeted to specific genes via interactions with other DNA-binding proteins (Pile and Wassarman, 2000). This study in *Drosophila* salivary glands showed that this complex is associated with hypoacetylated regions of chromosomes, yet it is not associated with actively transcribed regions, where RNA polymerase II is located (Pile and Wassarman, 2000). The authors infer from their data that the Rpd3/Sin3 complex regulates between 2% to 13% of genes in *Drosophila*, which is consistent with HDAC inhibition in mammalian tissue culture cells. A later study by this group shows that *Sin3*-deficient *Drosophila* cell lines had altered expression of 3% of genes (Pile et al., 2003). Of these genes, loss of Sin3 resulted in upregulation of genes involved in fatty-acid oxidation, the electron transport chain, and glycolysis. In addition, there was an increase in mitochondrial mass in these cells.

Pharmacological inhibition of HDACs is possible through multiple compounds, and the clinical effectiveness of these compounds has been shown for a variety of disorders. Valproic acid, sold under the brand name Depakote, is a drug

that is approved for treatment of epileptic seizures (Kostrouchova et al., 2007). Although valproic acid has many cellular targets that may be responsible for its anticonvulsant properties, its function as an inhibitor of Class I and Class II HDACs is thought to be important for the potential to treat malignancies (Gurvich et al., 2004). Suberoylanilide hydroxamic acid (SAHA), also known as Vorinostat or Zolinza, is another Class I and II HDAC inhibitor that was approved by the FDA for treatment of cutaneous T-cell lymphoma in 2006 (Marks and Breslow, 2007). The biology of HDACs and their inhibitors will continue to be an important topic of research due to the potential to treat disorders ranging from Alzheimer's disease to cardiovascular disorders (Kazantsev and Thompson, 2008; Zhao et al., 2012).

Sirtuins are another class of HDACs that have been studied extensively in relation to aging. In yeast it was first shown that deletion of *Sir2* led to a decreased life span whereas overexpression of *Sir2* led to an increased life span (Kaeberlein et al., 1999). Similarly, overexpression of *dSir2* in *Drosophila* either ubiquitously or only in the brain leads to a life span extension (Rogina and Helfand, 2004). In worms, it was shown that Sir-2.1 increases life span via activation of the *dFoxo* (Berdichevsky et al., 2006). In flies, Sir2 has been shown to interact with P53 to mediate the effects of life span extension (Bauer et al., 2009). Similarly in mice overexpression of *SIRT1*, the mammalian *Sir2* orthologue, resulted in many key features of CR (Bordone et al., 2007). The mice with the SIRT-knock-in allele had lower body weights, decreased white adipose tissue, and decreased plasma glucose amongst other characteristics. SIRT1 as a deacetylase can have many cellular targets. Previous studies have shown that SIRT1 can deacetylate eNOS and PGC-1α,

two key regulators of mitochondrial biogenesis (Mattagajasingh et al., 2007; Rodgers et al., 2005). This decrease in acetylation of PGC-1α leads to increased expression of PGC-1α's target genes in mammals. Mammalian Sir2 has been shown to deacetylate p53, a protein known as the "guardian of the genome" because of its role in preventing DNA mutations, and promote cell survival instead of apoptosis following different stressors such as hydrogen peroxide (Luo et al., 2001). Because of these roles of Sirtuins, they are particularly important since they have been found to mediate many beneficial changes associated with healthy aging.

Many chemical agents have been shown to increase the activity of Sir2, most notably resveratrol. Resveratrol is a polyphenol compound found in grapes and red wine that had previously been shown to have chemopreventive effects due to its properties as an antioxidant (Jang et al., 1997). Using a large chemical compound screen, Howitz et al. found that resveratrol increased the activity of Sir2 and led to a life span extension in yeast (2003). Interestingly, the life span was not increased in yeast lacking *Sir2*, and CR did not further increase the life span of wild type yeast treated with resveratrol. In *Drosophila*, resveratrol was shown to extend the life span of flies under normal conditions (Wood et al., 2004). However, resveratrol did not extend the longevity of flies on a CR diet or flies lacking functional *dSir2*. It has been shown in mice that resveratrol activates both SIRT1 and PGC-1α (Lagouge et al., 2006). Using compounds that are structurally similar to resveratrol, more potent activators of SIRT1 have been developed, and it is possible that they may be used to treat type II diabetes (Milne et al., 2007). Although it is unlikely that inhibiting sirtuins would be desired as a therapeutic considering the mainly

beneficial effects of sirtuins, inhibitors of this class of HDACs have also been developed as well (Grozinger et al., 2001). These results prove the importance of studying HDACs as they are able to modify life span of many organisms, and they are targetable by numerous drugs and compounds. Understanding the biology of HDACs and their role in aging could hold the key to increasing the number of healthy years we as humans are able to live.

1.3 Mitochondria and their relation to aging

Mitochondria are organelles in the cell who originally came from an ancestral bacteria closely related to *Alphaproteobacteria* that underwent an endosymbiotic event and coevolved to become a vital part of all known eukaryotic cells (Thrash et al., 2011). Mitochondria are typically taught as the powerhouses of the cell, known mainly for their role in ATP production for energy for the cell. This organelle possesses five complexes on the inner mitochondrial membrane that allows for large quantities of ATP production from intermediates that are generated from the citric acid cycle. The first four of these complexes (I, II, III, and IV) compose the respiratory chain, which transports electrons through the inner membrane to complex IV where they can join oxygen to form H_2O (Barrientos, 2002) (Fig. 1.1). Complex V uses a H^+ gradient created by the respiratory chain to convert ADP and P_i into ATP. However, the role of mitochondria is actually far more complex than simply facilitating ATP production, as they are involved in a variety of processes from signaling to anti-apoptotic mechanisms (Ruetenik and Barrientos, 2015).

Mitochondria were initially linked to aging because of their role in energy production and the ROS (reactive oxygen species) that were thought to be byproducts of this process (Guarente, 2008). During energy production, though, ROS are produced, which may be harmful to mitochondrial DNA and proteins, as well as other components of the cell (Lopez-Otin et al., 2013). Although much research has challenged the idea that ROS are not beneficial and entirely harmful, mitochondrial dysfunction and increased ROS production still remain prime candidates for causative agents of aging, even if not entirely responsible. In addition, many mitochondrial diseases show phenotypes that are similar to accelerated aging (Martikainen and Chinnery, 2015). Mitochondrial diseases are known to affect about 1 in 5000 adults, and they mainly target organs whose cells have a high energy demand such as the brain, skeletal muscles, and pancreas. Because of the role of mitochondria in both aging and diseases, understanding the biology of these organelles will be crucial to promoting healthier human lives.

As CR is a leading mechanism of life span extension and because mitochondria are involved in cellular energy production from nutrients, it was hypothesized early on that mitochondrial function may be a major effector of CR (Guarente, 2008). Many studies have examined the role of CR and its relation to mitochondria in *Drosophila* as well as yeast, worm, and mammalian model organisms. It was first shown in *S. cerevisiae* that CR extends life span through increased mitochondrial respiration (Lin et al., 2002). In this study yeast grown on 0.5% glucose had an increased rate of respiration compared to yeast grown on 2%

glucose, and deleting a component of the electron transport chain prevented this life span extension.

CR not only increases mitochondrial function, but it has also been shown to increase mitochondrial biogenesis as well in numerous species. In mice that were fed every other day rather than *ad libitum*, a variation of CR known to extend mice life span, it was shown that many genes involved in mitochondrial biogenesis were upregulated (Nisoli et al., 2005). These include peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (Tfam). This was true for multiple tissues including adipose tissue, brain, and liver, and all of these tissues exhibited increased respiration as well. This group also showed that these beneficial effects of CR depended on the expression of endothelial nitric oxide synthase (eNOS), which may activate SIRT1.

In fruit flies, mitochondrial biogenesis is equally as important to the response to CR and increasing life span extension. The *Drosophila* PGC-1α homologue, *spargel*, has been shown to be particularly important in healthy aging and the response to CR. Flies that overexpress *spargel* ubiquitously exhibited increased mitochondrial DNA and increased citrate synthase activity, but they had a reduced life span (Rera et al., 2011). To expand upon this finding, this group used drivers to induce *spargel* overexpression in specific tissues. They found that overexpression in the intestine and intestinal stem cells led to increased life span, increased mitochondrial enzyme activity, and decreased ROS production. Overexpression of *spargel* also led to the maintenance of proper differentiation of the intestinal stem

cell populations of aged *Drosophila*. Our lab confirmed these findings in a genetic model of CR, fruit flies with a mutation in *Indy* (Rogers and Rogina, 2014). It was found that *Indy* mutation resulted in flies with increased *spargel* expression, increased mitochondrial biogenesis, and maintained intestinal integrity. Consistently, life span extension was not observed in flies with mutations in both *Indy* and *spargel*, yet flies with only an *Indy* mutation experience an increased longevity.

Consistent with *Drosophila* and mammalian data, mitochondrial biogenesis is also increased in the skeletal muscles of humans who underwent a CR regimen for 6 months (Civitarese et al., 2007). Expression of genes such as PGC-1α, Tfam, and SIRT1 was increased in the CR group of obese humans compared to obese controls on a standard diet, and there was an increase in the mitochondrial DNA content, a commonly used measure of mitochondrial biogenesis. Taken together, the literature suggests that mitochondria are key mediators of CR in multiple organisms. Therefore, developing therapeutic targets to improve mitochondrial function and biogenesis could be effective in extending the healthspan of humans.

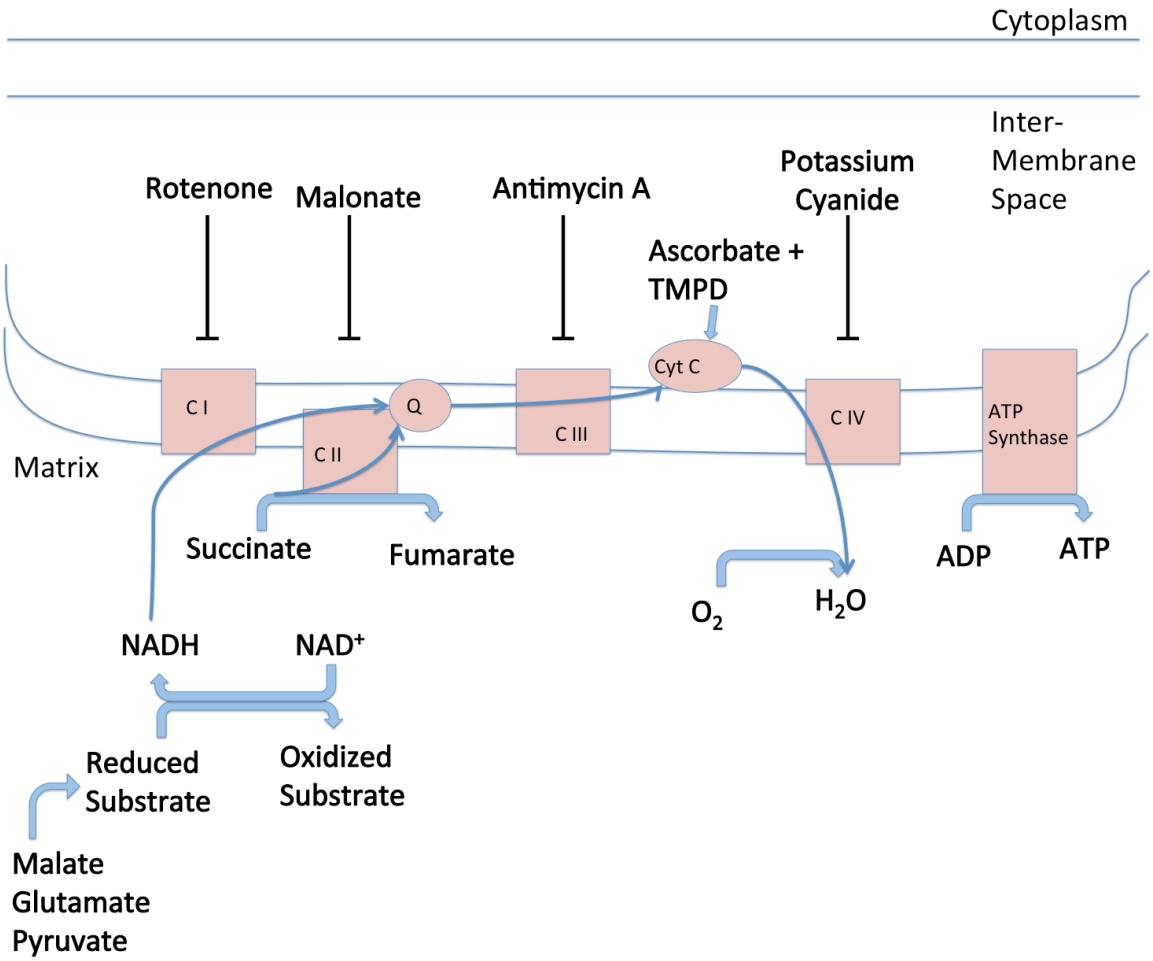

Figure 1.1 The mitochondria respiratory chain. Substrates from either the Krebs cycle or provided experimentally are shown. Electrons from NADH flow through Complex I, whereas substrates from $FADH_2$ (from succinate) flow through Complex II. O_2 is the final electron acceptor, when it is converted to H_2O. Not shown are protons, which are drawn from the mitochondrial matrix to the intermembrane space. From there they pass through ATP synthase to help catalyze the conversion of ADP + P_i to ATP. Specific inhibitors of Complexes I through IV are shown. Figure modified from Barrientos (2002).

1.4 Insulin Signaling and its relation to aging

The insulin-signaling pathway is a nutrient-sensing pathway that has evolved in multicellular organisms as a way to regulate nutrient homeostasis (Partridge et al., 2011). This pathway was first discovered to affect aging in *C. elegans* (Kenyon et al., 1993). The authors found that worms with mutations in the gene coding for insulin receptor (InR) lived twice as long as their controls. This initial report only referred to the gene coding for InR as *daf-2*, as its role in insulin signaling was not yet known. The authors also discovered that *daf-16* was required for the life span extension, and it would later be found that *daf-16* codes for the *C. elegans* orthologue of Forkhead box group O (Foxo) transcription factor (Ogg et al., 1997). Reports in *Drosophila* confirmed these results when it was found that mutations in either the gene coding for insulin receptor (InR) or insulin receptor substrate, known as *chico* in *Drosophila*, extended life span (Tatar et al., 2001; Clancy et al., 2001).

In *Drosophila*, insulin-like peptides (dIlps) are the circulating hormones that are orthologous to mammalian insulin (Broughton et al., 2005). There are eight of these peptides, and dIlps 2, 3, and 5 are made in the insulin producing cells of the fly brain. dIlp6 is produced in the fat body (Bai et al., 2012). Consistent with reduced insulin signaling extending life span, ablation of the insulin producing cells of the brain leads to extension of life span in fruit flies (Broughton et al., 2005). The role of each individual dIlp and its relation to aging is complex as there is functional overlap between the individual dIlps (Gronke et al., 2010).

Foxo transcription factors of different species have been shown to play an important role in aging (Kenyon, 2010). This transcription factor is a downstream

effector of the insulin signaling pathway, and it activates the expression of many genes involved in autophagy, stress response, and cell growth (Chang and Neufeld, 2010; Liu et al., 2014; Demontis and Perrimon, 2010). dFoxo has even been shown to be a feedback mechanism for regulation of dIlp2 production in the brain (Hwangbo et al., 2004). dFoxo is a transcriptional factor that is post-translationally modified in order to change its effects on gene activation (Kloet and Burgering, 2011). When insulin signaling is decreased, dFoxo is not phosphorylated by PKB, and it is able to enter the nucleus leading to expression of target genes. When insulin signaling is active, dFoxo is phosphorylated on three conserved residues, allowing 14-3-3 binding and cytoplasmic sequestration.

Downstream of the insulin signaling pathway, yet still partially independent, is the TOR signaling pathway. As this pathway is known to be involved in nutrient sensing, it has been the subject of much research relating to CR and aging. TOR signaling is activated by conditions of high energy availability, such as elevated ATP, and it is inhibited by signals of stress, such as ROS or hypoxia (Ruetenik and Barrientos, 2015). It has also been shown that although partially independent, there is overlap between the TOR pathway and the insulin pathway. For example, dFoxo has been shown to activate the transcription of *4E-BP* in *Drosophila* S2 cells (Puig et al., 2003). The TOR protein is negatively regulated by the Tsc1/Tsc2 complex, which are inactivated by Akt (Kapahi et al., 2004). This group found that overexpressing a dominant-negative form of *dTsc2* resulted in life span extension in *Drosophila*. This extension of longevity was shown to depend on the nutritional

status, in which this form of dTsc2 protected the flies against high calorie food. This provides further evidence to the link between nutritional status and TOR signaling.

TOR is a serine/threonine kinase, and as such TOR signaling influences the activity of a variety of proteins. TOR is known to be a regulator of protein synthesis through its activating effects of S6K and eukaryotic initiation factor 4E binding protein (4E-BP) (Katewa and Kapahi, 2011). However, these two proteins have an opposite effect on growth and translation. S6K is a known translation activator and promoter of growth (Kapahi et al., 2004). It is phosphorylated and positively regulated by the TOR complex. If flies have mutations in the *S6K* gene, they are developmentally delayed and have a smaller body size (Montagne et al., 1999). 4E-BP is a potent negative regulator of translation by its ability to bind and inhibit eIF4E, a eukaryotic initiation factor. When TOR signaling is active, the phosphorylated form of 4E-BP is unable to bind eIF4E, allowing translation to take place (Zid et al., 2009). Even though S6K and 4E-BP are known for having different effects on translation, their differing responses to phosphorylation via TOR allows them to have similar effects on translation. Interestingly though, the effects of 4E-BP are not to simply turn translation on or off globally. Zid et al. showed that in *Drosophila*, 4E-BP was upregulated in animals raised on CR diets (2009). This group found that while it globally decreased the rates of translation, there were classes of proteins such as mitochondrial proteins whose translation was upregulated. Therefore, the role of TOR signaling and its effects on translation should be investigated further to determine if it could be targeted to increase health span in humans.

The focus of this work is to investigate the mechanism by which Rpd3 affects the longevity of *Drosophila*. Previous research has shown the potential of mutation in Rpd3 to extend longevity, but it remained unclear as to how decreased Rpd3 led to this extension. Decreased Rpd3 levels were shown to affect *Drosophila* physiology in various mechanisms such as energy storage and stress resistance, but it was uncertain if these changes were consistent or not with CR. Mitochondrial biogenesis and respiration were analyzed since mitochondria are known to be affected by CR. Small changes may exist in mitochondrial function, but evidence of increased mitochondrial biogenesis was not found. However, the insulin signaling pathway was significantly affected. *Rpd3*-mutant flies had decreased levels of gene expression of components in the insulin signaling pathway as well as an increase in *dFoxo*, a transcription factor inversely related to insulin signaling that is a downstream effector of this pathway. mRNA sequencing results suggested that other pathways such as the immune system may be altered too in *rpd3* mutants. Taken together, our findings provide insight into the mechanism of life span extension seen in *rpd3*-mutant *Drosophila*.

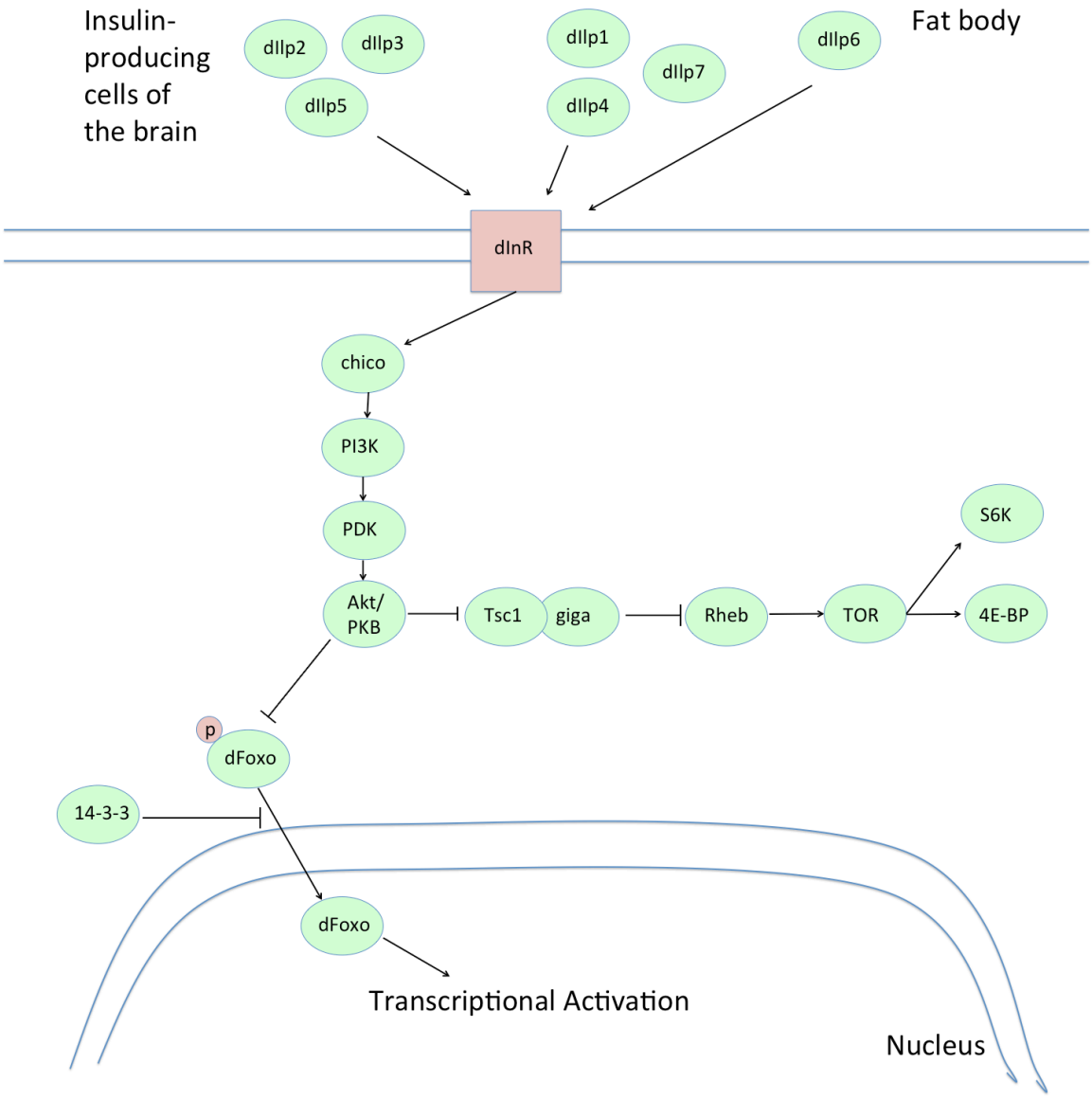

Figure 1.2 The *Drosophila* insulin signaling pathway. dIlps 1-7 activate the insulin receptor. The signaling cascade includes chico, the *Drosophila* insulin receptor substrate, PI3K, and PDK, which leads to activation of Akt (also known as PKB). Akt phosphorylates and inhibits dFoxo, allowing 14-3-3 proteins to bind and prevent translocation to the nucleus and therefore activation of transcription. Akt also inhibits the Tsc1/Tsc2 complex, a negative regulator of TOR signaling. Tsc2 is known as giga in *Drosophila.* Figure modified from Partridge et al. (2011).

Chapter 2: *rpd3*-Mutant *Drosophila* Experience Many Physiological Changes Consistent with Increased Longevity

2.1 Background and Significance

Many key physiological changes have been associated with animals that have an increased longevity. For example, increased stress resistance has been seen in many long-lived organisms (Bjedov et al., 2010; Yu and Chung, 2001). In particular, CR is known to cause an increase in stress response, potentially through the effects of 4E-BP (Tettweiler et al., 2005). dFoxo is known to activate *4E-BP* transcription, so this transcription factor may play a role as well.

Levels of nutrient storage have been shown to fluctuate due the nutritional status in many organisms. As an example, humans that are calorically restricted have lower levels of lipids in addition to other metabolic pathways (Fontana et al., 2004). However, one study in flies found that CR causes an increase in triglyceride levels in flies, but no change in the levels of glycogen or trehalose (Emran et al., 2014). Another study in *Drosophila* found that levels of triglycerides were decreased in CR flies (Wang et al., 2009). It has recently been discovered that the ratio of protein to carbohydrates has a major influence in response to CR (Taormina and Mirisola, 2014; Tatar, 2011). High protein diets seem to be detrimental to life span, yet high carbohydrate diets promote obesity. It is possible that flies are not affected as mammals are by obesity, due to the absence of some adverse cardiovascular effects such as atherosclerosis (Broughton et al., 2005).

Mobility has been shown to be reduced in species ranging from worms to humans as they age (Bansal et al., 2015). It has also been shown that CR increases

spontaneous locomotor activity as well (Parashar and Rogina, 2009). Therefore measuring the spontaneous activity of *Drosophila* is one indicator of their overall health, and could give clues as to whether *rpd3* mutation is a genetic form of CR. Our lab's initial study suggests *rpd3* mutation and CR overlap in mechanism of longevity extension, so we set out to test various aspects of physiology altered by CR (Rogina et al., 2002).

2.2 Rationale

There are many biological processes that gradually change as organism ages. These include stress resistance, changes in metabolic energy stores, and spontaneous locomotor activity. Many of these physiological changes are for the better in organisms that are calorically restricted. For example, CR in *Drosophila* was first discovered to extend life span as well as increase resistance to starvation (Chippindale et al., 1993). Since we hypothesized that *rpd3* mutation may extend longevity of fruit flies in a mechanism that overlaps with CR, we expected to see changes in these aspects of physiology.

Since our lab's first two publications on the effects of *rpd3* mutation, little has been done to determine the actual mechanism of lifespan extension (Rogina et al., 2002; Rogina and Helfand, 2004). Other groups have looked at the effect of *rpd3* mutation or inhibition on gene expression or in disease models of *Drosophila*, but no one has looked at the effects on aging (Cho et al., 2005; Pallos et al., 2008). Instead studies have been on amelioration of disease models or looking at the effects on transcription in fruit flies with altered Rpd3 activity. In addition to determining the

mechanism of *rpd3* mutation on lifespan, it is also important to determine the effects of decreased HDAC levels on physiology in general. HDAC inhibitors are being developed as treatments for cancer and neurological disorders, therefore it is important to understand the general biology and physiological consequences of reduction in the activity of Rpd3 or other HDACs (Kazantsev and Thompson, 2008; Drummond et al., 2005).

2.3 Results

2.3.1 *rpd3*-mutant flies have decreased levels of *rpd3* mRNA expression

Using lines we have previously shown to have an increased life span, we looked at levels of *rpd3* mRNA expression in flies that were 20 days of age (Rogina et al., 2002). At 20 days of age, we found a significant reduction in *rpd3* mRNA expression in male $rpd3^{def24}/+$ flies compared to *+/+* flies (Fig. 2.1A). At 20 days of age, $rpd3^{P\text{-}UTR}/CS$ males also had a significant reduction in *rpd3* levels (Fig. 2.1B). We also examined the levels of *rpd3* expression in a wild type strain of laboratory *Drosophila*, *Canton-S* (*CS*) flies, at different ages. Interestingly, we found a significant increase in *rpd3* mRNA expression at 40 and 60 days compared to younger flies (Fig. 2.1C). These results suggest that heterozygous mutation of *rpd3* may protect against the negative effects of a gradual increase in *rpd3* levels.

We also aimed to generate flies with *rpd3* expression inhibited in a tissue-specific manner using the GAL4-UAS system and flies with an RNAi construct targeted to *rpd3* (Dietzl et al., 2007). Using 1 strain of RNAi *Drosophila* from the Bloomington stock center and 2 strains from the Vienna stock center, we sought to

generate flies with reduced *rpd3* expression in muscle tissue. We crossed the *rpd3*-RNAi females with males expressing the myosin heavy chain (*Mhc-GS-Gal4*) driver (Katewa et al., 2012). In the line from the Bloomington stock center, *503*, we found that expression of *rpd3* mRNA was not significantly decreased in 20 day old flies raised on RU-486 food compared those raised on food with EtOH when measured by qPCR (Fig. 2.2A). In the two lines from the Vienna stock center, *30599* and *30600*, flies raised on food with RU-486 did not show significant reduction in *rpd3* expression compared to their littermates raised on food with EtOH (Fig. 2.2B, C). Since we did not see a reduction in *rpd3* levels like we did in $rpd3^{def24}/+$ or $rpd3^{P\text{-}UTR}/CS$ flies, further experiments were not performed on flies using the GAL4-UAS system.

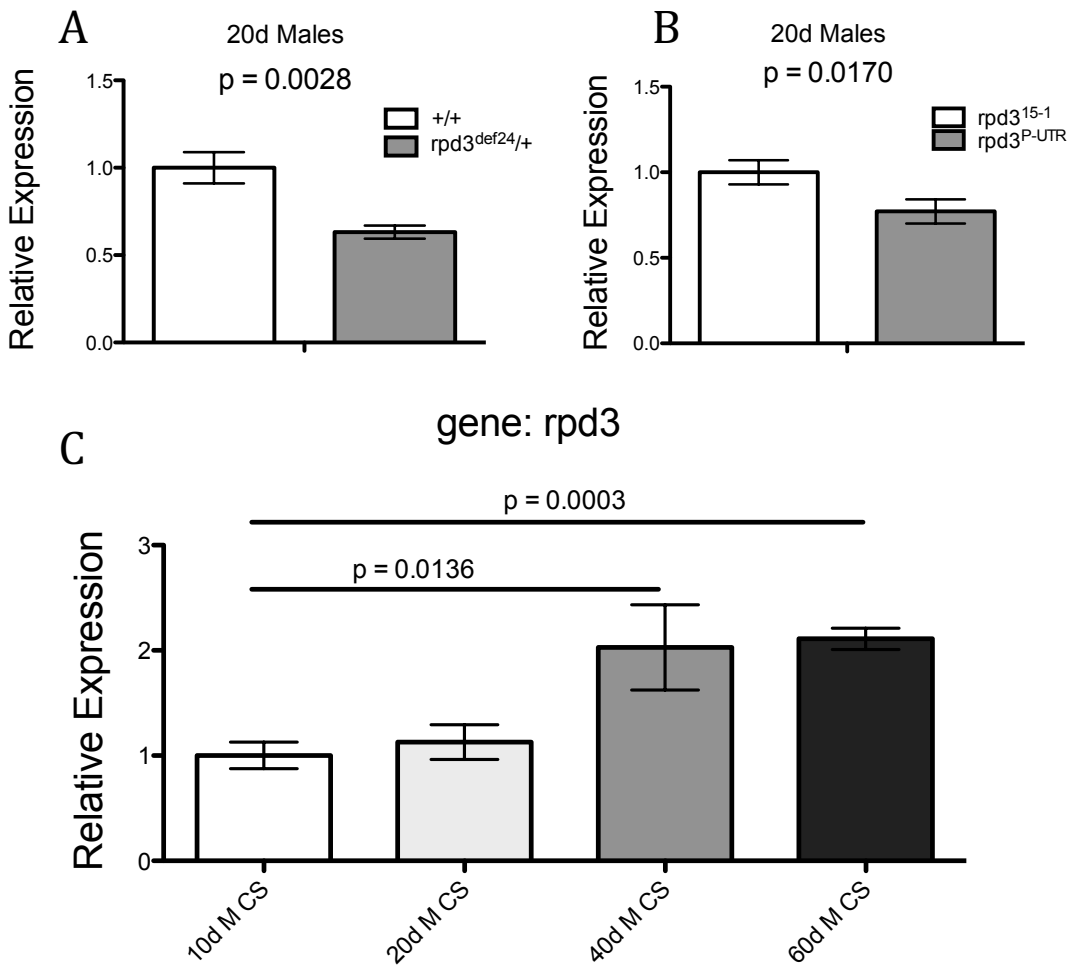

Figure 2.1 A) qPCR results showing *rpd3* expression in 20 day old *rpd3^{def24}/+* males compared *+/+* males. **B)** qPCR results showing *rpd3* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS*, and *dFoxo/yw* males. **C)** qPCR results showing *rpd3* expression in 10, 20, 40, and 60 day old *CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

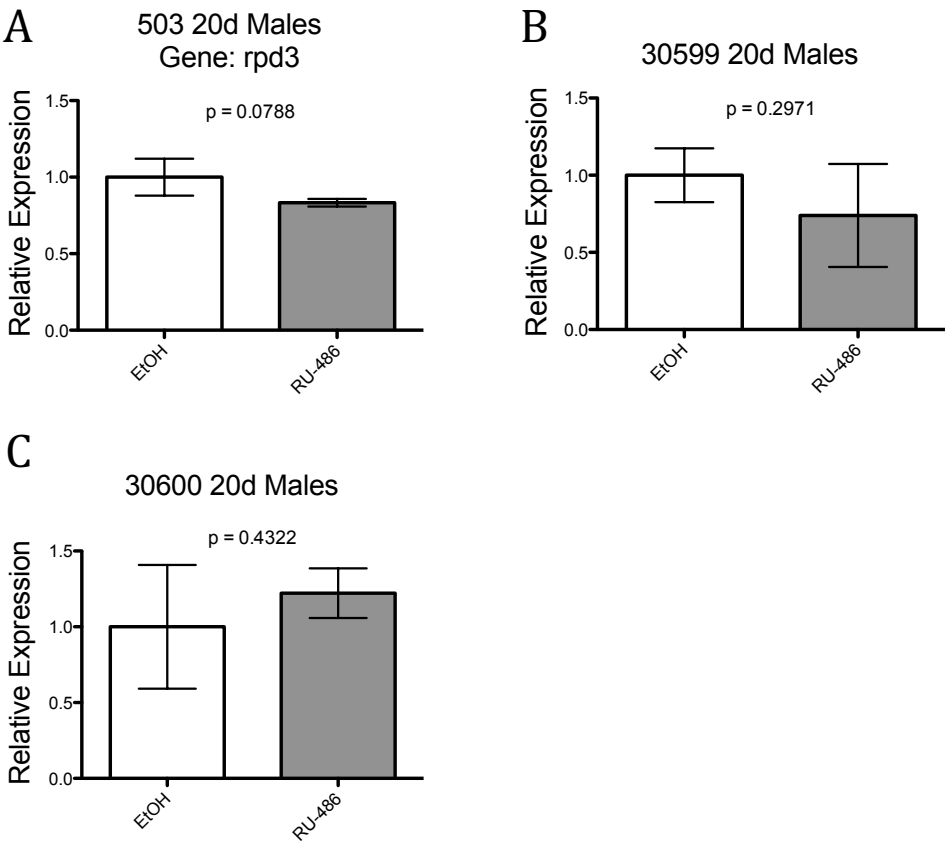

Figure 2.2 A) qPCR results for *rpd3* expression in 20 day old *Mhc-GS; rpd3^{RNAi}-503* males. **B)** qPCR results for *rpd3* expression in 20 day old *Mhc-GS; rpd3^{RNAi}-30599* males. **C)** qPCR results for *rpd3* expression in 20 day old *Mhc-GS; rpd3^{RNAi}-30600* males. In **A, B,** and **C**, the control group was raised on food containing EtOH, whereas the experimental group was raised on food containing RU-486. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

2.3.2 *rpd3* mutants are more resistant to oxidative stress

One hallmark of healthy aging and increased longevity in multiple species is a concurrent increase in stress resistance (Bansal et al., 2015). As expected, CR is known to increase stress resistance as well in multiple species (Finkel and Holbrook, 2000). To examine stress resistance in *rpd3* mutants, we exposed 10 or 40 day old flies to two forms of oxidative stress, hydrogen peroxide or the herbicide paraquat. At 10 days of age, both male and female $rpd3^{def24}/+$ flies were equally as resistant to hydrogen peroxide as their genetic controls (Fig. 2.3A). However at 40 days, male $rpd3^{def24}/+$ were more resistant to their genetic controls, and a similar increase was seen in females at this age (Fig. 2.3B). At 10 days of age $rpd3^{P\text{-}UTR}/CS$ males were equally as resistant to H_2O_2 as the control line of flies, $rpd3^{15\text{-}1}/CS$ (Fig. 2.3C). However, female $rpd3^{P\text{-}UTR}/CS$ flies at 10 days of age were more resistant than $rpd3^{15\text{-}1}/CS$ females. A similar trend was seen at 40 days, when male $rpd3^{P\text{-}UTR}/CS$ flies had a similar H_2O_2 resistance as their genetic controls, yet $rpd3^{P\text{-}UTR}/CS$ females were more resistant than $rpd3^{15\text{-}1}/CS$ females (Fig. 2.3D).

As previously mentioned stress resistance is a measure of aging, and therefore resistance is thought to decline gradually with age (Zou et al., 2000). We showed that as our groups of flies aged, there was a gradual decline in H_2O_2 resistance. In *+/+* males and females, the younger flies survived longer when exposed to H_2O_2 than older groups of flies for ages 10, 20, 30, and 40 days (Fig. 2.4A, B). This trend was also true for $rpd3^{def24}/+$ females (Fig. 2.4D). The only group who defied this trend was 40 day old male $rpd3^{def24}/+$ flies (Fig. 2.4C). They were more resistant to H_2O_2 than 20 or 30 day old flies, but they were not as resistant as 10 day

old flies. These results show that our flies behave as expected during aging studies and following exposure to H_2O_2.

We also exposed *rpd3*^{def24}*/+* flies to paraquat, an herbicide that has been widely used in *Drosophila* and worm studies to test oxidative stress resistance (Bansal et al., 2015). At 10 days of age male *rpd3*^{def24}*/+* and *+/+* flies had similar survivorships when exposed to paraquat (Fig. 2.5A). However, it is possible that female *rpd3*^{def24}*/+* flies survived slightly shorter than their genetic controls. At 40 days of age when exposed to paraquat, the survival curves were nearly identical *rpd3*^{def24}*/+* and *+/+* flies in both genders (Fig. 2.5B). It is interesting how the response would differ when the same flies are exposed to two different types of oxidative stress, hydrogen peroxide or paraquat. Taken together these data indicate that *rpd3* mutants do experience an overall resistance to stress at a later age in life, consistent with an extended lifespan. Nevertheless more experiments are needed to determine if *rpd3* mutation acts in a mechanism that overlaps with CR.

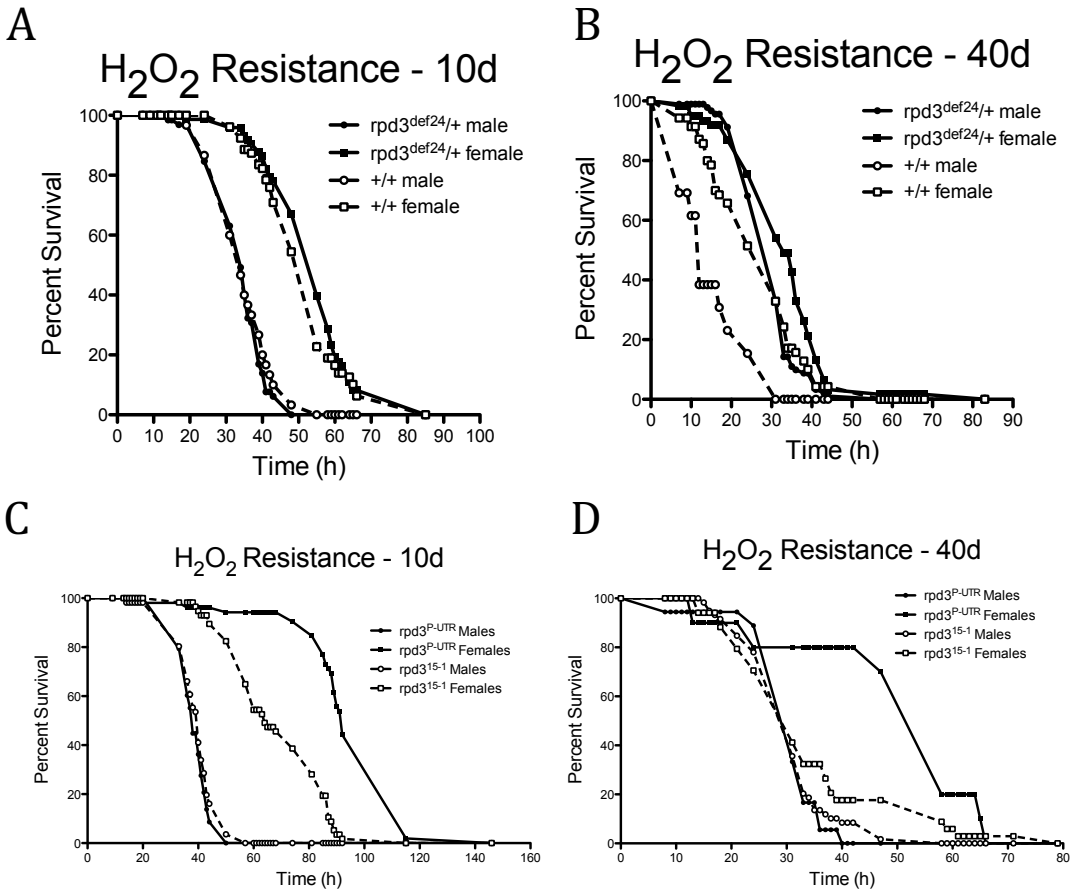

Figure 2.3 A) H_2O_2 resistance studies for 10 day old *rpd3^{def24}/+* and *+/+* flies. **B)** H_2O_2 resistance studies for 40 day old *rpd3^{def24}/+* and *+/+* flies. **C)** H_2O_2 resistance studies for 10 day old *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* flies. . **D)** H_2O_2 resistance studies for 40 day old *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* flies. Graphs are plotted as percentage of flies surviving as a function of time.

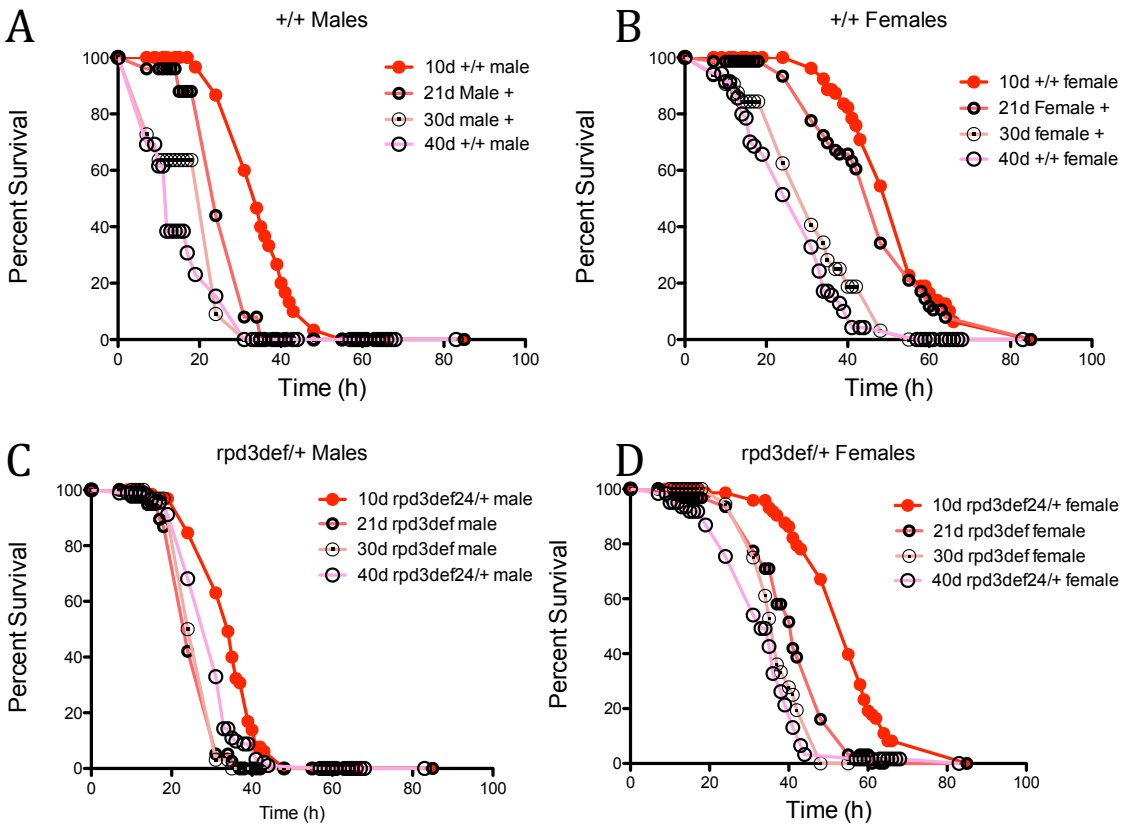

Figure 2.4 A) H_2O_2 resistance studies for 10, 20, 30, and 40 day old *+/+* males. **B)** H_2O_2 resistance studies for 10, 20, 30, and 40 day old *+/+* females. **C)** H_2O_2 resistance studies for 10, 20, 30, and 40 day old $rpd3^{def24}$*/+* males. . **D)** H_2O_2 resistance studies for 10, 20, 30, and 40 day old $rpd3^{def24}$*/+* females. Graphs are plotted as percentage of flies surviving as a function of time.

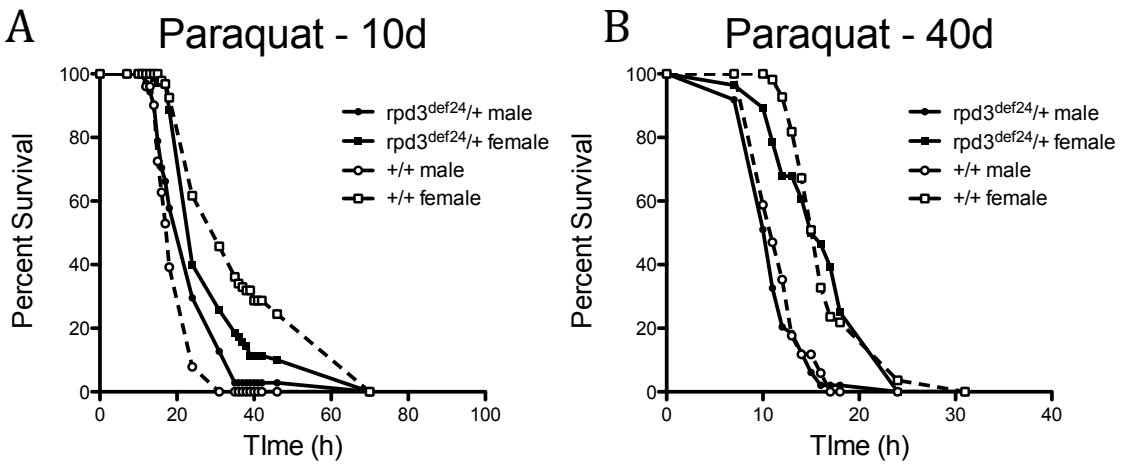

Figure 2.5 A) Paraquat resistance studies on 10 day old *rpd3^{def24}/+* and *+/+* flies. **B)** Paraquat resistance studies on 40 day old *rpd3^{def24}/+* and *+/+* flies. Graphs are plotted as percentage of flies surviving as a function of time.

2.3.3 *rpd3*-mutant flies are more resistant to starvation

Increased starvation resistance is a phenotype found in many long-lived animals, yet its modification by CR remains a debatable topic. The first *Drosophila* CR experiment found that both genders of flies had increased starvation resistance on low-calorie food, yet the increase was not as great in males (Chippindale et al., 1993). Yet, other studies indicate that CR decreases starvation resistance because of the reduction in energy stores (Wang et al., 2009). When $rpd3^{def24}/+$ flies were exposed to starvation, they were more resistant than their genetic controls at 10 days of age (Fig. 2.6A). The same results were seen at 40 days of age (Fig. 2.6B). At 10 days of age, $rpd3^{P\text{-}UTR}/CS$ females were more resistant to starvation than $rpd3^{15\text{-}1}/CS$ females while there was no difference between $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ males (Fig. 2.6C). At 40 days, the trend was the same with female $rpd3^{P\text{-}UTR}/CS$ flies surviving longer than their controls, and male $rpd3^{P\text{-}UTR}/CS$ and control flies showing a similar resistance to starvation (Fig. 2.6D). These studies show that *rpd3*-mutant flies have an increased resistance to starvation in most groups.

$rpd3^{def24}/+$ and $+/+$ flies were also raised on different food levels until 40 days of age and then exposed to starvation. We used 0.7N as a CR diet and 1.5N as a high calorie diet. Interestingly, male $rpd3^{def24}/+$ flies were more resistant to starvation than $+/+$ males at any food level (Fig. 2.7A). Unexpectedly, flies of both genotypes on corn food were more starvation resistant than the same flies on either 0.7N or 1.5N. Both of these trends were true for females as well (Fig. 2.7B). The $rpd3^{def24}/+$ females were more resistant to starvation than $+/+$ females at any food level, and females on corn were more resistant than those on CR or a high calorie

diet. Our starvation data show that *rpd3* mutants are more resistant than their genetic controls.

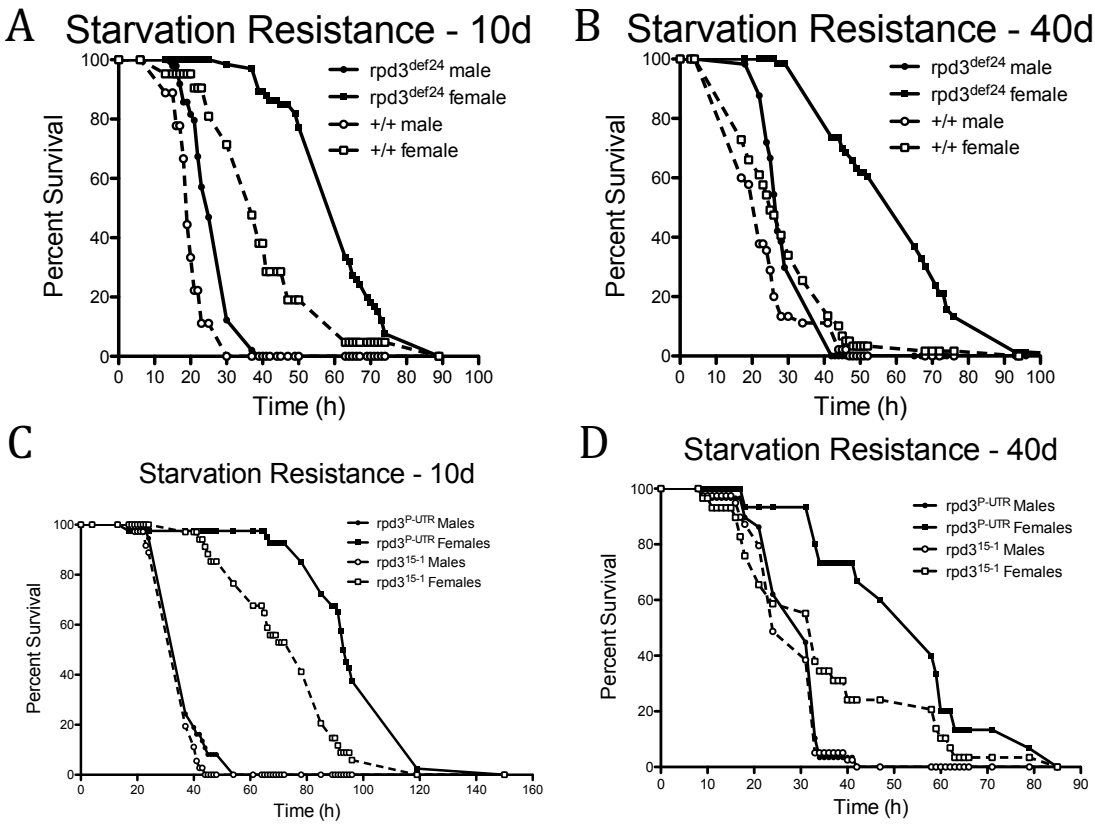

Figure 2.6 A) Starvation resistance studies for 10 day old *rpd3^{def24}/+* and *+/+* flies. **B)** Starvation resistance studies for 40 day old *rpd3^{def24}/+* and *+/+* flies. **C)** Starvation resistance studies for 10 day old *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* flies. . **D)** Starvation resistance studies for 40 day old *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* flies. Graphs are plotted as percentage of flies surviving as a function of time.

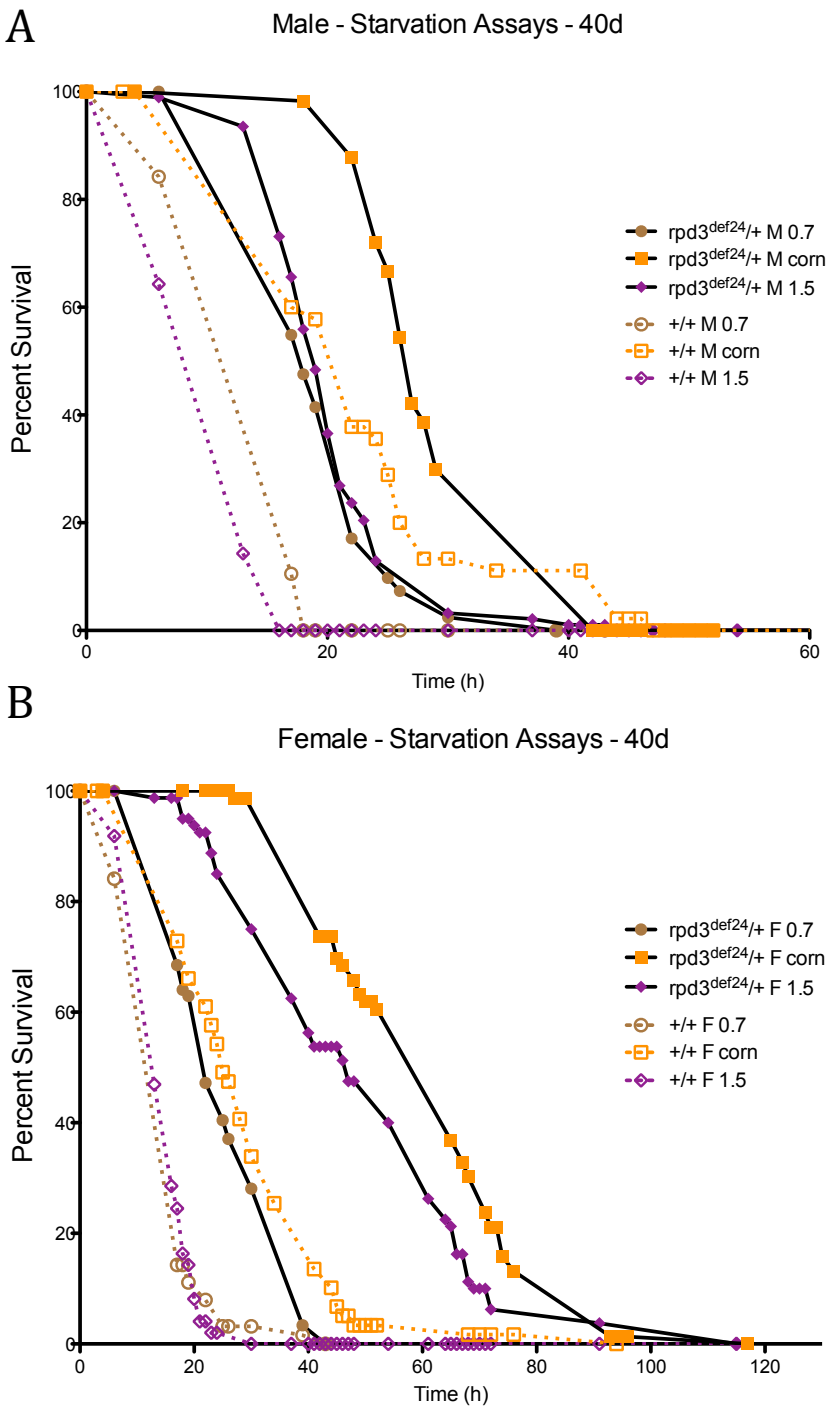

Figure 2.7 A) Starvation resistance studies for male 40 day old *rpd3^{def24}/+* and *+/+* flies on three different food levels, 0.7N, corn, and 1.5N. **B)** Starvation resistance studies for female 40 day old *rpd3^{def24}/+* and *+/+* flies on three different food levels, 0.7N, corn, and 1.5N. Graphs are plotted as percentage of flies surviving as a function of time.

2.3.4 *rpd3* mutants have altered biochemical energy stores

Organisms who are in a state of dietary restriction either by CR or by a genetically induced state of CR would be expected to experience a decrease in energy stores. CR organisms also weigh less compared to controls on a normal diet (Weindruch and Sohal, 1997). To test if *rpd3*-mutants were in a state of genetic CR, we measured total body levels of glucose, glycogen, trehalose, proteins, and triglycerides. In addition, triglycerides were also measured following a 24-hour period of starvation to test the mobilization of lipids following starvation.

The weight of *rpd3*-mutant flies and their controls were the first things to be measured. $rpd3^{def24}/+$ males weighed significantly more than *+/+* males at both 10 and 40 days of age (Fig. 2.8A) The same was true for females at both ages as well, with an even greater difference than males at 10 days. $rpd3^{P\text{-}UTR}/CS$ females had similar weights as $rpd3^{15\text{-}1}/CS$ females at both 10 and 40 days of age (Fig. 2.8B). Interestingly, male $rpd3^{P\text{-}UTR}/CS$ flies weighed significantly less than $rpd3^{15\text{-}1}/CS$ males at 10 days of age, even though the difference in the weights of the two groups is extremely small. The weights of 40 day old male $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies were not significantly different though. Taken together, the weights of groups of flies with *rpd3*-mutant alleles do not suggest that *rpd3* mutation is a genetic form of CR.

Carbohydrate metabolism has been shown to be altered in long-lived fruit flies (Broughton et al., 2005). At 10 days of age, $rpd3^{def24}/+$ males had the same quantity of glucose as their genetic controls, yet at 40 days, their total glucose levels were significantly increased compared to controls (Fig. 2.9A). Female $rpd3^{def24}/+$

flies had significantly decreased glucose levels compared to controls at 10 days, but at 40 days, their levels of glucose were much greater than the levels in control flies. Glycogen levels were not changed between *rpd3*^{def24}*/+* and *+/+* males at either 10 or 40 days (Fig. 2.9C). There was a trend towards increased glycogen levels in *rpd3*^{def24}*/+* flies at 40 days of age, though. In females, glycogen stores were also significantly decreased at 10 days in *rpd3*^{def24}*/+* flies, but they were increased significantly at age 40. Unlike mammals, *Drosophila* can synthesize trehalose, a form of carbohydrate storage used by insects mainly for flight, so we quantified the levels of this form of energy storage as well (Belgacem and Martin, 2006). At 10 days, there was no difference between males or females and their respective controls (Fig. 2.9E). At 40 days of age, both genders of *rpd3*^{def24}*/+* flies had increased levels of trehalose compared to their genetic controls. These results suggest that *rpd3*^{def24}*/+* flies are not calorically restricted genetically.

Carbohydrate stores were measured in *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* flies as well. Glucose levels were not different between *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* males at either 10 or 40 days, and the same was true for females (Fig. 2.9B). However, at 40 days levels of glucose in *rpd3*$^{P\text{-}UTR}$*/CS* males trended towards being significantly lower than in *rpd3*$^{15\text{-}1}$*/CS* males. Results were similar for glycogen and trehalose measurements in females, with there being no difference in *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* flies at either age, for both glycogen and trehalose (Figs. 2.9D, F). At 10 days, levels of both glycogen and trehalose were unchanged between *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* males. *rpd3*$^{P\text{-}UTR}$*/CS* males had significantly lower levels of glycogen, and their levels of trehalose trended towards being significantly lower. These results

suggest that age-related changes in carbohydrate storage may depend on both the genetic background and the type of mutation in *rpd3* mutants.

Protein storage is another form of energy storages that has been found to change during aging and in a state of CR. At 10 days the levels of proteins are the same in both males or female $rpd3^{def24}/+$ flies as their genetic controls (Fig. 2.10A). At 40 days of age, $rpd3^{def24}/+$ males have a significant increase in total levels of protein compared to *+/+* flies. There is no change in proteins between $rpd3^{def24}/+$ and *+/+* females at 40 days. For $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies, males have no difference in protein levels at 10 or 40 days of age (Fig. 2.10B). However, 10 day old, female $rpd3^{P\text{-}UTR}/CS$ flies have significantly lower levels of protein than 10 day, $rpd3^{15\text{-}1}/CS$ females. There is no change between proteins in 40 day old, female $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies.

Lipids are the form of energy storage that has the most energy per gram compared to carbohydrates or protein. Their levels are significantly reduced in many forms of caloric restriction, even in humans (Fontana et al., 2004). When triglyceride levels were measured from whole flies, $rpd3^{def24}/+$ and *+/+* males did not have significantly different levels at either 10 or 40 days of age, but there was a trend towards increased levels in $rpd3^{def24}/+$ males at both ages (Fig. 2.11A). Female $rpd3^{def24}/+$ flies had significantly increased triglyceride levels than *+/+* females at both ages. In $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies, triglyceride levels were not significantly different in either gender at 10 days (Fig. 2.11B). At 40 days of age, though, $rpd3^{P\text{-}UTR}/CS$ males had a significantly higher triglyceride level than $rpd3^{15\text{-}1}/CS$ males. The levels were unchanged in females at 40 days.

Triglycerides were also measured following a 24-hour period of starvation in *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* flies that were raised on 1.5N food. It has been found that CR flies have decreased levels of lipids following periods of starvation, while flies raised on a high-calorie diet maintain lipid levels following a period of starvation (Wang et al., 2009). In males at both 10 and 40 days, there was a trend towards increased triglyceride levels following starvation in *rpd3*$^{P\text{-}UTR}$*/CS* males compared to *rpd3*$^{15\text{-}1}$*/CS* males, but this was not significant (Fig. 2.11C). However, in females the levels of triglycerides following starvation were significantly increased in *rpd3*$^{P\text{-}UTR}$*/CS* flies compared to *rpd3*$^{15\text{-}1}$*/CS* flies at 10 and 40 days of age. The triglyceride stores of *Drosophila* depend on many factors throughout the life span, and our results suggest that triglycerides are generally increased in flies with an *rpd3* mutation.

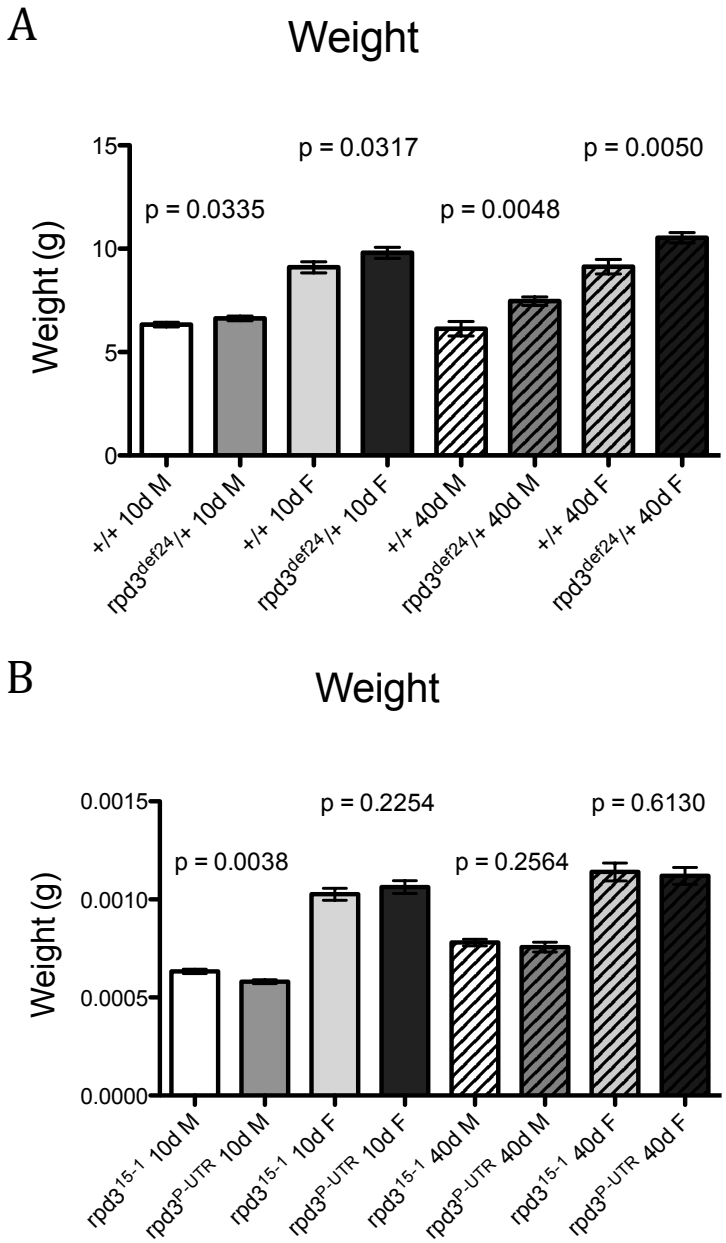

Figure 2.8 A) Average fly weight at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. **B)** Average fly weight at both 10 and 40 days for *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* males and females. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

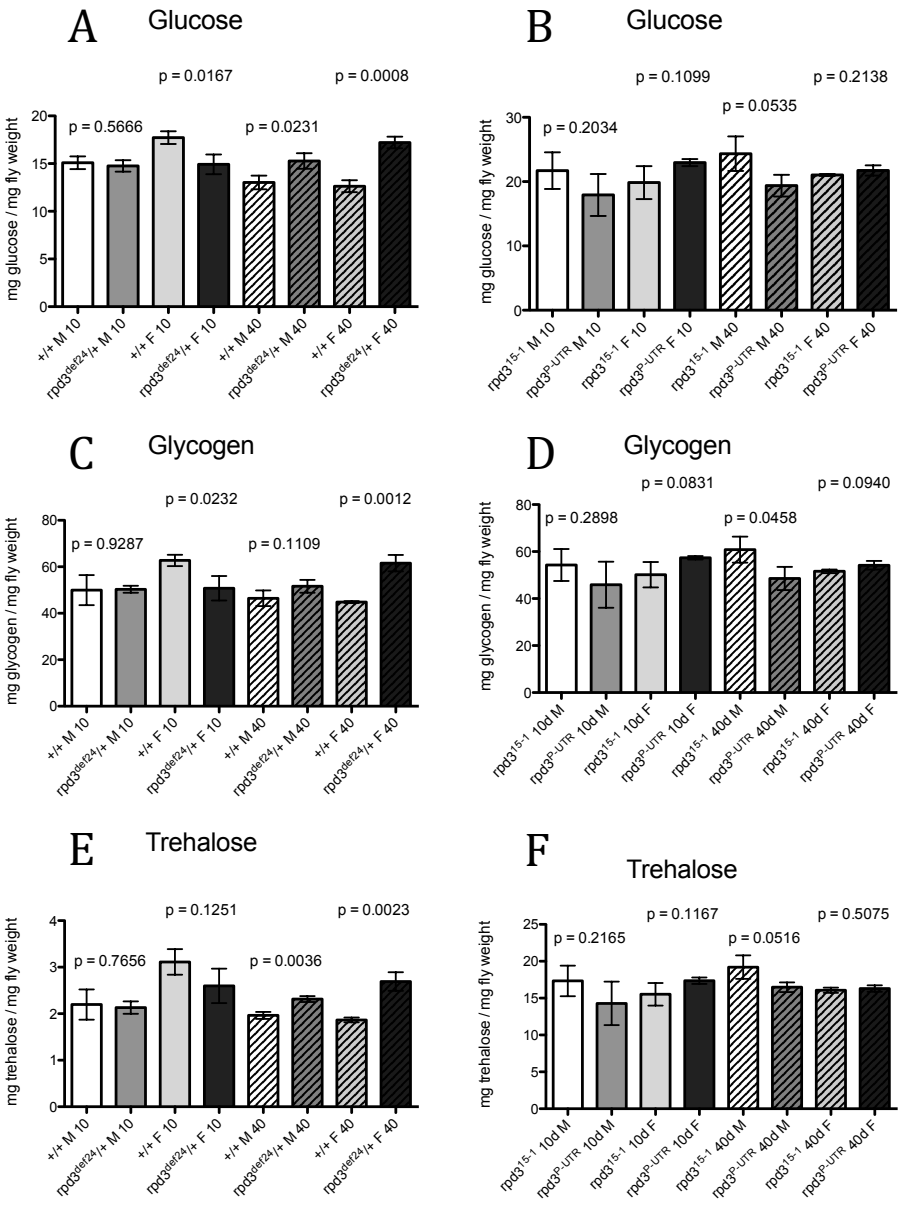

Figure 2.9 A) Average level of glucose at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. **B)** Average level of glucose at both 10 and 40 days for *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* males and females. **C)** Average level of glycogen at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. **D)** Average level of glycogen at both 10 and 40 days for *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* males and females. **E)** Average level of trehalose at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. **F)** Average level of trehalose at both 10 and 40 days for *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* males and females. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

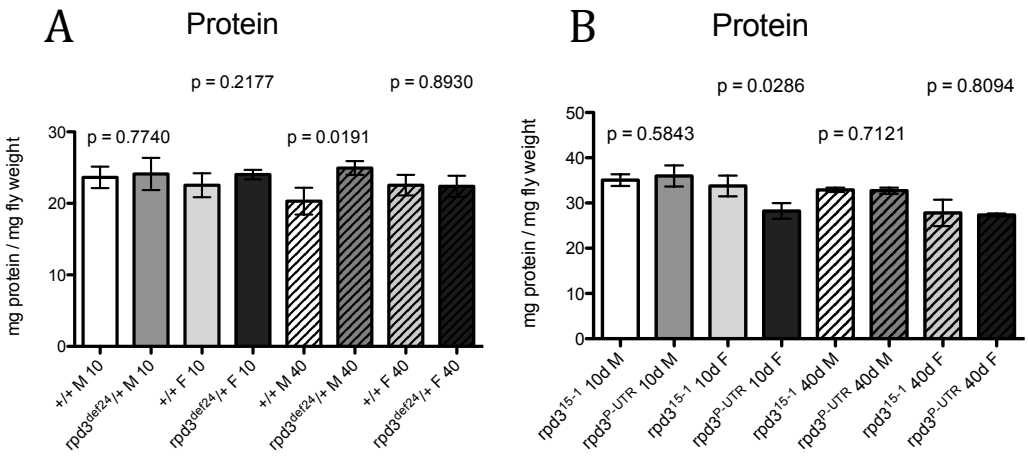

Figure 2.10 A) Average level of total protein at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. **B)** Average level of total protein at both 10 and 40 days for *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* males and females. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

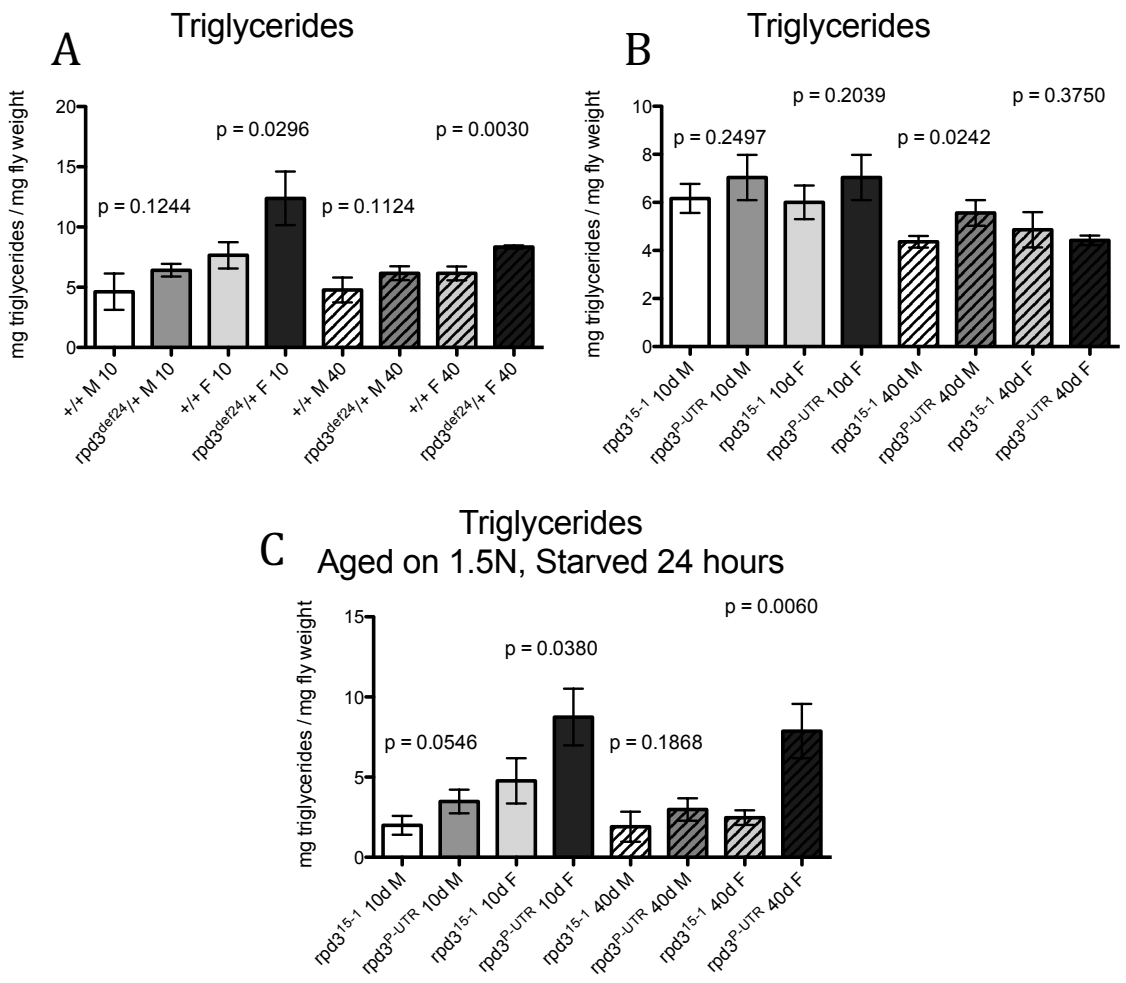

Figure 2.11 A) Average level of triglycerides at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. **B)** Average level of triglycerides at both 10 and 40 days for *rpd3$^{P\text{-}UTR}$/CS* and *rpd3$^{15\text{-}1}$/CS* males and females. **C)** Average level of triglycerides at both 10 and 40 days for *rpd3^{def24}/+* and *+/+* males and females. Flies for **C** were aged on 1.5N, high-calorie food and starved for 24 hours before processing for triglyceride analysis. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

2.3.5 *rpd3* mutation results in changes in spontaneous locomotor activity

Another hallmark of CR in multiple species is an increased spontaneous locomotor activity (Parashar and Rogina, 2009). This is thought to be due to an increased scavenging for food. To test if there is an overlap in the mechanism of CR and *rpd3* mutation, we examined the spontaneous physical activity of *rpd3* mutants and their controls. At 10 days of age, we found increases in peak activity in $rpd3^{def24}/+$ males (Fig. 2.12A). However, females showed little difference between $rpd3^{def24}/+$ flies and their controls at 10 days of age (Fig. 2.12B). It is possible that male $rpd3^{def24}/+$ flies have more spontaneous activity than their controls, yet the significance of this increase is still unknown.

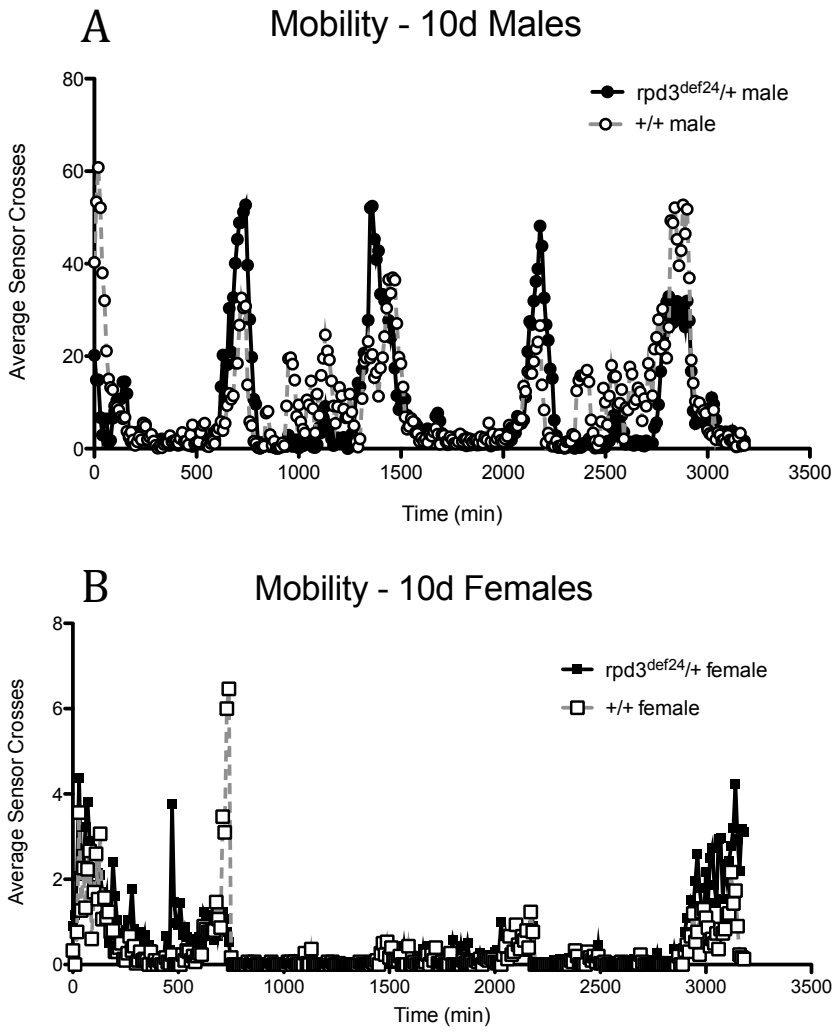

Figure 2.12 A) Total mobility shown as average beam interruptions as a function of time for 10 day old male *rpd3^{def24}/+* and *+/+* flies. **B)** Total mobility shown as average beam interruptions as a function of time for 10 day old female *rpd3^{def24}/+* and *+/+* flies.

2.4 Discussion

As expected, there were many physiological changes in *rpd3*-mutant flies. We showed that there was a decrease in *rpd3* mRNA expression in *rpd3*-mutant flies. Unfortunately, we were not able to generate lines with skeletal-muscle-specific knockdown of *rpd3*. Therefore we used our two previously published *rpd3*-mutant fly lines to test various aspects of physiology.

We showed that $rpd3^{def24}/+$ mutants were more resistant to oxidative stress at a later age, and that they were more resistant to starvation at both ages. These data are consistent with previously reported literature on stress resistance and long-lived *Drosophila* (Bjedov et al., 2010; Yu and Chung, 2001). Although we hypothesized that *rpd3* mutants live longer through mechanisms that overlap with CR, our starvation studies neither confirm nor deny this hypothesis due to conflicting reports in the literature.

Starvation resistance has been shown to be both increased and decreased in experimental groups on a CR diet. Therefore, the increase in starvation resistance that we see in *rpd3*-mutant flies could indicate that these flies are in a state of genetic CR. It is thought that the slight stress of CR on the body prepares the organism for further stress, such as starvation (Yu and Chung, 2001). For example, corticosterone levels are chronically increased in CR yet organisms still live longer. It is also possible that these flies are not in a state of CR, as indicated by their increased levels of biochemical energy stores.

For most forms of chemical energy storage that we measured in *Drosophila*, there were increases in either 40 day old male or female $rpd3^{def24}/+$ flies compared

to their controls. In addition, *rpd3*^{def24}*/+* flies weigh more than their controls in both genders and at both 10 and 40 days of age. These results would suggest that *rpd3* mutants are not in a state of genetic caloric restriction. Interestingly, these results are consistent with flies that have had their insulin producing cells in the brain ablated (Broughton et al., 2005). Flies in these experiments experienced increased levels of trehalose, glycogen, and lipids.

One group of experiments that is consistent with our initial CR and *rpd3* overlapping in mechanism hypothesis is the group of oxidative stress resistance experiments. We showed that *rpd3* mutants are more resistant to hydrogen peroxide at a later age, but not a younger age. This suggests that the *rpd3*^{def24}*/+* flies and *+/+* flies are equally as healthy at a young age, yet the *rpd3*^{def24}*/+* flies age at a slower rate. Although consistent with the fact that CR organisms also show increased stress resistance, it does not prove that *rpd3* mutants are in a state of caloric restriction.

Out of the oxidative stress experiments performed at different ages, we showed a gradual decline in survivorship with age in all groups except for 40 day old *rpd3*^{def24}*/+* males. These data show a gradual decline that is to be expected with age except for in this one group. However, this is not a perfect measurement, as these experiments were not performed at the exact same with the same working solution of hydrogen peroxide. This could account for the differences seen. Also, *rpd3*^{def24}*/+* males are the group with the greatest life span extension, so it is possible that they are the healthiest and most resistance to oxidative stress. More work will

need to be done to determine the downstream targets that are affected by *rpd3* mutation and mediate the changes seen in *rpd3* mutants.

2.5 Methods

2.5.1 Fly Stocks and Maintenance

Our lab has extensive experience working with and maintaining lines of *Drosophila* in aging research. 2 strains of *rpd3*-mutant *Drosophila melanogaster* were used as they have been in the past (Rogina et al., 2002; Mottus et al., 2000). $rpd3^{def24}/Sb,ser$ female virgins were crossed with *yw* males, to generate $rpd3^{def24}/+$ flies or *Sb,ser/+* flies. *Sb,ser/+* males and female virgins were crossed to generate *+/+* flies, which were used as the genetic control of $rpd3^{def24}/+$ flies. For the other *rpd3*-mutant allele, $rpd3^{P\text{-}UTR}/Sb,ser$ or $rpd3^{15\text{-}1}/Sb,ser$ males were crossed with *Canton-S* virgin females to generate $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies. The hypomorphic $rpd3^{P\text{-}UTR}$ allele has a P-element inserted in the 5'UTR region of the *rpd3* gene, which affects expression throughout the entire fly body. The control $rpd3^{15\text{-}1}$ allele has a P-element inserted 1.8 Kb upstream from the transcriptional start site, which only decreases expression in the eye (Mottus et al., 2000). $rpd3^{P\text{-}1.8}/Sb,ser$ is another name used for $rpd3^{15\text{-}1}/Sb,ser$ flies in literature.

Groups of 25 males and 25 females were collected 24 hours after eclosion. Unless indicated, flies were aged in vials containing normal corn food in a humidity-controlled incubator at 25°C on a 12-hour night/day cycle for their entire lifespan until time for experimentation. Normal *Drosophila* food consisted of water, inactive yeast, sucrose, agar, corn, and Tegosept (Methyl4-hydroxybenzoate, Sigma) as an

antifungal agent (Woods et al., 2014). For 0.7N food, the percentage of yeast and sugar were decreased by 30%, corn was left out of the recipe, but agar and Tegosept quantities were unchanged. For 1.5N food, the yeast and sugar were both increased by 50%, corn was left out of the recipe, but agar and Tegosept quantities were unchanged. Flies were passed into new vials of food every other day to prevent build up of bacteria.

For *UAS-rpd3*-RNAi lines, one line was acquired from the Bloomington stock center, *y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00164}attP2*, which we refer to as *503*. Two additional lines were acquired from the Vienna stock center, *w[1118]; P{GD4513}v30599/TM3*, and *w[1118]; P{GD4513}v30600*, which we refer to as *30599* and *30600*, respectively. These flies were backcrossed to *yw* flies, and then virgins were collected. We then crossed the *rpd3*-RNAi, virgin females with males expressing the myosin heavy chain (*Mhc-GS-Gal4*) driver (Katewa et al., 2012). Groups of 25 males and 25 females were raised either on corn food containing RU-486 (200 uM mifepristone, from Invitrogen) or EtOH at 25^{o}C on a 12 hour night/day cycle for their entire lifespan until time for experimentation.

2.5.2 Quantitative PCR (qPCR)

Flies were aged until the desired age, separated into groups of males or females, and then frozen and kept at -80^{o}C until dissection. Groups of 35 flies were dissected on a cold plate to ensure they stayed frozen, and each genotype had 3 biological replicates. Total RNA was isolated from groups of 35 heads and thoraces for $rpd3^{def24}$/+ or +/+ flies, or 35 thoraces for $rpd3^{P\text{-}UTR}$/*CS* and $rpd3^{15\text{-}1}$/*CS* flies. Total RNA was isolated from dissected tissue using Trizol as previously described (Wang

et al., 2009). cDNA was synthesized using a standard protocol, and qPCR was performed to determine changes in gene expression using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). TaqMan primers were obtained for *rpd3* from Invitrogen, and *ankryn* was used as an endogenous control. Gene expression was averaged between the biological replicates, and results are reported as relative mRNA expression as compared to the control group.

2.5.3 Oxidative Stress Resistance

Flies were aged until 10, 20, 30, or 40 days of age, and then they were either exposed to hydrogen peroxide or paraquat (Tettweiler et al., 2005). For both experiments, *Drosophila* were sorted on CO_2 pads into groups of 20 males or 20 females. They were placed on food and allowed to recover for at least 1 hour. Then they were switched to tubes containing only water and 2 filter papers for 6 hours. This brief starvation is thought to eliminate differences in the feeding state of the flies and prevent differential intake of the oxidative stressor. Following these 6 hours, flies were transferred to new tubes containing 300 ul of the hydrogen peroxide or paraquat working solution and 2 filter papers. For H_2O_2, the working solution consisted of 5% H_2O_2 and 5% sucrose diluted in distilled water. For paraquat, the working solution contained 20 mM paraquat and 5% sucrose diluted in distilled water. Dead flies were counted every hour or two during the day, and at one time point each late at night and early in the morning. Surviving flies were switched to new vials every 24 hours to prevent desiccation. Results are plotted as percent surviving verses time in hours.

2.5.4 Starvation Resistance

Flies were aged until 10 or 40 days of age, and then they were exposed to starvation without desiccation as described previously (Wang et al., 2009). For both experiments, *Drosophila* were sorted on CO_2 pads into groups of 20 males or 20 females. They were placed on food and allowed to recover for at least 1 hour. Then they were switched to tubes containing only 300 ul water and 2 filter papers. Dead flies were counted every hour or two during the day, and at one time point late at night and another early in the morning. Surviving flies were switched to new vials every 24 hours to prevent desiccation. Results are plotted as percent surviving verses time in hours.

2.5.5 Biochemistry of Molecular Energy Stores

Flies were aged under standard conditions until 10 or 40 days of age. Flies were separated into groups of 10, anesthetized using a CO_2 pad, and then weighed. Flies were then placed in 100 ul of homogenization buffer, and homogenized using a handheld, motorized homogenizer with Teflon pestles. Homogenization buffer is composed of 0.01M KH_2PO_4 and 1mM EDTA pH 7.4. 900 ul of additional homogenization buffer was then added to the samples and then they were spun for 2 minutes at 2000rpm and at 4^{o}C. Following this, 25 ul of samples were aliquoted in triplicate into 96-well plates, and these plates were frozen and stored at -80^{o}C until each experiment was performed.

The levels of each form of molecular energy storage were quantified as previously described (Clark and Keith, 1988). For glucose, PGO enzyme (Sigma #7119) plus color reagent was added to each homogenate on a 96-well plate, and

the optic density was read at 450 nm (Protocol from Sigma Kit 510). The procedure was the same for glycogen and trehalose except for the addition of amyloglucosidase and trehalase, respectively, before the addition of PGO. For quantification of total protein levels, BioRad protein solution (Bio-Rad Kit) was added to each homogenate, and the plate was read at 595 nm. For triglycerides, free glycerol and triglyceride reagents were added to each well containing homogenate, and the optic density was read at 540 nm (TRO100, Sigma). The results for each experiment were normalized per gram of fly weight, and the final results were statistically analyzed using the paired Student's T test.

2.5.6 Mobility

Spontaneous physical activity of *Drosophila* populations were measured as previously done in our lab (Woods et al., 2014 [see Chapter 6]; Parashar and Rogina, 2009). Flies were aged until the desired age for experimentation, and they were sorted into groups of 10 males or females on a CO_2 pad. They were placed in a glass vial containing food and allowed to recover for at least one hour before being placed in mobility monitors. The numbers of infrared beams interruptions were recorded hourly and at 3 different vertical levels of each glass vial containing the flies. The data were graphed as a function of time to see peaks in locomotor activity.

2.5.7 Statistical Analysis

Significance was determined using a two-tailed, unpaired t-test and expressed as P values. $P < 0.05$ is considered to be significant. Error bars represent standard deviation (SD).

Chapter 3: *rpd3*-Mutant *Drosophila* do not Experience an Increase in Mitochondrial Biogenesis and Function

3.1 Background and Significance

As previously discussed, mitochondria are important organelles that play an important role in the response to CR (Wallace, 2005). Our initial publication suggests that *rpd3*-mutant *Drosophila* experience an increase in life span potentially through a mechanism that overlaps with CR (Rogina et al., 2002; Rogina and Helfand, 2004). *rpd3*-mutant flies aged on 0.5N food did not experience a further increase in lifespan than the same flies aged on food with a normal calorie content. Also, *rpd3* mutants had increased expression of *dSir2* that was comparable to the increased *dSir2* expression seen in control flies aged on a CR diet.

Mitochondrial biogenesis is a process that has been increased in many organisms on CR. Our lab and others have shown the importance of increased biogenesis in healthy aging in *Drosophila* (Rera et al., 2011; Rogers and Rogina, 2014). Many genes regulate mitochondrial biogenesis, such as *PGC-1α* or *Sir2* (Lopez-Lluch et al., 2006). When this process is increased, it results in a larger pull of smaller mitochondria that are more energetically efficient and produce less ROS (Guarente, 2008). Increased respiration has also been shown to be important for the response to CR. Because of the relation to CR and aging, mitochondrial biogenesis and function were hypothesized to be altered in *rpd3* mutants.

3.2 Rationale

If *rpd3* mutation has its effects on longevity through a mechanism that overlaps with CR, it is possible that *rpd3*-mutant flies experience increased mitochondrial biogenesis and function. It has been shown in multiple species that mitochondria are critical to the response to CR, with extensions in life span not seen in organisms that are on CR regimens but have impaired respiration (Guarente, 2008). It has been hypothesized that increased mitochondrial biogenesis is beneficial because the larger number of newly generated mitochondria can protect against the damage that occurs to mitochondrial DNA and enzymes as they age. It is also possible that turnover via autophagy of mitochondria is increased as well in organisms on CR, leading to increased numbers of functional mitochondria and decreased numbers of damaged mitochondria. Consistently, autophagy has been shown to be necessary for the life span extension seen in CR in worms (Hansen et al., 2008). In addition to potential overlap of the mechanism of CR and *rpd3*-mutation, the Sin3 complex that relies on Rpd3 for its regulatory role has been shown to alter gene expression of mitochondrial proteins and other metabolic pathways in *Drosophila* tissue culture cells (Pile et al., 2003). The authors found that many genes involved in the electron transport chain, genes for antioxidant proteins, and genes involved in fatty acid oxidation were upregulated in cells lacking Sin3. Taken together, there is ample evidence to suggest *rpd3*-mutant *Drosophila* may experience an increase in mitochondrial biogenesis and function, consistent with their long life spans.

Mitochondria were initially believed to have an increased ROS production as their energy production increased, such as in increased respiration seen under conditions of CR (Guarente, 2008). However, one study showed that HeLa cells

cultured on serum from CR rats actually had a decrease in ROS production, even though there was no decrease in ATP production (Lopez-Lluch et al., 2006). Determining the mitochondrial respiration of *rpd3*-mutant *Drosophila* will provide insights to the mechanism of their life span extension.

3.3 Results

3.3.1 Mitochondrial biogenesis is not upregulated in *rpd3*-mutant flies

Increased mitochondrial biogenesis has been shown to be critical for organisms to respond to CR, and it is upregulated in a variety of species ranging from yeast to humans as a response to CR (Kayser et al., 2004; Civitarese et al., 2007). We measured biogenesis by a commonly used method, quantification of mitochondrial DNA in relation to nuclear DNA. This ratio has been shown to increase in organisms on CR (Lopez-Lluch et al., 2006). At 20 days of age, there is no difference between $rpd3^{def24}/+$ and *+/+* males in the ratio of a mitochondrial gene, *CytC*, to the quantity of a nuclear gene, *GAPDH* (Fig. 3.1A). The same results were seen at 40 days of age (Fig. 3.1B). Spargel (*Drosophila* PGC-1α homologue) is a transcription factor that is a main regulator of mitochondrial biogenesis, and the levels of this gene expression for *spargel* generally correlate with the level of mitochondrial biogenesis (Rera et al., 2011). We measured *spargel* expression in our flies as well. In males at 20 days of age, we found no change in mRNA expression of this gene between $rpd3^{def24}/+$ and *+/+* flies (Fig. 3.1C). The level of expression was not changed at 40 days of age either (Fig. 3.1D). These results suggest that mitochondrial biogenesis is not affected in *rpd3* mutants.

3.3.2 *rpd3*-mutant flies do not have increased levels of *dSir2* mRNA expression

As discussed previously, Sirtuins have been shown to be major factors that regulate mitochondrial biogenesis in CR and long-lived organisms (Guarente, 2008). We analyzed levels of *dSir2* mRNA expression in *rpd3*-mutant flies at 20 and 40 days of age. The levels of *dSir2* expression did not differ between $rpd3^{def24}/+$ and *+/+* males at either 20 or 40 days (Fig 3.2A). The levels of *dSir2* mRNA expression did decrease significantly though from 20 days to 40 days. In *Canton-S* flies, the levels of *dSir2* expression were not significantly different at any age (Fig. 3.2B). However, 40 day old males did show a trend towards increased expression compared to 10, 20, or 60 day old flies. These results differ from our initial study in which *dSir2* expression was increased in 10 day old *rpd3*-mutant flies compared to controls (Rogina et al., 2002).

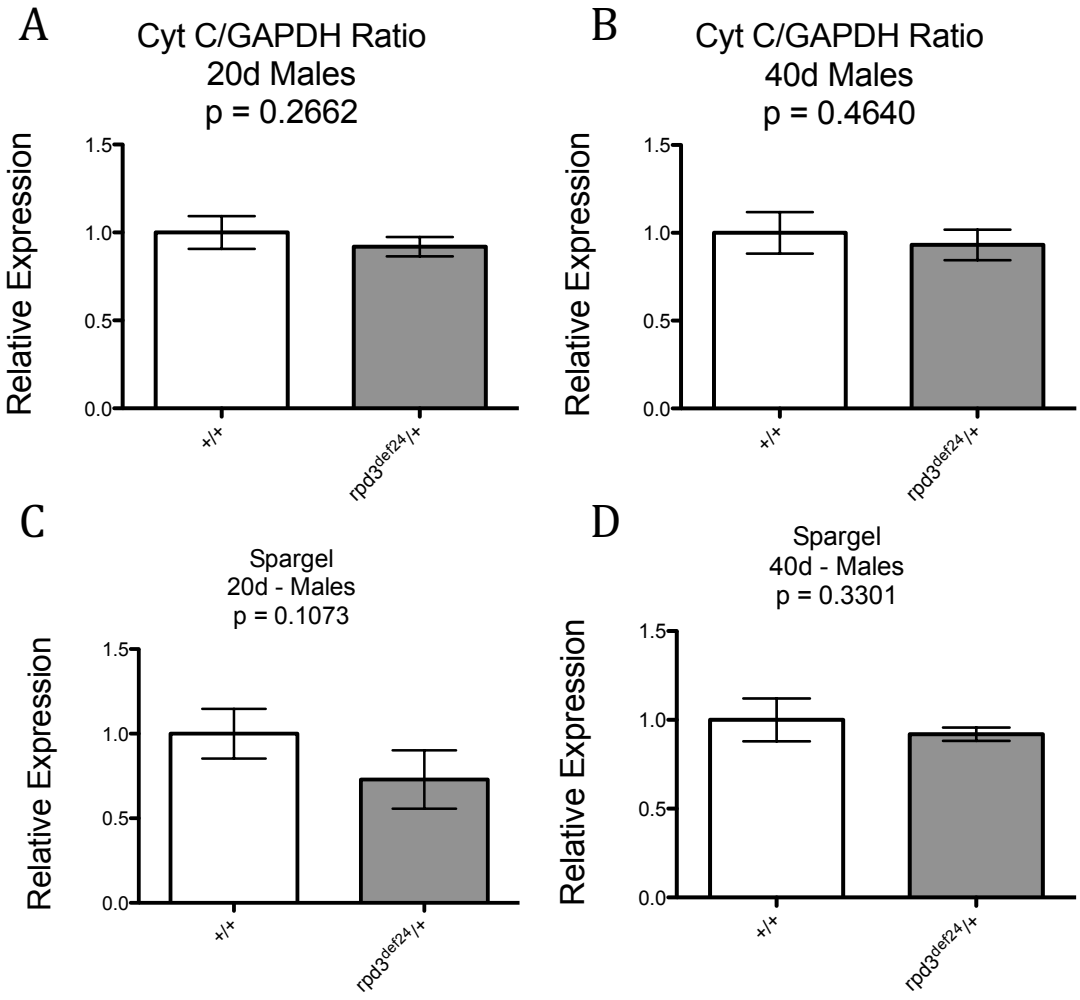

Figure 3.1 A) qPCR results for the ratio of *Cytochrome C* / *GAPDH* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **B)** qPCR results for the ratio of *Cytochrome C* / *GAPDH* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. **C)** qPCR results for *spargel (Drosophila PGC-1α)* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **D)** qPCR results for *spargel (Drosophila PGC-1α)* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

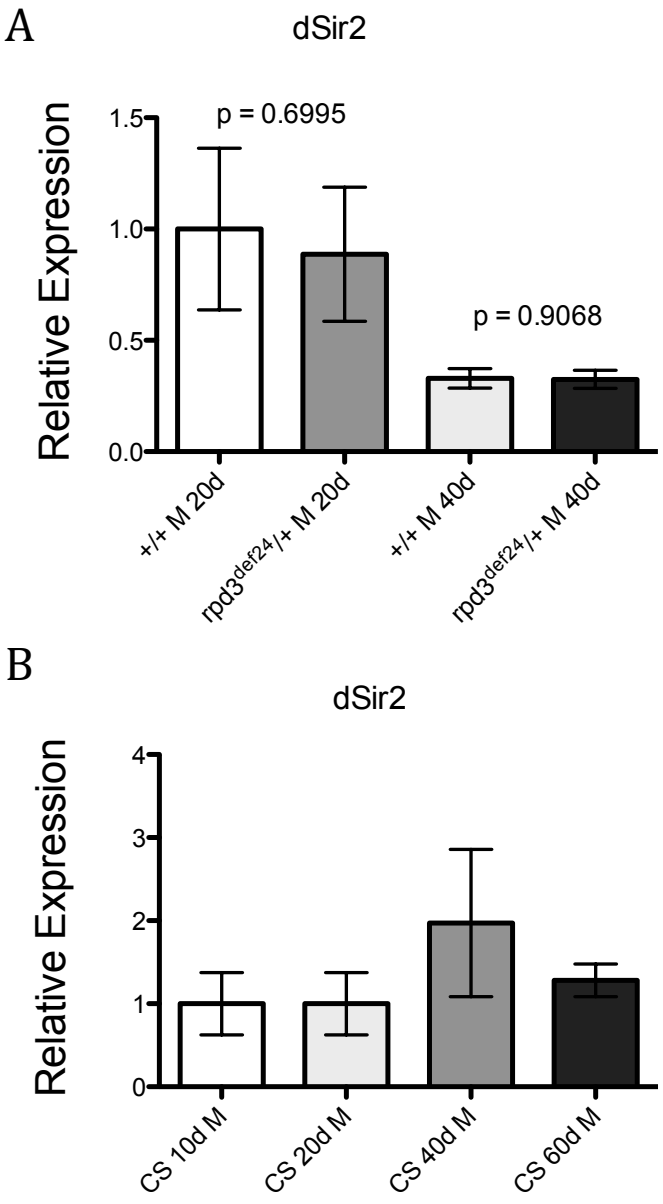

Figure 3.2 A) qPCR results for *dSir2* expression in 20 and 40 day old *rpd3^{def24}/+* and *+/+* males. **B)** qPCR results for *dSir2* expression in 10, 20, 40 and 60 day old *CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

3.3.3 *rpd3* mutants do not have increased mitochondrial numbers in skeletal muscle tissue

Although we did not see changes in mitochondrial DNA copy number or *spargel* mRNA expression, we wanted to be certain that mitochondria were not increased in the tissue of *rpd3* mutants. Using EM as our lab had previously done, we quantified the number of mitochondria in skeletal muscle of 40 day old flies (Rogers and Rogina, 2014). Consistent with our previous results, we found no difference between $rpd3^{def24}/+$ and *+/+* flies (Fig. 3.3) or between $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies (Fig. 3.4). These data confirm there is not an increase in mitochondrial biogenesis in *rpd3* mutants.

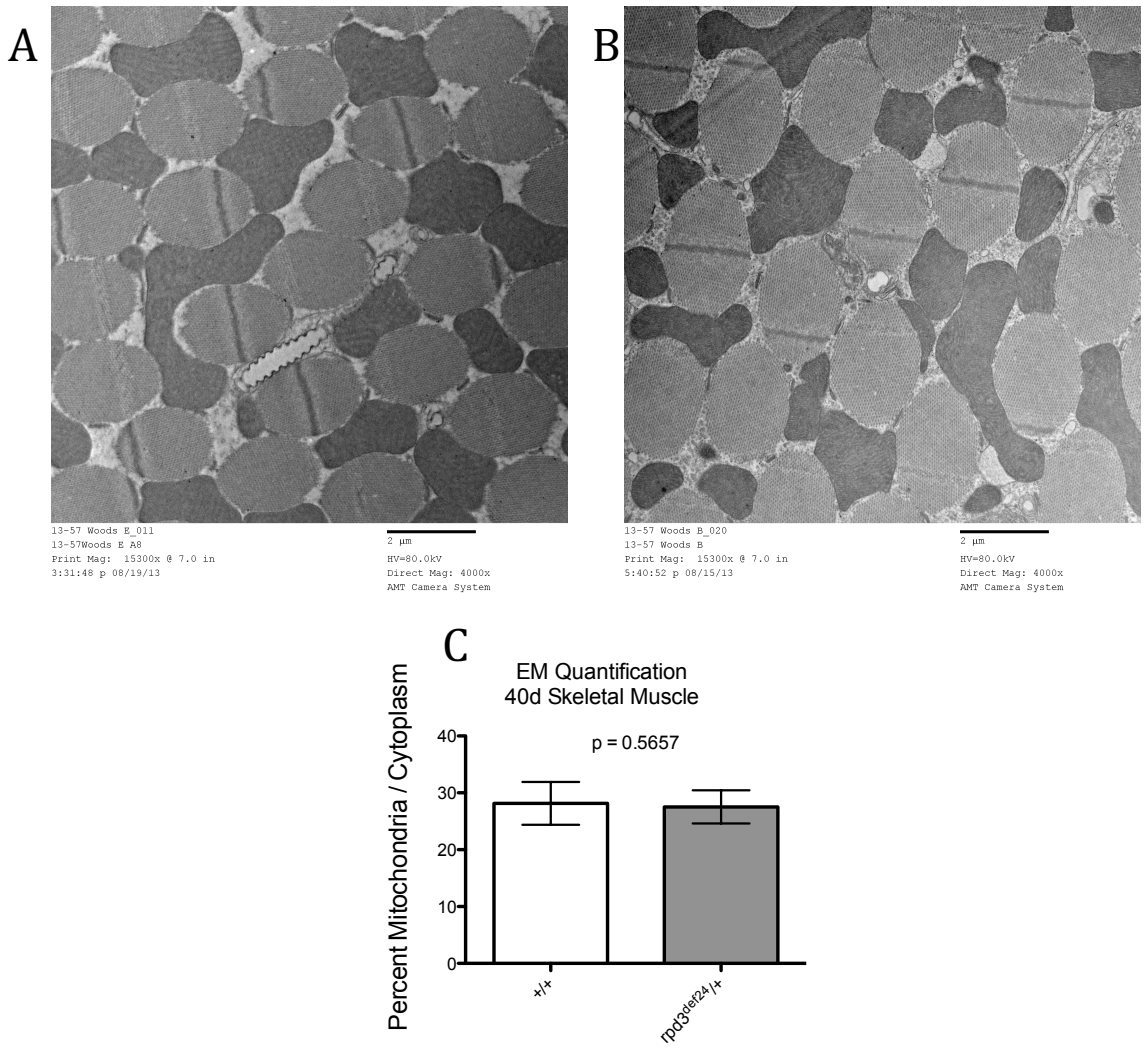

Figure 3.3 A) Representative electron micrograph of 40 day old *+/+* males. **B)** Representative electron micrograph of 40 day old *rpd3^{def24}/+* males. **C)** Quantification of percentage of cytoplasm occupied by mitochondria in 40 day old *rpd3^{def24}/+* and *+/+* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

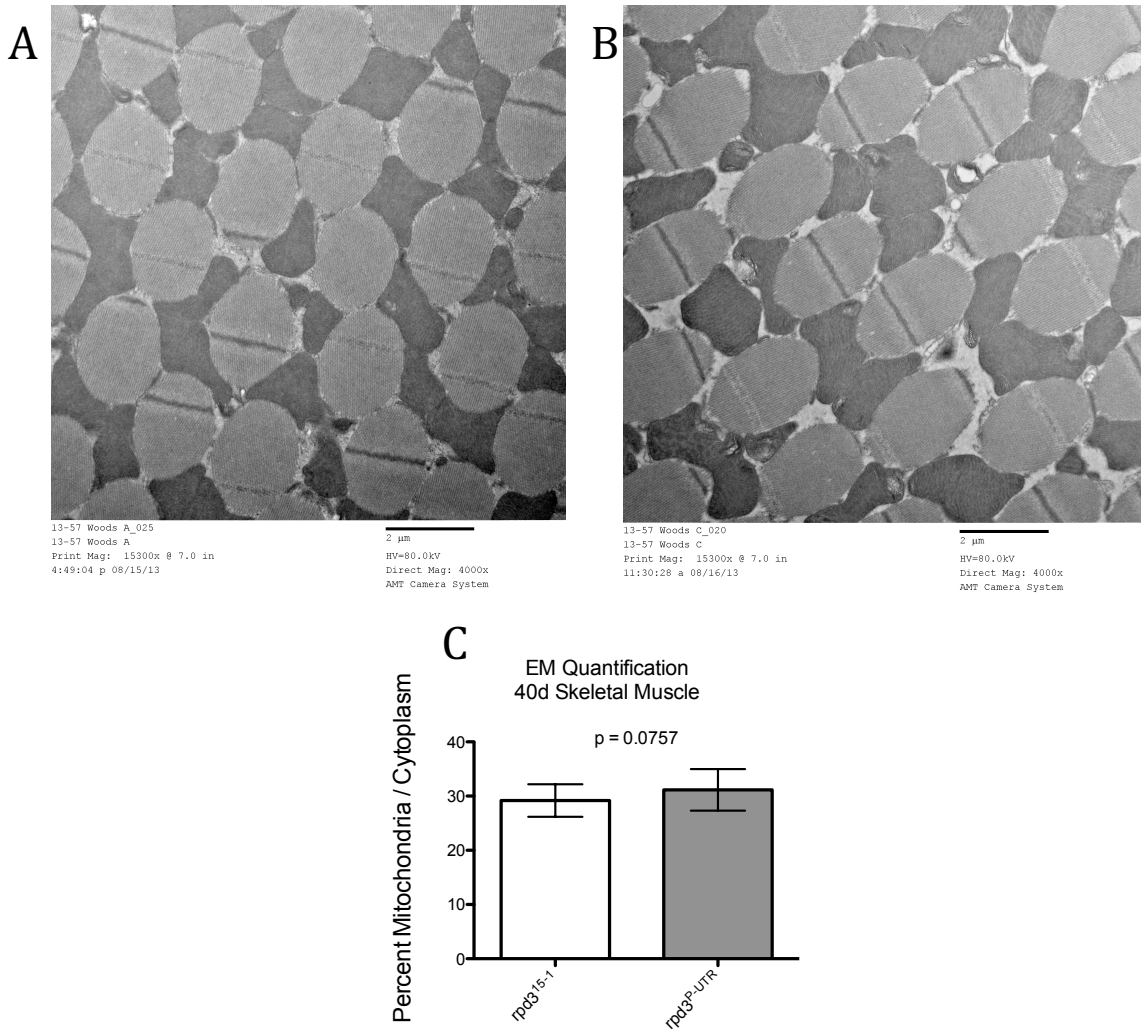

Figure 3.4 A) Representative electron micrograph of 40 day old *rpd3*$^{15\text{-}1}$*/CS* males. **B)** Representative electron micrograph of 40 day old *rpd3*$^{P\text{-}UTR}$*/CS* males. **C)** Quantification of percentage of cytoplasm occupied by mitochondria in 40 day old *rpd3*$^{P\text{-}UTR}$*/+* and *rpd3*$^{15\text{-}1}$*/CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

3.3.4 *rpd3*-mutant flies may have slight changes in mitochondrial respiration

Although we have shown that mitochondrial biogenesis is not affected by *rpd3* mutation, it is still possible that these flies have improved mitochondrial function. Increased respiration has been shown to be important for the response to CR (Guarente, 2008). To test this we isolated mitochondria and assessed their total respiration as well as the respiration of the individual complexes of the respiratory chain. First, we analyzed total respiration in $rpd3^{def24}/+$ and *+/+* flies at 40 days of age. For total respiration, we found that mitochondria isolated from the thoraces of male $rpd3^{def24}/+$ flies had an increased rate of respiration compared to mitochondria from *+/+* flies (Fig. 3.5A). However, mitochondria isolated from female $rpd3^{def24}/+$ flies had an decreased rate of respiration compared to mitochondria from *+/+* females. These results could partially explain why we see a greater life span extension in males compared to females in *rpd3* mutants.

Following this, we wanted to know if the individual complexes of the electron transport chain had altered function. It is known that each complex contributes differently to both energy production and ROS production (Wallace, 2005). After isolating mitochondria, malate and glutamate were added to the chamber to measure state 4 respiration of complex I (Barrientos, 2002). Following this, ADP is added to measure state 3 respiration of complex I. In both of these states, mitochondria from male $rpd3^{def24}/+$ flies had a decreased rate of respiration compared to mitochondria from *+/+* flies (Fig. 3.5B, C). Female $rpd3^{def24}/+$ mitochondria had decreased rates of complex I respiration in both states as well, yet the results were not statistically significant. To measure complex II, succinate was

added to the chamber. The rates of complex II respiration were unchanged between *rpd3*^{def24}*/+* and *+/+* flies in mitochondria from either males or females (Fig. 3.5D). For complex IV, ascorbate and TMPD are added to the chamber with isolated mitochondria. Like complex II, the rates of respiration did not differ between mitochondria from *rpd3*^{def24}*/+* and *+/+* flies in either males or females (Fig. 3.5E). Our results indicate that there are small changes in respiration in *rpd3* mutants, yet the total respiration and respiration of complex I seem to differ in the direction they are changed.

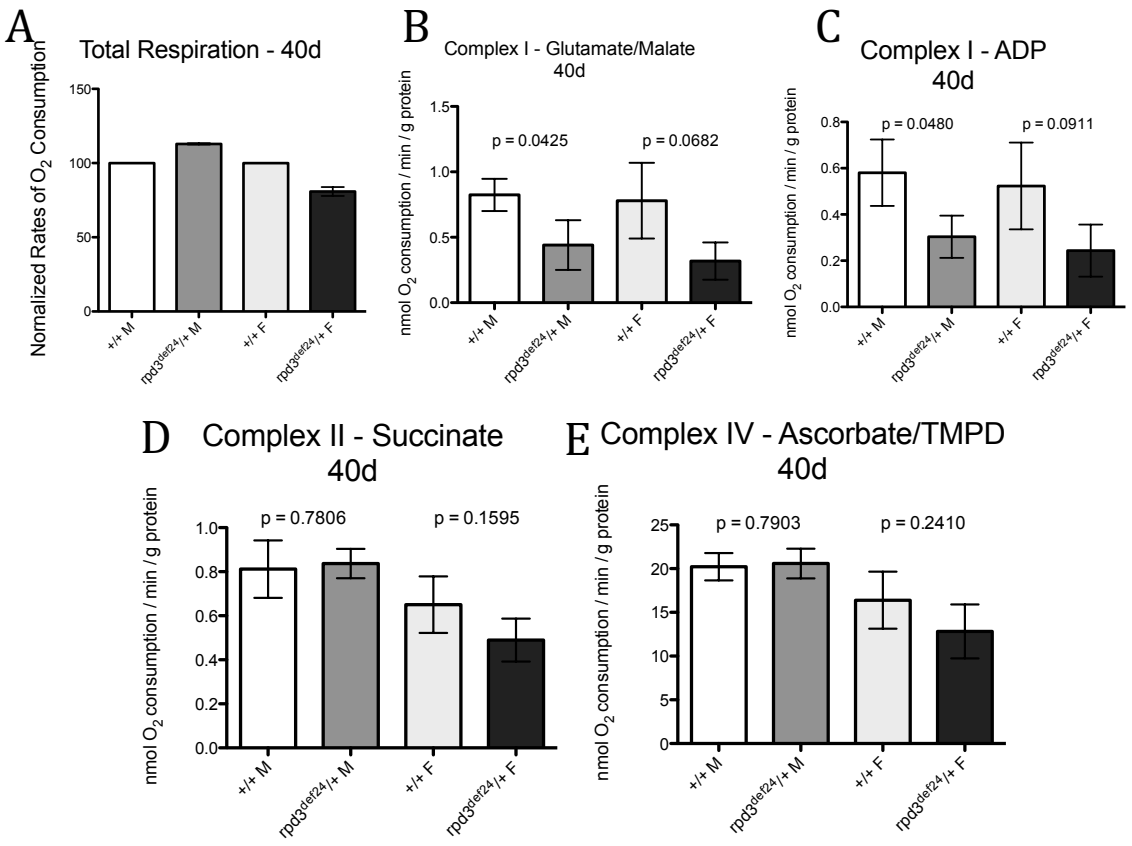

Figure 3.5 A) Total mitochondrial respiration in both male and female 40 day old *rpd3*^{def24}*/+* and *+/+* flies. **B)** Complex I respiration measured as oxygen consumption following the addition of glutamate/malate to the chamber in male and female 40 day old *rpd3*^{def24}*/+* and *+/+* flies. **C)** Complex I respiration measured as oxygen consumption after the addition of ADP following glutamate/malate to the chamber in male and female 40 day old *rpd3*^{def24}*/+* and *+/+* flies. **D)** Complex II respiration measured as oxygen consumption following the addition of succinate to the chamber in male and female 40 day old *rpd3*^{def24}*/+* and *+/+* flies. **E)** Complex IV respiration measured as oxygen consumption after the addition of ascorbate and TMPD to the chamber in male and female 40 day old *rpd3*^{def24}*/+* and *+/+* flies. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

3.4 Discussion

In these experiments, we have shown no evidence for increased mitochondrial biogenesis. We examined the quantity of mitochondrial DNA and levels of *spargel*, two commonly used methods for examining biogenesis, and found no changes from genetic controls. We also used electron microscopy to measure the percent volume of cytoplasm occupied by mitochondria in skeletal muscles of aged fruit flies. In both alleles of *rpd3* mutations, we saw no change in the number of mitochondria. Even though there is no increase in mitochondrial biogenesis, *rpd3*-mutant flies may still have an increase in life span due to unexamined aspects of mitochondrial function or signaling. For example, mitochondrial-derived peptides are a recently discovered class of retrograde signaling peptides encoded by short open reading frames in the mitochondrial DNA, one of which has been shown to alter insulin resistance in mice (Lee et al., 2015). Also, another group has found that *rpd3* inhibition does lead to increased citrate synthase activity (Chen et al., 2008). Citrate synthase catalyzes the first reaction in the TCA cycle, and has been used as an indicator of mitochondrial oxidative capacity (Bruce et al., 2004). Although we did not examine citrate synthase activity, we only found small changes in mitochondrial respiration that suggest the respiratory capacity is not as greatly altered as in Chen et al. (2008). Consistent with this, citrate synthase activity has been found to be correlated with increased *spargel* expression, a change we did not see in our *rpd3*-mutant flies (Mortensen et al., 2006).

We found no differences in the levels of *dSir2* mRNA expression in *rpd3*-mutant flies at 20 days or 40 days of age. These results differ from our original

studies, where it was found the *dSir2* expression increased in 10 day old males (Rogina et al., 2002). The original experiment was semi-quantitative PCR, so this could account for some differences. It would be interesting to do a more controlled time course study in which *dSir2* expression was quantified at regular intervals to determine what is really happening in *rpd3*-mutant flies. It would also be interesting to determine if *dSir2* expression is required for longevity extension by using the GAL4-UAS and RNAi lines of *Drosophila* to knock down *dSir2* expression in a temporally controlled and tissue-specific manner.

It has been shown that Complexes I and III of the electron transport chain are the major sources of ROS production in the mitochondria (Pamplona and Barja, 2007). Interestingly, we found that *rpd3*-mutant flies had a decreased rate of respiration compared to genetic controls at 40 days of age when measuring Complex I alone (Fig. 3.5). Potentially the decreased respiration of complex I could lead to less ROS production, which influences the longevity of *rpd3* mutants. Other studies have highlighted the differences between the different complexes of the respiratory chain. For example, a long-lived *C. elegans* mutant was found to have alterations in Complex I to III electron transfer due to alterations in the quinone pool, but Complex II was unaffected (Kayser et al., 2004). These results highlight the importance of looking at the different complexes when examining mitochondrial function. Potentially *rpd3* mutants may have changes in specific aspects of mitochondrial respiration that contribute to their increased longevity.

3.5 Methods

3.5.1 Fly stocks and Maintenance

The same 2 strains of *rpd3*-mutant *Drosophila melanogaster* as described in chapter 2 were used to determine the role of mitochondria in aging. Groups of 25 males and 25 females were collected 24 hours after eclosion. Unless indicated, flies were aged in vials containing normal corn food in a humidity-controlled incubator at 25°C on a 12-hour night/day cycle for their entire lifespan until time for experimentation. Normal *Drosophila* food consisted of water, inactive yeast, sucrose, agar, corn, and Tegosept (Methyl4-hydroxybenzoate, Sigma) as an antifungal agent. Flies were passed into new vials of food every other day to prevent build up of bacteria.

3.5.2 Mitochondria DNA Measurement

Total DNA was isolated from groups of 25 males at 20 or 40 days of ages in the genotypes $rpd3^{def24}/+$ or *+/+*. This was performed using the Invitrogen DNA Blood and Tissue Isolation kit (Life Technologies). DNA copy number was determined by qPCR as described below (see 3.5.3). Mitochondrial DNA to nuclear DNA ratios were determined by the ratio of a mitochondrial gene, *Cytochrome oxidase Subunit I (COI)*, to a nuclear gene, *GAPDH*, as previously described (Neretti et al., 2009). *Rpl32* was used as an endogenous control.

3.5.3 Quantitative PCR (qPCR)

Flies were aged until 20 or 40 days of age, separated into groups of males or females, and then frozen and kept at -80°C until dissection. Groups of 35 flies were dissected on a cold plate to ensure they stayed frozen, and each genotype had 3 biological replicates. Total RNA was isolated from groups of 35 heads and thoraces for $rpd3^{def24}/+$ or *+/+* flies using Trizol as previously described (Wang et al., 2009).

cDNA was synthesized using a standard protocol, and qPCR was performed to determine changes in gene expression using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). TaqMan primers were obtained for *spargel* and *dSir2* from Invitrogen, and *ankryn* was used as an endogenous control. Gene expression was averaged between the biological replicates, and results are reported as relative mRNA expression as compared to the control group.

3.5.4 Electron Microscopy

Fruit flies were aged to 40 days, and their thoraces were dissected and prepared for electron microscopy as previously described in our lab (Rogers and Rogina, 2014). Fly thoraces were dissected at 40 days of age and were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Post-fixation was conducted for 1 hour in 1% osmium tetroxide-0.8% potassium ferricyanide. Samples were stained with 1% aqueous uranyl acetate, dehydrated in a graded ethanol series, and embedded in Spurr low-viscosity epoxy resin. Thin sections of areas containing skeletal muscle were stained with uranyl acetate and lead citrate and examined using a Hitachi H7650 microscope. A minimum of 10 electron micrographs of skeletal muscle in cross section were taken for each sample. Images were processed and analyzed using ImageJ. Mitochondrial density was quantified by using the point counting method by applying a grid system to count the number of mitochondria present in a given image relative to cytoplasmic volume as previously described (Birkenfeld et al., 2011).

3.5.5 Mitochondrial Respiration

Fruit flies were aged until either 10 or 40 days of age, and then the thoraces were dissected in order to isolate mitochondria as described previously (Barrientos, 2002). Using the Hansatech Oxygen Electrode, mitochondrial respiration was analyzed in units of nmol O_2 consumption per ml. The rate per minute was calculated by recording for 10 minutes. To measure complex I respiration, glutamate and malate are added to the chamber, followed by the addition of ADP. For complex II, succinate and rotenone for inhibiting complex I are added. For complex IV, respiration is measured using ascorbate and TMPD plus antimycin A to inhibit upstream complexes. The protein concentration of each mitochondrial isolate was determined to ensure similar quantities were present in each preparation. Data are reported as the means +/- SD.

3.5.6 Statistical analysis

Significance was determined using a two-tailed, unpaired t-test and expressed as P values. $P < 0.05$ is considered to be significant. Error bars represent standard deviation (SD).

Chapter 4: Insulin and TOR signaling

4.1 Background and Significance

The insulin signaling pathway is a key nutrient sensing pathway in many animals, and it has been shown to be an important regulator of many aspects of aging (Kenyon, 2010). It was initially discovered in worms to be one of the greatest life span extensions due to a genetic mutation (Kenyon et al., 1993). In this study they found that mutating *daf-2*, now known as the gene for insulin receptor protein (InR), resulted in a great life span extension. This life span extension required the gene *daf-16*, which we now know is the gene for a Foxo protein homologue. Similarly in *Drosophila*, mutating either *dInR* or *chico*, the insulin receptor substrate, results in increased life span (Tatar et al., 2001; Clancy et al., 2001).

Since these initial discoveries, the roles of downstream mediators of the insulin signaling pathway have been under heavy investigation as a way to extend life span. The dFoxo protein is one of the main effector proteins of this pathway, as it is a transcription factor that can activate genes involved in many biological pathways, such as stress response (Liu et al., 2014). One study found that overexpressing *dFoxo* in the fat body of flies, which is an organ with functions similar to white adipose tissue and the liver of humans, led to an increase in life span (Giannakou et al., 2004). The same group later showed that *dFoxo* expression is not required for response to CR in fruit flies (Giannakou et al., 2008). Foxo3A, the human homologue of dFoxo, has been a gene linked to longevity in multiple cohorts of humans (Kenyon, 2010).

TOR signaling is partially downstream of insulin signaling, and it is also important as a nutrient-sensing pathway that stimulates growth when active (Katewa and Kapahi, 2011). In fruit flies it has been shown that inhibiting this pathway in multiple ways extends lifespan (Kapahi et al., 2004; Bjedov et al., 2010). Interestingly, a study in yeast has suggested that Rpd3 is required for the effects of TOR-mediated gene repression (Humphrey et al., 2004). Because of this, it is possible that the insulin and TOR signaling pathways are altered in *rpd3* mutant *Drosophila*.

4.2 Rationale

We initially hypothesized that the mechanism of longevity extension seen after *rpd3* mutation overlaps with the mechanism of CR. The insulin signaling pathway has been shown to be important for aging, life span extension, and the response to CR (Partridge et al., 2011). However, some studies have suggested that the effects on longevity of insulin signaling and CR may operate independently of each other (Giannakou et al., 2008). We started investigating the insulin signaling pathway following the results after the first experiment in this chapter suggested to us that this metabolic pathway may be altered in *rpd3* mutants.

The TOR signaling pathway is a known regulator of protein synthesis (Katewa and Kapahi, 2011). This process is known to be altered in calorically restricted organisms, with a general decrease in translation but upregulation of specific classes of proteins (Zid et al., 2010). Our lab also had initial microarray data suggesting that *thor* (*Drosophila 4E-BP* homologue) expression was altered in *rpd3*-

mutant *Drosophila*. Because of the relation to nutrient intake and longevity of both the insulin and TOR signaling pathways, we investigated if there are changes in these pathways in our long-lived *rpd3*-mutant flies.

4.3 Results

4.3.1 *rpd3*-mutant flies have altered gene expression of insulin signaling components

Following mitochondrial experiments that showed no difference in mitochondrial biogenesis, and only small differences in mitochondrial respiration, we wanted to examine if other pathways related to metabolism were altered in terms of gene expression. Our lab had designed a 96-gene plate that tested mRNA expression for up to 4 biological groups, and the genes on this plate represented many different metabolic pathways (TaqMan Custom Array, Applied Biosystems). We used cDNA from the heads and thoraces of 40 day old $rpd3^{def24}/+$ or *+/+* males in duplicate, and found potentially significant changes in expression of multiple genes (Fig. 4.1). Interestingly, many of these genes fell into the insulin signaling pathway. These included *dInR*, *chico*, and *dIlp3* for example.

Genes that are regulated by the TOR signaling pathway were also changed in *rpd3*-mutant *Drosophila*. These included *tsc1* and *thor*, the *Drosophila 4E-BP* homologue. Interestingly, as TOR signaling is known to regulate protein synthesis, two eukaryotic initiation factor genes were also decreased in *rpd3* mutants, *eIF4E-4* and *eIF4E-5*.

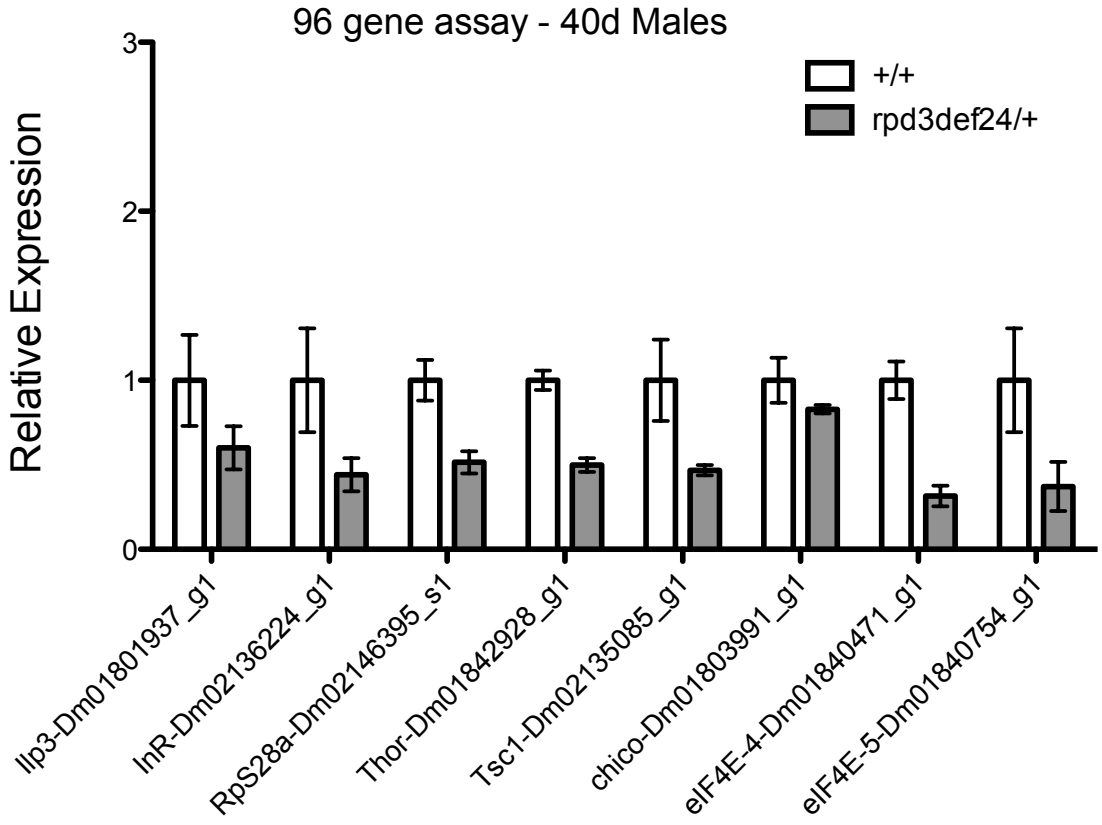

Figure 4.1 96-gene assay results showing relative mRNA expression of genes potentially changed in male, 40 day old *rpd3*^{def24}*/+* flies compared to *+/+* flies. Graphs are plotted as the means +/- standard deviation.

After finding that insulin signaling may be altered in *rpd3*-mutant *Drosophila*, we wanted to confirm these changes via standard qPCR. Using RNA isolated from the heads and thoraces of 40 day old $rpd3^{def24}/+$ or *+/+* males, we analyzed mRNA expression of many components of the insulin signaling pathway. Consistent with our 96-gene assay, the levels of *dIlp3* expression were significantly decreased (Fig. 4.2B). Interestingly, the levels of *dIlp2*, *dIlp5*, and *dIlp6* were all significantly increased (Figs. 4.2A, 4.2C, 4.2D). *dInR* and *chico* expression were both decreased in $rpd3^{def24}/+$ males compared to *+/+* flies. These results confirm the results seen from our 96-gene plate, and suggest that insulin signaling is decreased in aged *rpd3*-mutant flies.

We also wanted to know if insulin signaling was affected at a younger age in *rpd3*-mutant flies, so we performed qPCRs looking at the same genes in flies at 20 days of age. Interestingly, we found that out of the genes for dIlps, only *dIlp5* expression was significantly increased in $rpd3^{def24}/+$ flies compared to their genetic controls (Fig4.3C). mRNA expression of *dIlp2*, *dIlp3*, and *dIlp6* were not changed (Figs. 4.3A, 4.3B, 4.3D). *dInR* expression trended toward being decreased in $rpd3^{def24}/+$ flies, but it was not significant (Fig. 4.3E). *chico* expression was not altered (Fig 4.3F). Taken together it is possible that altered insulin signaling later in life is the key factor for life span extension in *rpd3*-mutant *Drosophila*.

The previous studies have been done in heads and thoraces combined, but dIlps 2, 3, and 5 are made in the insulin-producing cells of the brain (Broughton et al., 2005). Because of this we analyzed mRNA expression in RNA isolated only from the heads of 40 day old $rpd3^{def24}/+$ and *+/+* males. Interestingly, we found that

expression of all *dIlp* genes analyzed were increased significantly (Fig. 4.4A-D). The level of *dInR* mRNA expression was not changed in the head of $rpd3^{def24}/+$ and *+/+* males (Fig. 4.4E). Different from what we saw in the heads and thoraces, *chico* expression was increased in $rpd3^{def24}/+$ heads compared to *+/+* heads (Fig. 4.4F).

In our initial study on *rpd3* and life span extension, we published on two different mutant alleles of *rpd3* that extended longevity, $rpd3^{def24}/+$ and $rpd3^{P\text{-}UTR}$ (Rogina et al., 2002). We also examined the levels of gene expression of components in $rpd3^{P\text{-}UTR}$ flies as well, since a mutation in the same gene should lead to similar changes in an organism. We had to look at the thorax only for $rpd3^{P\text{-}UTR}$ flies since their controls, $rpd3^{15\text{-}1}$ flies, have the same mutation in the eyes only (Mottus et al., 2000). At 40 days of age, none of the genes we looked at were significantly different in $rpd3^{P\text{-}UTR}/CS$ flies compared to $rpd3^{15\text{-}1}/CS$ flies. These genes were *dIlp2, dIlp3, dIlp5, dIlp6, dInR,* and *chico* (Fig. 4.5A-F). These results differ greatly from $rpd3^{def24}/+$ flies.

We also examined gene expression of the insulin signaling pathway in the thoraces of 20 day old $rpd3^{P\text{-}UTR}/CS$ flies and their controls. Interestingly, we found that *dIlp3, dIlp5,* and *dIlp6* were all significantly decreased in $rpd3^{P\text{-}UTR}/CS$ flies compared to $rpd3^{15\text{-}1}/CS$ flies (Fig. 4.6B-D). However, we did not find significant differences in *dIlp2, dInR,* and *chico*, even though there was a trend towards decreased expression for all of these genes (Fig. 4.6A, E, F). Although these results differ from $rpd3^{def24}/+$ flies, it does indicate that insulin signaling may also be decreased in the $rpd3^{P\text{-}UTR}/CS$ mutants as well.

As previously mentioned, one of the major downstream effectors of insulin signaling is the Forkhead box Group O family of transcription factors (Kenyon, 2010). *Drosophila* only has one homologue of this protein, dFoxo, and this protein's activation is inversely regulated by the insulin signaling pathway (Partridge et al., 2011). If insulin signaling is actually decreased in *rpd3* mutants, we would expect to see increased dFoxo protein, increased activation of the protein, or increased gene expression of *dFoxo*. We first looked at gene expression throughout the life span of a laboratory control strain of *Drosophila*, *Canton-S*, using qPCR. We found that *dFoxo* expression was not significantly different at any age (Fig. 4.7A). However, the expression varied widely between biological replicates. In $rpd3^{def24}/+$ flies, there was not a significant increase in *dFoxo* expression at 20 days of age compared to genetic controls. (Fig. 4.7B). At 40 days, we did see a significant increase in *dFoxo* mRNA expression compared to controls. Consistently, $rpd3^{P\text{-}UTR}/CS$ flies had increased expression of *dFoxo* compared to $rpd3^{15\text{-}1}/CS$ flies at 40 days of age as well (Fig. 4.7C). The upregulation of *dFoxo* in *rpd3* mutants suggests that this may be a key mediator of longevity extension in these flies.

Another pathway that is downstream of the insulin signaling pathway, yet is partially independent, is the TOR signaling pathway (Katewa and Kapahi, 2011). This pathway is negatively regulated by the Tsc1/Tsc2 protein complex (Kapahi et al., 2004). Giga is the *Drosophila* Tsc2 homologue. It has also been shown to be a key mediator of the effects of CR (Hansen et al., 2007). Because of the relation to CR, insulin signaling, and longevity in general, we sought to examine the changes of this pathway in our *rpd3* mutants. In the heads and thoraces of 20 day old $rpd3^{def24}/+$

flies compared to *+/+* flies, we did not observe any differences in mRNA expression of *tsc1*, *giga* , or *tor* (Fig. 4.8A-C). However, at 40 days of age *tsc1* expression was significantly decreased in $rpd3^{def24}/+$ heads and thoraces compared to *+/+* flies (Fig. 4.8D). *giga* and *tor* expression were unchanged though (Fig. 4.8E, F). In heads alone at 40 days of age, *tsc1* was significantly decreased while *giga* was significantly increased (Fig. 4.8G, H). *tor* remained unchanged (Fig. 4.8I).

We also examined these genes in 20 and 40 day old $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies. In thoraces at 20 days of age, we found that both *tsc1* and *tor* mRNA expression were decreased in $rpd3^{P\text{-}UTR}/CS$ flies compared to $rpd3^{15\text{-}1}/CS$ flies (Fig. 4.9A, C). *giga* expression was not altered at 20 days though (Fig. 4.9B). At 40 days, *tsc1* expression was decreased as well in thoraces from $rpd3^{P\text{-}UTR}/CS$ flies compared to $rpd3^{15\text{-}1}/CS$ flies (Fig. 4.9D). *giga* expression was increased in thoraces from 40 day old $rpd3^{P\text{-}UTR}/CS$ flies relative to $rpd3^{15\text{-}1}/CS$ flies (Fig. 4.9E). mRNA expression of *tor* was decreased in $rpd3^{P\text{-}UTR}/CS$ flies, but these results were not significant (Fig. 4.9F). Taken together, these results on *tsc1, giga,* and *tor* all indicate that this signaling pathway is alternatively regulated in *rpd3*-mutant *Drosophila*.

TOR signaling has downstream effectors that cause the decrease in translation that is seen in CR (Katewa and Kapahi, 2011). The main two proteins that mediate the effects of TOR are 4E-BP, a negative regulator of translation, and S6K, a kinase shown to phosphorylate ribosomal subunits (Zid et al., 2009; Stewart et al., 1996). We looked the expression of *thor*, the *Drosophila 4E-BP* homologue, and *S6K* to determine if they were changed in our *rpd3* mutants. The mRNA expression of *thor* was not altered in the heads and thoraces of 20 day old

rpd3^{def24}*/+* flies compared to *+/+* flies, yet there was a trend towards decreased expression (Fig. 4.10A). However, there was decreased expression in 40 day old *rpd3*^{def24}*/+* flies compared to *+/+* flies (Fig. 4.10B). In the thoraces of *rpd3*$^{P\text{-}UTR}$*/CS* flies, *thor* expression was decreased at both 20 days and 40 days of age when compared to their genetic controls, but the reduction was not significant at 40 days (Fig. 4.10D). For *S6K*, *rpd3*^{def24}*/+* flies were found to have increased expression at 20 days, but this only trended towards significance, and expression was unchanged at 40 day of age (Fig. 4.10E). In *rpd3*$^{P\text{-}UTR}$*/CS* thoraces at 40 days, expression of *S6K* was increased although not significantly (Fig. 4.10C). The changes seen in *S6K* and *thor* suggest that protein translation may be increased in *rpd3*-mutant flies, which may mediate some aspects of life span extension.

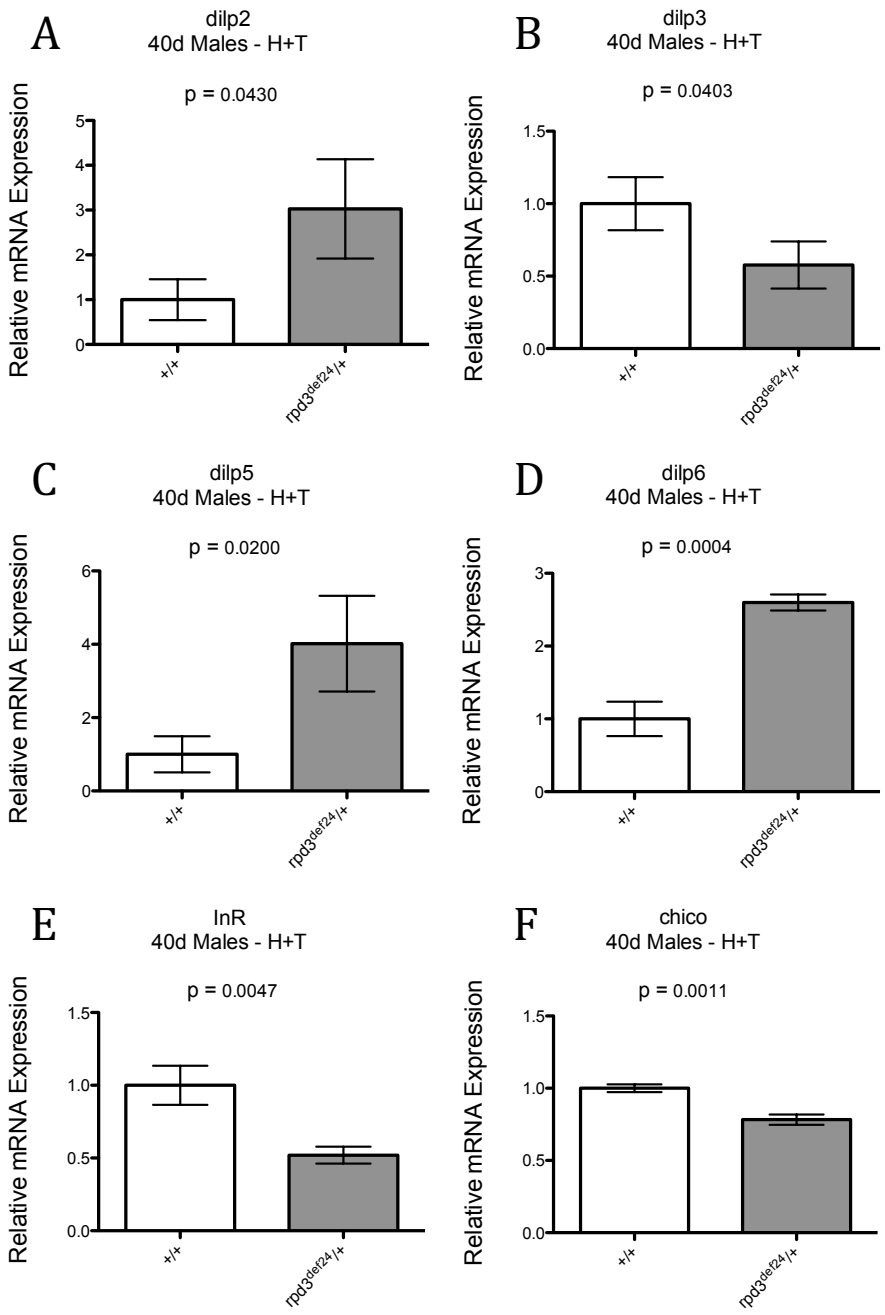

Figure 4.2 A) qPCR results for *dIlp2* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. **B)** qPCR results for *dIlp3* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. **C)** qPCR results for *dIlp5* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. **D)** qPCR results for *dIlp6* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. **E)** qPCR results for *dInR* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. **F)** qPCR results for *chico* expression in 40 day old *rpd3^{def24}/+* and *+/+* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

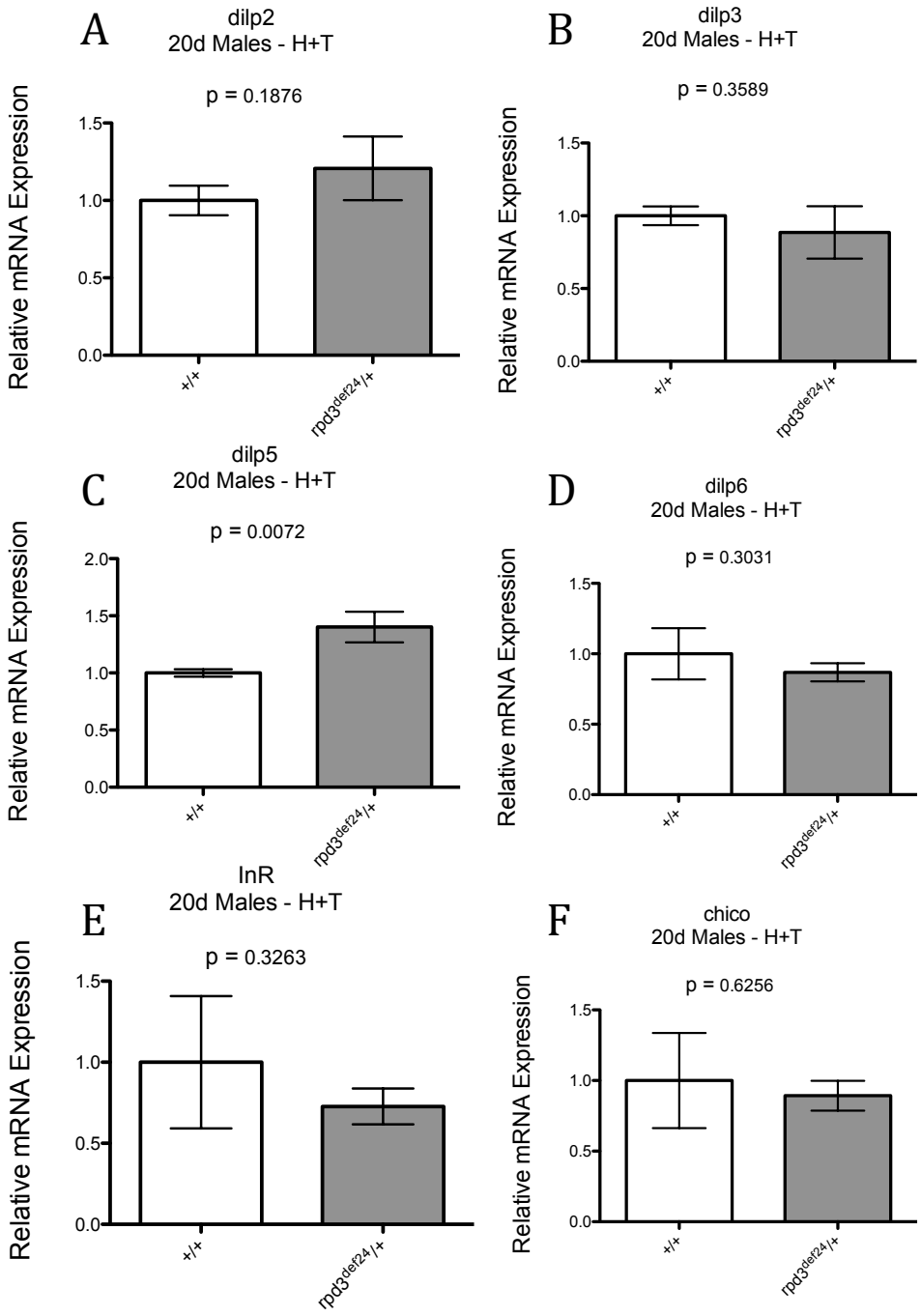

Figure 4.3 A) qPCR results for *dIlp2* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **B)** qPCR results for *dIlp3* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **C)** qPCR results for *dIlp5* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **D)** qPCR results for *dIlp6* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **E)** qPCR results for *dInR* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. **F)** qPCR results for *chico* expression in 20 day old *rpd3^{def24}/+* and *+/+* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

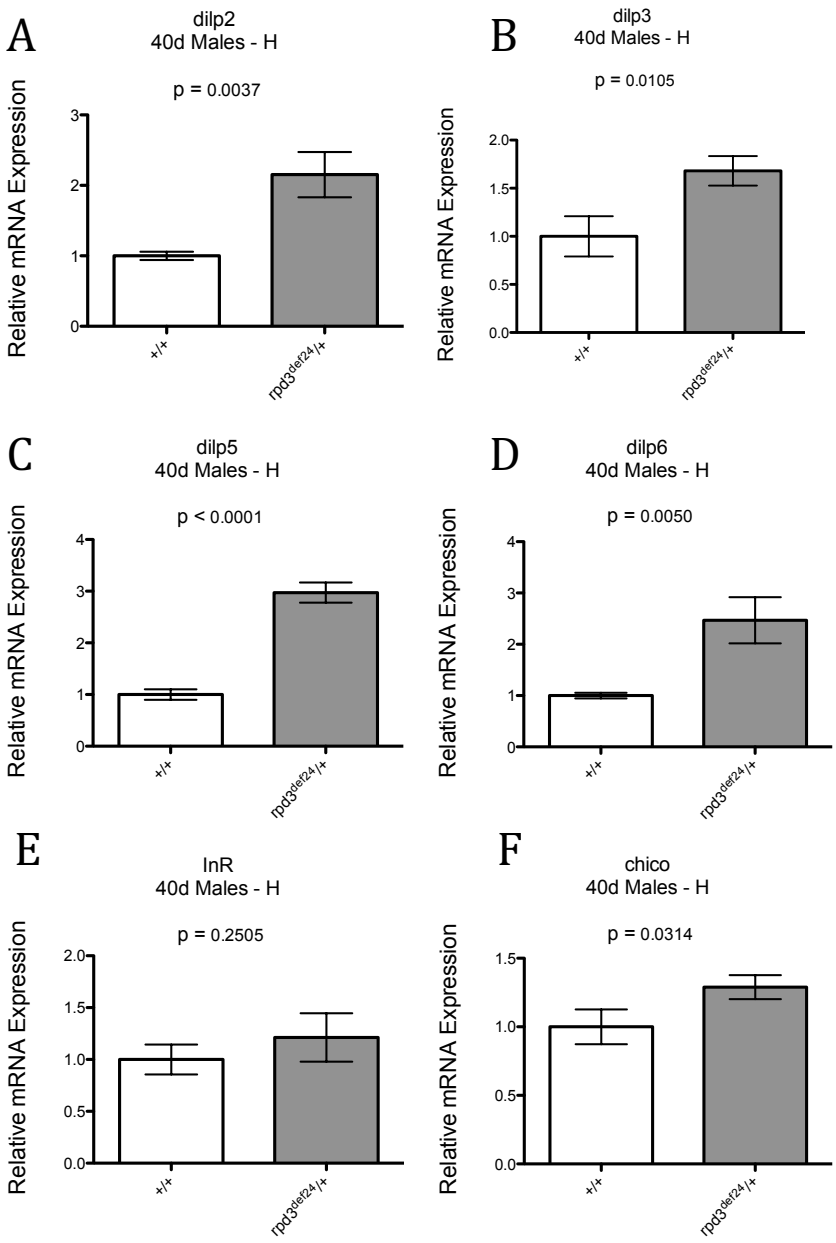

Figure 4.4 A) qPCR results for *dIlp2* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **B)** qPCR results for *dIlp3* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **C)** qPCR results for *dIlp5* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **D)** qPCR results for *dIlp6* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **E)** qPCR results for *dInR* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **F)** qPCR results for *chico* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

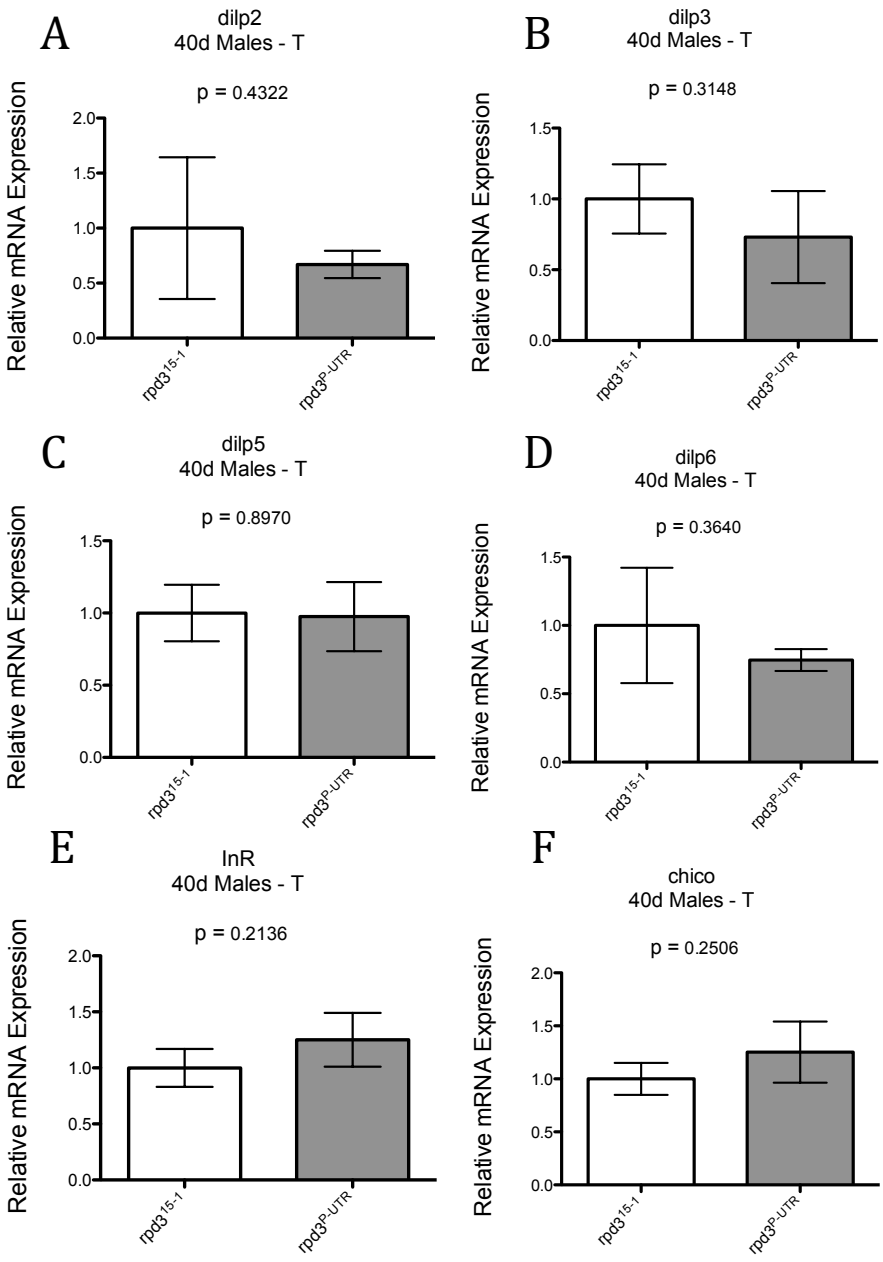

Figure 4.5 A) qPCR results for *dIlp2* expression in 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **B)** qPCR results for *dIlp3* expression in 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **C)** qPCR results for *dIlp5* expression in 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **D)** qPCR results for *dIlp6* expression in 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **E)** qPCR results for *dInR* expression in 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **F)** qPCR results for *chico* expression in 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

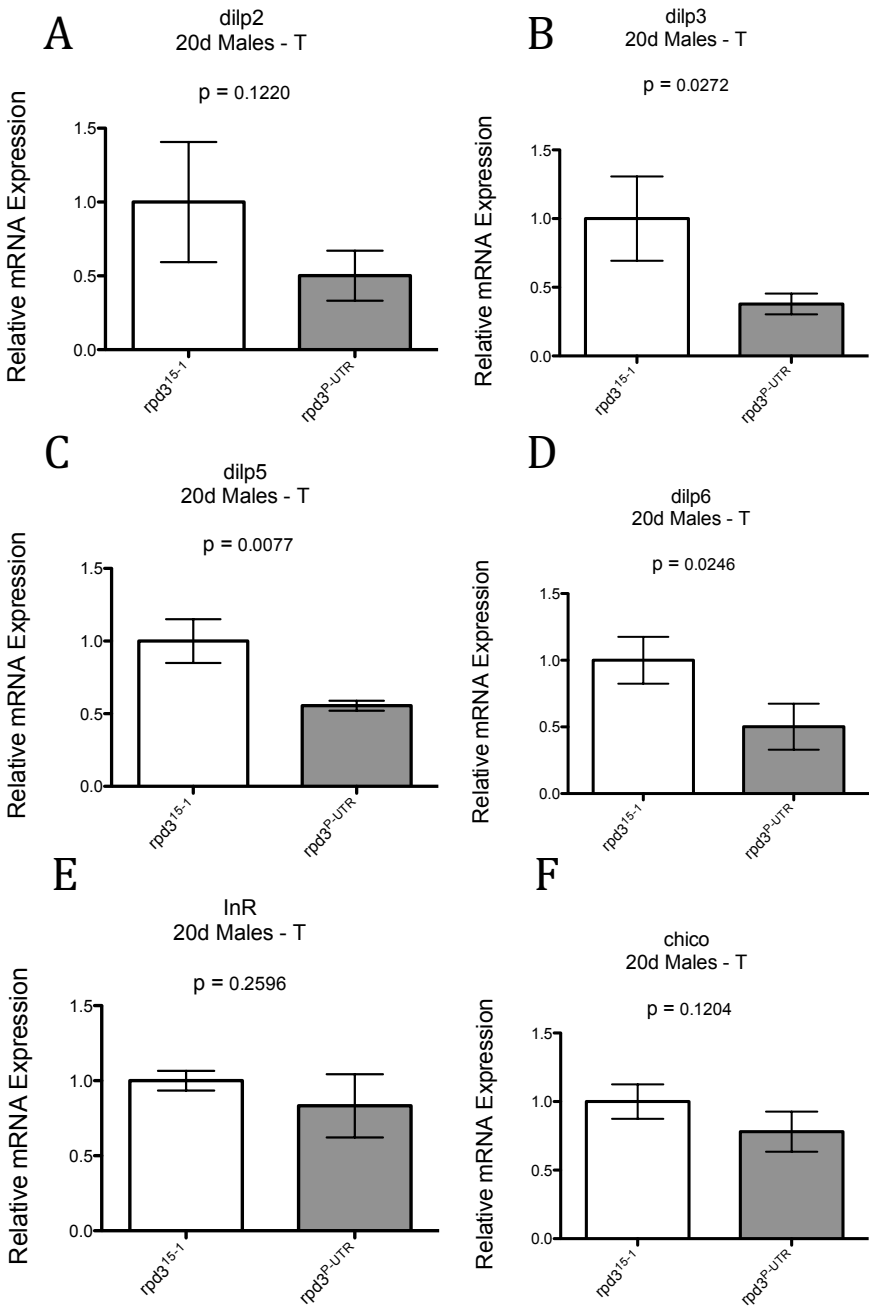

Figure 4.6 A) qPCR results for *dIlp2* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **B)** qPCR results for *dIlp3* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **C)** qPCR results for *dIlp5* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **D)** qPCR results for *dIlp6* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **E)** qPCR results for *dInR* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **F)** qPCR results for *chico* expression in 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

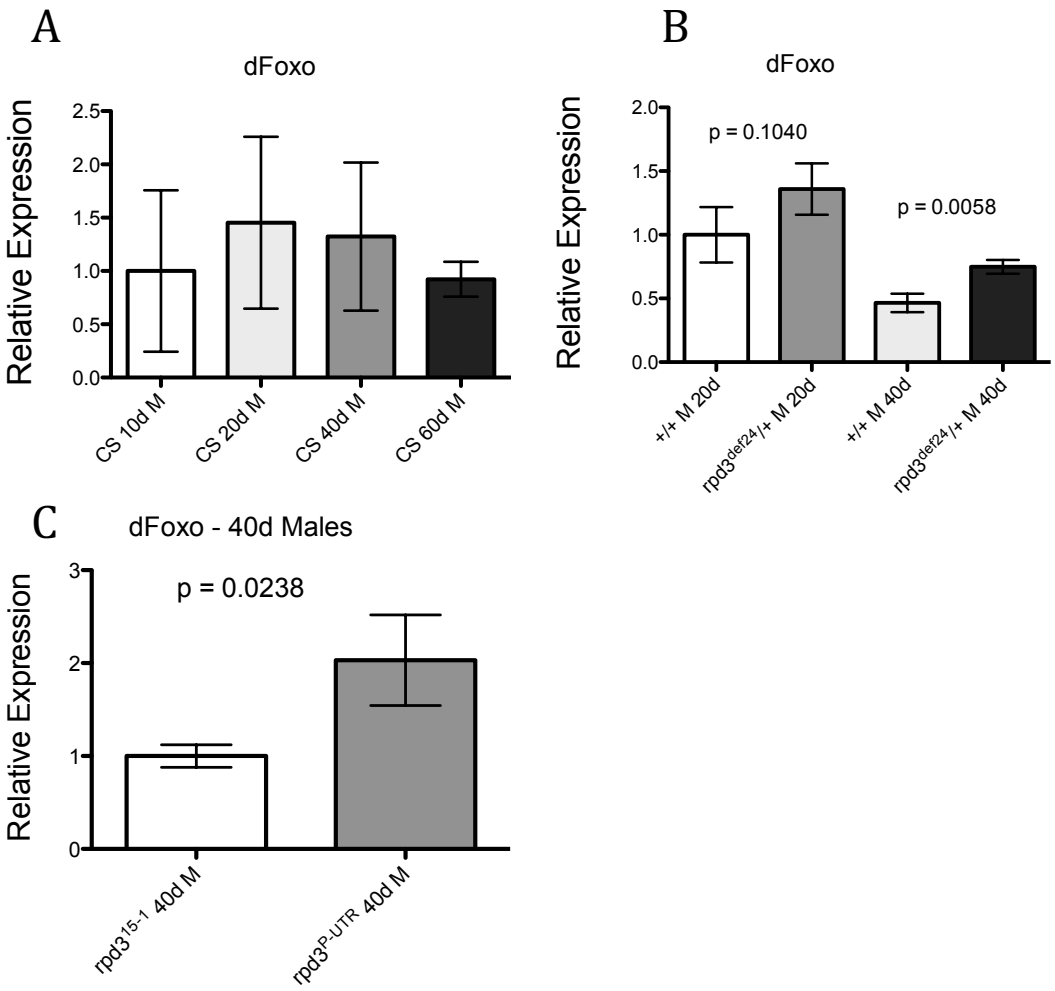

Figure 4.7 A) qPCR results for *dFoxo* expression in 10, 20, 40 and 60 day old *CS* males. **B)** qPCR results for *dFoxo* expression in 20 and 40 day old $rpd3^{def24}$/+ and +/+ males. **C)** qPCR results for *dFoxo* expression in 40 day old $rpd3^{P\text{-}UTR}$/*CS* males and $rpd3^{15\text{-}1}$/*CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

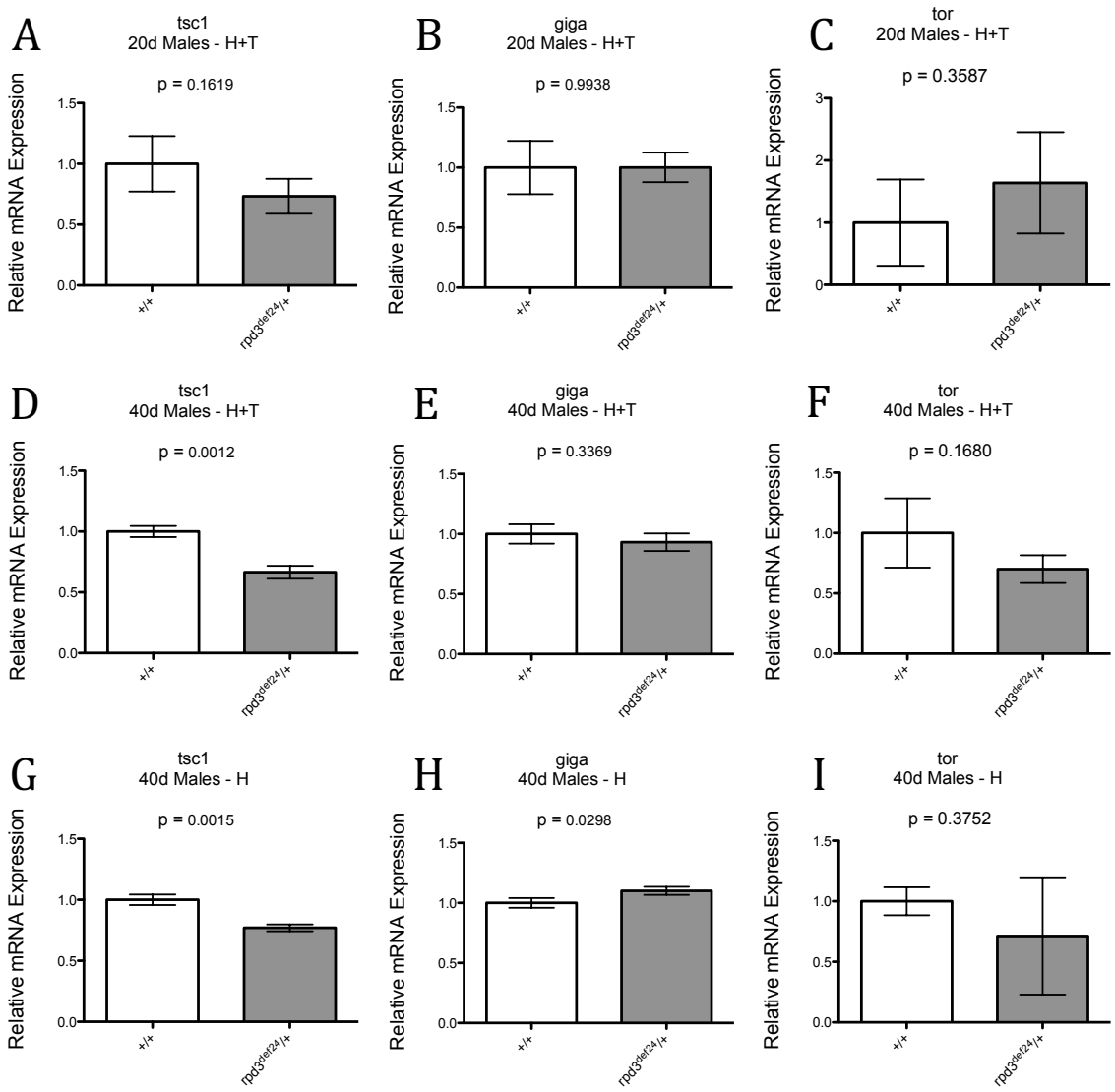

Figure 4.8 A) qPCR results for *tsc1* expression in heads and thoraces of 20 day old *rpd3^{def24}/+* and *+/+* males. **B)** qPCR results for *giga* expression in heads and thoraces of 20 day old *rpd3^{def24}/+* and *+/+* males. **C)** qPCR results for *tor* expression in heads and thoraces of 20 day old *rpd3^{def24}/+* and *+/+* males. **D)** qPCR results for *tsc1* expression in heads and thoraces of 40 day old *rpd3^{def24}/+* and *+/+* males. **E)** qPCR results for *giga* expression in heads and thoraces of 40 day old *rpd3^{def24}/+* and *+/+* males. **F)** qPCR results for *tor* expression in heads and thoraces of 40 day old *rpd3^{def24}/+* and *+/+* males. **G)** qPCR results for *tsc1* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **H)** qPCR results for *giga* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. **I)** qPCR results for *tor* expression in heads of 40 day old *rpd3^{def24}/+* and *+/+* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

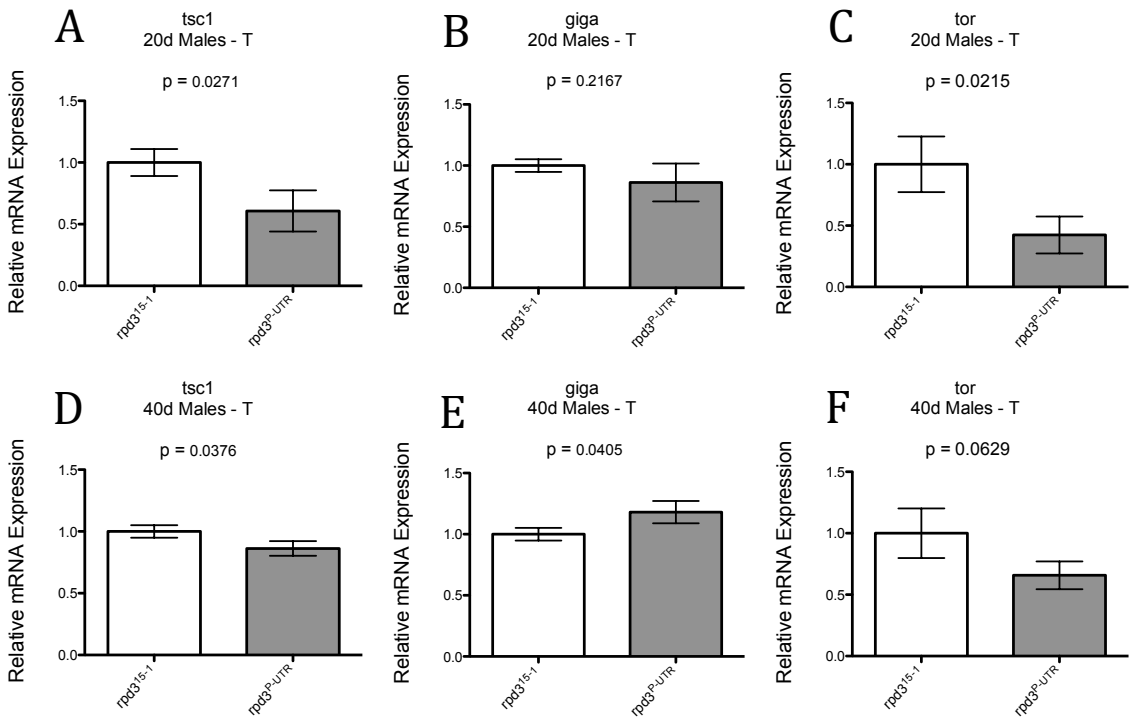

Figure 4.9 A) qPCR results for *tsc1* expression in thoraces of 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **B)** qPCR results for *giga* expression in thoraces of 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **C)** qPCR results for *tor* expression in thoraces of 20 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **D)** qPCR results for *tsc1* expression in thoraces of 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **E)** qPCR results for *giga* expression in thoraces of 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. **F)** qPCR results for *tor* expression in thoraces of 40 day old *rpd3$^{P\text{-}UTR}$/CS* males and *rpd3$^{15\text{-}1}$/CS* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

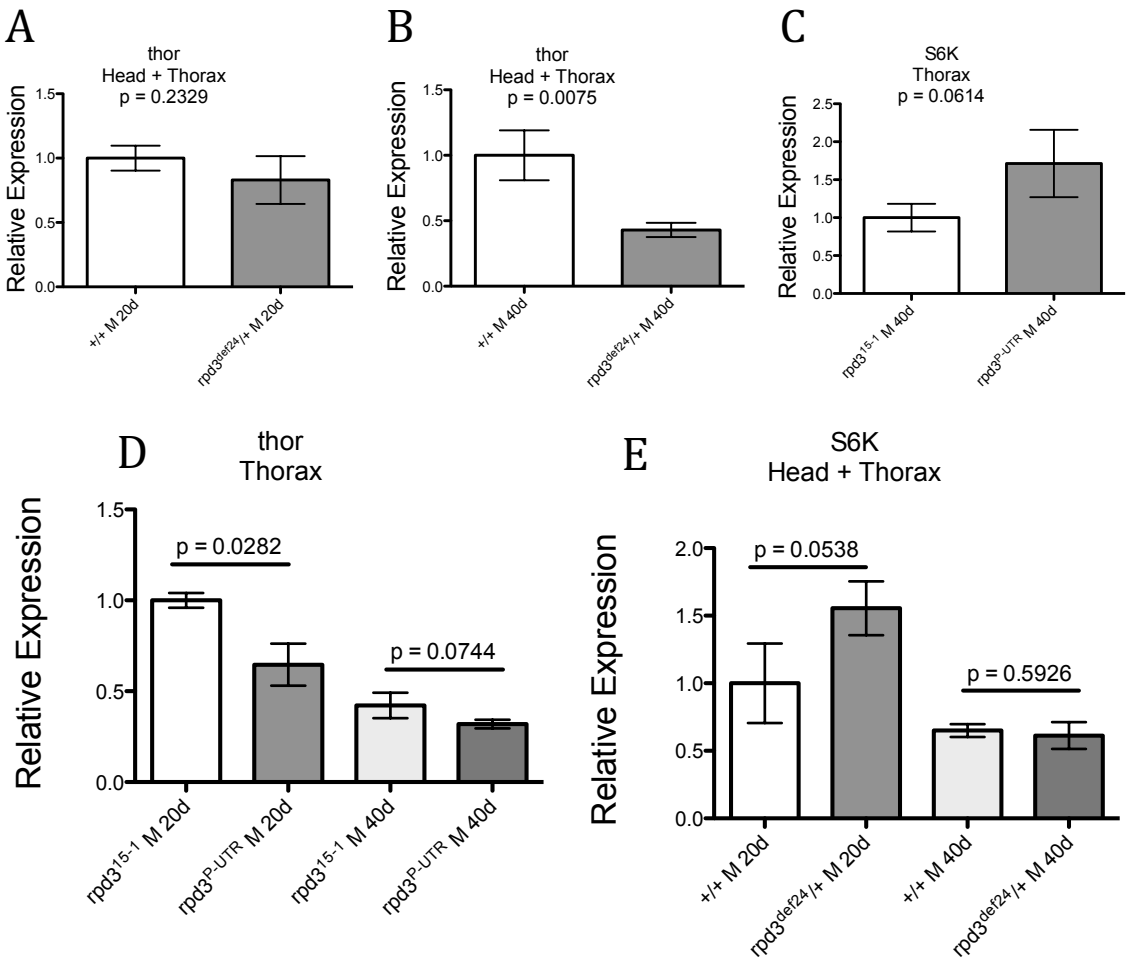

Figure 4.10 A) qPCR results for *thor* expression in 20 day old $rpd3^{def24}$/+ and +/+ males. **B)** qPCR results for *thor* expression in 40 day old $rpd3^{def24}$/+ and +/+ males. **C)** qPCR results for *S6K* expression in 40 day old $rpd3^{P\text{-}UTR}$/*CS* males and $rpd3^{15\text{-}1}$/*CS* males. **D)** qPCR results for *thor* expression in 20 and 40 day old $rpd3^{P\text{-}UTR}$/*CS* males and $rpd3^{15\text{-}1}$/*CS* males. **E)** qPCR results for *S6K* expression in 20 and 40 day old $rpd3^{def24}$/+ and +/+ males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

4.3.2 dFoxo partially mediates the effects of life span extension in *rpd3*-mutant flies

In life span experiments, double mutational studies are extremely helpful in determination of the mechanism of longevity extension by a single mutation (Min et al., 2008). If the addition of a second mutation causes no further longevity extension, then it is possible that the mechanism of the first mutation works either downstream or upstream of the second gene that was mutated. Conversely, if the second mutation still provides a further increase in longevity, those two pathways should be independent of each other. To test if *dFoxo* is a downstream mediator of longevity extension seen in *rpd3* mutants, we generated flies that were heterozygous mutants for *dFoxo* as well.

First we wanted to confirm that these flies had the expected changes in gene expression. We found that $rpd3^{def24}/yw$ flies and $rpd3^{def24}/dFoxo$ double mutants had decreased expression of *rpd3* in their thoraces at 40 days of age compared to *dFoxo/yw* flies (Fig. 4.11A). $rpd3^{def24}/dFoxo$ double mutants and *dFoxo/yw* flies had decreased levels of *dFoxo* mRNA compared to $rpd3^{def24}/yw$ flies (Fig. 4.11B). Consistent with increased *dFoxo* expression in *rpd3* mutants, we also saw a significant increase in the expression of *dFoxo* in $rpd3^{def24}/dFoxo$ double mutants than *dFoxo/yw* flies. Since the results of these qPCRs indicated the expected changes, we performed life span studies on these three lines of flies. Interestingly, we found that $rpd3^{def24}/yw$ males had the longest mean survival (Fig. 4.12). *dFoxo/yw* flies had the shortest mean survival as expected, and $rpd3^{def24}/dFoxo$ double mutant flies had a mean survival between the two genotypes with only one

mutation. These results suggest that *dFoxo* is partially responsible for the life span extension seen in *rpd3* mutants.

4.3.3 *dFoxo* mutations result in altered stress resistance

Since *rpd3* mutation leads to increased oxidative stress and starvation resistance we also wanted to test the resistance of our $rpd3^{def24}/dFoxo$ double mutants and *dFoxo/yw* mutants. When exposed to H_2O_2 at 40 days of age, $rpd3^{def24}/yw$ flies had the longest mean survival as expected (Fig. 4.13A). Consistent with life span studies and expected results, $rpd3^{def24}/dFoxo$ double mutants had a mean survival between the $rpd3^{def24}/yw$ and *dFoxo/yw* flies. When exposed to starvation at 40 days of age, $rpd3^{def24}/yw$ flies once again had the longest mean survival as expected (Fig. 4.13B). The results for *dFoxo* mutants were also similar too, with the mean survival of double mutants between $rpd3^{def24}/yw$ and *dFoxo/yw* flies. These results support the hypothesis that *dFoxo* partially mediates the beneficial effects of *rpd3* mutation on stress resistance and life span.

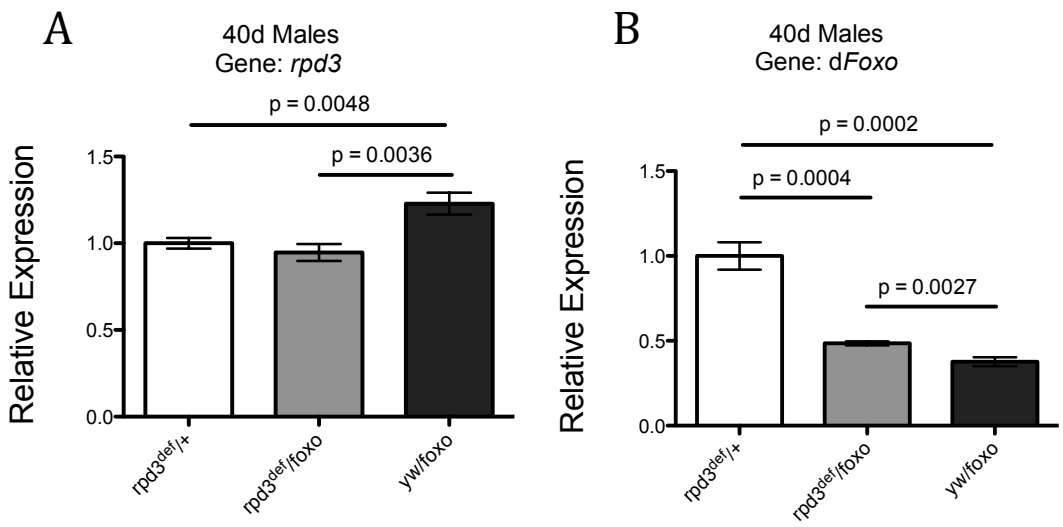

Figure 4.11 A) qPCR results for *rpd3* expression in 40 day old *rpd3*^{def24}*/yw*, *rpd3*^{def24}*/dFoxo*, and *dFoxo/yw* males. **B)** qPCR results for *dFoxo* expression in 40 day old *rpd3*^{def24}*/yw*, *rpd3*^{def24}*/dFoxo*, and *dFoxo/yw* males. Graphs are plotted as the means +/- standard deviation. Statistical analysis done using the Student's T-test.

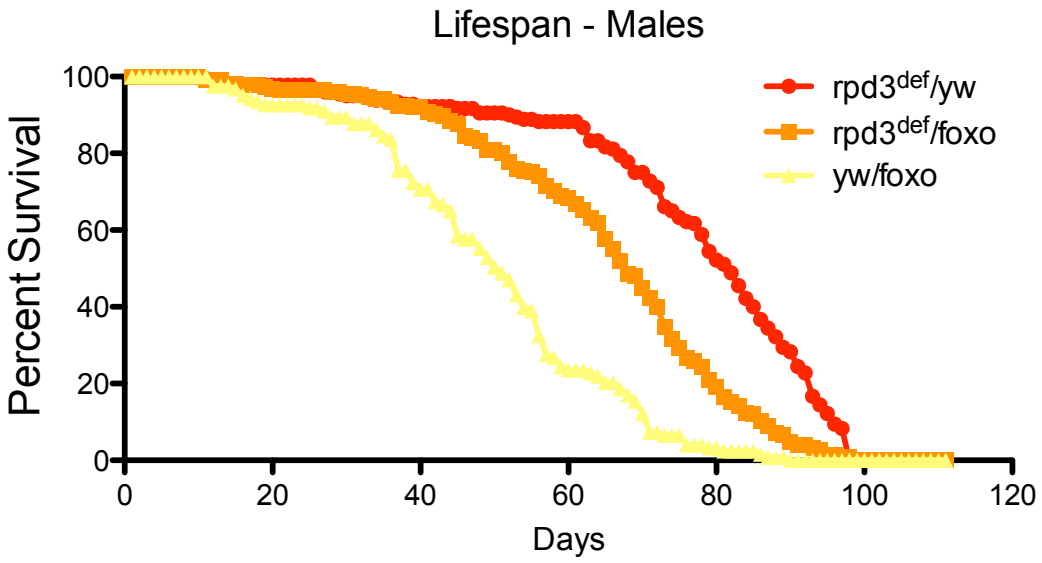

Figure 4.12 Life span curves of *rpd3^{def24}/yw*, *rpd3^{def24}/dFoxo,* and *dFoxo/yw* flies. Deaths during the first 10 days are excluded because they are not truly representative of deaths due to aging. The results are plotted as the percentage of flies surviving as a function of time in days.

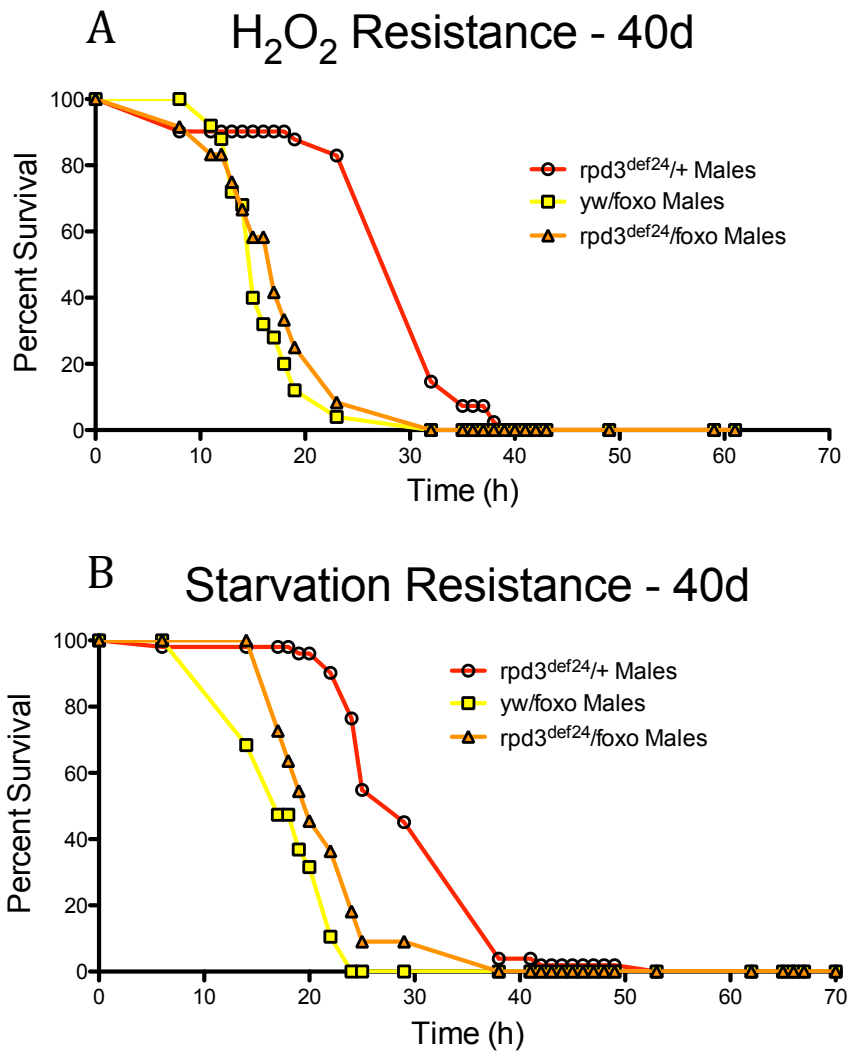

Figure 4.13 A) H_2O_2 resistance studies for 40 day old *rpd3^{def24}/yw*, *rpd3^{def24}/dFoxo*, and *dFoxo/yw* males. **B)** Starvation resistance studies for 40 day old *rpd3^{def24}/yw*, *rpd3^{def24}/dFoxo*, and *dFoxo/yw* males. Graphs are plotted as percentage of flies surviving as a function of time.

4.3.4 dFoxo protein is not affected in *rpd3*-mutant *Drosophila*

Although we showed that *dFoxo* mRNA expression is increased in *rpd3*-mutant *Drosophila*, we also wanted to see if these effects could be seen at the protein level as well. For dFoxo to have its positive effects on gene regulation as a transcription factor, it must be activated and translocated to the nucleus where it can access target genes (Villa-Cuesta et al., 2010). dFoxo is negatively regulated by phosphorylation by Akt, and it remains in the cytoplasm when inactive (Puig et al., 2003). Once dephosphorylated, dFoxo is activated, and it translocates to the nucleus to increase transcription.

We first wanted to look at the total levels of dFoxo protein. When we tried to look at the levels of dFoxo in proteins isolated from the thoraces of $rpd3^{def24}/+$ flies, *+/+* flies, $rpd3^{P\text{-}UTR}/CS$ flies, and $rpd3^{15\text{-}1}/CS$ flies, we saw no difference in the level of dFoxo protein (Fig. 4.14A). Next we tried to determine if dFoxo was localized to the nucleus or the cytoplasm. As previously mentioned, when dFoxo is dephosphorylated it can move to the nucleus from the cytoplasm to activate transcription (Puig et al., 2003). When we used a kit to separate nuclear and cytoplasmic fractions and stained for dFoxo, we could not determine any differences in the quantities of this protein in the nucleus versus the cytoplasm of $rpd3^{def24}/+$ and *+/+* flies (Fig. 4.14B). Since it is known that phosphorylation deactivated dFoxo and sequesters it to the cytoplasm, we tried to stain for a phosphorylated form of dFoxo. Again, we were unable to see differences between the $rpd3^{def24}/+$ and *+/+* flies, or between $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies (Fig. 4.14C).

Following this, we tried to examine the activation status of dFoxo by staining sections of *Drosophila* thoraces to determine if we could see nuclear localization of dFoxo as others have previously seen (Wang et al., 2009). Using 40 day old $rpd3^{def24}/+$ and $+/+$ flies we saw no clear nuclear localization of dFoxo (Fig. 4.15A, B). Taken together, our experiments on the dFoxo protein suggest that while we see changes in the levels of mRNA expression, those changes may not exist at the level of the protein.

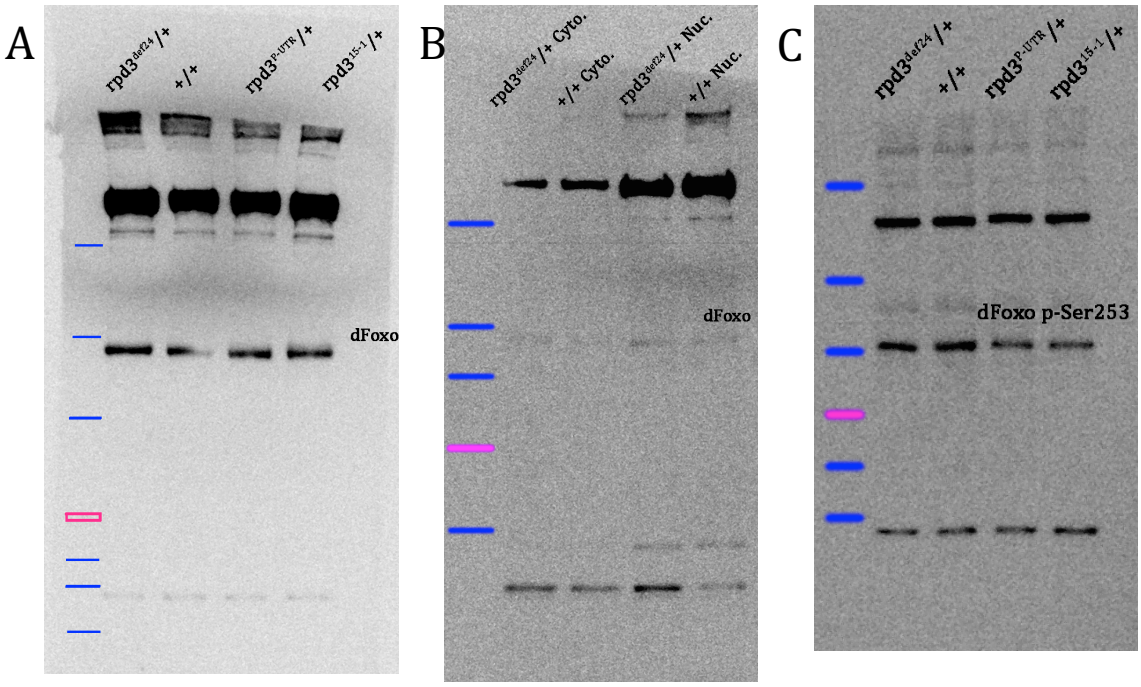

Figure 4.14 A) Western blot staining for dFoxo in 40 day old *rpd3^{def24}/+* males, *+/+* males, *rpd3$^{P\text{-}UTR}$/CS* males, and *rpd3$^{15\text{-}1}$/CS* males. **B)** Western blot staining for dFoxo in the cytoplasmic and nuclear fractions of 40 day old *rpd3^{def24}/+* males and *+/+* males. **C)** Western blot staining for phosphorylated Serine 253 dFoxo in 40 day old *rpd3^{def24}/+* males, *+/+* males, *rpd3$^{P\text{-}UTR}$/CS* males, and *rpd3$^{15\text{-}1}$/CS* males.

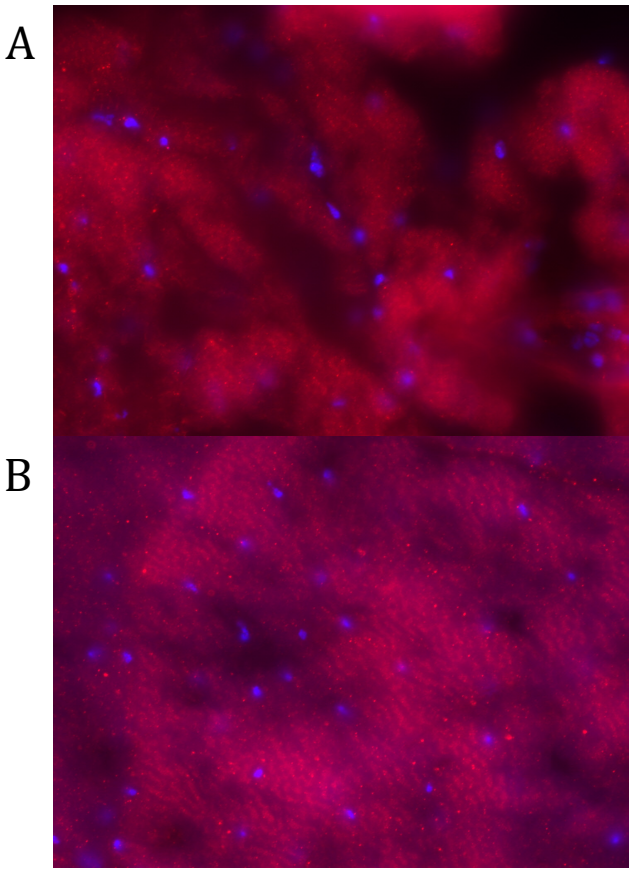

Figure 4.15 A) dFoxo staining (red) in 40 day old *+/+* males. **B)** dFoxo staining (red) in 40 day old *rpd3*^{def24}*/+* males. Nuclei are stained with DAPI (blue).

4.4 Discussion

Through the initial experiment with a 96-gene plate, we discovered that *rpd3*-mutant flies have altered expression of many genes related to the insulin signaling pathway. We confirmed these results using standard qPCR to show that insulin signaling is reduced in *rpd3* mutants. Consistent with these data, *dFoxo* expression was increased in these flies at a later age. However, we could not prove that there were changes in dFoxo levels or that this protein had post-translational modifications that primed it for activating transcription.

One interesting finding is that the changes in insulin signaling gene expression are drastically different in $rpd3^{def24}/+$ flies and their controls versus $rpd3^{P\text{-}UTR}/CS$ flies and their genetic controls. We found significant changes in many genes in $rpd3^{def24}/+$ flies but not in $rpd3^{P\text{-}UTR}/CS$ flies. One possible explanation is that the genetic background of these strains of flies is different. Genetic background has been shown to have different effects on longevity in other *Drosophila* genetic mutants (Rogina et al., 2000; Toivonen et al., 2007; Neretti et al., 2009; Toivonen et al., 2009). $rpd3^{def24}/+$ flies are generated by crosses with the *yw* strain of flies, but $rpd3^{P\text{-}UTR}/CS$ flies are generated using *Canton-S* flies. It is possible that decreased levels of Rpd3 affect these genetic backgrounds differently. Also, it could be possible that the heads of $rpd3^{def24}/+$ flies are accounting for most of the changes since head tissue was excluded in all RNA isolation from $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies. However, heads are relatively small compared to thoraces, and therefore contain much less genetic material. Also, we saw differences in expression of *dIlp3* and *chico* between heads alone or heads and thoraces of $rpd3^{def24}/+$ flies. Even

though we do not see decreased expression of components of the insulin signaling pathway in *rpd3*$^{P\text{-}UTR}$*/CS* flies, we do see increased expression of *dFoxo* in these flies, indicating that dFoxo may still be responsible for the changes we see in life span in flies of both *rpd3* mutant alleles (Fig. 4.7).

Interestingly, the levels of *dIlp2*, *dIlp5*, and *dIlp6* mRNA expression were all increased. The different functions of the dIlps have been hard to elucidate because of their functional overlap (Gronke et al., 2010). A recent review has tried to highlight the individual functions of these proteins (Kannan and Fridell, 2013). Potentially the levels of expression of genes for these three dIlps are increased because the insulin-producing cells are trying to compensate for a global decrease in insulin signaling. If target tissues have reduced levels of dInR and chico, as we have shown at the transcription level, then the concentrations of dIlps would still not be able to induce the effects of insulin signaling in these tissues.

Our life span experiments on *rpd3*^{def24}*/dFoxo* flies confirmed the involvement of dFoxo in life span extension in *rpd3* mutants. We showed that double mutants also had a decreased survival when exposed to oxidative or starvation stress compared to *rpd3* mutants alone. These results suggest that dFoxo partially mediates the life span extension in *rpd3*-mutant flies. Since it is known that both *rpd3* and *dFoxo* mutations affect fly longevity in opposite manners, it is possible that double mutant flies have altered gene expression from what would be seen in single mutants of either gene (Rogina et al., 2002; Junger et al., 2003). An obvious hypothesis would be that beneficial changes gene expression in *rpd3* mutants are mediated by dFoxo's positive effects on transcription, and these genes are not

activated in double-mutant flies. It could also be that these two proteins regulate synergistically beneficial sets of genes, and the beneficial effects of one set are removed when one of the genes is mutated. Either way, it seems that while dFoxo must be present for the full life span extension seen in *rpd3* mutants, it is not fully responsible for mediating this extension. This is to be expected, as there is probably not one single gene or protein that is responsible for the effects of decreased levels of *rpd3*.

We tried multiple methods to look at nuclear localization of dFoxo proteins, but were not successful. Staining for dFoxo, Western blots for phosphorylated dFoxo, and Western blots using nuclear and cytoplasmic fractions that were stained for dFoxo all failed to show differences in dFoxo activation. A colleague informed us that in his lab, they are unable to see changes in dFoxo localization unless they starve fruit flies (Villa-Cuesta et al., 2010). Another previous report in flies showed that dFoxo was localized to the nucleus in the fat body of CR flies following starvation (Wang et al., 2009). One of the main limitations of *Drosophila* research compared to research in humans and mice is the relatively lower amount of antibodies that can target specific post-translational modifications. Although we tried two different antibodies that targeted mammalian phosphorylated Foxo3A, only one of these antibodies recognized the protein. There are still methods we could try to determine if dFoxo is active though. For one, we could still try to determine if dFoxo's target genes show increased expression, indicating that dFoxo is exerting its positive effects on transcription. Also, we could starve flies briefly

before looking at the nuclear localization of dFoxo, but this method would introduce a potentially confounding variable.

Although it was not analyzed in great depth, it is possible that the TOR signaling pathway also plays a role the longevity of *rpd3* mutants. One consistent finding we had was the decrease in expression of *tsc1* in both lines of flies (Fig. 4.8, 4.9). *tsc1* codes for the tuberous sclerosis 1 protein, which is a negative regulator of TOR signaling. It has been shown that overexpressing the Tsc1 protein in *Drosophila* leads to a life span extension (Kapahi et al., 2004). This is due to the inhibition of TOR signaling. Using rapamycin to inhibit TOR signaling has also been show to increase life span (Bjedov et al., 2010). Although decreased *tsc1* expression would not be consistent with an increased life span in *rpd3* mutants due to decreased TOR signaling, it would be consistent with a previous study in yeast that shows Rpd3 is required to mediate the effects of TOR inhibition

We initially hypothesized that *4E-BP* would be increased, as it is a main negative regulator of protein synthesis (Katewa and Kapahi, 2011). Other groups have shown that 4E-BP is responsible for mediating some of the effects of CR (Zid et al., 2009). dFoxo is known to be a positive regulator for the transcription of 4E-BP as well (Demontis and Perrimon, 2010). However we found that *4E-BP* mRNA expression was decreased in *rpd3*-mutant flies. Our 96-gene plate did show that 2 other genes involved in the eukaryotic translation initiation complex, *eIF4E-4* and *eIF4E-5*, were increased in 40 day old *rpd3* mutants. Like in previous studies, it is possible that translation is changed only in specific classes of proteins (Zid et al.,

2009). More research would be needed to clarify the role of TOR signaling, 4E-BP, and protein synthesis in the longevity extension seen in *rpd3*-mutant *Drosophila*.

4.5 Methods

4.5.1 Fly Stocks and Maintenance

The same 2 strains of *rpd3*-mutant *Drosophila melanogaster* as described in chapter 2 were used to determine the role of insulin signaling in aging. $dFoxo^{c01841}$ were kindly provided by the Exelixis center at Harvard Medical School. These flies were backcrossed to the *yw* strain for 10 generations to eliminate difference in genetic background. Groups of 25 males and 25 females were collected 24 hours after eclosion. Unless indicated, flies were aged in vials containing normal corn food in a humidity-controlled incubator at 25°C on a 12-hour night/day cycle for their entire lifespan until time for experimentation. Normal *Drosophila* food consisted of water, inactive yeast, sucrose, agar, corn, and Tegosept (Methyl4-hydroxybenzoate, Sigma) as an antifungal agent. Flies were passed into new vials of food every other day to prevent build up of bacteria.

4.5.2 96-gene plate

Our lab designed a Taqman Custom Array card (Applied Biosystems) to test mRNA expression of 96 different genes for 4 samples. This 384-well microfluidic card allows for 384 simultaneous real-time PCR reactions, similar to what is described in 4.5.3. We used cDNA isolated from the heads and thoraces of 40 day old $rpd3^{def24}/+$ or *+/+* males, with 2 biological replicates each. 4 genes on the plate were

endogenous controls, and the other 92 were members of various metabolic pathways, such as protein translation, insulin signaling, and mitochondrial function.

4.5.3 Quantitative PCR (qPCR)

Flies were aged until the desired age, separated into groups of males or females, and then frozen and kept at -80°C until dissection. Groups of 35 flies were dissected on a cold plate to ensure they stayed frozen, and each genotype had 3 biological replicates. Total RNA was isolated from groups of 35 heads and thoraces for *rpd3*^{def24}*/+* or *+/+* flies, 35 heads for *rpd3*^{def24}*/+* or *+/+* flies, or 35 thoraces for *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* flies. Total RNA was isolated from groups of 35 thoraces of 40 day old male *rpd3*^{def24}*/yw*, *dFoxo/yw*, and *rpd3*^{def24}*/dFoxo* flies as well. Total RNA was isolated from dissected tissue using Trizol as previously described (Wang et al., 2009). cDNA was synthesized using a standard protocol, and qPCR was performed to determine changes in gene expression using a 7500 Fast Real-Time PCR System (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). TaqMan primers were obtained for *dIlp2, dIlp3, dIlp5, dIlp6, dInR, chico, tsc1, giga, dFoxo, Tor, 4E-BP (Thor),* and *S6K* from Invitrogen, and *ankryn* was used as an endogenous control. Gene expression was averaged between the biological replicates, and results are reported as relative mRNA expression as compared to the control group.

4.5.4 Life span experiments

After flies were generated that contained mutations in both *rpd3* and *dFoxo*, life span experiments were done as we have previously done in our lab (Rogina et al., 2002). We used *rpd3*^{def24}*/yw*, *dFoxo/yw*, and *rpd3*^{def24}*/dFoxo* flies for these experiments. Groups of 25 males and 25 females were collected 24 hours after

eclosion. Flies were aged in vials containing normal corn food in a humidity-controlled incubator at 25°C on a 12-hour night/day cycle. The flies were passed into new vials and the number of dead flies was counted daily. Mortality during the first 10 days of life was excluded as these deaths are considered to be non-aging related.

4.5.5 Western blots

40 day old flies were dissected on CO_2 and placed in tubes containing Kinase Lysis Buffer. The tissue was homogenized as was done for RNA isolation using Teflon pestles. Following this, protein concentrations were quantified using the BioRad protein assay (BioRad Protein Determination Kit). Lysates were cleared by centrifugation, run on a Lithium Dodecyl Sulfate (LDS) PAGE using NuPAGE NOVEX 4-12% gradient gels (Life Technologies) and transferred overnight onto nitrocellulose membranes in transfer buffer (20mM CAPS pH 11, 20% MeOH). Western blotting was performed using standard procedures with washes done in TBST. Membranes were blocked in 5% milk for an hour followed by overnight, 1:500 primary antibody incubation at 4°C. Secondary antibody incubation was done for an hour at RT, at a concentration of 1:5,000. Secondary antibodies were labeled with Horseradish Peroxidase. Blots were imaged using the Kodak Image Station 4000 MM following application of Enhanced Chemiluminensence reagent (Perkin Elmer). The dFoxo antibody was a gift from Mark Tatar, and the two anti-phospho-Foxo3A antibodies were gifts from Cell Signaling Technology to test if they would work in *Drosophila* tissue. To separate cytoplasmic and nuclear fractions, we used Active Motif's Nuclear Extract Kit (#40010). The protocol was followed as in

the manual, starting with the "Preparation of Nuclear Extract from Tissue" section and using quantities of solution as specified for the weight of 35 male *Drosophila* thoraces.

4.5.6 Immunohistochemistry

Immunohistochemistry was performed as previously described in *Drosophila* tissue (Wang et al., 2009; Rogers and Rogina, 2014). Flies were aged until 40 days of age, and then dissected on CO_2, and then fixed in 4% paraformaldehyde. Following dehydration steps, they were embedded in Tissue-Tek O.C.T. Compound, frozen, and stored at -20^{o}C until sectioning on a cryostat. The thoraces were sectioned and placed on charged, glass slides. These slides were stained using anti-dFoxo at a 1:300 dilution overnight, followed by secondary antibody for an hour. Staining for nuclei was done using DAPI (Invitrogen) diluted in PBT and 2% donkey serum for 10 minutes. Imaging was done using a Zeiss Oberserver.Z1 microscope. The dFoxo antibody was a gift from Mark Tatar.

4.5.7 Oxidative Stress Resistance

Flies were aged until 40 days of age, and then they were exposed to hydrogen peroxide (Tettweiler et al., 2005). For these experiments, *Drosophila* were sorted on CO_2 pads into groups of 20 males or 20 females. They were placed on food and allowed to recover for at least 1 hour. Then they were switched to tubes containing only water and 2 filter papers for 6 hours. This brief starvation is thought to eliminate differences in the feeding state of the flies and prevent differential intake of the oxidative stressor. Following these 6 hours, flies were transferred to new tubes containing hydrogen peroxide working solution and 2 filter papers. For H_2O_2,

the working solution consisted of 5% H_2O_2 and 5% sucrose diluted in distilled water. For paraquat, the working solution contained 20 mM paraquat and 5% sucrose diluted in distilled water. Dead flies were counted every hour or two during the day, and at one time point each late at night and early in the morning. Surviving flies were switched to new vials every 24 hours to prevent desiccation. Results are plotted as percent surviving verses time in hours.

4.5.8 Starvation Resistance

Flies were aged until 40 days of age, and then they were exposed to starvation without desiccation as described previously (Wang et al., 2009). For both experiments, *Drosophila* were sorted on CO_2 pads into groups of 20 males or 20 females. They were placed on food and allowed to recover for at least 1 hour. Then they were switched to tubes containing only 300 ul water and 2 filter papers. Dead flies were counted every hour or two during the day, and at one time point late at night and another early in the morning. Surviving flies were switched to new vials every 24 hours to prevent desiccation. Results are plotted as percent surviving verses time in hours.

4.5.9 Statistical analysis

Significance was determined using a two-tailed, unpaired t-test and expressed as P values. $P < 0.05$ is considered to be significant. Error bars represent standard deviation (SD).

Chapter 5: mRNA Sequencing

5.1 Background and Significance

Recently, next generation sequencing has become an important tool for biologists. With a single experiment it is possible to analyze changes in mRNA expression, differential splicing, and mutations in the genome (Trapnell et al., 2012). The cost of sequencing is rapidly declining, and this type of experiment will soon be a necessity in both laboratories studying genetics and medical diagnostic laboratories (Biesecker and Green, 2014). Analysis of the transcriptome has provided valuable insight into genetic changes during *Drosophila* development, so it makes sense that it could do the same for the aging process (Graveley et al., 2011). Using mRNA sequencing it may be possible to discover other pathways that are affected in our long-lived *rpd3* mutants.

5.2 Rationale

Because aging is a complex process, we wanted to perform high-throughput sequencing to determine if there are other cellular or signaling pathways that could account for the longevity extension seen in *rpd3*-mutant flies. We initially hypothesized that *rpd3* mutation would cause changes in mitochondrial biogenesis and function, yet we found that there are actually differences in the insulin signaling pathway. Many other pathways have been shown to be important in *Drosophila* aging, such as antioxidants, the JNK pathway, and even signals from the reproductive system (Finkel and Holbrook, 2000; Kenyon, 2010; Lopez-Otin et al., 2013). We found that the insulin signaling pathway through the actions of dFoxo

may only be partially responsible for the life span extension seen in *rpd3*-mutant *Drosophila*. By performing mRNA sequencing, we sought to discover other molecular pathways that may be responsible for the effects on longevity due to *rpd3* mutation.

5.3 Results

RNA was isolated from young and old *rpd3*-mutant males and their genetic controls. Heads and thoraces were separated for $rpd3^{def24}/+$ and *+/+* flies, but only the thoraces were used for $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies. After RNA isolation and library preparation, mRNA sequencing was performed on an Illumina NextSeq. The reads were mapped to the *Drosophila* genome using TopHat, and the alignment files were analyzed for differential expression using Cuffdiff. Although we compared all groups, only the differences in 40 day old mutants and their controls are presented here.

When we compared the heads of $rpd3^{def24}/+$ and *+/+* males at 40 days of age, we found that expression of 311 genes was significantly changed based on Cuffdiff analysis. For the thoraces of the same genotypes and age, we found 371 genes that were differentially expressed. For thoraces of 40 day old $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies, 217 genes were differentially expressed. These lists of genes were analyzed using DAVID bioinformatics resources (Huang et al., 2009). Functional annotation charts for each group are shown, with only the most significant 50 notations displayed (Tables 5.1, 5.2, and 5.3).

Finally we compared the lists of altered genes in thoraces of 40 day old *rpd3*^{def24}*/+* and *rpd3*$^{P\text{-}UTR}$*/CS* and their respective controls using Enrichment Map software. This plug-in for the Cytoscape network visualization software allows comparison of gene sets to determine if there is overlap (Merico et al., 2010). As expected, there was overlap in the pathways associated with the immune system (Fig. 5.1).

Category	Term	Count	%	PValue
GOTERM_CC_FAT	GO:0005811~lipid particle	40	13.986	5.31E-20
SP_PIR_KEYWORDS	Secreted	32	11.189	5.98E-18
SP_PIR_KEYWORDS	oxidoreductase	45	15.734	1.58E-14
SP_PIR_KEYWORDS	signal	39	13.636	1.97E-14
GOTERM_BP_FAT	GO:0055114~oxidation reduction	49	17.133	7.89E-14
GOTERM_CC_FAT	GO:0022626~cytosolic ribosome	21	7.3427	3.23E-13
GOTERM_BP_FAT	GO:0022904~respiratory electron transport chain	18	6.2937	8.67E-13
GOTERM_CC_FAT	GO:0005576~extracellular region	45	15.734	1.81E-12
GOTERM_BP_FAT	GO:0022900~electron transport chain	19	6.6434	2.28E-12
GOTERM_BP_FAT	GO:0042773~ATP synthesis coupled electron transport	17	5.9441	5.76E-12
SMART	SM00708:PhBP	11	3.8462	1.37E-11
GOTERM_BP_FAT	GO:0006119~oxidative phosphorylation	21	7.3427	2.87E-11
GOTERM_BP_FAT	GO:0042775~mitochondrial ATP synthesis coupled electron	16	5.5944	3.01E-11
GOTERM_CC_FAT	GO:0005746~mitochondrial respiratory chain	18	6.2937	5.00E-11
GOTERM_CC_FAT	GO:0070469~respiratory chain	18	6.2937	9.18E-11
GOTERM_CC_FAT	GO:0044445~cytosolic part	21	7.3427	1.73E-10
GOTERM_BP_FAT	GO:0006091~generation of precursor metabolites and ener	25	8.7413	2.49E-10
GOTERM_BP_FAT	GO:0019731~antibacterial humoral response	11	3.8462	2.74E-10
GOTERM_BP_FAT	GO:0045333~cellular respiration	18	6.2937	3.25E-10
INTERPRO	IPR006625:Insect pheromone/odorant binding protein PhBF	11	3.8462	3.86E-10
UP_SEQ_FEATURE	signal peptide	39	13.636	4.36E-10
GOTERM_CC_FAT	GO:0044455~mitochondrial membrane part	21	7.3427	4.67E-10
SP_PIR_KEYWORDS	ribosomal protein	20	6.993	5.89E-10
GOTERM_BP_FAT	GO:0015980~energy derivation by oxidation of organic com	18	6.2937	1.22E-09
KEGG_PATHWAY	dme03010:Ribosome	21	7.3427	1.47E-09
SP_PIR_KEYWORDS	antibiotic	8	2.7972	2.34E-09
INTERPRO	IPR006170:Pheromone/general odorant binding protein, PB	11	3.8462	3.14E-09
SP_PIR_KEYWORDS	Antimicrobial	9	3.1469	7.63E-09
GOTERM_MF_FAT	GO:0003735~structural constituent of ribosome	21	7.3427	1.28E-08
GOTERM_CC_FAT	GO:0033279~ribosomal subunit	21	7.3427	1.75E-08
GOTERM_BP_FAT	GO:0006952~defense response	20	6.993	2.34E-08
INTERPRO	IPR005521:Attacin, C-terminal region	6	2.0979	3.30E-08
GOTERM_MF_FAT	GO:0005550~pheromone binding	8	2.7972	8.07E-08
SP_PIR_KEYWORDS	ribonucleoprotein	16	5.5944	8.42E-08
GOTERM_CC_FAT	GO:0019866~organelle inner membrane	23	8.042	9.63E-08
GOTERM_CC_FAT	GO:0005743~mitochondrial inner membrane	22	7.6923	1.07E-07
SP_PIR_KEYWORDS	innate immunity	11	3.8462	1.10E-07
SP_PIR_KEYWORDS	disulfide bond	22	7.6923	1.49E-07
GOTERM_CC_FAT	GO:0005840~ribosome	21	7.3427	1.50E-07
SP_PIR_KEYWORDS	immune response	11	3.8462	1.59E-07
GOTERM_CC_FAT	GO:0022625~cytosolic large ribosomal subunit	12	4.1958	2.30E-07
GOTERM_BP_FAT	GO:0009617~response to bacterium	13	4.5455	3.43E-07
GOTERM_BP_FAT	GO:0006959~humoral immune response	13	4.5455	5.18E-07
GOTERM_BP_FAT	GO:0042742~defense response to bacterium	12	4.1958	5.71E-07
GOTERM_BP_FAT	GO:0019730~antimicrobial humoral response	12	4.1958	6.64E-07
GOTERM_BP_FAT	GO:0045087~innate immune response	13	4.5455	1.61E-06
GOTERM_CC_FAT	GO:0044429~mitochondrial part	30	10.49	1.98E-06
GOTERM_CC_FAT	GO:0005740~mitochondrial envelope	23	8.042	1.98E-06
GOTERM_CC_FAT	GO:0031966~mitochondrial membrane	22	7.6923	2.47E-06
INTERPRO	IPR016040:NAD(P)-binding domain	15	5.2448	5.56E-06

Table 5.1 Functional annotation chart of 40 day old heads of *rpd3^{def24}/+* and *+/+* males.

Category	Term	Count	%	PValue
GOTERM_CC_FAT	GO:0005576~extracellular region	53	15.543	1.31E-19
GOTERM_CC_FAT	GO:0005615~extracellular space	20	5.8651	1.22E-13
UP_SEQ_FEATURE	signal peptide	40	11.73	3.97E-13
SP_PIR_KEYWORDS	signal	40	11.73	2.69E-12
SP_PIR_KEYWORDS	Secreted	28	8.2111	4.27E-12
GOTERM_BP_FAT	GO:0006952~defense response	23	6.7449	6.33E-11
SP_PIR_KEYWORDS	antibiotic	9	2.6393	1.80E-10
GOTERM_CC_FAT	GO:0044421~extracellular region part	22	6.4516	1.93E-10
GOTERM_BP_FAT	GO:0019731~antibacterial humoral response	11	3.2258	2.22E-10
SP_PIR_KEYWORDS	Antimicrobial	10	2.9326	1.44E-09
GOTERM_MF_FAT	GO:0005550~pheromone binding	9	2.6393	2.36E-09
GOTERM_BP_FAT	GO:0042742~defense response to bacterium	13	3.8123	4.98E-08
GOTERM_MF_FAT	GO:0005549~odorant binding	17	4.9853	5.21E-08
GOTERM_BP_FAT	GO:0019730~antimicrobial humoral response	13	3.8123	5.89E-08
SP_PIR_KEYWORDS	innate immunity	12	3.5191	6.92E-08
INTERPRO	IPR005521:Attacin, C-terminal region	6	1.7595	8.08E-08
SP_PIR_KEYWORDS	immune response	12	3.5191	1.04E-07
GOTERM_BP_FAT	GO:0045087~innate immune response	14	4.1056	1.76E-07
GOTERM_BP_FAT	GO:0009617~response to bacterium	13	3.8123	2.72E-07
GOTERM_BP_FAT	GO:0006955~immune response	18	5.2786	3.52E-07
GOTERM_BP_FAT	GO:0006959~humoral immune response	13	3.8123	4.11E-07
SMART	SM00505:Knot1	6	1.7595	5.24E-07
GOTERM_BP_FAT	GO:0055114~oxidation reduction	36	10.557	6.52E-07
INTERPRO	IPR003614:Knottin	6	1.7595	7.21E-07
PIR_SUPERFAMILY	PIRSF014263:Drosophila hypothetical protein EG_34	9	2.6393	8.11E-07
GOTERM_MF_FAT	GO:0030246~carbohydrate binding	19	5.5718	9.48E-07
GOTERM_BP_FAT	GO:0006022~aminoglycan metabolic process	16	4.6921	9.49E-07
SP_PIR_KEYWORDS	disulfide bond	23	6.7449	1.07E-06
GOTERM_BP_FAT	GO:0006030~chitin metabolic process	14	4.1056	2.18E-06
GOTERM_BP_FAT	GO:0005976~polysaccharide metabolic process	16	4.6921	2.46E-06
GOTERM_BP_FAT	GO:0019236~response to pheromone	7	2.0528	3.12E-06
INTERPRO	IPR006170:Pheromone/general odorant binding pro	9	2.6393	3.16E-06
SMART	SM00587:CHK	10	2.9326	3.53E-06
UP_SEQ_FEATURE	disulfide bond	22	6.4516	4.95E-06
SMART	SM00708:PhBP	8	2.346	5.78E-06
INTERPRO	IPR004119:Protein of unknown function DUF227	10	2.9326	6.30E-06
INTERPRO	IPR015897:CHK kinase-like	10	2.9326	6.30E-06
INTERPRO	IPR006625:Insect pheromone/odorant binding prote	8	2.346	9.02E-06
GOTERM_MF_FAT	GO:0001871~pattern binding	14	4.1056	1.03E-05
GOTERM_MF_FAT	GO:0030247~polysaccharide binding	14	4.1056	1.03E-05
INTERPRO	IPR008176:Gamma thionin	5	1.4663	1.70E-05
GOTERM_BP_FAT	GO:0050832~defense response to fungus	7	2.0528	2.31E-05
GOTERM_MF_FAT	GO:0008061~chitin binding	12	3.5191	3.19E-05
GOTERM_BP_FAT	GO:0009620~response to fungus	7	2.0528	3.63E-05
SP_PIR_KEYWORDS	oxidoreductase	32	9.3842	3.86E-05
PIR_SUPERFAMILY	PIRSF036514:alpha-crystallin-related small heat sho	5	1.4663	6.91E-05
INTERPRO	IPR005520:Attacin, N-terminal region	4	1.173	7.61E-05
INTERPRO	IPR005520:Attacin, N-terminal	4	1.173	7.61E-05
SMART	SM00494:ChtBD2	11	3.2258	9.91E-05
INTERPRO	IPR002557:Chitin binding protein, peritrophin-A	11	3.2258	1.78E-04

Table 5.2 Functional annotation chart of 40 day old thoraces of *rpd3^{def24}/+* and *+/+* males.

Category	Term	Count	%	PValue
GOTERM_CC_FAT	GO:0005576~extracellular region	28	14.21	2.16E-10
GOTERM_CC_FAT	GO:0005615~extracellular space	13	6.599	7.88E-10
GOTERM_BP_FAT	GO:0006952~defense response	16	8.122	3.78E-09
SP_PIR_KEYWORDS	innate immunity	10	5.076	7.10E-08
SP_PIR_KEYWORDS	immune response	10	5.076	9.94E-08
GOTERM_CC_FAT	GO:0044421~extracellular region part	14	7.107	1.07E-07
GOTERM_CC_FAT	GO:0005624~membrane fraction	12	6.091	1.70E-07
GOTERM_BP_FAT	GO:0045087~innate immune response	11	5.584	2.15E-07
INTERPRO	IPR017972:Cytochrome P450, conserved site	11	5.584	2.48E-07
GOTERM_CC_FAT	GO:0005626~insoluble fraction	12	6.091	2.50E-07
GOTERM_CC_FAT	GO:0000267~cell fraction	12	6.091	3.30E-07
GOTERM_BP_FAT	GO:0009617~response to bacterium	10	5.076	6.70E-07
GOTERM_BP_FAT	GO:0019731~antibacterial humoral response	7	3.553	8.72E-07
KEGG_PATHWAY	dme00903:Limonene and pinene degradation	10	5.076	9.36E-07
INTERPRO	IPR017973:Cytochrome P450, C-terminal region	10	5.076	1.39E-06
INTERPRO	IPR001128:Cytochrome P450	10	5.076	1.54E-06
INTERPRO	IPR013172:DIM, Drosophila melanogaster	5	2.538	1.81E-06
SP_PIR_KEYWORDS	microsome	10	5.076	2.13E-06
GOTERM_BP_FAT	GO:0042742~defense response to bacterium	9	4.569	2.29E-06
GOTERM_CC_FAT	GO:0005792~microsome	10	5.076	3.12E-06
GOTERM_CC_FAT	GO:0042598~vesicular fraction	10	5.076	3.12E-06
SP_PIR_KEYWORDS	Secreted	15	7.614	3.18E-06
UP_SEQ_FEATURE	metal ion-binding site:Iron (heme axial ligand)	10	5.076	4.81E-06
PIR_SUPERFAMILY	PIRSF000051:cytochrome P450 CYP3A5	8	4.061	7.21E-06
GOTERM_MF_FAT	GO:0020037~heme binding	11	5.584	8.83E-06
GOTERM_MF_FAT	GO:0046906~tetrapyrrole binding	11	5.584	8.83E-06
GOTERM_BP_FAT	GO:0006955~immune response	12	6.091	8.92E-06
COG_ONTOLOGY	Secondary metabolites biosynthesis, transport, a	10	5.076	9.59E-06
SP_PIR_KEYWORDS	Monooxygenase	10	5.076	1.42E-05
UP_SEQ_FEATURE	signal peptide	18	9.137	2.11E-05
SMART	SM00020:Tryp_SPc	13	6.599	2.81E-05
SP_PIR_KEYWORDS	antibiotic	5	2.538	2.99E-05
SP_PIR_KEYWORDS	heme	10	5.076	3.88E-05
GOTERM_MF_FAT	GO:0009055~electron carrier activity	12	6.091	4.02E-05
INTERPRO	IPR005521:Attacin, C-terminal region	4	2.03	4.12E-05
GOTERM_CC_FAT	GO:0019898~extrinsic to membrane	11	5.584	4.55E-05
INTERPRO	IPR001254:Peptidase S1 and S6, chymotrypsin/H	13	6.599	8.66E-05
GOTERM_MF_FAT	GO:0008236~serine-type peptidase activity	14	7.107	1.10E-04
GOTERM_MF_FAT	GO:0017171~serine hydrolase activity	14	7.107	1.17E-04
GOTERM_MF_FAT	GO:0004252~serine-type endopeptidase activity	13	6.599	1.79E-04
INTERPRO	IPR018114:Peptidase S1/S6, chymotrypsin/Hap,	11	5.584	1.81E-04
SP_PIR_KEYWORDS	signal	18	9.137	1.92E-04
GOTERM_BP_FAT	GO:0019730~antimicrobial humoral response	7	3.553	2.48E-04
SP_PIR_KEYWORDS	Antimicrobial	5	2.538	2.75E-04
SP_PIR_KEYWORDS	endoplasmic reticulum	10	5.076	2.87E-04
INTERPRO	IPR002401:Cytochrome P450, E-class, group I	7	3.553	4.69E-04
GOTERM_BP_FAT	GO:0006959~humoral immune response	7	3.553	6.26E-04
GOTERM_BP_FAT	GO:0055114~oxidation reduction	19	9.645	7.32E-04
INTERPRO	IPR005520:Attacin, N-terminal	3	1.523	9.81E-04
INTERPRO	IPR005520:Attacin, N-terminal region	3	1.523	9.81E-04

Table 5.3 Functional annotation chart of thoraces of 40 day old *rpd3*$^{P\text{-}UTR}$*/CS* and *rpd3*$^{15\text{-}1}$*/CS* males.

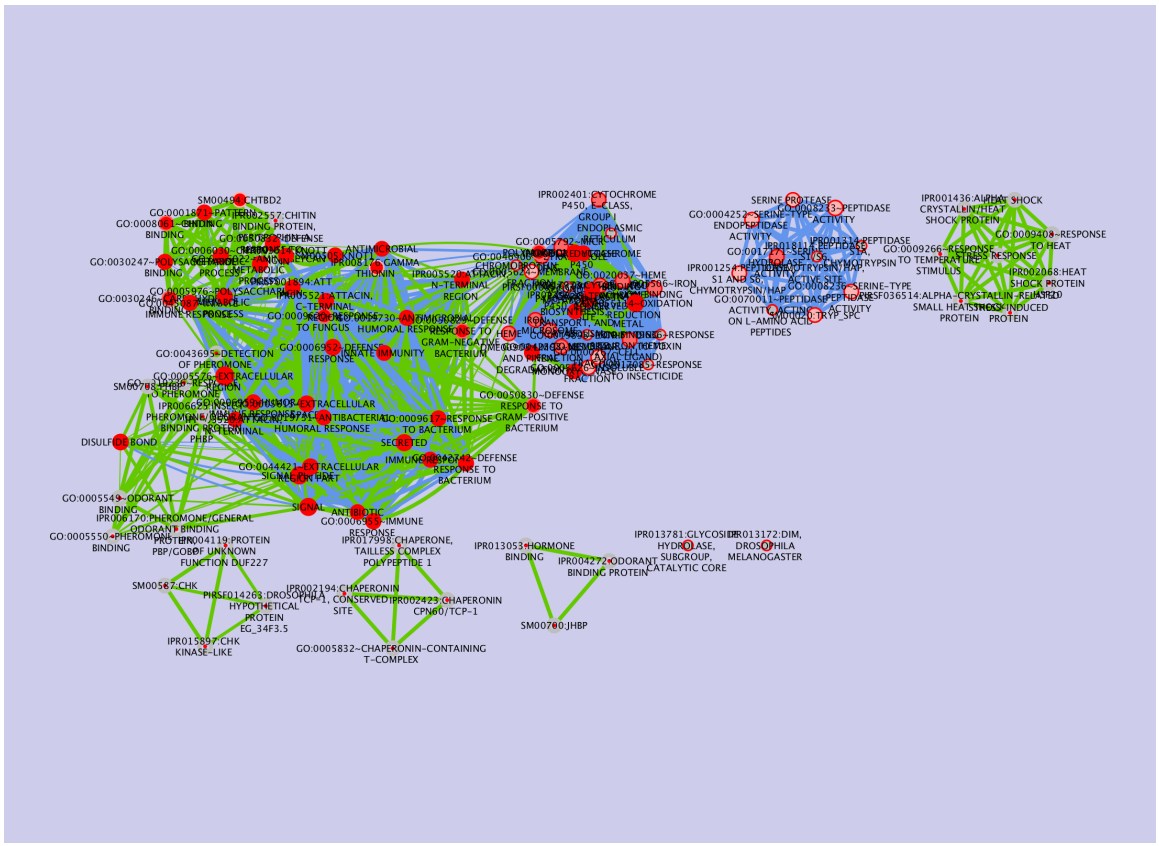

Figure 5.1 Enrichment map analysis of pathways altered in 40 day old *rpd3*^{def24}*/+* and *rpd3*$^{P\text{-}UTR}$*/CS* and their respective controls.

5.4 Discussion

Interestingly, we did not find changes in the insulin signaling pathway as we had found via qPCR. This highlights both the importance of verifying sequencing results, and the limitations that are still present in mRNA sequencing data analysis. RNA sequencing still has many sources of bias that can come from the library preparation protocol or from the way data is analyzed (Trapnell et al., 2012). For example, our data are measured in terms of fragments per kilobase of transcript per million mapped reads (FPKM), a unit of normalization for sequencing data whose consistency has been called into question recently (Wagner et al., 2012; Dillies et al., 2012). Newer methods of alignment and expression analysis for sequencing data are constantly under development, such as the kallisto alignment program under development by the Pachter lab (Bray et al., 2015). It is possible that these newer analysis tools might confirm the changes in genes of the insulin signaling, and they might discover more pathways altered by *rpd3* mutation.

A common group of genes that is changed in *rpd3* mutants compared to their controls is a group of genes related to the immune system. *Drosophila*'s immune system differs from that of humans in that only innate immunity is present (Hoffmann, 2003). Similar to mammals though, the *Drosophila* immune system has been implicated in aging and has been shown to increase expression of many antimicrobial proteins as age progresses (Zerofsky et al., 2005). Interestingly, one study proposed that *Drosophila* are able to clear infections better as they age, yet they are unable to deal with the secreted factors from bacteria as they age (Ramsden et al., 2008). The potential effects of *rpd3* mutation and its alteration of

the immune system would need to be studied in further detail to determine if the immune system is responsible for any effects on longevity extension.

5.5 Methods

5.5.1 Fly Stocks and Maintenance

The same 2 strains of *rpd3*-mutant *Drosophila melanogaster* as described in chapter 2 were used for sequencing. Groups of 25 males and 25 females were collected 24 hours after eclosion. Unless indicated, flies were aged in vials containing normal corn food in a humidity-controlled incubator at 25°C on a 12-hour night/day cycle for their entire lifespan until time for experimentation. Normal *Drosophila* food consisted of water, inactive yeast, sucrose, agar, corn, and Tegosept (Methyl4-hydroxybenzoate, Sigma) as an antifungal agent. Flies were passed into new vials of food every other day to prevent build up of bacteria.

5.5.2 RNA Isolation and Library Preparation

Flies were aged until the desired age, separated into groups of males or females, and then frozen and kept at -80°C until dissection. Groups of 35 flies were dissected on a cold plate to ensure they stayed frozen, and each genotype had 2 biological replicates. Total RNA was isolated from groups of 35 heads or 35 thoraces for $rpd3^{def24}/+$ or +/+ flies, and 35 thoraces for $rpd3^{P\text{-}UTR}/CS$ and $rpd3^{15\text{-}1}/CS$ flies. Total RNA was isolated from dissected tissue using Trizol as previously described (Wang et al., 2009). Sequencing libraries were prepared as described in Brown et al. (2014). Briefly, stranded paired-end RNA was prepared using the Illumina TruSeq stranded sample preparation kit.

5.5.3 mRNA Sequencing

Following library preparation, libraries were sequenced using an Illumina NextSeq 500 as previously described (Brown et al., 2014).

5.5.4 Analysis using the Tuxedo Suite

Results were analyzed as previously described by another lab (Trapnell et al., 2012). Briefly, data were converted into .fasq files following sequencing. The reads were mapped to the *Drosophila* reference genome (Release 6.03) using TopHat. The alignment files were then provided to Cuffdiff. Cuffdiff calculates expression levels and tests the statistical significance of changes between groups.

5.5.5 DAVID Analysis

Lists of genes that had were significantly different FPKM values were put into the online DAVID software (Huang et al., 2009). This software allows for functional classification of gene lists, and can generate enrichment scores to determine the importance of a gene group in a particular study. Enrichment Map software was used to compare the pathways found to be altered by DAVID software between $rpd3^{def24}/+$ and $rpd3^{P\text{-}UTR}/CS$ flies and their controls as described (Merico et al., 2010).

Chapter 6: Determination of the Spontaneous Locomotor Activity in Drosophila Melanogaster

Video Article

Determination of the Spontaneous Locomotor Activity in Drosophila Melanogaster

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URL: http://www.jove.com/video/51449

DOI: doi:10.3791/51449

Keywords: Investigative Techniques, Life Sciences (General), Behavioral Sciences, Drosophila melanogaster, Fruit flies, Spontaneous physical activity, Mobility, Fly behavior, Locomotor Activity

Date Published: 2/25/2014

Citation: Woods, J.K., Kowalski, S., Rogina, B. Determination of the Spontaneous Locomotor Activity in Drosophila Melanogaster. J. Vis. Exp. (), e51449, doi:10.3791/51449 (2014).

Abstract

Drosophila melanogaster has been used as an excellent model organism to study environmental and genetic manipulations that affect behavior. One such behavior is spontaneous locomotor activity. Here we describe our protocol that utilizes Drosophila population monitors and a tracking system that allows continuous

monitoring of the spontaneous locomotor activity of flies for several days at a time. This method is simple, reliable, and objective and can be used to examine the effects of aging, sex, changes in caloric content of food, addition of drugs, or genetic manipulations that mimic human diseases.

Video Link

The video component of this article can be found at

http://www.jove.com/video/51449/

Introduction

Fruit flies, *Drosophila melanogaster*, have been used as a valuable model organism to study mechanisms underlying complex behaviors, such as learning and memory, social interaction, aggression, drug abuse, sleep, sensory function, courtship, and mating (Ali et al., 2011; Jones and Grotewiel, 2011). One behavior that has been studied through multiple protocols is spontaneous locomotor activity. Negative geotaxis was one of the first methods developed for measuring *Drosophila* activity, and this protocol involves measuring the percentage of flies that reach a certain height of the vial after flies were shaken to the bottom of the container (Ali et al., 2011; Grotewiel et al., 2005). This method has advantages of being straightforward, inexpensive, and since it does not require any special equipment it can be performed in any laboratory. It has been used as a valuable screening tool to study effects of different genetic manipulations on fly mobility (Grotewiel et al., 2005). However, it is time and labor intensive and has the possibility of bias due to variable shaking of the vials and human recordings.

The negative geotaxis method was improved upon by development of the Rapid Iterative Negative Geotaxis (RING) method (Gargano et al., 2005; Nichols et al., 2012) which takes photographs of the fly vials following shaking of the flies to the bottom. The advantage of this protocol is its sensitivity and the possibility of testing a large number of fly vials at the same time. However, this protocol still has the potential for human error, and only measures negative geotaxis. Other laboratories have used simple observation in culture vials to determine locomotor activity (Long and Rice, 2007).

Recently several video recording systems for measuring fly locomotor activity have been developed. One video monitoring protocol provides time for adjustment before recording (Slawson et al., 2009). The method described by Slawson et al., also uses an air pulse to stop movement until the start of recording, which could potentially be a stressor to the animals (2009). This method provides information on average speed, max speed, time spend in motion, etc. Another three-dimensional tracking system measures the maximal velocity of individual flies during ~0.2 seconds of free flight takeoff (Marden et al., 2003). A three-dimensional video monitoring protocol uses flies expressing GFP and multiple cameras fitted with filters allowing for detection of fluorescence to determine fly mobility (Grover et al., 2008). Flies in this protocol tend to exhibit cylindrical flight patterns, which is potentially due to the shape of Drosophila culture vials (Grover et al., 2009). This method was improved by using a dome that allows measuring spontaneous movement of two flies (Ardekani et al., 2013). A high-throughput method that uses a camera to automatically monitor and quantify the individual and social behavior of

Drosophila has been also described (Branson et al., 2009). Zou et al. developed a behavioral monitor system (BMS) that uses two computer-assisted cameras to record lifetime behavior and movements such as resting, moving, flying, eating, drinking, or deaths of individual tephritid fruit flies (2011). Several other video systems have been developed to monitor fly behavioral activity (Valente et al., 2007; Inan et al., 2011).

Here we describe a method for quantifying *Drosophila* activity that utilizes population monitors. These monitors are housed in temperature- and humidity-controlled incubators at 25 °C on a 12 hour day-night light cycle. Each population monitor has infrared beams placed in rings positioned at three different heights. Every time a fly moves across the rings it interrupts the infrared beam, which is recorded by a microprocessor that independently records and counts the activity of flies within the vial. A microprocessor uploads the total activity within the vial to the computer at user-defined intervals that could vary from 1 second to 60 minutes. The method described here provides ample time for flies to adjust to the new environment and allows for simultaneous measuring of the spontaneous locomotor activity of as many as 120 populations of flies. In addition, we describe preparation of the food, fly maintenance, setting up the mobility population monitors in temperature controlled incubators, and potential factors that may affect results. This method can be used to study how different environmental or genetic modifications affect spontaneous locomotor activity of the flies.

Protocol

Note: The *Canton-S* strain is the standard wild-type background line obtained from the Bloomington Stock Center.

1. Food Preparation and Recipe for 1,000 ml of Food

Note: This section describes the protocol for food preparation. Large metal pots are used to prepare about 18 L of food at a time. The protocol described here is downsized and uses 1,000 ml H_2O. Food is autoclaved twice.

1. Mix 113 g sucrose and 28 g brewers yeast in 643 ml water. Leave ingredients on a hot plate set at 25 °C with a stir bar to mix throughout for 15 minutes.

2. Autoclave food solution for 20 min.

3. Mix 49 g cornmeal and 8.1 g agar in 268 ml water and add to the autoclaved food mixture described in step 1.2. Mix well with a large spoon or a whisk.

4. Autoclave food mixture for another 20 min.

5. Place the food on a plate and let cool down with constant mixing with a stir bar. If additional solutions should be added to food, such as mifepristone (RU486), keep the food on a hot plate set up at 60°C and add solution when the food reaches the required temperature.

6. Dissolve 2.4 g tegosept in 10.7 ml 100% EtOH and keep on a cold plate with a stirrer to completely dissolve and mix for about 15 min.

7. Add tegosept solution to food when the temperature of food is 60 °C and mix well.

8. Use a pump or a food dispenser to pour about 10 ml of food into a wide vial. By using a food dispenser one can pour food simultaneously into 100 wide, plastic vials (1 tray) at a time.

9. Cover the vials with Kimwipes and cheese cloth and leave food at room temperature for 12-24 hr to cool down. Keep the food at 4 °C and use within 3-4 weeks. Warm up the food to room temperature before use for fly work.

2. Preparation of Glass Vials

1. Prepare food according to the protocol listed in step 1.

2. Aliquot 5 ml of food into each narrow, glass vial, which is the correct size for the population monitors. This amount of food should be low enough to be below the lowest ring of the population monitor.

3. After the food cools down to room temperature cover the vials with sponge plugs and keep them at 4 °C for up to 2 weeks. Because the amount of food in a vial is rather low, it is best to use the food within a week or two to prevent any drying.

4. Warm up the vials to room temperature before use.

3. Maintenance of the Parental Flies

1. Grow the flies in wide plastic vials with standard laboratory food and keep the vials in a humidified, temperature-controlled environmental chamber at 25 °C on a 12 hr light/dark cycle. The daylight period starts at 6:00 AM in this laboratory.

2. In the morning clear adult flies from the vials from which parental flies will be collected.

3. Collect newly eclosed flies and separate them by gender on a CO_2 pad within 8 hr after eclosion to make sure that the female flies are virgins. Flies start to mate 8 hr after eclosion.

4. When the virgin male and female flies are between 5 and 10 days of age, put 10 males and 10 female flies in a vial with standard food and several grains of active yeast on top.

Note: Control the density of the larvae by using the same number of flies and keeping them in a vial for two days. Addition of active yeast promotes egg production.

5. Keep the flies to mate and lay eggs in a temperature-controlled environmental chamber at 25 °C with a 12 hr light/dark cycle for 2 days. Set up 5-10 vials of parental flies.

6. Pass the flies to a new plastic vial every other day and keep the vials with the eggs in an incubator at 25 °C.

4. Collection of Experimental Flies

1. After 9 days flies will start to eclose from the vials where the parental flies laid eggs (described in step 3.6.). Clear and discard the flies that eclosed during the first day and return the vials to incubator. Most of the flies eclosed on day 1 are females. A more synchronized population of flies will eclose on day 2.

2. Within 24 hr place newly eclosed flies on CO_2 pads and collect 25 male and 25 female flies per vials with a paintbrush or metal spoon. Keep flies on CO_2 pads for a short period of time to minimize any effects of CO_2. Write down the day of eclosion on the vial. Assemble at least 5 replicate vials for experimental and for control groups.

3. Keep the vials in temperature-controlled environmental chambers at 25 °C with a 12 hr light/dark cycle.

4. Pass the flies to a new plastic vial every other day using a funnel.

5. Age the flies until the desired age for experimentation is reached.

5. Setting Up the Mobility Monitors

1. Place the population monitors in a temperature-controlled incubator.

2. Connect each monitor with a 4-wire telephone cable to the Power Supply Interface Unit (PSIU) via 5-way splitters (multi-line), which can connect up to 5 individual monitors to one opening in the PSIU. See Figure 1A and B *(6.1A and 6.1B in this dissertation)*.

3. Connect the PSIU to a line power outlet (100-240 V). Plug the power supply output connector into one of the 2 mating PSIU jacks. The adjacent green light illuminates green when connected properly.

4. Connect the PSIU to the Universal Serial Bus (USB) hardware. Connect the USB cable between the USB hardware with a Macintosh or a Windows PC for data recording. It would be best to have a computer dedicated only for data collection since collection runs for days at a time.

5. Download the USB software (PSIUdrivers.zip). USB driver software is used by the Power Supply interface and needs to be downloaded only once. It synthesizes a data link between the computer program and the PSIU/activity monitors. For a PC use a COM port and for a Macintosh use a simple serial port.

6. Download the computer program for Macintosh OSX (Intel) or for Windows PC (XP/Vista/7) programs by following instructions provided by manufacturer Notes 308.pdf.

7. Start the computer program and set up the program by clicking on the Preferences, Lights or Monitors. The program will run until the user selects “quit” to stop the program. If the computer program or the computer is shut down the monitors will continue to count beam interruptions, but the counts will not be recorded until the program is re-launched. In that case the first reading will include all the counts since the last time the PSIU sent the data to computer.

8. Select the Preferences tab and choose the Serial Port, PSIU for Macintosh and COM for the PC.

9. Select the reading interval that ranges from seconds, minutes, or an hour.

10. Select the monitors: Each monitor has its unique number that is given by the manufacturer. Select the Monitor Range that corresponds to the numbers given to the monitors by the manufacturer.

11. The Lights box: Make sure that all the monitors are properly connected, which is marked by a green light next to the monitor number on the software. A red light indicates that the connection is lost, and a black box indicates that the system is off or improperly set up.

6. Setting Up the Experiment

1. Remove glass vials containing food from 4 °C and let them warm to room temperature.

2. Separate male and female flies of the same age on CO_2 pad. For aging studies it is possible to start mobility studies as early as 3 days of age.

3. Put 10 male or 10 female flies into each glass vial containing food. Use at least three vials for each experimental and control line of flies and for each gender.

4. Keep the vials on their side until the flies recover from CO_2 to ensure the flies do not get stuck in the food. Separate flies at about 8:00 AM and leave them for about 2 hr at room temperature to recover from CO_2.

5. Place the vials inside the population monitors housed in the incubators.

6. Discard the data collected within the first 24 hr after the flies are put into the incubator to let them adjust to the novel environment.

7. Pass the flies after 3 or 4 days to new vials to avoid drying of the food. If flies are prone to death or are age 40 days or older, pass the flies after 2 days and use data collected for day 2. Also, use more than three vials per group to ensure adequate replicates. Data from vials with dead flies should be disregarded and not included in analysis.

7. Running the Activity Monitors and Calculating the Total Spontaneous Activity

1. Select preferences - the interval for data collection.

Note: The computer program allows collection of the data at intervals ranging from 1 second to 60 minutes. 10 and 30 minute periods have been found to provide adequate information about mobility without having an overwhelming number of time points. At the selected time period, the program will send the current total count for each monitor to the computer and start counting again from zero. The computer program stores the data in a new folder created by the computer data system. The data collected in each monitor are stored separately, and individual text documents are created for each vial. The data are continuously collected as long as the program operates.

2. At the end of the experiment, scan the data using the FileScan110X for Macintosh OSX (Intel) or SystemMB108 for Windows PC (XP/Vista/7) program.

Note: The Scan program eliminates duplicate readings and makes sure that the recordings are complete.

3. Save the data collected within a specific time and period of days. Choose an experimental name and copy the files from the computer data folder for analysis.

Note: At this time, activity intervals can be changed and converted to different ones. The original data will stay stored in the computer data folder and can be retrieved as long as they are not deleted.

8. Data Analysis

1. Copy the data collected in the text files into columns of Excel spreadsheets for data analysis. Data collected by this software are in columns, which contain numbers representing total activity in a single monitor over a period of time selected by the investigator.

Note: Data collected for each monitor are in separate text files. There are 32 columns for each monitor. The first six columns are empty and contain only 0; next three contain the data collected at the bottom ring, in the middle, and at the top ring. The rest of the channels can be deleted since they do not contain any data. Each ring outputs a single value per time. See screen shot of the raw data in Figure 2.

2. Calculate the total activity within a desired period of time for each monitor that represents the sum of activity collected at three different heights of infrared beams.

Note: The time period can range from several hours, 24 hours or several days.

3. Determine the average locomotor activity and the standard deviation between the 3 monitors that represent 3 biological replicates.

Note: The data can be analyzed for statistical significance by using a number of tests. A two-tailed Students's t-test, a one-way analysis of variance (ANOVA) and a Tukey HSD post-hoc test could be used to determine the effects of several environmental or genetic manipulations on 24 hours of spontaneous locomotor activity (Parashar and Rogina, 2009). There are a number of other programs that can be used and have been previously published (Kaneuchi et al., 2003).

Representative Results

The spontaneous locomotor activity in Drosophila depends on fly gender (**Figure 3A**) *(Fig. 6.3A in this dissertation)*, calorie content of the food (**Figure 3B**) *(Fig. 6.3B in this dissertation)* and the light/dark cycle. Once the light is switched off fly activity dramatically decreases. **Figure 3A** *(Fig. 6.3A in this dissertation)* illustrates 24 hours of locomotor activity recordings of male and female flies. An asterix on the x-axis marks the time when the light was switched off and the transition to dark cycle. **Figure 3B** *(Fig. 6.3B in this dissertation)* illustrates the standard deviation between the average spontaneous locomotor activity collected in three population monitors for Male flies age 3 days on corn food. The data collected for the spontaneous physical activity during the 24 hours can be also expressed as the total activity per fly during a 24 hour period, **Figure 3C** *(Fig. 6.3C in this dissertation)***.**

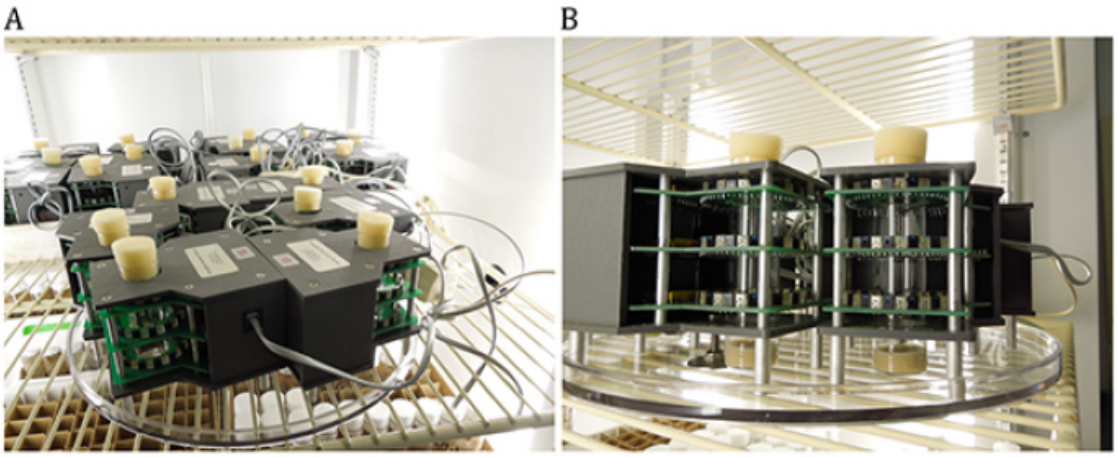

Figure 6.1: Population monitor setup for monitoring of spontaneous locomotor activity of flies. **A)** Several population monitors are connected with a 4-wire telephone cable to 5-way splitters and placed in a temperature-controlled incubator. **B)** Higher magnification of two population monitors, which show placement of the vials within the population monitors and three rings with infrared beams positioned at three different heights. Click here to view larger image.

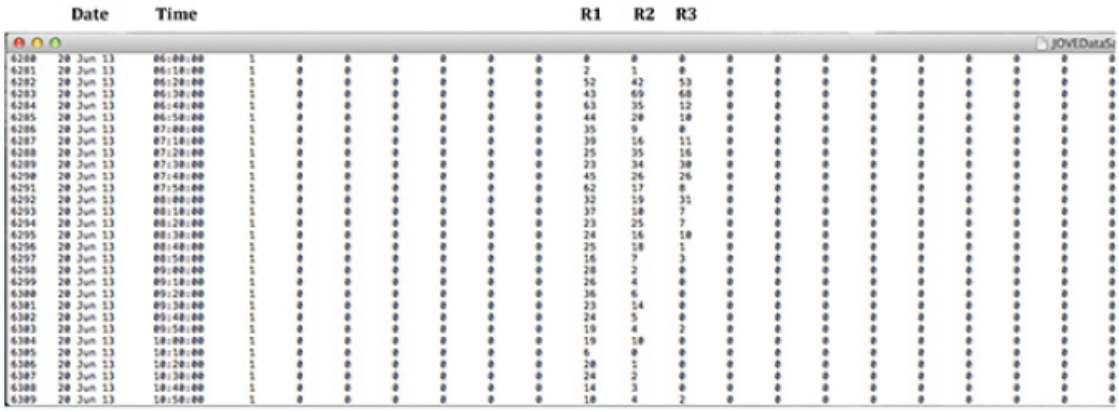

Figure 6.2: Screen shot of the raw data generated by the software showing Date, Time and data collected in Rings 1, 2, and 3. R stands for Ring. Click here to view larger image.

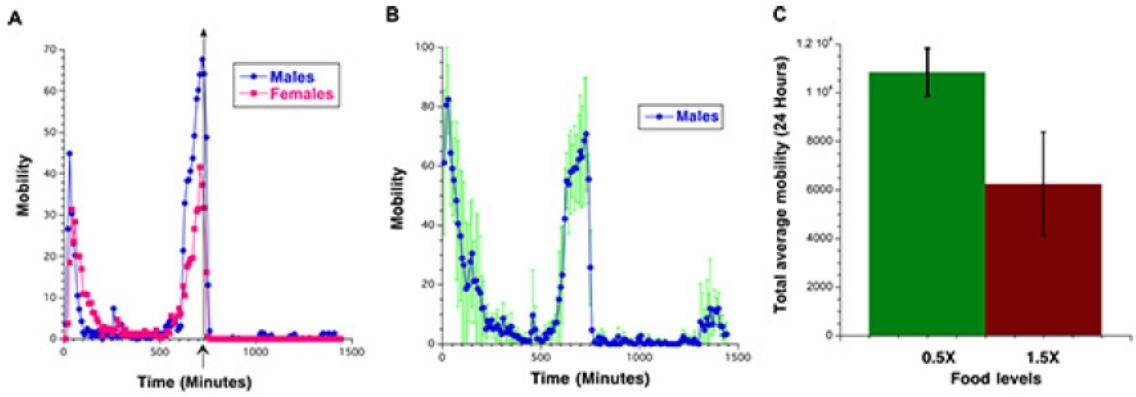

Figure 6.3: A) Average spontaneous locomotor activity of male (Black) and female (Magenta) flies during 24 hours on standard laboratory diet. The data are collected in 10 minute bins and represent average activity per fly calculated as average activity between three vials each containing 10 flies. **B)** Average spontaneous locomotor activity of male flies during 24 hours on standard laboratory diet. The data are collected in 10 minute bins and represent average activity per fly calculated as average activity between three vials. Standard deviations are marked in green. **C)** Total activity of 20 days old male flies on low-calorie (0.5X) (Green) and high-calorie (1.5X) (Brown) food over 24 hours. Click here to view larger image.

Discussion

Spontaneous locomotor activity of flies is influenced by many factors such as age, genetic background, and gender (Jones and Grotewiel, 2011; Zou et al., 2013; Carey et al., 2006; Rhodenizer et al., 2008). In addition, environmental factors such as caloric content of the food, temperature of the environment, addition of different drugs, and day/night light cycle can affect fly activity. For instance, male flies of the same age have a higher spontaneous physical activity compared to females (**Figure 1**) *(Fig. 6.1 in this dissertation)*. Therefore, flies of the same age and gender should be compared to each other. When examining the effect of genetic manipulations on fly activity, such as overexpression or loss of function of a particular gene, the experimental and control flies must be in the same genetic background to remove any potential effects of different genetic background or second site modifiers. This can be achieved by backcrossing experimental female flies to w^{1118} or *yw* males for 10 generations. After 10 generations of backcrossing, w^{1118} or *yw* flies could be used as a genetic control. Another way to control for the genetic background is to use the inducible GAL4 GeneSwitch (GAL4-GS)-UAS binary system, which allows overexpression or down-regulation (RNAi) of the gene of interest in a time and tissue-specific manner in flies fed food with the addition of mifepristone (RU486) (Osterwalder et al., 2001; Dietzl et al., 2007). RU486 is necessary for GAL4 to dimerize and bind to the UAS sequence. Genetic controls are sibling flies kept on food with the addition of EtOH (Ru486 diluent).

Various methods have been used to record *Drosophila* mobility. The method described here is simple, reliable, more informative, and has less potential of bias

compared to other methods used to determine *Drosophila* mobility, such as negative geotaxis. It has the advantage of objective simultaneous recording of multiple populations of flies for a long period of time in standard culture conditions. Measuring locomotor activity by using population monitors can be useful for studying how different caloric contents of the food affect fly activity or to study genetic mechanisms underlying increased activity of flies on CR (Parashar and Rogina, 2009). Similarly, this system has been used to study the effects of different genetic mutations, aging, or the addition of different drugs on fly spontaneous physical activity. Use of individual tubes instead of population monitors allows measuring H_2O_2 resistance in different genotypes of flies, studying circadian rhythms *in vivo*, analyzing sleep behavior, and others (Kaneuchi et al., 2003; Chiu et al., 2010; Pfeiffenberger et al., 2010A; Pfeiffenberger et al., 2010B).

Like any method, there are limitations to this monitoring system. When monitoring flies for a long period of time, there is a potential for fly death, especially if using aged flies. Using only healthy flies will help prevent this. We also try to use more than 3 biological replicates per group if the flies are old or prone to dying. One solution is to keep flies only for 2 days in the mobility monitors and use data collected during day 2, after flies have adjusted to the environment. If death occurs we do not use the data collected for the vial in calculations. Although we have been using vials positioned only vertically in the Trikinetics activity monitors, there is a possibility to place the vials horizontally. We choose to place vials vertically because the food is at the bottom of the vial, which is similar to standard incubator culture conditions. This allows flies to have more space to walk up and down the vials, and

it is more similar to negative geotaxis experiments. The humidity of the incubator should also be monitored if food desiccation becomes a problem (Pfeiffenberger et al., 2010). This system provides data in terms of average activity, and does not provide specific details about the nature of the activity. In addition, if two flies cross the beam at the same time, it will only be recorded as one interruption. The protocol described here is useful for quantifying total activity, but other protocols could provide useful data if more precise information such as flight trajectory or velocity are desired (Zou et al., 2011; Valente et al., 2007; Ardekani et al., 2013).

Following this experiment, differences in spontaneous locomotor activity due to genetic or environmental manipulations will be known. A future modification of this protocol could be to analyze the different levels of activity of flies at the top, middle, and bottom rings of the population monitors. This would determine whether the fly populations spend most of their time at the bottom of the vial near the food or at the top. The protocol in its current form allows for accurate, simultaneous quantification of spontaneous locomotor activity of *Drosophila* experimental and control populations.

Disclosures

We have nothing to disclose.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (AG023088 to B.R.).

References

(References from this paper have been combined with the final references for this dissertation.)

Chapter 7: General Discussion and Future Directions

7.1 General Discussion

Since our lab first published the initial report on longevity extension in *rpd3*-mutant *Drosophila*, little work had been done to determine the mechanism of life span extension. Our lab's initial two papers suggested that *rpd3* and CR had overlapping mechanisms of life span extension (Rogina et al., 2002; Rogina and Helfand, 2004). Therefore, we investigated two biological processes known to play a role in health aging, mitochondrial function and insulin signaling, in addition to many other aspects of physiology in these fruit flies. Our significant findings are summed up in Fig. 7.1.

We found that many components of the insulin signaling pathway were altered. Expression of *dInR* and *chico* were both decreased, and expression of *dFoxo* was increased. Our experiments using double mutants for *rpd3* and *dFoxo* suggest that the longevity extension and increased stress resistance of *rpd3*-mutant flies are at least partially mediated by an increase in *dFoxo*. Similarly, it has been shown in flies and in mammalian cells that an overexpression of *dFoxo* mediates an increased stress resistance (Junger et al., 2003). However, the phenotype of *rpd3*-mutant flies does not completely mimic that of flies with decreased insulin signaling. For example, *rpd3* mutants have an increased body weight compared to flies with overexpressed *dFoxo* in the fat body, and they are not sterile like *chico* mutants. These results illustrate the complicated interactions between *rpd3*, its downstream targets, and longevity extension.

Unexpectedly, many of the results from our experiments contradicted the hypothesis that *rpd3* mutation and CR work through similar mechanisms to induce life span extension in fruit flies. We found that *rpd3* mutants did not have an increase in *dSir2* mRNA expression as previously reported (Fig. 3.2). However, the initial experiment was done on 10 day old flies, and it was semi quantitative PCR (Rogina et al., 2002). Using quantitative PCR on flies 20 or 40 days of age, it was found that there was no difference in *dSir2* expression in *rpd3* mutant males and their genetic controls. As flies age, we see changes in oxidative stress resistance and altered gene expression that becomes more apparent in later ages. This could be viewed from two different perspectives. On one hand, it could be thought that changes seen at later ages are the changes that mediate the longer lifespan and increased stress resistance. An example of this would be how *dFoxo* expression is significantly increased at a later age but not at a younger age. However, it is also possible that beneficial changes seen at a young age leave a lasting mark on an organism that can cause it to have an increased life span or increased stress resistance at a later age.

One interesting piece of data is a gradual increase in *rpd3* mRNA expression levels in a laboratory wild-type strain, *Canton-S* (Fig. 2.1). This is consistent with the fact that keeping levels of *rpd3* expression artificially low via heterozygous mutation would result in an increased life span. However, previous yeast studies showing an increased life span following *rpd3* deletion also showed a gradual decline in *rpd3* mRNA expression throughout yeast life span (Kim et al., 1999). Many differences in yeast and fruit fly biology and aging studies could explain these

results. For one, yeast life span experiments are a measure of replicative life span rather than absolute life span (Lu et al., 2011). Although many aging studies in yeast have been very informative, being a single cell organism precludes experiments involving interactions between different organ systems amongst other things.

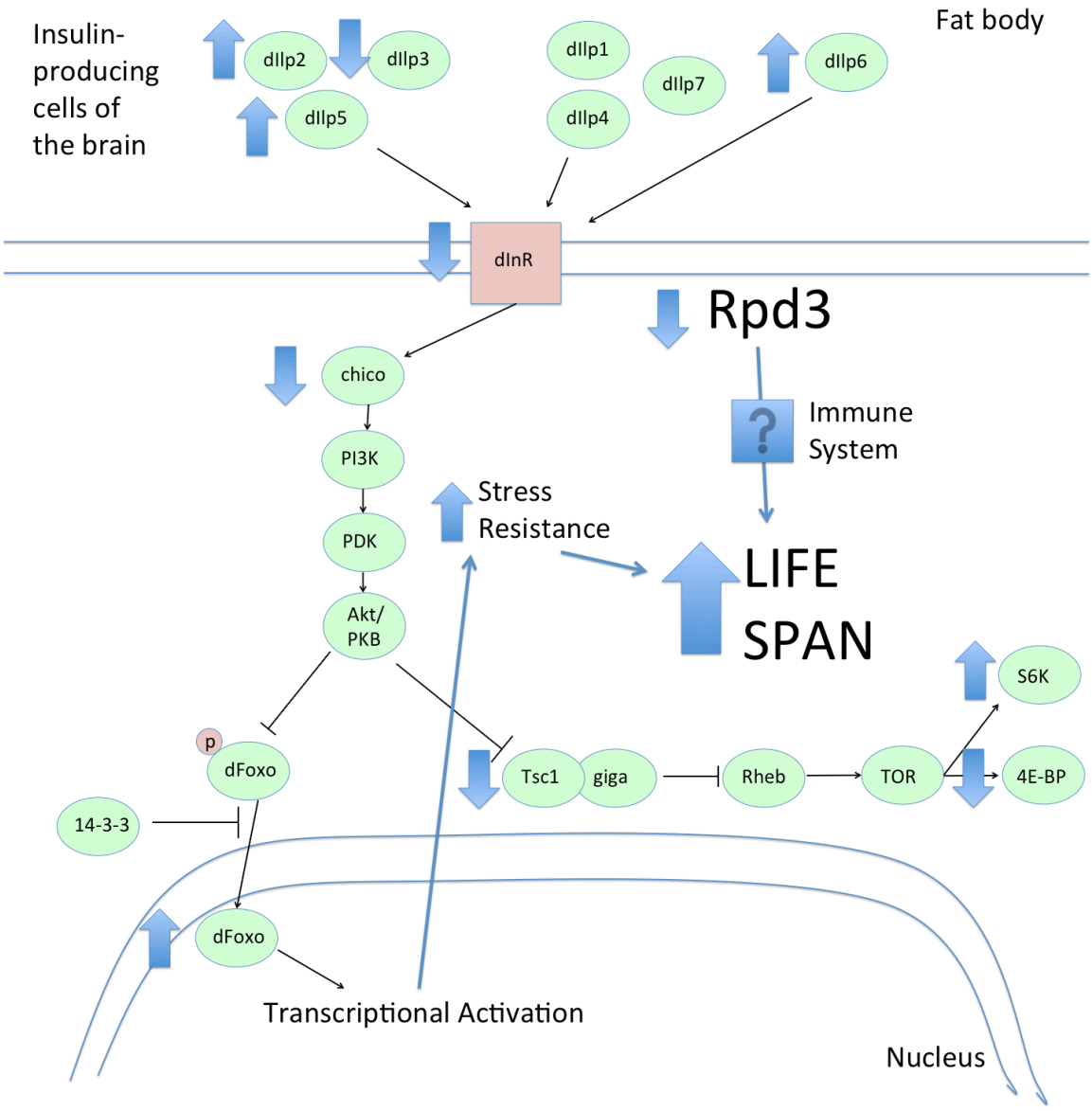

Figure 7.1 Alterations seen in long-lived *rpd3*-mutant *Drosophila*. We saw decreased expression of the genes in the insulin signaling, consistent with increased levels of *dFoxo*. TOR signaling was altered in *rpd3* mutants as well. As it is known that dFoxo is important in the response to oxidative stress, we suggest that dFoxo partially mediates the increased stress resistance in *rpd3* mutants. Our sequencing results indicate that the immune system may also be altered in *rpd3*-mutant flies, and this may contribute to the longevity extension.

7.2 Future Directions

Although the role of *rpd3* mutation in *Drosophila* aging has been examined in great detail in this work, many possible directions remain for future experiments. Mitochondrial biogenesis may not be changed by the parameters we have measured, but it would be interesting to confirm these data by looking at other activators of mitochondrial biogenesis. We did not find any changes in the levels of *spargel* mRNA expression, but other regulators of biogenesis exist. The roles of Tfam and NRF-1 and -2 could be evaluated, although it is unlikely that these will be changed since we found no differences in mitochondrial DNA or mitochondrial density via electron microscopy.

Increased mitochondrial function and biogenesis as the main factors in longevity extension of *rpd3*-mutant *Drosophila* have been ruled out, yet other aspects of metabolism may contribute to life span extension of these flies. $rpd3^{P\text{-}UTR}/CS$ females had a much greater quantity of triglycerides following starvation that $rpd3^{15\text{-}1}/CS$ flies, which is consistent with their increased starvation resistance. This aspect of metabolism has been shown to play a key role in longevity in *Drosophila* (Giannakou et al., 2004). It is possible that lipid metabolism may be a pathway that mediates the effects of *rpd3* mutation on longevity in addition to the insulin-signaling pathway. We only looked at triglyceride levels in *rpd3* mutants, and it may be possible that more specific aspects of lipid metabolism and storage are altered in these long-lived flies. For example, it would be interesting to study tissue-specific knockdown of *rpd3* in the oenocytes, cells that have been shown to be important for lipid accumulation during periods of fasting (Gutierrez et al., 2007).

Insulin signaling has shown to have significant changes at the mRNA expression level in long-lived *rpd3* mutants. However, we are still unclear about the functional consequences of having reduced insulin signaling at the protein level. Although we found *dFoxo* mRNA expression to be increased in *rpd3*-mutant flies, we could not confirm that levels of dFoxo protein were also increased. Furthermore, we were unable to confirm the activation status of dFoxo. It has been shown in the literature that protein levels and mRNA levels are not entirely in line with each other (Vogel and Marcotte, 2013). In this review, the authors suggest that 40% of the changes in protein concentration can be known by the mRNA concentration. The other 60% is due to post-transcriptional regulation or other confounders.

The insulin signaling pathway is a cellular pathway in cells of target tissues that responds to hormonal signals from other parts of the body such as the insulin-producing cells of the brain or the fat body. In our *rpd3*-mutant flies, we found significant differences in gene expression depending on the tissue analyzed. For example, mRNA expression of both *dIlp3* and *chico* were significantly increased in the heads only in *rpd3* mutants (Fig. 4.4D, F) but decreased when analyzed in the heads and thoraces combined (Fig. 4.2D, F). Sequencing results also showed differences in changes in gene expression of $rpd3^{def24}/+$ flies in the head versus the thorax. These results highlight the tissue specificity of gene expression, and indicate that it would be of interest to look at specific tissues that relate to aging. Our lab has shown that the intestine is an organ in *Drosophila* whose decline is associated with advanced age, and other labs have indicated that the fat body and brain are also organs whose function are altered in aging (Rogers and Rogina, 2014; Giannakou et

al., 2004; Broughton et al., 2005). Although we did not have luck using *rpd3* RNAi lines with the MHC driver (Fig. 2.2), it is possible that other drivers could lead to effective knockdown in other tissues. Also, it has been shown that overexpression of *dicer-2,* a gene coding for an endoribonuclease, has led to a more robust knockdown in *Drosophila* RNAi lines (Dietzl et al., 2007). Using these techniques it would be interesting to see if a reduction of *rpd3* targeted to a specific tissue would result in life span extension or altered insulin signaling.

Our data only indicate that dFoxo is partially responsible for life span extension. If dFoxo were to be fully responsible for mediating the effects of life span extension in *rpd3* mutants, we would expect the longevity curve of $rpd3^{def24}$*/dFoxo* double mutant flies to overlap with that of *dFoxo/yw* flies. This suggests that there are other pathways at play in *rpd3* mutants. One possibility that we also show changes in is the TOR signaling pathway. We see a consistent, significant reduction of *tsc1* in both alleles of *rpd3* mutants. However, previous reports in *Drosophila* show that *dTsc1* overexpression result in life span extension (Kapahi et al., 2004). The Tsc1/Tsc2 complex is a known inhibitor of TOR signaling, and therefore TOR signaling may be more active in *rpd3*-mutant flies. This would be consistent with the increased weights and energy stores we see in $rpd3^{def24}$*/+* flies compared to their controls. It would be interesting to examine the role of *dTsc1* mutations or overexpression in *rpd3* mutants on life span or stress resistance would be additive or detrimental to *rpd3*-mutant flies.

Our sequencing results indicate the role of the immune system may be another factor in the longevity extension seen in *rpd3*-mutant *Drosophila*. Changes

in the immune system have been indicated as a prognostic indicator of longevity in humans, so it would be of interest to determine how this pathway is affected in *Drosophila* by *rpd3* mutation (Larbi et al., 2008). The immune system in *Drosophila* is simpler from that of humans, because flies only have the innate immune system (Hoffmann, 2003). Although other groups have studied the *Drosophila* immune system and its changes during aging, none have studied its relation to HDACs during this process (Zerofsky et al., 2005; Ramsden et al., 2008). It would be interesting to generate double mutants as we did with *rpd3* and *dFoxo* to determine if the immune system plays a role in longevity extension in *rpd3* mutants

We have sought to determine what are physiological and genetic changes that result in longevity extension in *rpd3*-mutant *Drosophila*. Our results indicate that they have altered stress response, energy storage, and insulin signaling, but small if any changes in mitochondrial biogenesis and function. The question of whether reduction of *rpd3* is a form of genetic CR seems to be unlikely, yet there are some overlapping changes in *rpd3* mutants such as increased mobility and stress resistance. It will be interesting to see if other signaling pathways or other aspects of physiology are changed in these fruit flies. Although aging is a very complex process with both genetic and environmental factors playing a role, studies on *rpd3* mutants will be important to determine how HDAC1 could be targeted to promote an increased health span in humans.

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