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Mechanisms Associated with Aging and Age-Related Disease in Drosophila

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MECHANISMS ASSOCIATED WITH AGING AND AGE-RELATED DISEASE IN DROSOPHILA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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I would like to thank my mentor Dr. Mike Grotewiel for his patience in answering all of my questions and for insightful discussions on my research projects. The vigorous training in the laboratory with designing and performing experiments, interpreting results, and writing manuscripts has helped prepare me for a future career in research. Mike has continuously supported me throughout my graduate studies and has never hesitated to provide guidance when asked how I can improve my work. His dedication to training students and encouragement for high quality work is greatly appreciated. Mike has really inspired me to strive for excellence in my research and my writing and I could not have been successful as a graduate student without his support.

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### List of Abbreviations

- **Age-related locomotor impairment** .... **ARLI**
- **CDGSH iron sulfur domain 2** .... **CISD2**
- **Dorsocross 3** .... **Doc3**
- **Endoplasmic reticulum intermembrane small protein** .... **ERIS**
- **Huntington disease** .... **HD**
- **infantile neuronal ceroid lipofuscinosi** .... **INCL**
- **juvenile neuronal ceroid lipofuscinosi** .... **JNCL**
- **Methionine sulfoxide reductase A** .... **MSRA**
- **Neuronal ceroid lipofuscinosi** .... **NCL**
- **Superoxide** .... **O$_2^-$**
- **Hydroxyl radical** .... **OH$^-$**
- **4-phenylbutyrate** .... **PBA**
- **Protein carboxyl methyltransferase** .... **PCMT**
- **phosphatidylinositol 3-kinase** .... **PI3K**
- **phosphoinositide-dependent kinase 1** .... **PDK1**
- **6-phosphofructo-2-kinase** .... **Pfrx**
- **Phospholipid hydroperoxide glutathione peroxidase** .... **PHGpx**
- **Palmitoyl protein thioesterase 1** .... **PPT1**
Rapid iterative negative geotaxis ................................................................. RING
Reactive oxygen species .................................................................................... ROS
Spinocerebellar ataxia 3 ..................................................................................... SCA3
Superoxide dismutase ......................................................................................... SOD
Thioredoxin reductase ......................................................................................... TRXR
Ubiquitin interacting motif .................................................................................. UIM
Ubiquitin protease domain .................................................................................. UPD
Unfolded protein response .................................................................................... UPR
Wolfram Syndrome .............................................................................................. WFS
Wolfram Syndrome 2 ........................................................................................... wfs2
Abstract

MECHANISMS ASSOCIATED WITH AGING AND AGE-RELATED DISEASE IN DROSOPHILA

Melanie Ann Jones, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010.

Director: Dr. Michael Grotewiel
Associate Professor, Department of Human and Molecular Genetics

Aging is an intrinsic process that is independent of obvious disease. In contrast to normal aging, age-related diseases are conditions that typically manifest at advanced ages, are associated with explicit pathology and cause disability and premature death. We used Drosophila as a model to investigate the molecular-genetic mechanisms associated with aging and age-related disease. Age-related locomotor impairment (ARLI) is a serious condition for the elderly and greatly impacts their quality of life. Toward identifying genes and mechanisms that influence ARLI, we performed a forward genetic screen using Drosophila mutants. This screen identified a loss of function mutant in PDKI, a component of the insulin signaling pathway. Additional loss of function mutants in the insulin signaling pathway genes PI3K Dp110, and AKT also delayed ARLI. These results suggest a role for insulin signaling in ARLI. Wolfram Syndrome (WFS) is
a progressive neurodegenerative disease that is caused by mutations in the genes \textit{WFS1} and 
\textit{CISD2}. The function of \textit{CISD2}, the most recently identified gene has not been fully resolved. 
We used RNAi to knockdown \textit{wfs2}, the fly ortholog of \textit{CISD2} to identify genes and pathways 
associated with \textit{wfs2} that will provide insight into the normal function of this gene. Through a 
targeted genetic screen in the \textit{Drosophila} eye we identified that \textit{wfs2} interacts with two 
lysosomal storage disease genes \textit{PPT1} and \textit{CLN3}. These results suggest that WFS and 
lysosomal storage diseases may be influenced by common molecular-genetic mechanisms. 
Furthermore, \textit{wfs2} may play a role in the neurodegenerative pathways associated with lysosomal 
storage disease. Oxidative stress is associated with aging and age-related disease. To identify 
genes that can protect against endogenous oxidative stress we performed a candidate suppressor 
screen. This screen revealed that expression of wild-type \textit{Ataxin-3} suppressed the short lifespan 
of \textit{Sod2} knockdown flies. The ubiquitin associated function of \textit{Ataxin-3} was determined to be 
important for this suppression. Interestingly, \textit{Ataxin-3} expression also extended the short 
lifespan due to knockdown of thioredoxin reductase in muscle. These results suggest that 
\textit{Ataxin-3} expression may play a protective role against enhanced endogenous oxidative stress due 
to reduced function of a number of antioxidant enzymes.
Aging, Age-Related Disease, and the *Drosophila* Model

1. *Aging and age-related disease in humans*

Aging is an intrinsic process that is independent of obvious disease. Aging results in reduced reproduction and an increased risk of death (Finch et al., 1990). Functional senescence is the progressive age-dependent decline in function and is a fundamental feature of aging (Arking, 1998). Functional senescence manifests as defects in behavior, reproduction, cardiac function, and immune system function (Lakatta, 1993; Burns, 2004). Age-related behavioral changes in humans include a progressive decline in locomotor ability, olfactory ability, memory function, and circadian rhythmicity (Grady, 1998; Nusbaum, 1999; Yoon et al., 2003; Onder et al., 2005). Age-related functional declines are particularly important because towards the end of life older adults are more concerned with retaining their functional capacity as opposed to prolonging their lifespan (Phelan et al., 2004). In contrast to normal aging, age-related diseases are conditions that typically manifest at advanced ages, are associated with explicit pathology and cause disability and premature death. A combination of environmental and genetic factors predisposes humans to age-related diseases such as diabetes, obesity, cardiovascular disease, cancer and neurodegeneration. There are currently no interventions that can stop or reverse the effects of aging in humans. With the improvement in health care humans are continuing to live longer and the number of individuals that will be affected by age-related functional declines and age-related diseases will continue to increase as the population ages.

2. *The utility of Drosophila as a model organism to study aging and age-related disease*

Model organisms that are commonly used to study aging and age-related disease include *C. elegans, Drosophila melanogaster*, and mice. However, *Drosophila* has several features that set
this model organism apart from other model systems with regards to aging and age-related disease. Most tissues in the fly are post-mitotic which is important for aging studies because there is evidence that functional senescence is due to the inability to maintain homeostasis in existing cells (Helfand and Rogina, 2003). Most genes in Drosophila have homologs in mammals and the physiology between flies and mammals is generally conserved (Adams et al., 2000). Therefore, the molecular-genetic mechanisms associated with aging and age-related diseases are likely to be conserved in flies and vertebrates. In addition, flies have a quick generation time, develop to adulthood quickly, have a relatively short lifespan (50-80 days), show functional declines quickly and have powerful genetic strategies to manipulate the genome. These features are all beneficial for studying aging and age-related disease (Grotewiel et al., 2005a). Furthermore, pathways and processes involved in many human conditions can be elucidated via genetic screens. Genetic screens can be performed using behavioral assays which aids in the discovery of genes and pathways that accelerate or delay physiological changes that are associated with aging. Genetic screens using the Drosophila eye can aid in the identification of interacting genes which can provide insight into the molecular and cellular mechanisms underlying age-related disease. Therefore, the use of genetic screens may lead to the discovery of therapeutics or interventions that can improve the quality of life for aging humans. Drosophila are also a powerful model organism for identifying the key organ systems that fail with age and the pathophysiological changes that underlie many age-related functional declines and age-related diseases.

3. The fly as a model for age-related behavioral declines
Commonalities in the biological and physiological systems between flies and humans allows for the opportunity to investigate the molecular-genetic basis for age-related behavioral declines. Many of the behavioral changes associated with aging in humans are also observed in Drosophila including a decline in locomotor ability with age, a decrease in olfactory abilities, and a decline in memory function (Fresquet and Medioni, 1993; Cook-Wiens and Grotewiel, 2002). Assays to quantitate age-related behavioral declines are readily available in flies (Grotewiel et al., 2005). It is suggested that specific organ systems are involved that contribute to the functional declines observed in flies and not a generalized deterioration in physiological functioning since some behaviors do not appear to senesce in Drosophila (Cook-Wiens and Grotewiel, 2002). Therefore, flies are a good model system for the elucidation of mechanisms that influence behavioral declines with age.

4. The fly as a model for age-related diseases

Drosophila are very useful to delineate the mechanisms associated with age-related disease because of the conservation of gene function and physiology between flies and humans. In addition, genetic pathways are well conserved thereby allowing for the investigation of pathways involved in the pathology of age-related diseases. Flies have been successfully used to study a number of age-related diseases including cancer, heart failure, diabetes, and neurodegenerative disease. Mechanisms underlying human carcinogenesis are being elucidated using the fly (Froldi et al., 2008). This is possible because the pathways involved in tumorigenesis are conserved between flies and humans (Brumby and Richardson, 2005). Drosophila are also used to investigate mechanisms involved in altered cardiac function that leads to heart failure in humans (Wolf and Rockman, 2008). The mechanisms involved in lipotoxicity progression in humans are
conserved in *Drosophila* thereby making the fly useful for the study of diabetes (Kuhnlein, 2010). Flies have also been used successfully to model many neurodegenerative diseases in humans including Parkinson and Alzheimer diseases (Cauchi and van den Heuvel, 2006). This is very important because not only have the molecular and cellular mechanisms of neurodegenerative diseases been elucidated by using this model organism but the discovery of drug targets and therapeutics have been revealed. Therefore, the study of age-related diseases in *Drosophila* has the potential to identify therapeutic interventions for treatment that could improve the quality of life for patients afflicted with these conditions.

5. **Aims of this dissertation**

This dissertation describes how I used the *Drosophila* model to investigate molecular-genetic mechanisms associated with aging and age-related disease. The following introductory sections provide a review of the literature relevant to each specific aim in this dissertation. The aim of Chapter 2 was to identify and characterize P element insertion mutants with a delay in age-related locomotor impairment (ARLI) with the overall goal of identifying genes and genetic pathways that forestall ARLI. A more complete understanding of the genetic basis of ARLI will facilitate the development of interventions that can improve the quality of life in humans with ARLI. The background for this research is reviewed below under the heading Age-Related Locomotor Impairment and summarizes the significance of ARLI in humans, the utility of flies to model ARLI and the assays used to record locomotor behavior in *Drosophila*. The aim of Chapter 3 was to use RNAi to decrease the expression level of *wfs2*, the fly ortholog of *CISD2* to provide insight into the normal function of *wfs2*. Furthermore, the use of *Drosophila* to identify genes and pathways that interact with *wfs2* will further elucidate the possible function or
function(s) of \textit{wfs2} and has implications for the development of treatments or therapies for Wolfram Syndrome patients. The background for this research is reviewed below under the heading Wolfram Syndrome: \textit{Drosophila} as a genetic model to provide insight into \textit{wfs2} function. A brief overview of Wolfram Syndrome is provided along with a summary of what is currently known about the function of \textit{CISD2}, the most recently discovered gene implicated in Wolfram Syndrome. The aim of Chapter 4 was to identify genes that can mitigate the short lifespan of a model of endogenous oxidative stress via \textit{Sod2} knock-down. The identification of genes and a better understanding of how they suppress the negative consequences of endogenous oxidative stress has implications for the treatment and management of conditions associated with aging and age-related disease where oxidative stress is a component. The background for this research is reviewed below under the heading Oxidative Stress in Aging and Age-Related Disease and summarizes the role oxidative stress has on aging and age-related disease and the role of antioxidants in protecting against oxidative stress.

**Age-Related Locomotor Impairment**

1. \textit{Introduction}

   One of the most devastating age-related behavioral changes in humans is the progressive decline in the ability to walk. In order to maintain a high quality of life, mobility is crucial because daily activities and therefore independence rely on locomotor skills (Pahor et al., 2006). Walking speed may represent a general measure of overall health and can be an indication of risk for future disabilities (Onder et al., 2005). One of the key features of locomotor impairment in elderly individuals is reduced speed of walking (Kozakai et al., 2000; Laufer, 2005; Espeland et
al., 2007). In fact, one of the most common complaints in elderly individuals is a decrease in mobility (Kozakai et al., 2000; Laufer, 2005; Espeland et al., 2007; Cohen-Mansfield and Frank, 2008). It is estimated that at least 20% of elderly uninstitutionalized people have trouble walking, need someone to help them walk, or require the aid of a walking device (Ostchega et al., 2000). Locomotor impairment has many negative consequences within the aging population including an increase in falls, hospitalizations, and even future requirements for a caregiver (Montero-Odasso et al., 2005). An increased fear of falling also leads to further decreases in mobility (Chamberlin et al., 2005). Individuals with locomotor impairment are also at greater risk for depression (Braam et al., 2005). Locomotor impairment is also linked to a number of comorbidities including osteoporosis, arthritis, congestive heart failure, muscle pain, stroke, and dementia (Duxbury, 2000). Ultimately, locomotor impairment can lead to an increase in mortality (Newman et al., 2006). Therefore, locomotor impairment is a very serious complication associated with aging. The molecular genetic mechanisms associated with age-related locomotor impairment currently remain unclear in any animal.

2. Age-related locomotor impairment in flies

The progressive locomotor decline associated with age in humans is also observed in 

*Drosophila*. Locomotor decline is associated with a loss of muscle mass, balance, and speed that is observed across humans and rodents (Giladi et al., 2005) (Altun et al., 2007). Like humans, walking speed declines with age in flies (Rhodenizer et al., 2008). Additionally, age-dependent decreases in muscle mass are also observed in flies (Baker, 1976). This suggests that physiological changes that contribute to locomotor decline might be common across organisms and suggests there are evolutionarily conserved mechanisms involved that impact locomotor
impairment across age. Investigation of locomotor senescence in *Drosophila* has the potential to
develop interventions that may improve the quality of life for aging humans and to potentially
delay the onset of cormobidities that are associated with locomotor impairment.

3. History of using flies to assess locomotor behavior

Locomotor behavior can be readily assessed in *Drosophila* and has been studied for several
decades. A number of assays were developed to study the effect of age on locomotor function
including exploratory activity, negative geotaxis, and the power tower. An introduction to the
different locomotor assays are discussed below with a more thorough review of the rapid
iterative negative geotaxis (RING) assay that was developed in our laboratory.

A. Exploratory Activity

To assess locomotor behavior via exploratory activity, individual flies are placed in a circular
arena and their movement within the arena is recorded (Le Bourg, 1983). As the flies age, the
distance walked and the number of flies that do not move when placed in the arena increases (Le
Bourg, 1983; Le Bourg and Minois, 1999). However, it was determined that 30 day old flies
placed in the arena may be initially less active but after time the activity levels in aged flies are
comparable to young flies (Martin et al., 1999). Furthermore, there are reports that declines in
this behavior are not observed between males and females of the same genetic background, and
exploratory activity can actually increase during the first five weeks of age in males in certain
genetic backgrounds (Le Bourg, 1987; Fernandez et al., 1999). These complexities decrease the
effectiveness of this assay in measuring locomotor behavior across age.

B. Single Fly Negative Geotaxis Assay
The single fly negative geotaxis assay has reproducibly demonstrated that climbing ability decreases with age (Arking and Wells, 1990; Orr and Sohal, 1994; Benguria et al., 1996; Le Bourg and Minois, 1999; Minois et al., 2001; Cook-Wiens and Grotewiel, 2002; Kang et al., 2002; Goddeeris et al., 2003). For this assay, individual flies are placed in a cylinder and are shaken or banged to the bottom. The fly is observed for a period of time and the maximum height reached in centimeters is recorded. Other variations of this assay are also used to measure climbing ability in *Drosophila* (Minois et al., 2001). One benefit of using the single fly assay is the ability to follow the locomotor behavior of a single fly across age. In contrast, there are many disadvantages to using single fly assays to assess locomotor behavior in *Drosophila*. Single fly assays tend to be very labor intensive and tedious. These limitations to the single fly assay are problematic when performing small experiments if the goal is to measure negative geotaxis behavior across age. Additionally, this design is virtually impossible for high-throughput studies aimed at identifying genes involved in age-related locomotor decline. Furthermore, single fly assays require either single flies being housed separately throughout an aging experiment or anesthetizing the flies to place them in vials before performing the experiments. This results in a very expensive and time consuming regimen for each aging experiment. Furthermore, repeatedly anesthetizing the flies could alter the behavior of the flies during the assay or during the aging of the flies. All of these issues limit the utility of this assay.

C. Rapid Iterative Negative Geotaxis (RING) Assay

To improve the assessment of locomotor behavior in flies our lab developed an assay called rapid iterative negative geotaxis (RING) that measures locomotor performance on groups of flies (Gargano et al., 2005). The RING assay consists of an apparatus that contains five tubes with 25
flies placed in each tube. The apparatus is rapped against a table three times to bring all the flies to the bottom of the container. This stimulation initiates an escape response in the flies that manifests as climbing the tube walls. A digital image is taken of the flies four seconds after initiating the behavior and the vertical distance climbed for each fly is determined using Scion image. Five vials for each genotype are tested with multiple genotypes tested simultaneously in the apparatus. Each test consists of 5 trials that include 5 digital images recording the behavior with testing performed weekly. The experimenter is blinded to the genotypes during testing and during the analysis of data, thus eliminating potential bias. The lab determined that the data generated by the RING assay is highly reproducible, the number of trials has no effect on behavioral performance, the density of flies placed in the vial has a negligible effect on performance and sex has no uniform effect on locomotor behavior across age (Gargano et al., 2005). The lab also determined that negative geotaxis behavior is insensitive to circadian rhythm unlike spontaneous locomotor activity (Allada et al., 2001; Gargano et al., 2005). Therefore, the RING assay has many features that make it suitable for identifying flies with altered locomotor decline.

The ability of the fly to climb is principally a legged locomotor behavior with jumping or flying having little if any impact on the distance climbed (Rhodenizer et al., 2008). Further assessment of data generated from the RING assay revealed that there are two main changes that contribute to senescence of locomotor behavior in flies. As flies age their walking speed decreases and their latency to start climbing the tube walls increases (Rhodenizer et al., 2008). The lab also determined that the main contributor to age-related reduction in the distance climbed is a decrease in the climbing speed, which is a conserved feature of humans and flies during aging.
(Rhodenizer et al., 2008). Flies also climb at linear rates regardless of age and qualitative changes in the locomotor behavior with age are not obvious (Rhodenizer et al., 2008). There are many benefits to the RING assay including the ability to accurately measure locomotor performance across age in groups of flies that is highly reproducible. The high-throughput nature of the RING assay makes it very valuable for genetic screens. One limitation of our RING assay is that it measures locomotor behavior across populations and not in single flies. Therefore, it is impossible to follow locomotor decline across age on a single fly with the RING assay.

D. Power Tower (Exercise Training Drosophila)

Recently, a modification of the RING assay was developed and is called the power tower (Piazza et al., 2009). This assay was developed to mimic the physiological responses that are produced in vertebrates during endurance exercise training. The power tower consists of a series of racks of tubes that are placed on a platform. The platform rises and drops inducing the negative geotaxis response. The machine was designed to repeat this process indefinitely, thereby controlling the duration and frequency of exercise (continuous climbing). Interestingly, flies placed in the power tower demonstrated a significant improvement in mobility compared to unexercised flies. However, the increase in climbing ability was observed only when the flies were exercised early in life while exercising the flies later in life (3 or 5 weeks of age) had no discernable effect (Piazza et al., 2009). This assay can be useful for the identification of loci that can modulate the physiological response to exercise. This is relevant for humans because a reduced incidence of age-related diseases and slower declines in mobility and cardiovascular function are correlated with exercise training (Bauman, 2004; Ascensao et al., 2007; Saraceni and Broderick, 2007).
With this assay it is unclear if the force of the repeated drops by the machine damages the flies even though the arrest rate between flies placed on the power tower and naïve flies is similar (Piazza et al., 2009).

E. Conclusions

The identification of genes and pathways that impact ARLI is now feasible with the creation of these locomotor assays. With the development of the RING assay it is now possible to screen a large number of genotypes in a timely manner that is more accurate and reproducible. The high-throughput capacity of the RING assay allows for the ability to quickly assess the effects of various manipulations of genes on locomotor decline across age. Elucidation of genes and mechanisms that influence locomotor decline discovered through the use of these assays are described below.

4. Mechanisms associated with age-related locomotor impairment

Locomotor behavior studies in *Drosophila* have generally focused on whether strains and mutants that are long-lived also have a delay in locomotor impairment. If so, the data would suggest there are common mechanisms that influence both longevity and locomotor impairment. Our work along with others have revealed a number of genes that influence locomotor impairment and are associated with a number of pathways or processes including alterations in insulin signaling, environmental sensing, protein damage repair, muscle structure, antioxidant function, and drug treatment. These mechanisms are discussed in more detail below.
One pathway known to influence lifespan is the insulin signaling pathway (Tatar et al., 2003b). There is evidence that loss of function mutations in the insulin signaling pathway influences ARLI in a number of species. In C. elegans *daf-2* (insulin receptor) mutants have delayed ARLI (Huang et al., 2004). In *Drosophila, chico* mutants tested using our RING assay have delayed ARLI (Gargano et al., 2005). Although humans with polymorphisms within the insulin signaling pathway components have a longer lifespan (Suh et al., 2008; Willcox et al., 2008) it is unclear whether individuals with these polymorphisms or others retain their locomotor ability longer than the general population.

There is evidence that alteration in environmental sensing can positively impact ARLI. In *Drosophila or83b* (an odorant receptor) mutants have delayed ARLI compared to controls (Rhodenizer et al., 2008). Or83b oligomerizes with other receptors to form functional receptors for detecting odorants (Larsson et al., 2004). Our results using the RING assay suggests that olfactory cues can influence this aspect of aging.

There is also evidence that genes involved in muscle structure may alter ARLI. The X-linked gene myospheroid in *Drosophila* encodes βPS (β position-specific), the major β-integrin in flies (MacKrell et al., 1988). During development integrins function to attach the ends of muscles to the tendon matrix and play a role in the assembly of muscle structure (Bokel and Brown, 2002). The lab found that three independent loss of function alleles in myospheroid resulted in delayed ARLI (Goddeeris et al., 2003). This suggests that alterations in muscle structure during development may impact ARLI.
Cellular repair processes decline with age and are associated with senescence (Knight, 2000). Reduced functioning of antioxidant enzymes that reduce oxidative stress on the organism can have a negative impact on functional status. For example, ubiquitous knock-down of the antioxidant enzymes SOD1 (cytoplasmic Sod) or SOD2 (mitochondrial Sod) via RNAi accelerates ARLI (Martin et al., 2009a; Martin et al., 2009b). A complete loss of SOD2 activity in mice also causes disturbed locomotor function (Lebovitz et al., 1996). In contrast, overexpression of enzymes that function to repair damaged proteins can positively impact locomotor behavior. For example, ubiquitous overexpression of protein carboxyl methyltransferase (PCMT), a protein damage repair enzyme enhanced locomotor activity in Drosophila (Chavous et al., 2001). Another enzyme that repairs oxidative damage to proteins is peptide methionine sulfoxide reductase A (MSRA). Overexpression of MSRA in the nervous system in flies delayed the onset of reduced physical activity levels associated with age compared to controls (Ruan et al., 2002). This fly was also found to be resistant to oxidative stress raising the question of whether there may be a connection between enhanced oxidative stress resistance and delayed ARLI (Ruan et al., 2002). However, there is evidence that oxidative stress resistance can be uncoupled from ARLI. For example, resistance to oxidative stress with no delay in age-related locomotor impairment was observed in Methuselah mutants (Cook-Wiens and Grotewiel, 2002). Furthermore, a delay in age-related locomotor impairment without resistance to oxidative stress was observed in loss of function mutants in the β-integrin myospheroid (Goddeeris et al., 2003). These studies suggest that enhanced or proper functioning of enzymes that decrease oxidative stress and repair damaged proteins are important in sustaining locomotor ability across age.
Currently, there are no drug treatments to delay the onset of ARLI. However, there is evidence that inhibiting histone deacetylase by using the drug 4-phenylbutyrate (PBA) can have beneficial effects on age-related locomotor impairment. Flies fed PBA maintained their climbing ability with age compared to controls (Kang et al., 2002). It has been suggested that PBA treatment can activate repair mechanisms and possibly inhibit the accumulation of damage with age thereby having a beneficial effect on locomotor ability (Kang et al., 2002).

5. Connections between age-related locomotor impairment and longevity

There are a number of mechanisms that appear to influence age-related locomotor decline in Drosophila. Many of these mechanisms were first identified as extending lifespan. Studying ARLI has provided insight into whether the ability to maintain locomotor function across age is mechanistically tied to lifespan. There is evidence that lifespan and ARLI can be experimentally uncoupled. For example, the long lived Drosophila mutant methuselah extends lifespan (Lin et al., 1998) but does not alter ARLI (Cook-Wiens and Grotewiel, 2002). We observed the same effect when placing flies under dietary restriction (Bhandari et al., 2007). In contrast, mutations in myospheroid have an increase in lifespan and delayed ARLI (Goddeeris et al., 2003). Additionally, pan-neuronal overexpression of MSRA increases lifespan and enhances locomotor behavior in aged flies (Ruan et al., 2002). Long-lived chico mutants (Clancy et al., 2001; Tu et al., 2002) also have delayed ARLI (Gargano et al., 2005). These studies suggest ARLI may be driven by mechanisms that are independent or distinct from those that impact lifespan. Therefore, an extension of lifespan may not positively impact all aspects of aging including ARLI. Further study to elucidate which mechanisms differ between lifespan and delayed ARLI
will provide insight into genetic or cellular pathways that positively influence each aspect of aging independently.

6. Summary

ARLI is very detrimental to the population and causes an increase in morbidity and mortality. *Drosophila* have proven to be a valuable model organism to study locomotor impairment across age. The development of the RING assay to conduct large scale genetic screens to assess locomotor behavior across age has the potential to identify molecular genetic mechanisms and processes associated with this aspect of aging. The identification of genes that influence ARLI will provide a more complete understanding of mechanisms and pathways that impact locomotor behavior across age and has implications for future interventions and therapies to improve the quality of life in elderly humans.

Wolfram Syndrome: *Drosophila* as a genetic model to provide insight into wfs2 function

1. Introduction

Neurodegenerative diseases including Parkinson disease, Alzheimer disease and polyglutamine diseases have all been successfully modeled in *Drosophila* (Cauchi and van den Heuvel, 2006). Many neurodegenerative disease genes are structurally and functionally conserved in flies, raising the possibility that discoveries identified in flies could be applicable to humans. *Drosophila* are very useful in investigating the molecular-genetic mechanisms underlying these neurodegenerative diseases because phenotypes can be readily observed and assessed (Reiter et al., 2001). In addition, modifier screens are commonly used to identify interacting genes and pathways that may be involved in disease pathology. The eye is commonly used for modifier
screens in *Drosophila* because variations in eye morphology are easily observed under the light microscope. In addition, severe degeneration of the eye does not negatively impact survival. Therefore, *Drosophila* are very beneficial in delineating the molecular and cellular processes underlying neurodegenerative disease. The following is a review of the literature on the rare neurodegenerative disease called Wolfram Syndrome.

2. *Wolfram Syndrome*

Wolfram Syndrome (WFS) is a rare autosomal recessive progressive neurodegenerative disease that affects roughly 1 in 770,000 people in the UK (Barrett et al., 1995). The prevalence of this disorder in the Lebanese population is higher with 1 in 68,000 individuals affected due to the high rates of consanguinity (Medlej et al., 2004). The four main features of this disorder include diabetes mellitus, diabetes insipidus, optic atrophy and deafness (DIDMOAD) (Wolfram, 1938). However, the only two features required for the Wolfram Syndrome diagnosis are diabetes mellitus and optic atrophy (Barrett et al., 1995). Diabetes mellitus is a progressive disorder caused by deterioration in pancreatic β-cell function and insulin resistance (Bollyky et al., 2008). Diabetes mellitus is the first manifestation of this disorder and is due to the degeneration of the pancreatic β-cells along with a decrease in the level of circulating insulin (Barrett et al., 1995). Initial symptoms of optic atrophy are typically mild and include loss of both color and peripheral vision (Barrett and Bundey, 1997). Optic atrophy is progressive and patients usually go blind within eight years after the initial diagnosis (Barrett and Bundey, 1997). Diabetes insipidus is only seen in about 73% of patients but usually manifests in the second decade of life (Barrett et al., 1995). Diabetes insipidus is caused by inadequate or impaired release of the antidiuretic hormone (ADH) from the hypothalamus (Makaryus and McFarlane, 2006). High frequency
sensorineural hearing loss manifests in the second or third decade of life and is seen in about 62% of patients (Barrett et al., 1995). Numerous other features are also associated with this disorder. Psychiatric illness is sometimes observed and includes suicidal behavior (Strom et al., 1998). Renal-tract abnormalities (Barrett et al., 1995) can also be observed. Neurological manifestations include ataxia of the trunk, loss of gag reflex, loss of olfaction, seizures, and involuntary eye movement (Rando et al., 1992; Barrett et al., 1995). Atrophy of visual pathways, the brainstem, or the cerebellum is also observed (Pakdemirli et al., 2005; Ito et al., 2007).

Gastrointestinal manifestations are less common but do occur in some patients and include bowel dysmotility and muscle weakness in the stomach that results in the difficulty of passing food through the stomach (Barrett et al., 1995; Medlej et al., 2004). All features that are associated with this disorder have differing ages of onset (Table 1). Patients usually die within the 3rd decade of life due to respiratory failure associated with brainstem atrophy (Scolding et al., 1996).

3. Wolfram Syndrome is caused by loss of function mutations in WFS1 and CISD2

Wolfram Syndrome is a genetically heterogeneous disorder with causative mutations identified in two genes. The first gene identified was WFS1 and it is reported that 90% of Wolfram Syndrome patients have mutations in this gene (Khanim et al., 2001). This gene is widely expressed with abundant expression in the brain, pancreas, heart, and muscle. The WFS1 protein product wolframin is well studied. Wolframin is a transmembrane protein that is localized to the ER and plays a role in ER calcium homeostasis. For example, knock-down of
Table 1: Clinical features of Wolfram Syndrome, prevalence and median age of onset. Data collected from a number of sources and reviewed in (Kumar, 2009).

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Prevalence</th>
<th>Average onset (age)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Diabetes mellitus</em></td>
<td>100%</td>
<td>6 years</td>
</tr>
<tr>
<td><em>Optic Atrophy</em></td>
<td>100%</td>
<td>11 years</td>
</tr>
<tr>
<td><em>Diabetes Insipidus</em></td>
<td>73%</td>
<td>14 years</td>
</tr>
<tr>
<td><em>Deafness</em></td>
<td>62%</td>
<td>16 years</td>
</tr>
<tr>
<td><em>Urological abnormalities</em></td>
<td>58%</td>
<td>3rd decade</td>
</tr>
<tr>
<td><em>Neurological complications</em></td>
<td>62%</td>
<td>4th decade</td>
</tr>
<tr>
<td><em>Psychiatric or behavioral disorder</em></td>
<td>60%</td>
<td>Variable</td>
</tr>
<tr>
<td><em>Gastrointestinal tract problems</em></td>
<td>20-30%</td>
<td>Variable</td>
</tr>
</tbody>
</table>
WFS1 in HEK293 cells results in lower levels of ER calcium supporting a role of Wolframin in ER calcium uptake (Takei et al., 2006). Wolframin is also predicted to play a role in the ER stress response. The unfolded protein response (UPR) is activated by ER stress and serves to limit the amount of accumulated misfolded proteins in the ER (Zhang and Kaufman, 2006). The UPR consists of three branches IRE1, PERK, and ATF6. The UPR is controlled by these three genes which become activated by the accumulation of unfolded or misfolded proteins in the ER. (Zhang and Kaufman, 2006). WFS1 was discovered to be a novel downstream component of IRE1 and PERK signaling in the UPR (Osman et al., 2003). Additionally, WFS1 deficiency in beta-cells causes chronic ER stress via upregulation of ER stress markers (Fonseca et al., 2005). An enhanced ER stress response was also reported in pancreatic islets cells isolated from wfs1 deficient mice (Yamada et al., 2006). Therefore, some insight into the function of WFS1 has been elucidated by a number of studies.

The second and more recently discovered gene associated with Wolfram Syndrome is CDGSH iron sulfur domain 2 (CISD2). Mutations in this gene were identified in three unrelated consanguineous Jordanian families with Wolfram Syndrome (Amr et al., 2007). A splice site mutation was identified in these families that cause a frameshift in the transcript that generates a stop codon that is proposed to eliminate the full-length transcript (Amr et al., 2007). Additional phenotypes are observed in these individuals that are unique to mutations in this gene including bleeding tendency and defective platelet aggregation with collagen (al-Sheyyab et al., 2001). Peptic ulcer disease with gastrointestinal tract bleeding is also a common finding in patients with CISD2 mutations (Ajlouni et al., 2002). CISD2 is expressed in a wide variety of tissues including the pancreas and brain (Amr et al., 2007). CISD2 transcripts were found in platelets,
which explains the bleeding tendency (Peri et al., 2003). *CISD2* is not expressed in fetal liver, skeletal muscle, or cartilage (Amr et al., 2007). The *CISD2* gene product ERIS (endoplasmic reticulum intermembrane small protein) also localizes to the ER and contains a single transmembrane domain and a single iron sulfur domain. Unlike *WFS1*, knock-down of *CISD2* in patient insulinoma cells and lymphoblastoid cells did not have an upregulation of UPR markers compared to controls suggesting *CISD2* may not play a role in the UPR (Amr and Shiang, personal communication). Additional analysis using co-immunoprecipitation studies suggested that Wolframin does not interact with ERIS (Amr et al., 2007). Interestingly, *CISD2* was recently discovered to function within the Beclin-1 mediated autophagy pathway suggesting a role of *CISD2* in the regulation of autophagy (Chang et al., 2009). Despite several advances in understanding ERIS (the *CISD2* gene product), its function or function(s) have not been fully resolved.

4. *Animal Model to investigate the physiological function of CISD2*

Recently, a *CISD2* knock-out mouse was created that recapitulates many of the features of Wolfram Syndrome in humans including reduced lifespan, progressive neurodegeneration, and impaired glucose tolerance (Chen et al., 2009). This group localized ERIS primarily to the outer mitochondrial membrane which differs from previous reports of *CISD2* being primarily localized to the ER (Amr et al., 2007; Wiley et al., 2007). In addition, these mice have mitochondrial degeneration that is accompanied by cell death caused by autophagy and precedes the muscle and nerve degeneration observed in these mice (Chen et al., 2009). Given the phenotypes of these knock-out mice the authors suggest Wolfram Syndrome is a mitochondrial mediated disorder. The tissues affected in this disorder have a high energy demand supporting this idea.
However, controversy exists in previous clinical studies as to whether there is a mitochondrial component to Wolfram Syndrome. One study identified abnormal mitochondria in a muscle biopsy of a patient, suggesting that a mitochondrial defect could be causative for Wolfram Syndrome (Bundey et al., 1992). However, other clinical studies found no evidence of mitochondrial deficiency in patients (Hofmann et al., 1997a; Barrett et al., 2000). Therefore, this mouse model supports a mitochondrial component to this disease but further study is needed to solidify whether Wolfram Syndrome is in fact a mitochondrial mediated disorder.

5. Summary

The biochemical properties of the \textit{CISD2} gene product are starting to be elucidated. Although the mouse knock-out model provides some insight into the physiological role of \textit{CISD2} the function or function(s) of this gene have not been fully resolved. Furthermore, the genes and pathways associated with \textit{CISD2} have yet to be identified. Insight into the molecular mechanisms that contribute to Wolfram Syndrome can be gained through the study of the normal function of \textit{CISD2} in \textit{Drosophila}. This has implications for the diagnosis and treatment of patients with this disorder along with other individuals afflicted with diabetes and neurodegeneration.

\textbf{Oxidative Stress in Aging and Age-Related Disease}

1. Introduction

Oxidative stress has long been implicated in the aging process and plays a contributing factor in age-related disease. Oxidative stress is caused by the imbalance between the production of prooxidants and antioxidant defense mechanisms. Mitochondria are the major producers of
superoxide, a reactive oxygen species (ROS) that is formed as a byproduct of cellular respiration (Fridovich, 2004). The generation of ROS from mitochondria is primarily determined by metabolic conditions, can be produced at high rates and is therefore mostly associated with damaging processes. Mitochondria consume more than 90% of cell O$_2$ and the electron transport chain continuously reduces one O$_2$ molecule into two H$_2$O molecules with at least 0.1% or more of O$_2$ being reduced to superoxide (O$_2^-$)(Chance et al., 1979; Fridovich, 2004). Redox reactions involving O$_2^-$ generates other reactive oxygen species including the hydroxyl radical (OH$^-$) that is highly reactive and results in oxidative damage to macromolecules (Chance et al., 1979).

There are number of antioxidant enzymes that limit the damage from ROS and function to protect cells from oxidative stress. These enzymes include superoxide dismutases, catalase, and glutathione peroxidases which all convert ROS to less reactive products. Superoxide dismutases are responsible for capturing the superoxide produced and converting it to H$_2$O$_2$ and O$_2$ (Halliwell and Gutteridge, 1999). The two main superoxide dismutases are Sod1 that is located in the cytosol and Sod2 that is located in the mitochondrial matrix (Landis and Tower, 2005). Additional antioxidant systems include catalase, glutathione peroxidase and thioredoxin peroxidase that are responsible for converting H$_2$O$_2$ to H$_2$O and O$_2$ (Matthews et al. 2000). All of these enzymes function to protect cells from oxidative stress. These antioxidant enzymes are conserved between flies and humans thereby making Drosophila a good model system to study antioxidant function (Missirlis et al., 2001). Enhanced functioning of antioxidant enzymes has beneficial effects on lifespan in Drosophila. Although overexpression of Sod2 during development and adulthood does not extend lifespan in Drosophila there is an extension of mean lifespan by 33% in Drosophila when this enzyme is conditionally overexpressed only during
adulthood (Paul et al. 2007 and Sun et al. 2002). Overexpression of Sod1 alone or Sod1 and catalase also extends lifespan in *Drosophila* (Sun and Tower 1999, Orr and Sohal 1994). Therefore, antioxidant enzyme function is very important for protecting against oxidative stress and in preserving lifespan.

2. **Oxidative stress in aging**

Accelerated aging and shortened lifespan are associated with the cumulative oxidative damage to DNA, lipids and proteins by reactive oxygen species (ROS) (Beckman and Ames, 1998). This damage causes the deterioration of cells, tissues and organ systems that culminates in functional senescence and a shortened lifespan (Harman, 1956). Oxidative damage is associated with age-related declines in behavior and function (Joseph et al., 1999; Liu et al., 2003). ROS content rises with age in a number of organisms and the rate at which aging occurs is correlated with the rate of ROS production (Sohal et al., 1990b; Sohal and Sohal, 1991; Sohal et al., 1994; Sohal et al., 1995). The number of oxidized proteins in humans and flies increases with age along with the accumulation of oxidative damage to macromolecules (Starke-Reed and Oliver, 1989; Orr and Sohal, 1994; Sohal et al., 2002). With age, mitochondria have increased damage to their genome and the inner membrane and have a decline in respiratory activity. Consequently, these changes further lead to an increase in ROS generation by the mitochondria and causes further oxidative damage to the organism (Hillered and Ernster, 1983; Sohal et al., 1994). Oxidative damage is even detectable in young animals suggesting that this damage accumulates for years until functional status is impaired (Agarwal and Sohal, 1994). In addition, oxidative damage to mitochondrial DNA in brain and heart tissue and an increase in oxidative damage to DNA, lipids, and proteins in skeletal muscle is observed in older humans (Mecocci et al., 1999; Barja
and Herrero, 2000). In fact, it is estimated that in some tissues with age there is oxidative
damage to at least 30% of total cellular protein (Starke-Reed and Oliver, 1989). Additionally,
survival in *Drosophila* declines with age when exposed to paraquat, a free radical inducer,
demonstrating that the ability to counteract exogenous oxidative stress also declines with age
(Bonilla et al., 2002). This evidence highlights the significance of oxidative damage on
decreased functioning and increased mortality with age.

A number of mechanisms are suggested to contribute to the accumulation of oxidative damage
with age. During aging, the function of some antioxidants decreases while others remain
unchanged or actually increase suggesting that aging is not due to a global deterioration in
antioxidant function (Sohal et al., 1990a; Sohal et al., 1990b; Sohal and Weindruch, 1996).
However, an increase in mitochondrial ROS production is associated with increased oxidative
damage (Sohal and Weindruch, 1996). Activity of enzymes involved in repairing oxidative
damage to DNA (Petropoulos et al., 2001) and proteins (Chen et al., 2002) decreases with age.
For example, activity of MSRA and several mitochondrial DNA glycosylases decline with age in
a number of tissues (Petropoulos et al., 2001; Chen et al., 2002; Imam et al., 2006). This
suggests that the inability to repair damaged proteins and DNA contributes to the accumulation
with age. The organelle that is responsible for removing proteins with oxidative damage is the
proteasome and the activity of this organelle decreases with age in a number of tissues (Shibatani
et al., 1996; Shang et al., 1997; Bulteau et al., 2000; Keller et al., 2000; Petropoulos et al., 2000;
Louie et al., 2002; Carrard et al., 2003; Husom et al., 2004; Viteri et al., 2004). Consequently,
reduced function of macromolecules that repair oxidatively damaged proteins and DNA and

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degradative systems that remove oxidatively damaged proteins contribute to the accumulation of oxidative damage associated with aging.

3. Oxidative Stress and Age-Related Disease

Oxidative stress is also thought to contribute to a number of human age-related diseases including sarcopenia, cancer, diabetes, coronary artery disease and neurodegenerative diseases (McFarland et al., 2002, 2007; Pieczenik and Neustadt, 2007). In rats, aged muscle undergoing sarcopenia had increased rates of electron transport chain abnormalities suggesting that mitochondrial abnormalities are associated with muscle fiber loss (Bua et al., 2002). There is also an association with increased oxidative damage and cancer. Mice that have a 50% decrease in function of Sod2 show an increase in oxidative damage and an increase in the incidence of cancer (Van Remmen et al., 2003). Diabetic patients are reported to have an increase in oxidative damage compared to individuals without this condition (Akkus et al., 1996; Atalay et al., 1997). Defective oxidative phosphorylation results in an increase in oxidative damage and contributes to artherosclerosis during aging (Bogliolo et al., 1999). Defective oxidative phosphorylation is also associated with hypertrophic cardiomyopathy (Zeviani et al., 1995).

Oxidative stress is also implicated in a number of human neurodegenerative diseases. Tissues involved in neurodegenerative diseases are very susceptible to oxidative stress due to the high energy demand that is required for proper functioning. Huntington disease (HD) brain mitochondria from patients show an increase in oxidative damage (Polidori et al., 1999). There is also an increase in production of ROS in transgenic mouse models of HD (Perez-Severiano et al., 2004). An increase in oxidative damage but a decrease in DNA repair mechanisms that remove
oxidized bases in DNA is observed in Alzheimer disease patients (Lovell et al., 1999). Mitochondrial and oxidative stress are both implicated in the pathogenesis of Parkinson’s disease (Greenamyre and Hastings, 2004). Oxidative stress increases ischemic injury after a stroke and is believe to be due to reduced oxygen levels during the stroke that results in an increase in superoxide production (Gilgun-Sherki et al., 2002). Friedrich’s Ataxia is also associated with oxidative stress where loss of the protein Frataxin causes iron overload that inactivates mitochondrial enzymes and oxidizes cellular components (Schon and Manfredi, 2003).

A number of animal models for these diseases exhibit sensitivity to exogenous oxidative stress. A *Drosophila* model of Alzheimer disease is sensitive to H$_2$O$_2$ compared to controls (Rival et al., 2009). Familial forms of Parkinson disease are associated with the genes Pink1 and Parkin. Mutations in these genes in *Drosophila* predisposes to sensitivity to the exogenous oxidative stressor paraquat (Pesah et al., 2004; Clark et al., 2006). A *Drosophila* model for Friedrich ataxia demonstrated that reduced Frataxin levels via RNAi caused sensitivity to oxidative stress (Llorens et al., 2007). Flies expressing the amyloid β$_{1-42}$ peptide have an increased sensitivity to H$_2$O$_2$ compared to control flies (Rival et al., 2009). This evidence demonstrates a reduced ability to handle exogenous oxidative stress in these diseases. Therefore, oxidative stress plays a significant role in the progression of many age-related diseases.

4. *Consequences of reduced antioxidant function*

A reduction in the function of antioxidant enzymes results in detrimental effects to the organism and can lead to reduced lifespan. Both *Sod1* and *Sod2* null flies have reduced lifespan (Phillips et al., 1989; Duttaroy et al., 2003). *Sod1* knock-out mice have a 30% reduction in mean lifespan.
(Elchuri et al. 2005) and motor impairments (Shefner et al., 1999). Decreased expression of Sod2 in Drosophila via Sod2 RNAi increases the amount of endogenous oxidative stress in adults (Kirby et al., 2002). Mice null for Sod2 have motor impairments, neurodegeneration, dilated cardiomyopathy, motor disturbances and perinatal death (Li et al., 1995; Lebovitz et al., 1996). Reduction of Sod2 and thioredoxin reductase (TrxR) in Drosophila decreases lifespan (Kirby et al., 2002; Missirlis et al., 2002). A shortened lifespan is observed in Drosophila with decreased function of catalase and in mice with knock-out of catalase (Mackay and Bewley, 1989; Ho et al., 2004). Disruption of the peroxidase PHGpx (phospholipid hydroperoxide glutathione peroxidase) that is responsible for reducing peroxidized lipids results in embryonic lethality in mice (Imai et al., 2003). A number of age-related diseases in humans also have reduced antioxidant function. Diabetic patients have decreased Sod and catalase levels due to hyperglycemia (Akkus et al., 1996; Atalay et al., 1997). Many tumors have a reported reduction in Sod2 expression levels and transformation of tumors with Sod2 cDNA reverses the malignant phenotype (Xu et al., 1999). Coronary heart disease is also associated with reduced intracellular Sod levels (Wang et al., 1998). This highlights the detrimental effects due to elevated levels of endogenous oxidative stress due to decreased function of antioxidant enzymes. Oxidative stress is a component of many diseases and is not classified as a disease itself. No clinical therapies currently exist that use SOD or any other antioxidant enzymes to aid in the clearance of superoxide production. Therefore, there is a need to identify genes than can protect against endogenous oxidative stress.

5. Summary
These studies highlight the importance of these antioxidant enzymes for the continuous surveillance in the cell to decrease oxidative stress on the organism. The consequence of decreased functioning of these enzymes reduces survival and functioning of the organism. Loss of function of these enzymes not only impacts aging but also the underlying pathology of a number of age-related and neurodegenerative diseases. Although lifespan has been well studied with respect to alterations in antioxidant function, it remains to be determined whether manipulation of certain genes can counteract endogenous oxidative stress on the organism. Many of these antioxidant enzymes are conserved in *Drosophila* (Jacobs et al., 2004) making this model organism very useful in the study of mechanisms that can mediate endogenous oxidative stress. Studies that identify, investigate and provide a better understanding of the genetic mechanisms that suppress the negative consequences of endogenous oxidative stress have implications in the treatment and management of diseases associated with the accumulation of oxidative damage. The modulation of oxidative stress has the potential to delay or decrease the severity for a number of age-related diseases and neurodegenerative conditions.
Chapter 1

Materials and Methods

1.1 Fly husbandry, fly strains, and genetics

All flies for all studies were reared at 25°C and 60% relative humidity under a 12-hour light/dark cycle on standard *Drosophila* medium (10% sucrose, 2% yeast, 3.3% cornmeal, 1% agar, 0.2% Tegosept) supplemented with active yeast. For all aging studies, adult flies (1-3 days old) were briefly anesthetized with carbon dioxide (CO$_2$) and then placed into fresh food vials at 25 flies per vial. Adult flies were transferred to fresh food vials twice weekly for all studies. **Age-Related Locomotor Impairment Project:** EP lines were obtained from the *Drosophila* Stock Center (Bloomington, IN, USA). pGawB lines were kindly provided by Laurent Seroude (Queens University, Kingston, ON, Canada). Our standard laboratory stock, *w*, contains the *w*$_{118}$ allele in the Canton-S genetic background. All EP and pGawB element chromosomes identified in the primary and secondary screens were backcrossed for seven generations to *w*[cs] prior to testing in the confirmation phase of the screen. **Wolfram Syndrome Project:** da-Gal4, mef2-Gal4, 188Y-Gal4, appl-Gal4, and all *PPTI* modifiers were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). UAS-RNAi transgenic lines for *wfs2* (33925 and 33926, a.k.a. WFS2.1 and WFS2.2, respectively), *CLN3* (5322) and *CLN7* (5089 and 5090) were purchased from the Vienna
Drosophila Research Center (Vienna, Austria). The GMR-WFS2.1 and the GMR-WFS2.2 double transgenic flies were created by recombining the GMR-Gal4 element and the wfs2 RNAi transgenes onto the same chromosome and were confirmed by PCR with primers that detected both transgenes independently (Table 2). PPT1 loss of function mutants (PPT1-A179T and PPT1-S77F) were provided by Robert Glaser (Wadsworth Center, Albany NY). CLN3 overexpression strains (UAS-CLN3 #4, GMR-CLN3 #8) were provided by Richard Tuxworth (Kings College London, London UK). Sod2 Candidate Suppressors Project: Sod2 was knocked-down by using the Gal4/UAS system (Brand and Perrimon, 1993) to express Sod2 inverted repeat (Sod2-IR) transgenes previously described (Kirby et al., 2002). The UAS-Sod2-IR24, UAS-Sod2-IR15 and daughterless-Gal4 (da-Gal4) lines were provided by John Phillips (University of Guelph). UAS-DJ-1β-IR1 (transformant 17214) and UAS-DJ-1β-IR2 (transformant 17215) (Vienna Drosophila RNAi Center, Vienna, Austria). Flies with a second chromosome harboring the Actin-Gal4 and UAS-Sod2-IR15 transgenes and flies with a third chromosome harboring the da-Gal4 and UAS-Sod2-IR15 transgenes were generated through recombination and confirmed by PCR with primers that detected both transgenes independently (Table 2). The Actin-Gal4::Sod2-IR15 chromosome and the da-Gal4::Sod2-IR15 chromosome are homozygous lethal and are therefore maintained in a heterozygous state over a balancer chromosome (CyO or Sb).

1.2 Negative Geotaxis, Lifespan, and Bang Sensitivity

Negative geotaxis (startle-induced climbing) was analyzed in Rapid Iterative Negative Geotaxis (RING) assays as described previously (Gargano et al., 2005). Briefly, negative geotaxis was assessed in 125 animals per genotype with 25 flies per vial at the indicated ages. Statistical
Table 2: PCR primers to confirm recombinant flies. Primers used to confirm GMR-WFS2.1 and GMR-WFS2.2 and Actin-Gal4::Sod2-IR15, da-Gal4::Sod2-IR15 recombinants.

<table>
<thead>
<tr>
<th>Transgene or Gene</th>
<th>Primer</th>
</tr>
</thead>
</table>
| Actin-Gal4        | 5’-GGGTTTTGTTGGGTATCTTCATC-3’  
                 | 5’-CATTAGTGCCACTGACCCCGTC-3’ |
| Da-Gal4           | 5’-GGGTTTTGTTGGGTATCTTCATC-3’  
                 | 5’-CATTAGTGCCACTGACCCCGTC-3’ |
| Gmr-Gal4          | 5’-GGGTTTTGTTGGGTATCTTCATC-3’  
                 | 5’-CATTAGTGCCACTGACCCCGTC-3’ |
| UAS-Sod2-IR       | 5’-GAACACGTCGCTAAGCGAAAGC-3’   
                 | 5’-GCAGAAATTTTACGGGCGACGAAC-3’ |
| UAS-WFS2.1-IR     | 5’-CGCGAATTCTCAAGGATTTGTTTGCCCTGATCC-3’  
                 | 5’-GAGGCGCTTCGTCTACGGAGCGAC-3’ |
| UAS-WFS2.2-IR     | 5’-CGCGAATTCTCAAGGATTTGTTTGCCCTGATCC-3’  
                 | 5’-GAGGCGCTTCGTCTACGGAGCGAC-3’ |
treatment of data (one-way analysis of variance) was performed using JMP 5.01. Lifespan was assessed as previously described (Martin et al., 2009b). Log-rank tests on lifespan data were performed with JMP 5.01. Bang sensitivity (seizure susceptibility) was assessed by determining the climbing latency (i.e. time to recovery) in groups of 25 flies after being vortexed in a vial for 15 seconds at the highest setting using a Diagger Vortex Genie 2 (Fergestad et al., 2006).

1.3 PCR and inverse PCR

**Age-Related Locomotor Impairment Project:** Confirmation of previously mapped P-element insertions was performed using primers that anneal to genomic DNA flanking the insertion sites and to the P-elements (Table 3) using standard protocols. The locations of previously unmapped PGawB elements were identified through inverse PCR (Dalby et al., 1995). Genomic DNA was isolated from a sample of 50 flies from each pGawB line using a DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA was digested with DpnII or MspI and then self-ligated with T4 DNA ligase (Invitrogen). Ligation products were used as templates for inverse PCR using primers (5’-CCCGACGGACATGCTAAGG-3’, 5’-CTCATAATATTAATTAGACGAAATTATTTTTAAAG-3’) that anneal to the 3’ end of PGawB. Amplification products from inverse PCR reactions (i.e. DNA flanking the pGawB insertions) were sequenced using a primer (5’-CGACAAGAATACTATTTCCCTTCAC-3’) that anneals near the 3’ end of pGawB.

1.4 Quantitative real-time PCR

mRNA expression was assessed via quantitative real-time PCR (qRT-PCR) studies as previously described (Jones et al., 2009). Briefly, groups of 25 male flies frozen at -80°C. Total RNA was
<table>
<thead>
<tr>
<th>Transposon</th>
<th>Gene or Allele</th>
<th>5' Primer</th>
<th>3' Primer</th>
</tr>
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<tr>
<td>PDK1</td>
<td>EP837</td>
<td>5'-TTGGCCTTATGATGTTGTGTTG-3'</td>
<td>5'-CAATCGGTTAAATACCTTTGAACAC-3'</td>
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<td>Pfrx</td>
<td>EP1150</td>
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<td>5'-GAGGTAAGCTACAATCGACACAAAC-3'</td>
</tr>
<tr>
<td>Doc3</td>
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<td>5'-CGTGCCTCCGAAATTACAGC-3'</td>
<td>5'-CATAGGGGATCTGTGGTAGAC-3'</td>
</tr>
<tr>
<td>m6/HLHm7</td>
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<td>5'-GTTAGCTGCGACGTTGAGTGG-3'</td>
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<tr>
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<td>e00368</td>
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<td></td>
<td>e03435</td>
<td>5'-CAGACGCACTTCTCTCTG-3'</td>
<td>5'-GAGCTGCGCCAAAATCGAC-3'</td>
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<tr>
<td>Akt</td>
<td>c02098</td>
<td>5'-CGATACAATCGAATCACAGTCC-3'</td>
<td>5'-CTGCTTGCGCGGATATGGCTGC-3'</td>
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<tr>
<td>EY10012</td>
<td>5'-CGGTTATGAGCAGTTATCG-3'</td>
<td>5'-GCTGTACCAATGGTGCTATCT-3'</td>
<td></td>
</tr>
<tr>
<td>EP and pGawB</td>
<td>5'-CGACCGGGACCACCTTTATGTTATTTACATTCATG-3'</td>
<td>5'-CGACCGGGACCACCTTTATGTTATTTACATTCATG-3'</td>
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<tr>
<td>pBac</td>
<td>5'-CAGTGACACTTACCACCGCATTTGACAAGCAC-3'</td>
<td>5'-CCTCGATATACAGACCGATAAACACAT-3'</td>
<td></td>
</tr>
</tbody>
</table>
isolated using TRIZOL (Invitrogen) and reverse transcribed using oligo(dT) primers and
Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed using an Applied
Biosystems Fast 7500 system. All assays were performed in triplicate with three independent
RNA and cDNA isolations and normalized to Actin5c mRNA expression. Age-Related
Locomotor Impairment Project: Expression of PDK1, Pfix, Doc3, m6, HLHm7 and CG14045
was detected using SYBR Green (Quanta Biosciences) with custom primers. For detection of
Dp110, a Taqman assay was used (#Dm02142683_g1) with RPII140 (#Dm02134593_g1) as
endogenous control. Wolfram Syndrome Project: Expression of wfs2, 4E-BP, PPT1 and CLN3
were visualized with SYBR Green (Quanta Biosciences) with custom primers. ~800 fly heads
were used for the PPT1 and CLN3 mRNA expression experiments. Expression levels are
relative to the GMR-Gal4/+ control fly. Sod2 Candidate Suppressors Project: Expression of
Sod2, DJ-1α, DJ-1β and Ataxin-3 was detected using SYBR Green (Quanta Biosciences) with
custom primers. Primer information is listed in Table 4.

1.5 Stress Tests

Age-Related Locomotor Impairment Project: Paraquat Survival: Males and females were
collected for each genotype under brief CO₂ anesthesia and given a period of 24 hours to
recover. Flies were starved for 6 hours and then placed in vials with Whatman filter disks
saturated with 300 µl of 40mM paraquat in 5% sucrose. The vials were placed in a humidified
box at room temperature and the number of surviving flies in each vial was recorded after 18
hours. Each paraquat experiment was repeated 3 times with 6 vials of 25 flies per genotype and
sex. Wolfram Syndrome Project: All flies for stress tests were collected at 1-3 days of age and
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK1</td>
<td>5'-CGCGACTCATCTATATCGATCC-3'</td>
<td>5'-GTCAGACGCCGTCGAAGCTGTG-3'</td>
</tr>
<tr>
<td>Pfrx</td>
<td>5'-CTACAGACGAGCGAAGACG-3'</td>
<td>5'-GACAGACGTCAGCTCATTCC-3'</td>
</tr>
<tr>
<td>Doc3</td>
<td>5'-CACGGGAAAAAACTGGCAATC-3'</td>
<td>5'-CTGGCCGAGATCGAGGTT-3'</td>
</tr>
<tr>
<td>m6</td>
<td>5'-ACAGCTCAAGCGACACG-3'</td>
<td>5'-CGAGTGCCGATAGAAGCTG-3'</td>
</tr>
<tr>
<td>HLHm7</td>
<td>5'-AGTCACCAGTGACGATCCTC-3'</td>
<td>5'-CAGATGGGTCATCAGTGTG-3'</td>
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<td>CG14045</td>
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<td>5'-GCAAATAGCAGCGTCGC-3'</td>
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<td>Akt</td>
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<td>5'-GTTTACGGGAGAGATGTTGG-3'</td>
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<td>wfs2</td>
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<td>5'-CGGTCCCGACGACTCG-3'</td>
</tr>
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<td>PPT1</td>
<td>5'-GTTCCTTTATCGATCGCTC-3'</td>
<td>5'-GAGGAACCTTATCACACTCG-3'</td>
</tr>
<tr>
<td>CLN3</td>
<td>5'-GTCAGGTGCTTCTTGTCC-3'</td>
<td>5'-GGAAGTCTGATCGC-3'</td>
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<tr>
<td>4E-BP</td>
<td>5'-CATGCAGCAAACTGCAAATC-3'</td>
<td>5'-CCGAGAAGAAACAAAGGTTG-3'</td>
</tr>
<tr>
<td>Sod2</td>
<td>5'-ACGAGATGTCATCCTGCTC-3'</td>
<td>5'-CAAGAAGTCGGGCACACTGC-3'</td>
</tr>
<tr>
<td>DJ-1α</td>
<td>5'TGTATCAAGTGCAATCACCAGG-3'</td>
<td>5'-GGAGAAGCTTCACTCGG-3'</td>
</tr>
<tr>
<td>DJ-1β</td>
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<tr>
<td>Ataxin-3</td>
<td>5'-CTGACCAACTCTCTGCAGATG-3'</td>
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<tr>
<td>Actin5c</td>
<td>5'-AGCGCGGTCTTCTCACCAC-3'</td>
<td>5'-GTGGGCTCTGCTGACTAAGT-3'</td>
</tr>
</tbody>
</table>
were tested for stress sensitivity at 1 and 6 weeks of age. In each experiment, the number of
dead flies was recorded for each stress test every 4-8 hrs until all flies were dead. Three vials of
25 flies each were tested for each group. For starvation studies, flies were housed in food vials
containing 1% agar. For desiccation studies, flies were housed in empty vials placed in a box
with desiccant. To assess thermal stress, flies were placed in vials containing 1% agar with 5%
sucrose in a 36°C incubator. Hyperoxia studies were performed by placing flies in standard food
vials in an air tight container charged with 95% O₂ twice daily. All drug tests compared survival
in drug-treated and vehicle-treated food vials. For Tunicamycin treatment, flies were placed into
vials with food pre-treated with 100µl 2mM Tunicamycin in 95% ethanol or 95% ethanol
(vehicle). Flies were exposed to paraquat, FeCl₃, and H₂O₂ by placing them in vials with 2
Whatman paper discs treated with 300µl of 5% sucrose (vehicle) or 5% sucrose supplemented
with 40mM paraquat, 200mM FeCl₃, or 30% H₂O₂, respectively. Survival in different genotypes
or treatment groups was analyzed with log-rank tests performed using JMP 5.01.

Sod2 Candidate Suppressors Project: Adult males (1-3 days old) were collected with brief CO₂
anesthesia and allowed to recover for 12 hours in food vials. For paraquat treatment flies were
starved for 6 hours before being transferred to vials containing Whatman No. 3 filter paper with
5% sucrose alone or with 5% sucrose supplemented with 300µl of 20 mM paraquat. For H₂O₂
treatment flies were transferred to vials containing Whatman No. 3 filter paper with 5% sucrose
alone or with 5% sucrose supplemented with 300µl of 15% H₂O₂. Flies were maintained at 85%
relative humidity/23°C throughout the experiments. For hyperoxia treatment flies were
transferred to fresh food vials and placed in an air tight container charged with 95% O₂ 3x daily.
Surviving flies were counted every 4-8 hours after initiating paraquat, H₂O₂, or hyperoxia
treatment. Each genotype was represented by 100-125 flies with 25 flies/vial in each experiment.

1.6 PPT1 activity assay

PPT1 enzyme activity levels were measured as described previously in (Buff et al., 2007). Briefly, a single fly head was placed in a well of a 96 well plate on ice with ~15 heads used per genotype. Heads were crushed by a pestle in 30µl solution consisting of 20µl H2O and 10µl of the PPT1 fluorogenic substrate (4-MU-6S-palm-β-glc) and incubated for 2 hours at 30°C. PPT1 activity was measured by the absorbance change at 460 nm. PPT1 loss of function flies were used as a negative control. PPT1 activity data were analyzed with one-way ANOVA using JMP 5.01.

1.7 SOD activity

Groups of 25 adult males (0-3 days old) per genotype were collected under brief CO2 anesthesia and homogenized in extraction buffer (50 mM potassium phosphate/0.1 mM EDTA/2% Triton-X-100, pH 7.8) on ice. To rupture mitochondria, samples were sonicated for 20 sec and then incubated at 4°C for 45 min. Ruptured mitochondrial samples were centrifuged at 16,000 x g for 15 min at 4°C and the resulting supernatant was stored at 4°C. Protein concentration was determined using the Lowry method (DC Protein Assay, Bio-Rad). Samples containing equal amounts of protein were electrophoresed by Discontinuous Native PAGE (4% stacking gel (pH 6.8), 20% resolving gel (pH 8.8) in sample buffer (0.5 M Tris-HCl/50% glycerol/0.01% bromophenol blue) at 80-100 V. SOD activity was measured colorimetrically using a modified version of an “in-gel” assay previously described (Kirby et al., 2002). Briefly, gels containing
electrophoresed protein samples were incubated in 2.5 mM nitroblue tetrazolium and 50 mM potassium phosphate buffer for 20 min in the dark under gentle agitation. Gels were washed briefly in 50 mM phosphate buffer and then incubated in 28 mM N,N,N’,N’-tetramethylethlenediamine/28 µM riboflavin/50 mM potassium phosphate for 15 min in the dark with gentle agitation. Following a brief wash in 50 mM potassium phosphate buffer, gels were exposed to white light for ~15 min for full color development. In-gel SOD activity was quantified by densitometry using Alpha Imager software (Alpha Innotech Corp., San Leandro, CA).

1.8 Western blots

Protein was isolated from 25 flies per genotype by homogenization in radioimmunoprecipitation RIPA lysis buffer containing protease inhibitor cocktail (Roche 1:25 dilution in lysis buffer). Samples were sonicated, incubated on ice for 45 minutes and centrifuged at 16,000 x g for 15 minutes at 4°C. Supernatants were transferred to a new tube and protein concentration was measured using the DC Protein Assay (Bio-Rad). Protein extracts were electrophoresed via SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Western blots were probed with a rabbit anti-mouse ERIS polyclonal antibody (ProteinTech, 1:1000 dilution in 5% BSA in tris-buffered saline solution containing 0.1% Tween-20 (TBST) to detect Drosophila WFS2 (dWFS2) or a mouse anti-α tubulin monoclonal antibody (Sigma, 1:1000 dilution in 5% milk in TBST) to detect the loading control. Expression of dWFS2 and α-tubulin was visualized with goat anti-rabbit IgG-HRP (BioRad, 1:10,000 dilution in 5% milk in TBST) and goat anti-mouse IgG-HRP (Santa Cruz, 1:10,000 dilution in 5% milk in TBST), respectively,
in conjunction with Western Lightning chemiluminescence reagent plus (PerkinElmer). Western blot experiments were repeated three times with independent protein extracts.

1.9 Eye area measurements

The area of the eye was measured using Scion Image. 5 fly heads were cut off for each genotype and a line was manually drawn around the perimeter of the eye. The area within this perimeter in mm$^2$ was determined by Scion image. Flies that were collected for each genotype were generally the same whole body size and flies with obvious differences in whole body size were discarded.
Chapter 2

A forward genetic screen in *Drosophila* implicates insulin signaling in age-related locomotor impairment

1. Introduction

Aging in humans and other species is characterized by a number of progressive functional declines (Arking, 1998). Age-related locomotor impairments (ARLI) are particularly problematic in this regard. Patients with impaired locomotor abilities have reduced physical activity levels (Pahor et al., 2006), are less independent and require more clinical care (Boyd et al., 2005), are at much greater risk for being institutionalized (von Bonsdorff et al., 2006), and are at greater risk for depression (Braam et al., 2005). Additionally, ARLI is associated with an increased risk for falling and skeletal fractures as well as a variety of co-morbidities including coronary artery and cerebrovascular diseases, obesity, diabetes and hypertension (Boyd et al., 2005; Hillsdon et al., 2005; Onder et al., 2005; Volpato et al., 2005; Cesari et al., 2006a; Cesari et al., 2006b). ARLI is therefore a significant clinical challenge for elderly patients and their caregivers.

ARLI occurs in rodents (Forster et al., 1996) and flies (Grotewiel et al., 2005) in addition to humans (Walston et al., 2006). ARLI is associated with age-dependent decreases in muscle mass and walking speed in vertebrates (Arking, 1998; Woo et al., 1999) as well as in *Drosophila* (Baker, 1976; Sohal, 1985; Rhodenizer et al., 2008). Additionally, oxidative damage might be an underlying cause of ARLI in humans (Nikolic et al., 2005) and flies (Bhandari et al., 2007).
These parallels raise the possibility that ARLI might be driven by common mechanisms in invertebrates and mammals.

Our laboratory has developed an assay called Rapid Iterative Negative Geotaxis (RING) for assessing locomotor function in flies (Gargano et al., 2005). Negative geotaxis (i.e. startle-induced climbing) is a legged locomotor behavior that becomes impaired with age due to an age-dependent decrease in climbing speed (Rhodenizer et al., 2008). Studies from our laboratory using RING assays show that flies with mutations in the insulin signaling gene *chico*, the metabolite transporter gene *Indy*, and the olfactory receptor gene *Or83b* have slowed ARLI (Gargano et al., 2005; Martin and Grotewiel, 2006; Rhodenizer et al., 2008). Additional studies from our laboratory show that RNAi-mediated knock-down of SOD2, a major mitochondrial antioxidant, accelerates ARLI (Bhandari et al., 2007). These studies support a role for insulin signaling, metabolism, environmental sensing and oxidative damage in ARLI.

A more complete understanding of the genes and genetic pathways that influence ARLI would greatly facilitate the development of interventions that mitigate the effects of age on locomotor ability. To this end we performed a forward genetic screen for P-element insertions that forestall ARLI in *Drosophila*. We identified a number of transposon insertion strains with delayed ARLI including a strain with a P-element insertion in the gene *phosphoinositide-dependent kinase 1* (*PDK1*) that reduced expression of the gene’s mRNA. Additional studies with this and other loss of function alleles in *PDK1* in conjunction with loss of function alleles in two other insulin signaling genes, *Dp110* and *Akt*, confirmed a role for insulin signaling in ARLI.
2. Results

2.1 Identification of delayed ARLI mutants

We performed a forward genetic screen to identify P-element insertions in *Drosophila* that delay age-related locomotor impairment (ARLI). We used a Rapid Iterative Negative Geotaxis (RING) assay previously developed by our laboratory (Gargano et al., 2005) to assess ARLI throughout the screen and for all follow-up studies. We tested only males in our screen toward limiting the potential confounding effects of reproduction on aging in female flies (Chapman et al., 1995; Sgro and Partridge, 1999; Partridge et al., 2005). We screened a total of 729 EP (Rorth, 1996) and 364 pGawB (Seroude et al., 2002) transposon insertions. Although EP and pGawB elements have various uses in *Drosophila*, we focused on their ability as transposons to disrupt the function of a gene when inserted nearby (Stoltzfus et al., 2003). We went through two rounds of screening to isolate P element insertions that consistently had delayed locomotor impairment across age. The putative insertions that consistently had delayed locomotor decline were backcrossed to our w[cs] control fly for 7 generations to control for genetic background effects on behavior and aging (Cook-Wiens and Grotewiel, 2002; Goddeeris et al., 2003; Gargano et al., 2005; Bhandari et al., 2006; Bhandari et al., 2007). After testing to confirm that the locomotor behavior was consistently delayed after backcrossing we were left with 7 P element insertions strains for further study (Figure 1A and B).

We mapped or confirmed the location of the transposon insertions in all of the strains with delayed ARLI via inverse PCR or standard PCR, respectively (primer information provided in Table 3). EP837 is inserted in the transcription unit of *phosphoinositide-dependent kinase 1* (*PDK1*, a.k.a. *CG1210*, Figure 2A), a gene within the canonical insulin signaling pathway.
Figure 1. Delayed ARLI in backcrossed EP and pGawB insertion lines. Negative geotaxis was measured at weekly intervals in control (w[cs]) and the indicated EP (837, 1150 and 1174) and pGawB (DJ708, DJ913, DJ996 and DJ1026) insertion lines (A and B). Negative geotaxis declined with age in all strains tested (two-way ANOVA, \( p < 0.0001 \)). All EP and pGawB insertion lines shown performed significantly better than w[cs] controls (individual two-way ANOVAs, \( p < 0.0001 \)). Data (mean ± SEM, \( n = 10–15 \)) are from 2 to 3 independent experiments. Experiments were performed by Devin Rhodenizer and Pretal Patel.
Figure 2. Transposon insertion sites in flies with delayed ARLI. In all panels, transcription units are indicated by black boxes, protein-coding exons by open boxes, and untranslated regions by grey-filled boxes. P-elements are depicted as triangles with arrows indicating their 5’-3’ orientation. Scale bars in the upper right-hand corner of each panel are 1 kb. Schematics of exons, introns and transcripts are based on information from FlyBase (http://flybase.org). (A) PDK1 with EP insertions 837 and 3553 in addition to GT1 insertion BG02759. (B) Pfrx with EP insertion 1150. (C) Doc3 with pGawB insertion DJ708. (D) m6 and HLHm7 region with pGawB insertions DJ996, DJ1026 and DJ913. (E) CG14045 with EP insertion 1174. (F) Dp110 with PiggyBac insertions c00368 and e03435. (G) Akt with PiggyBac insertion c00298. All pGawB insertion sites were identified by inverse PCR. All other transposon insertion sites were confirmed by PCR using genomic DNA as template.
EP1150 is inserted within 6-phosphofructo-2-kinase (Pfrx, a.k.a. CG3400, Figure 2B). Pfrx encodes a key, highly conserved enzyme (6-phosphofructo-2-kinase) involved in gluconeogenesis (Rider et al., 2004). DJ708 is inserted upstream of Dorsocross3 (Doc3, a.k.a. CG5093, Figure 2C), a gene that encodes a T-box transcription factor involved in wingless and decapentapalegic signaling (Reim et al., 2003; Hamaguchi et al., 2004; Reim and Frasch, 2005). Three pGawB transposon insertions (DJ913, DJ1026 and DJ996) are inserted between the genes m6 and HLHm7 (Figure 2D). The m6 locus encodes a 70 amino acid protein of unknown function found only in the Drosophila genus (FLYBASE.BIO.INDIANA.EDU). HLHm7 (a.k.a. E(spl)m7 or m7) encodes a helix-loop-helix transcription factor of the enhancer of split family (Klambt et al., 1989; Ligoxygakis et al., 1999). EP1174 (Figure 2E) is inserted near CG14045 which encodes a predicted GTPase activating protein (GAP) for Rho G proteins (FLYBASE.BIO.INDIANA.EDU). Based on BLAST searches (Altschul et al., 1997), the GAP encoded by CG14045 is found in insects but not other animals.

We performed quantitative real-time PCR (qRT-PCR) analyses to determine whether the EP and pGawB insertions that delayed ARLI were associated with altered expression of nearby genes (primer information provided in Table 4). Compared to w[cs] controls, expression of PDK1 and Pfrx were decreased by 25% in EP837 and 92% in EP1150, respectively (Table 5). In contrast, expression of Doc3 was increased by ~8-fold in DJ708 (Table 5). Thus, EP837 and EP1150 are partial loss of function mutations in PDK1 and Pfrx, respectively, whereas DJ708 is a gain of function allele of Doc3. These three alleles are designated PDK1\textsuperscript{EP837}, Pfrx\textsuperscript{EP1150} and Doc3\textsuperscript{DJ708} hereafter. We analyzed PDK1\textsuperscript{EP837} and other insulin signaling mutants as described below. The lab is currently analyzing Pfrx\textsuperscript{EP1150} and Doc3\textsuperscript{DJ708} and results will be published at a later date.
**Table 5: Gene expression in transposon insertion lines.** Expression of genes (column 1) harboring transposon alleles (column 2) was assessed via qRT-PCR. Data (mean ± S.E.M., n in parentheses) are percent change in expression (column 3) relative to expression in w[cs] controls. Negative and positive values indicate decreases and increases in expression, respectively. All of the transposons significantly altered expression of their respective genes (column 4, one-sample t-test).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Expression</th>
<th>p Value</th>
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<tr>
<td>PDK1</td>
<td>EP837</td>
<td>-25.3 ± 3.5 (11)</td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td>EP3553</td>
<td>-8.0 ± 2.9 (6)</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>BG02759</td>
<td>-21.4 ± 2.1 (5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Pfrx</td>
<td>EP1150</td>
<td>-92.3 ± 0.9 (3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Doc3</td>
<td>DJ708</td>
<td>818 ± 118 (6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Dp110</td>
<td>c00368</td>
<td>-15.9 ± 5.1 (6)</td>
<td>0.026</td>
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<tr>
<td></td>
<td>e03435/+</td>
<td>-27.5 ± 2.1 (3)</td>
<td>0.006</td>
</tr>
<tr>
<td>Akt</td>
<td>c02098/+</td>
<td>-38.6 ± 4.3 (4)</td>
<td>0.003</td>
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</table>
Despite repeated efforts, we did not consistently detect a change in expression of \textit{CG14045} in EP1174 adult flies. Similarly, we did not find a consistent change in expression of either \textit{m6} or \textit{HLHm7} in DJ913, DJ996 or DJ1026 adult flies. It is possible that expression of these genes might be altered during a limited number of developmental stages or altered in a subset of tissues. Nevertheless, we provide preliminary data on these P-element insertion strains in the interest of fully reporting the results of our screen and because these strains facilitated our investigation of the relationship between paraquat resistance and ARLI.

\subsection*{2.2 Insulin signaling and delayed ARLI}

PDK1 is a component of the canonical insulin signaling pathway (Taniguchi et al., 2006). The insulin signaling pathway is conserved across species (Figure 3). The insulin signaling pathway is stimulated when insulin or insulin like peptides bind the cell surface receptor InR. InR then phosphorylates its substrate chico. The insulin signaling pathway continues down a phosphorylation cascade through PI3K, PDK1, AKT, and FOXO. When FOXO is phosphorylated by AKT it is sequestered in the cytoplasm. When FOXO is not phosphorylated (i.e. under conditions of low insulin signaling) it translocates to the nucleus, thereby regulating expression of a number of genes. The delayed ARLI in \textit{PDK1^EP837} flies (Figure 1A) suggested that insulin signaling might influence this aspect of aging. We explored this possibility by further investigating the role of \textit{PDK1} and other insulin signaling genes.

To address whether the EP837 insertion was responsible for the delayed ARLI in \textit{PDK1^EP837} flies, we assessed negative geotaxis across age in revertant flies (\textit{PDK1^EP837rv4} and \textit{PDK1^EP837rv9}) with wild type chromosomes derived from precise excision of the EP837 transposon insertion.
Figure 3. The insulin signaling pathway in *Drosophila*, *C. elegans* and Mammals.
Precise excision of EP837 returned *PDK1* expression to normal in both revertant lines (one sample t tests, n = 5-6, n.s.). ARLI was indistinguishable in *w[cs]* and both revertant lines, whereas it was significantly delayed in *PDK1*<sup>EP837</sup> flies compared to these three controls (Figure 4). These results demonstrate the EP837 insertion in *PDK1* causes delayed ARLI. *PDK1* functions immediately downstream of phosphatidylinositol 3-kinase (PI3K) and immediately upstream of AKT in the canonical insulin signaling pathway (Taniguchi et al., 2006). Based on the reduced expression of *PDK1* in EP837 flies (Table 5), we reasoned that additional partial loss of function alleles in *PDK1* and other insulin signaling genes might also delay ARLI. We therefore characterized two additional transposon insertions in *PDK1* (EP3553 and BG02759, Figure 2A), two transposon insertions in *Dp110* (a.k.a. *CG4141*, the gene that encodes the catalytic subunit of PI3K, Figure 2F), and a transposon insertion in *Akt* (a.k.a. *CG4006*, the gene that encodes AKT, Figure 2G). The insertion sites for all transposons in or near these genes were confirmed by standard PCR. All transposons were subsequently backcrossed for seven generations to *w[cs]* to control for genetic background effects on behavior and aging. qRT-PCR studies indicated that these additional five transposon insertions cause partial loss of function in their respective genes (Table 5). These strains are designated as *PDK1*<sup>EP3553</sup>, *PDK1*<sup>BG02759</sup>, *Dp110*<sup>00368</sup>, *Dp110*<sup>e03435</sup>, and *Akt*<sup>e02098</sup>.

To address whether additional partial loss of function alleles in *PDK1* and whether partial loss of function in *Dp110* and *Akt* delay ARLI, we assessed negative geotaxis across age in *w[cs]* controls and *PDK1*<sup>EP3553</sup>, *PDK1*<sup>BG02759</sup>, *Dp110*<sup>00368</sup>, *Dp110*<sup>e03435/+</sup>, and *Akt*<sup>e02098/+</sup> flies. We evaluated males and females in these studies because mutations in insulin signaling genes have sex-specific effects on life span (Burger and Promislow, 2004). Negative geotaxis across age
Figure 4. **ARLI in PDK1<sup>EP837</sup> and revertants.** Negative geotaxis in w[cs], the revertants PDK1<sup>EP837rv4</sup> and PDK1<sup>EP837rv9</sup>, and PDK1<sup>EP837</sup> flies. Age and genotype affected negative geotaxis (two-way ANOVA, p<0.0001 for both factors). The w[cs] control (open circles) and revertants (PDK1<sup>EP837rv4</sup>, open squares; PDK1<sup>EP837rv9</sup>, open triangles) performed indistinguishably, while the PDK1<sup>EP837</sup> (filled circles) flies performed better (Tukey HSD p<0.05). Data (mean ± SEM) are from two experiments with 5 vials of 25 flies per genotype in each experiment.
declined in all genotypes as expected (Figure 5). Negative geotaxis was elevated relative to 
\( w^{cs} \) controls in \( PDK1^{EP3553} \) and \( PDK1^{BG02759} \) males (Figure 5A) and females (Figure 5B). Negative geotaxis across age was also elevated in \( Dp110^{c00368} \) males and females and in 
\( Dp110^{c03435/+} \) females (Figure 5C and 5D). \( Akt^{c02098/+} \) males and females also had enhanced negative geotaxis across age (Figure 5E and 5F). Our studies show that partial loss of function mutations in multiple insulin signaling genes delay ARLI in flies.

2.3 Paraquat resistance and delayed ARLI

Increased resistance to exogenously applied oxidative stress is associated with extended life span in \( Drosophila \) (Lin et al., 1998; Tatar et al., 2001; Wang et al., 2004; Walker et al., 2006). We investigated whether the delayed ARLI in the transposon insertion strains used in our studies might be associated with altered oxidative stress resistance and assessed survival in flies fed paraquat, a strong oxidative stressor (Kirby et al., 2002). Paraquat survival was enhanced in all the transposon insertion lines recovered from our screen including the EP837 P element insertion (Figure 6A). In contrast, additional studies with other alleles of \( PDK1 \) and alleles of \( Dp110 \) and \( Akt \) revealed that only \( PDK1^{BG02759} \) males had enhanced paraquat survival compared to \( w^{cs} \) control flies (Figure 6B). In females, none of the additional \( PDK1, Dp110 \) or \( Akt \) mutants tested had significantly altered paraquat survival (Figure 6C). Although elevated paraquat survival was observed in many transposon lines with delayed ARLI, enhanced resistance to this oxidative stressor is not required for the preservation of locomotor function across age.
Figure 5. Delayed ARLI in insulin signaling mutants. Negative geotaxis across age in males (A, C, and E) and females (B, D and F) flies with mutations in PDK1 (A and B), Dp110 (C and D) and Akt (E and F). Age and genotype affected negative geotaxis in all experiments (individual two-way ANOVAs, p<0.0001 for both factors). Individual Tukey HSD multiple comparison tests revealed that negative geotaxis was increased compared to w[cs] in all insulin signaling mutants except Dp110\textsuperscript{e03435}/+ males (C). Data (mean ± SEM) are compiled from two experiments with 5 vials of 25 flies per genotype in each experiment.
Figure 6. Paraquat survival in EP and pGawB insertion lines with delayed ARLI. Survival at 18 hours in the presence of 40mM paraquat was assessed in the indicated genotypes. (A) Genotype had a significant effect on survival of males (one-way ANOVA, p=0.0006, n=3). All transposon alleles survived significantly longer than $w_{cs}$ (*Bonferroni’s multiple comparison test, p<0.05). (B) Paraquat survival in male insulin signaling mutants was significantly affected by genotype (one-way ANOVA, p=0.0111). Only $PDK1^{BG02759}$ flies survived longer than $w_{cs}$ controls (*Bonferroni’s multiple comparison test, p<0.05). (C) Genotype had no significant effect on paraquat survival in female insulin signaling mutants (one-way ANOVA, n.s.).
3. Discussion

Aging is associated with progressive impairments in a number of physiological and cognitive functions (Arking, 1998). Among the functional deficits that occur during aging, ARLI is particularly significant because it is associated with increased rates of falls, fractures and institutionalization in humans (Boyd et al., 2005; Pahor et al., 2006; von Bonsdorff et al., 2006). Identifying genes and genetic pathways that influence age-related changes in locomotor ability and other functions is critical for developing treatment strategies that mitigate the effects of age on health status. Here, we report the results of our studies in Drosophila that investigate the genetic basis of ARLI.

Through a forward genetic strategy, we identified seven transposon insertion lines with age-dependent preservation of negative geotaxis, a startle-induced locomotor behavior (Rhodenizer et al., 2008). One of the lines with delayed ARLI has a transposon insertion in PDK1 that reduces expression of this gene. Removal of the transposon insertion via precise excision restores normal expression of PDK1 and eliminates the delayed ARLI phenotype in these flies suggesting that PDK1 and, more generally, insulin signaling influence this aspect of aging. Consistent with this possibility, ARLI is delayed in flies with other partial loss of function alleles of PDK1 and in flies with partial loss of function mutations in two additional insulin signaling genes, Dp110 and Akt. Furthermore, our laboratory previously reported that mutation of chico, which encodes an insulin receptor substrate in Drosophila, also forestalls ARLI (Gargano et al., 2005). All of these data support a role for the insulin signaling pathway in regulating the effects of age on locomotor function in flies.
Disruption of insulin signaling has a number of positive effects on aging in diverse species. An extensive literature demonstrates that loss of function mutations in key genes within the pathway extend life span in worms, flies and mice (Kenyon, 2001; Tatar et al., 2003a). Additionally, extended life span in humans is associated with polymorphisms in the genes that encode the insulin/insulin like growth factor receptor (Suh et al., 2008) and FOXO3A (Willcox et al., 2008), a downstream effector of the pathway. Importantly, though, the effects of the insulin signaling pathway on aging extend beyond its influence on life span. Mutations that disrupt insulin signaling in C. elegans delay senescence of pharyngeal pumping and body movements (Huang et al., 2004) and age-related decline in associative memory (Murakami et al., 2005). Additionally, genetic manipulations that blunt insulin signaling delay cardiac aging in Drosophila (Wessells et al., 2004). The studies reported here and our previous studies on chico mutants (Gargano et al., 2005) demonstrate that the insulin signaling pathway also influences ARLI in flies. Given that the insulin signaling pathway appears to have conserved effects on life span in diverse species, it would be extremely interesting to determine whether this pathway is also involved in the preservation of locomotor function in mammals including humans.

Data from our screen also suggest that genes outside of the insulin signaling pathway might be important for ARLI. Three strains with delayed ARLI contain P-elements inserted between the genes m6 and HLHm7. Additionally, other delayed ARLI strains contain P-element insertions near the genes CG14045, Pfrx and Doc3. While it is tempting to speculate that m6, HLHm7, CG14045, Pfrx or Doc3 might influence ARLI, these effects must be formally validated before definitive connections can be made regarding the role of these genes in locomotor senescence.
Oxidative damage is thought to play a role in ARLI in humans (Nikolic et al., 2005) as well as flies (Bhandari et al., 2007). Consistent with this possibility, many of the transposon insertions with delayed ARLI have elevated resistance to the oxidative stressor paraquat. Several other insertion lines with delayed ARLI, however, have no change in their paraquat survival. These data indicate that while enhanced resistance to paraquat can accompany preservation of locomotor function across age, the enhanced resistance is not required. Additional studies will be necessary to fully delineate the role of oxidative stress resistance in ARLI. Determining whether overexpression of antioxidant genes that compat oxidative stress forestalls ARLI will be important for assessing if this mechanism is important for preserving locomotor function across age.

A number of manipulations that extend life span in *Drosophila* and other species have sex-specific effects (Burger and Promislow, 2004). For example, mutations in *Drosophila* that blunt insulin signaling produce a larger life span increase in females as compared to males (Tatar et al., 2001; Tu et al., 2002). Additionally, dietary restriction (defined as reduced food intake without malnutrition) causes a greater extension of life span in female versus male flies (Magwere et al., 2004). Interestingly, preservation of locomotor function across age does not strictly follow this same pattern. While the effects of mutations in *Dp110* and *Akt* are more robust in females than in males, the opposite is true for mutations in *PDK1*. Additional studies will be necessary to formally address whether genetic and other manipulations that preserve locomotor or other functions across age have predictable sex-specific effects in flies and mammals as found for life span. For example, testing of females and males in learning and memory assays, odor avoidance
assays, circadian behavior, reproduction, and cardiac senescence will reveal whether these age-related changes have sex-specific effects.
Chapter 3

Wolfram syndrome 2 (\textit{wfs2}) is a novel genetic modifier of the lysosomal storage disease genes \textit{PPT1} and \textit{CLN3} in \textit{Drosophila}

1. Introduction

Wolfram Syndrome is an autosomal recessive neurodegenerative disease that affects 1 in 770,000 people in the UK (Barrett et al., 1995). Affected individuals present with diabetes mellitus, diabetes insipidus, optic atrophy and deafness (DIDMOAD) (Wolfram, 1938). Other features of this syndrome include psychiatric illness (Strom et al., 1998) and renal-tract abnormalities (Barrett et al., 1995). Patients usually die within the 3\textsuperscript{rd} decade of life due to respiratory failure associated with brainstem atrophy (Scolding et al., 1996). Mutations in two genes, \textit{WFS1} and \textit{CISD2} (Strom et al., 1998; Amr et al., 2007) and are known to cause Wolfram Syndrome. \textit{WFS1} encodes wolframin, a transmembrane protein that localizes to the ER. Wolframin is important for intracellular calcium homeostasis and is a downstream component of IRE1 and PERK signaling in the unfolded protein response (Osman et al., 2003; Fonseca et al., 2005).

\textit{CISD2}, the second Wolfram Syndrome locus, was more recently identified (Amr et al., 2007). A splice site mutation in \textit{CISD2} that eliminates the full-length transcript was identified in three Jordanian families with Wolfram Syndrome (Amr et al., 2007). The \textit{CISD2} gene encodes ERIS (endoplasmic reticulum intermembrane small protein), a protein with a single predicted transmembrane domain and a single predicted iron-sulfur domain (Amr et al., 2007; Wiley et al.,
ERIS localizes to the ER and, like wolframin, could be involved in regulating the unfolded protein response (Amr et al., 2007). A more recent study found that knock-out of Cisd2 in mice recapitulates at least some features of Wolfram Syndrome in humans (Chen et al., 2009). For example, Cisd2 knock-out mice exhibit neurodegeneration in conjunction with shortened lifespan. These mice also have mitochondrial degeneration, suggesting that ERIS is important for mitochondrial integrity and that mitochondrial dysfunction might contribute to the pathology of Wolfram Syndrome (Chen et al., 2009). It was also recently discovered that Cisd2 functions within an autophagy pathway mediated by Beclin-1 (Chang et al., 2009). Despite these and other advances in understanding ERIS (the Cisd2 gene product), however, its function or functions have not been fully resolved.

Here, we describe genetic studies in the fruit fly, Drosophila melanogaster, to provide insight into the function of wfs2, the fly orthologue of CISD2. We investigated whether decreased expression of wfs2 had deleterious consequences in Drosophila. In addition, we investigated whether knocking down wfs2 expression in the eye modified the phenotype of a number of different genetic models related to neurodegeneration. Although knockdown of wfs2 by itself in flies had no obvious effect on a number of divergent phenotypes, we found that decreased expression of wfs2 modified the degenerative eye phenotypes caused by overexpression of two lysosomal storage disease genes, PPT1 and CLN3. Additional studies indicated that PPT1 and CLN3 are functionally related. Our data support a model in which wfs2 might function in concert with PPT1 and CLN3 under normal or possibly pathological states.

2. Results
2.1 Identification and manipulation of Drosophila wfs2

BLASTp searches identified Drosophila CG1458 as a potential orthologue of human CISD2. The CG1458 predicted gene product is 46% identical and 68% similar overall to human ERIS. Both proteins are very similar in size (135 and 133 amino acids, respectively), have the same predicted topology (Figure 7A), and contain a single predicted transmembrane domain (Figure 7A) and a single predicted CDGSH iron sulfur domain at the same positions (Figure 7B). The conservation in predicted primary amino acid sequences, protein sizes, transmembrane domain number and positions, and overall topologies indicate that CG1458 is likely orthologous to human CISD2. Considering these data and that we found no other predicted Drosophila proteins with significant homology to ERIS, we have designated CG1458 as wfs2.

To manipulate wfs2 expression, we used the Gal4-UAS system to drive two wfs2 RNAi transgenes (obtained from the Vienna Drosophila Research Center). We performed quantitative real-time PCR (qRT-PCR) analyses to determine if these RNAi transgenes knocked down wfs2 mRNA expression. da-Gal4-driven ubiquitous expression of UAS-wfs2-RNAi transgenes 33925 (WFS2.1) and 33926 (WFS2.2) decreased wfs2 mRNA levels by at least 95%. Additionally, a band consistent with dWFS2 protein was readily detectable in extracts from control flies on Western blots using an ERIS polyclonal antibody (Figure 8A). This band was undetectable, however, in flies with ubiquitous expression of wfs2 RNAi (Figure 8A). These qRT-PCR and Western blot results indicate that expression of the wfs2 RNAi transgenes causes a strong loss of function in wfs2, although they do not preclude the possibility that some residual expression of wfs2 remains in these animals.

2.2 Knockdown of wfs2 alone does not have obvious detrimental effects in Drosophila
**Figure 7. Conserved structure of ERIS and dWFS2.** (A) Hydropathy plots for human ERIS (i) and dWFS2 (ii). Amino termini are on the left. Predicted transmembrane domains (TMpred at embnet) are indicated by arrows. (B) Comparison of the primary amino acid sequences for human ERIS and dWFS2. Gaps are represented by asterisks (*). Identical amino acids are in bold. The single underline represents the predicted transmembrane domains. The double underline represents the CDGSH domains.

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<tr>
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<th>Human</th>
<th>Drosophila</th>
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<tr>
<td>i.</td>
<td>MVLESVARIVKVQLPAYLRLPVPESTGFARLTVESWLRLLPLGLV</td>
<td>M**EPISHLVKSSLPNYLSLPVPEDSGGWFKLSFKDLALIPPTVV</td>
</tr>
<tr>
<td>ii.</td>
<td></td>
<td></td>
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<tr>
<td>(B)</td>
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</tr>
<tr>
<td>Drosophila</td>
<td>77</td>
<td>135</td>
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Figure 8. Ubiquitous knockdown of wfs2 is not associated with obvious detrimental effects. (A) Knockdown of dWFS2 protein. dWFS2 protein was robustly detected with anti-CISD2 antisera in extracts from control animals (da/+), WFS2.1/+ and WFS2.2/+ whereas it was undetectable in extracts from wfs2 knockdown flies (da/WFS2.1 and da/WFS2.2). Top panel, dWFS2; bottom panel, α-tubulin loading control. (B) Expression of wfs2 RNAi transgenes using GMR-Gal4 had no effect on the external eye morphology. Light microscope images of eyes in representative flies with genotype (i) GMR-Gal4/+ control, (ii) GMR-Gal4/WFS2.1, and (iii) GMR-Gal4/WFS2.2. (C) Survival under normal housing conditions in males was not altered by ubiquitous expression of WFS2.1 RNAi (da-Gal4/WFS2.1) compared to controls (WFS2.1/+; da-Gal4/+). (log-rank test, n.s.) (D) Ubiquitous knockdown of wfs2 (da-Gal4/WFS2.1) did not alter locomotor performance (negative geotaxis) across age compared to controls (da-Gal4/+, WFS2.1/+) (two-way ANOVA, n.s.).
Toward investigating the role of *wfs2* in *Drosophila*, we determined whether knocking down its expression in several tissues via the Gal4-UAS system (Brand and Perrimon, 1993) led to obvious phenotypes in flies reared and aged under normal housing conditions. Expression of *wfs2* RNAi in the eye via GMR-Gal4 (Freeman, 1996) had no discernable effect on external eye morphology (Figure 8B). Similarly, knockdown of *wfs2* throughout the body (Figure 8C and D, da-Gal4 and Actin-Gal4, Table 6), in the musculature (mef2-Gal4, Table 6), or in the nervous system (188Y-Gal4 and Appl-Gal4, Table 6) had no obvious effects on lifespan or age-related locomotor impairment. Additionally, knockdown of *wfs2* ubiquitously (da-Gal4), in the musculature (mef2-Gal4) or in the nervous system (Appl-Gal4) did not lead to a change in bang sensitivity, an index of seizure susceptibility (Fergestad et al., 2006), in 1-8 week-old flies (Table 6). Ubiquitous knockdown of *wfs2* via da-Gal4 had no significant effect on expression of 4E-BP at 1 or 8 weeks of age (Table 6), suggesting that insulin signaling was not impaired in these animals (Fuss et al., 2006). Thus, knockdown of *wfs2* via a number of Gal4 drivers does not appear to have major negative consequences on the measures we assessed in flies housed under normal laboratory conditions.

To address whether *wfs2* might be important for stress sensitivity, we evaluated whether ubiquitous knockdown of *wfs2* altered survival when flies were exposed to thermal, desiccation, starvation, oxidative (hyperoxia, paraquat and H₂O₂), FeCl₃ (iron overload) and tunicamycin (ER) stress. We assessed survival of flies at 1 and 6 weeks of adulthood to address the possibility that effects of *wfs2* knockdown could manifest with age. Although we occasionally saw subtle effects of ubiquitous knockdown of *wfs2* on stress sensitivity in individual experiments, these effects were not consistently observed (Table 6). Additionally, expression of

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</tr>
<tr>
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<td>Week 1</td>
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<td><em>da, Actin, mef2, 188Y, Appl</em></td>
<td>Weeks 1 and 6</td>
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<tr>
<td>External Eye Morphology</td>
<td>GMR</td>
<td>Weeks 1 and 6</td>
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</table>
wfs2 RNAi selectively in the nervous system and musculature had no consistent effect on sensitivity to exogenous stressors (Table 6). Knockdown of wfs2, therefore, had no discernable effect on sensitivity to any of the stressors we tested.

2.3 Targeted genetic analysis identifies a novel interaction between wfs2 and the lysosomal storage disease gene PPT1

Given that mutations in CISD2 cause neurodegeneration in Wolfram Syndrome, we postulated that wfs2 might interact with genes known or predicted to cause neuropathology in flies. We therefore assessed whether GMR-driven expression of wfs2 RNAi modified the phenotypes in several genetic models of neurodegeneration (autosomal dominant retinitis pigmentosa, ataxia telangiectasia, Parkinson disease, Alzheimer disease, etc.). Additionally, we determined whether GMR-driven expression of wfs2 RNAi led to a synthetic phenotype in conjunction with altered cellular processes associated with pathology (oxidative stress, apoptosis and autophagy) (Table 7). We evaluated external eye morphology at 1 and 6 weeks of age to address the possibility that wfs2 knockdown could have age-dependent modifier effects.

Knockdown of wfs2 appeared to modify the external eye morphology in two strains that overexpressed wild-type palmitoyl-protein thioesterase 1 (PPT1), but had no discernable effect in any of the other strains tested. Overexpression of PPT1 in the Drosophila eye causes blackened ommatidia thought to be indicative of apoptosis (Korey and MacDonald, 2003). Mutations in PPT1 cause infantile neuronal ceroid lipofuscinosi (INCL), a severe pediatric neurodegenerative disease resulting in death by 10 years of age (Vesa et al., 1995).
Table 7. Candidate interactors for *wfs2*. Candidates (UAS transgenes or mutants, column 1) associated with several different human diseases or pathological processes (column 3) were crossed to flies expressing *wfs2* RNAi in the eye (GMR-WFS2.1). The effects of *wfs2* RNAi on the external eye morphology associated with expression of the transgenes in column 1 were assessed at 1 and 6 weeks of age (see main text).

<table>
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<td>Origin</td>
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<td>Oxidative damage</td>
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We pursued the potential interaction between \textit{wfs2} and \textit{PPT1} by performing a series of larger single-blind studies that included more than 300 eyes per genotype. As previously reported (Korey and MacDonald, 2003), we found that overexpression of \textit{PPT1} via two independent transgenic lines (PPT1-2.1 and PPT1-8.1) in the fly eye led to a blackened ommatidia phenotype that exhibited variable expressivity (Figure 9A). We characterized the phenotype in each eye as none (no black ommatidia), mild (a few black ommatidia), moderate (a patch of black ommatidia) or severe (black ommatidia throughout the eye) (Figure 9A i-iv, respectively). We then compiled the data for each genotype so that the overall phenotype was represented in a histogram (Figure 9B-E). This single-blind experimental design allowed us to formally compare the phenotypes between strains using logistical regression.

These larger studies confirmed that \textit{wfs2} knockdown (via the WFS2.1 RNAi transgene) partially suppressed the black ommatidia phenotype caused by expression of two independent \textit{PPT1} transgenes (Figure 9B, PPT1-2.1; Figure 9C, PPT1-8.1). Similarly, knockdown of \textit{wfs2} via the WFS2.2 RNAi transgene led to a partial suppression of the black ommatidia phenotype in flies overexpressing \textit{PPT1} (Figure 9D, PPT1-2.1; Figure 9E, PPT1-8.1). In all four combinations of \textit{wfs2} RNAi and \textit{PPT1} transgenes, the partial suppression of black ommatidia by \textit{wfs2} knockdown manifested as a leftward shift in the histograms (Figure 9B-9E; \textit{PPT1} overexpression alone, white bars; \textit{PPT1} overexpression with \textit{wfs2} knockdown, black bars).

To address the possibility that expression of \textit{wfs2} RNAi transgenes suppressed the severity of black ommatidia simply by reducing the expression or function of \textit{PPT1}, we performed qRT-PCR to evaluate \textit{PPT1} mRNA expression and PPT1 enzyme assays to evaluate activity of PPT1.
Figure 9. *wfs2* RNAi in the eye suppresses the effects of *PPT1* overexpression. (A) GMR-Gal4-driven expression of two independent UAS-*PPT1* transgenes (PPT1-2.1 and PPT1-8.1) caused black ommatidia that varied in severity. Each eye was scored into one of four categories: none (i), mild (ii), moderate (iii) and severe (iv). At least 300 eyes were assessed blind for each genotype. (B-E) Expression of *wfs2* RNAi suppressed the black ommatidia phenotype caused by *PPT1* overexpression. GMR-Gal4-driven expression of PPT1-2.1 alone or with WFS2.1 (B), PPT1-8.1 alone or with WFS2.1 (C), PPT1-2.1 alone or with WFS2.2 (D), and PPT1-8.1 alone or with WFS2.2 (E). Data are the percentage of eyes in each category in *PPT1* overexpressors (open bars) and *PPT1* overexpression with *wfs2* knockdown (closed bars). In all cases the severity of the black ommatidia was decreased by expression of *wfs2* RNAi (individual logistic regression, p<0.05, n = 310-801). Data are compiled from two independent experiments.
We used fly head extracts for these studies because GMR-Gal4 drives expression in the eye (a major portion of the head) and therefore data from these extracts would be relevant for the eye phenotypes we characterized. Expression of wfs2 RNAi had no discernable effect on PPT1 mRNA expression (Figure 10A). Additionally, although expression of the WFS2.1 RNAi transgene led to a modest but statistically significant decrease in PPT1 enzyme activity (Figure 10B), expression of the WFS2.2 RNAi transgene did not alter PPT1 enzyme activity. Thus, co-expression of the WFS2.1 and WFS2.2 RNAi transgenes did not consistently alter PPT1 enzyme activity. The most parsimonious interpretation of our qRT-PCR and PPT1 enzyme studies is that the wfs2 RNAi-mediated suppression of black ommatidia is unlikely to be due to decreased expression or function of PPT1. Therefore, we conclude that the suppression of black ommatidia in PPT1 overexpressors is due to decreased function of wfs2.

2.4 wfs2 genetically modifies a second lysosomal storage disease gene, CLN3

Given that wfs2 is a genetic modifier of PPT1, a lysosomal storage disease gene, we postulated that wfs2 may interact with additional genes associated with lysosomal storage diseases. To investigate this possibility, we assessed the effect of wfs2 knockdown on the external eye morphology of flies with altered expression of or mutations in genes associated with several different lysosomal storage diseases (Table 8). In these studies knockdown of wfs2 appeared to enhance the small eye phenotype (Tuxworth et al. 2009) caused by CLN3 overexpression (Figure 11A). CLN3 is a transmembrane protein localized to the lysosomal membrane. Mutations in CLN3 are responsible for the juvenile form of neuronal ceroid lipofuscinosis (JNCL), the most prevalent form of the neuronal ceroid lipofuscinosis diseases.
Figure 10: Knockdown of \( wfs2 \) does not alter \( PPT1 \) mRNA expression or enzyme activity.
GMR-Gal4 was used to drive UAS-\( PPT1 \) and UAS-\( wfs2 \) RNAi transgenes. (A) \( PPT1 \) mRNA expression. Total fly head mRNA was isolated from \( PPT1 \) overexpression flies with and without \( wfs2 \) RNAi. \( PPT1 \) mRNA expression was measured by qRT-PCR. \( wfs2 \) RNAi did not affect \( PPT1 \) mRNA expression levels (one-way ANOVA \( p>0.05 \)). Fold change of expression levels is relative to the GMR-Gal4/+ control. (B and C) \( PPT1 \) enzyme activity in fly heads. Fly heads were isolated from \( PPT1 \) overexpression flies with and without \( wfs2 \) RNAi. \( PPT1 \) enzyme activity was measured as described in Methods. (B) Co-expression of the WFS2.1 RNAi transgene with either \( PPT1 \)-2.1 or \( PPT1 \)-8.1 significantly decreased \( PPT1 \) enzyme activity, though the effect was small (Tukey HSD, \( p<0.05 \)). (C) Co-expression of the WFS2.2 RNAi transgene with either \( PPT1 \)-2.1 or \( PPT1 \)-8.1 did not affect \( PPT1 \) enzyme activity (Tukey HSD, n.s.)
Table 8. Lysosomal storage disease candidate interactors. Each lysosomal storage disease strain (column 2) was crossed to flies expressing WFS2.1 RNAi in the eye via GMR-Gal4.

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Figure 11. Knockdown of wfs2 enhances the small eye phenotype caused by CLN3 overexpression. (A) Light microscope images of GMR/+ control (i), CLN3/+ control (ii), GMR/CLN3 (iii), GMR-WFS2.1/CLN3 (iv), and GMR-WFS2.2/CLN3 (v). (B) Quantitation of eye area in the indicated genotypes. Area was measured from 5 eyes for each genotype. Eye area was decreased in GMR/CLN3 flies (open bar) compared to controls (grey bars) (one-way ANOVA, p<0.0001; Tukey HSD, p<0.05). Eye area was further decreased in GMR-WFS2.1/CLN3 and GMR-WFS2.2/CLN3 flies (black bars) compared to GMR/CLN3 flies (open bar) (Tukey HSD, p<0.05). (C) CLN3 mRNA expression. Total fly head mRNA was isolated from flies overexpressing CLN3 alone or in conjunction with wfs2 RNAi. CLN3 mRNA expression was measured by qRT-PCR. CLN3 was robustly expressed in GMR/CLN3 and GMR/CLN3/WFS2.1 flies compared to GMR/+, CLN3/+ and GMR/WFS2.1 controls (one-way ANOVA, p<0.0001; Tukey HSD, p<0.05).
ANOVA, p<0.02 Tukey HSD p<0.05, n = 3). *CLN3* induction was statistically indistinguishable in GMR/CLN3 (open bar) and GMR-WFS2.1/CLN3 flies (black bar) (Tukey HSD, n.s.).
We pursued the potential interaction between wfs2 and CLN3 by performing additional experiments in which we measured the area of the eye using Scion image. Consistent with the original description (Tuxworth et al., 2009), we found that CLN3 overexpression significantly reduced the size of the eye (Figure 11A and B). We also found that expression of two independent wfs2 RNAi transgenes further reduced the size of the eye in flies with CLN3 overexpression (Figure 11A and B). In addition to small eye size, overexpression of CLN3 causes a rough eye phenotype (Tuxworth et al., 2009), but this did not appear to be altered by wfs2 knockdown (Figure 11A). To address whether the enhanced small eye phenotype could be due to increased CLN3 expression, we performed qRT-PCR studies on mRNA isolated from fly heads. We found that wsf2 knockdown did not significantly alter CLN3 mRNA expression (Figure 11C). These studies identify wfs2 as a genetic modifier of the CLN3 overexpression phenotype in flies.

2.5 Knockdown of wfs2 does not interact with loss of function in PPT1 or CLN3 or with genetic modifiers of these genes

Since reduced function of PPT1 or CLN3 alone does not cause obvious changes in external eye morphology (Hickey et al., 2006; Tuxworth et al., 2009), we postulated that wfs2 knockdown in conjunction with PPT1 or CLN3 loss of function might lead to a synthetic phenotype. Knockdown of wfs2 in the eye in PPT1 mutant or CLN3 knockdown flies, however, did not lead to an obvious change in the external morphology of the eye. We also postulated that wfs2 may work in concert with previously identified genes that genetically interact with PPT1 and CLN3 (Buff et al., 2007; Tuxworth et al., 2009). We therefore evaluated the external eye morphology in flies harboring previously reported genetic modifiers of PPT1 and CLN3 (Table 9) alone and
Table 9. Modifiers of \textit{PPT1 overexpression} and \textit{CLN3 overexpression}. \textit{PPT1} modifiers were obtained from the \textit{Drosophila} Stock Center (Bloomington, IN). \textit{CLN3} modifiers were provided by Jennifer Treisman (Skirball Institute, NYU Medical Institute).

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with wfs2 knockdown. External eye morphology was normal in flies with PPT1 or CLN3 modifiers with or without reduced wfs2. These studies suggest that reduced function of wfs2 does not interact with PPT1 or CLN3 loss of function manipulations or genetic modifiers of PPT1 or CLN3. Our studies do not formally rule out these possibilities, however, since our interpretation is based on the lack of a synthetic phenotype.

2.6 A novel interaction between PPT1 and CLN3

PPT1 and CLN3 are the causative genes in two different lysosomal storage diseases in humans (Vesa et al., 1995; Munroe et al., 1997). Our studies in flies indicate that both genes exhibit a genetic interaction with wfs2, suggesting that PPT1 and CLN3 may be functionally linked. To address this possibility, we evaluated external eye morphology in flies expressing several different combinations of PPT1 and CLN3 transgenes. We first asked whether overexpression of PPT1 could modify the phenotype of eyes with overexpression of CLN3. Based on visual observation, we found no evidence that PPT1 overexpression altered the rough or small eye phenotypes due to CLN3 overexpression (Figure 12A). Eyes with concurrent overexpression of both PPT1 and CLN3 did exhibit a decrease in pigmentation compared to eyes overexpressing either gene alone (Figure 12A). Although this decrease in pigmentation is consistent with the possibility that PPT1 and CLN3 function in concert, it is also consistent with simple additive pathology. Additionally, we found no evidence that PPT1 loss of function altered the rough or small eye phenotypes due to CLN3 overexpression (low pressure SEM, data not shown). Thus, alteration of PPT1 expression does not appear to modify the effects of CLN3 overexpression in the Drosophila eye.
Figure 12. A genetic interaction between *PPT1* and *CLN3*. (A) Light microscope images of GMR/CLN3 (i), GMR PPT1-2.1, PPT1-8.1/+ (ii), and GMR PPT1-2.1, PPT1-8.1/CLN3 (iii). *PPT1* overexpression did not alter the small or rough eye phenotypes caused by *CLN3* overexpression. (B and C) Quantitation of black ommatidia due to *PPT1* overexpression. (B) *CLN3* overexpression (black bars) suppressed the black ommatidia phenotype in *PPT1* overexpression flies (open bars) (logistic regression, *p*<0.05, *n* = 456-666. (C) Expression of *CLN3* RNAi (black bars) enhanced the black ommatidia phenotype in *PPT1* overexpression flies (open bars) (logistic regression, *p*<0.05, *n* = 579-666. Data are the percentage of eyes in each category. Data are compiled from two independent experiments.
We next addressed the possibility that \( CLN3 \) overexpression could modify the black ommatidia phenotype in eyes with \( PPT1 \) overexpression. We used the same single-blind subjective quantitation of black ommatidia resulting in histograms as described for studies summarized in Figure 8. Interestingly, we found that \( CLN3 \) overexpression partially suppressed the severity of black ommatidia caused by \( PPT1 \) overexpression (Figure 12B, leftward shift in black bars). Conversely, \( CLN3 \) knockdown via RNAi enhanced the severity of black ommatidia caused by \( PPT1 \) overexpression (Figure 12C, rightward shift in black bars). These data indicate that \( CLN3 \) modifies the \( PPT1 \) black ommatidia phenotype in \( Drosophila \). Furthermore, these data raise the possibility that \( CLN3 \) might normally function as a negative regulator of the \( PPT1 \) pathway.

2.7 A model for the coordinated function of \( wfs2 \), \( PPT1 \) and \( CLN3 \)

Our data support a model in which \( wfs2 \), \( PPT1 \), and \( CLN3 \) function in concert (Figure 13). In our model, \( wfs2 \) is normally a positive regulator of the pathway leading from \( PPT1 \) overexpression to black ommatidia while it is normally a negative regulator of the pathway leading from \( CLN3 \) overexpression to reduced eye size. Additionally, \( CLN3 \) normally antagonizes the \( PPT1 \) pathway leading to black ommatidia in our model. Importantly, the arrows in our model (Figure 13) could represent a number of genes involved in the degenerative eye phenotypes caused by overexpression of \( PPT1 \) or \( CLN3 \). Our studies are the first to support a model for the coordinated function of \( wfs2 \), \( PPT1 \) and \( CLN3 \) in any species.

3. Discussion

Wolfram syndrome is caused by mutations in \( WFS1 \) (Strom et al., 1998) and \( CISD2 \) (Amr et al., 2007). Although we are beginning to better understand the biochemical properties of the \( CISD2 \)
Overexpression of CLN3 and PPT1 led to eyes with reduced size or black ommatidia, respectively. wfs2 is a negative regulator of the CLN3 pathway and is a positive regulator of the PPT1 pathway. CLN3 is a negative regulator of the PPT1 pathway. As for the CLN3 small eye phenotype, alteration of PPT1 expression had no effect on the size of the eye suggesting that PPT1 functions upstream of CLN3. Arrows in the model could represent the function of a number of genes and are not meant to indicate direct physical interactions between the gene products from CLN3, PPT1 or wfs2.
gene product (Amr et al., 2007), the genes and genetic pathways associated with \textit{CISD2} have not been characterized. Here, we present the first genetic analysis of \textit{wfs2}, the \textit{Drosophila} orthologue to human \textit{CISD2}.

We used RNAi to knockdown expression of \textit{wfs2} and asked whether this manipulation led to obvious phenotypes in \textit{Drosophila}. Surprisingly, flies with strong loss of function in \textit{wfs2} alone appeared remarkably healthy under standard housing conditions and when subjected to various exogenous stressors. While we do not currently understand why flies with \textit{wfs2} knockdown were seemingly unperturbed, several possibilities exist. One possibility is that \textit{wfs2} knockdown could be deleterious only under prescribed environmental conditions such as in the presence of certain microbial pathogens as found in mouse models of cystic fibrosis (Davidson et al., 1995). Another possibility is that \textit{wfs2} has an important but very narrowly defined function in \textit{Drosophila}. Given these first two possibilities, it is conceivable that we have not examined the consequences of reduced \textit{wfs2} function under the required environmental conditions or that we simply have not performed the key set of experiments to assess the narrow function of \textit{wfs2}.

Another possibility is that there is a functionally redundant gene in flies that can compensate for reduced expression of \textit{wfs2}. Although it is difficult to formally exclude this possibility, \textit{wfs2} is the only \textit{CISD2} orthologue in flies and, importantly, reduced expression of \textit{wfs2} modifies \textit{PPT1} and \textit{CLN3} pathology. Thus, if there is a functionally redundant or compensatory gene in flies, it is not structurally homologous to \textit{wfs2} and it cannot compensate for reduced \textit{wfs2} expression in all experimental conditions. A final possibility is that a low level of \textit{wfs2} expression, which could exist in our \textit{wfs2} knockdown flies, might be sufficient for supporting the functions that we have assessed. This last possibility, if found to be true, would mirror findings with catalase loss
of function mutants in which 1% of wild-type expression is sufficient for viability under normal housing conditions (Mackay and Bewley, 1989). While additional studies are required to more comprehensively address the effects of \textit{wfs2} loss of function in \textit{Drosophila}, our results suggest that decreased expression of \textit{wfs2} alone does not have broad deleterious consequences in this organism. Further assessment by placing \textit{wfs2} knockdown flies in different environments like excess cold or heat may trigger a physiological response that may result in detrimental phenotypes or sensitivity to stresses.

Toward identifying genes that function in concert with \textit{wfs2}, we determined whether knockdown of \textit{wfs2} modified the eye phenotype in several previously described models of neurodegeneration in \textit{Drosophila}. We found that knockdown of \textit{wfs2} suppresses the black ommatidia phenotype caused by \textit{PPT1} overexpression while it enhances the small eye phenotype caused by overexpression of \textit{CLN3} in flies. Through follow-up studies we also found that altered \textit{CLN3} expression modifies the severity of black ommatidia caused by \textit{PPT1} overexpression. Our studies support a novel model in which \textit{wfs2}, \textit{PPT1} and \textit{CLN3} function in concert.

Mutations in \textit{PPT1} and \textit{CLN3} cause infantile neuronal ceroid lipofuscinosi (INCL) and juvenile neuronal ceroid lipofuscinosi (JNCL), respectively. Mutations in nine genes result in different forms of neuronal ceroid lipofuscinosi (NCL) with varying ages of onset (Mole, 2004). All NCLs are characterized by progressive mental and motor deterioration, seizures, vision loss and premature death. Additionally, all NCLs have characteristic inclusions in tissues due to the accumulation of substrates for these enzymes and are therefore classified as lysosomal storage diseases (Mitchison et al., 1998). It would be interesting to determine whether \textit{Drosophila wfs2}
interacts with lysosomal storage disease genes in addition to \textit{PPT1} and \textit{CLN3}. Furthermore, it would be interesting to explore whether \textit{CISD2} has comparable interactions with mammalian \textit{PPT1}, \textit{CLN3} and potentially other lysosomal storage disease genes.

Palmitoylation is a common post-translational protein modification that can affect protein localization, function, association with membranes, trafficking and activity (Smotrys and Linder, 2004). The \textit{PPT1} enzyme removes palmitoyl moieties from cysteine residues, thereby ensuring efficient degradation of de-palmitoylated proteins in the lysosome (Zeidman et al., 2009). Interestingly, the dWFS2 protein has a single predicted palmitoylation site on cysteine 60 (Figure 7B). This predicted palmitoylation site, however, is not found in the human ERIS protein. Furthermore, we could not find other predicted palmitoylation sites in human ERIS using CSS Palm 2.0 (Ren et al., 2008). Although this suggests human ERIS is not palmitoylated, the absence of a consensus sequence for protein palmitoylation makes the prediction of palmitoylation sites difficult (Smotrys and Linder, 2004; Nadolski and Linder, 2007). Future studies that examine whether ERIS is de-palmitoylated by \textit{PPT1} or other enzymes and, if so, whether the palmitoylation status of ERIS is linked to neurotoxicity could shed considerable light on the molecular basis for Wolfram Syndrome and possibly some lysosomal storage diseases.

Mutations in \textit{CLN3} cause juvenile neuronal ceroid lipofuscinosis (JNCL), the most prevalent form of the NCLs. The clinical features of JNCL overlap with INCL except death occurs typically around the end of the second decade of life (Hofmann and Peltonen 2001). \textit{CLN3} is a transmembrane protein localized to the lysosomal membrane. Although the biochemical
function of *CLN3* has not been fully resolved due to its hydrophobic nature (Rakheja et al., 2008), it is predicted to participate in lysosomal pH homeostasis and to play a role in membrane trafficking through regulation of vesicle fusion and transport (Rakheja et al., 2008). Studies in *Drosophila* suggest that *CLN3* might be involved in the Notch and JNK signaling pathways and in sphingolipid synthesis or trafficking (Persaud-Sawin et al., 2007; Tuxworth et al., 2009). Future studies that examine whether *wfs2* is involved in sphingolipid metabolism or JNK signaling could provide insight into the normal biochemical function of *wfs2* as well as Wolfram Syndrome pathology.

Interestingly, *CISD2* is predicted to function within the Beclin-1 mediated autophagy pathway (Chang et al., 2009). It was determined that knock-down of *CISD2* enhanced autophagy in starved cells (Chang et al., 2009). During the process of autophagy, organelles and proteins are delivered to the lysosome for degradation which is accomplished through autophagosome-lysosome fusion (Luzio et al., 2007). There is evidence that disturbed autophagy is associated with lysosomal storage diseases (Kiselyov et al., 2007; Settembre et al., 2008). Reduced autophagosome-lysosome fusion was determined to be a contributor to the neurodegeneration observed in a knock-in mouse model of JNCL (Cao et al., 2006). Additionally, defective autophagocytic proteolysis was proposed to contribute to INCL pathology due to loss of PPT1 function (Hofmann et al., 1997b). Therefore, a relationship may exist between *CISD2*, alterations in autophagy and lysosomal storage diseases. Future investigation into whether deficiency of *CISD2* influences autophagy in the lysosomal storage diseases INCL and JNCL will provide insight into the role *CISD2* has with regard to these and maybe other lysosomal storage diseases.
CLN3 overexpression suppresses the black ommatidia phenotype in PPT1 overexpressors whereas CLN3 loss of function enhances the black ommatidia phenotype. These genetic interactions indicate that CLN3 and PPT1 might function in a common genetic pathway. Interestingly, CLN3 and PPT1 have been implicated in both sphingolipid metabolism (Persaud-Sawin et al., 2007) and JNK signaling (Buff et al., 2007; Tuxworth et al., 2009), and disruption of sphingolipid metabolism and JNK signaling have been implicated in neurodegeneration (Kacher and Futerman, 2006; Johnson and Nakamura, 2007). Thus, it is possible that changes in either sphingolipid metabolism or JNK signaling might be important for the CLN3- and PPT1-induced degenerative phenotypes in flies. Our studies provide the rational framework for investigating the connections between PPT1, CLN3, sphingolipid metabolism and JNK signaling within the context of neurodegeneration.

Our studies in Drosophila reveal novel genetic interactions between wfs2, PPT1 and CLN3. Our studies support several avenues of future research that could provide insight into molecular mechanisms associated with Wolfram Syndrome. Additionally, our studies suggest that there could be previously unappreciated mechanistic connections between Wolfram Syndrome and lysosomal storage diseases.
Chapter 4

Knockdown of *DJ-1B* and expression of wild-type *Ataxin-3* suppresses the short lifespan in a *Drosophila* model of elevated endogenous oxidative stress

1. Introduction

Oxidative stress is caused by the imbalance between the production of prooxidants and antioxidant defense mechanisms. Mitochondria are the major producers of superoxide, a reactive oxygen species (ROS) that is formed as a byproduct of cellular respiration (Fridovich, 2004). The generation of ROS from mitochondria is primarily determined by metabolic conditions, can be produced at high rates and is therefore mostly associated with damaging processes. Mitochondria consume more than 90% of cell O\(_2\) with at least 0.1% or more of O\(_2\) being reduced to superoxide (O\(_2^-\)) (Chance et al., 1979; Fridovich, 2004). Redox reactions involving O\(_2^-\) generates other reactive oxygen species including the hydroxyl radical (OH\(^-\)) that is highly reactive and results in oxidative damage to macromolecules (Chance et al., 1979).

There are a number of antioxidant enzymes that protect cells from oxidative stress (Figure 14). Superoxide dismutase (SOD) enzymes catalyze the dismutation of superoxide to oxygen and hydrogen peroxide. Hydrogen peroxide is subsequently reduced to water by catalase or a number of peroxidases (Halliwell B, 1999). Peroxidases require an additional substrate that can be oxidized in concurrence with reduction of their ROS substrate (Matthews CK, 2000). For example, the enzyme thioredoxin reductase (TrxR) reduces oxidized thioredoxin (TRx) so it can be used by thioredoxin peroxidase (TPx) (Missirlis et al., 2003). Additionally, reduction of
Figure 14. The antioxidant enzymes (SOD, CAT, and TrxR) protect against oxidative stress. See text for description of each antioxidant enzyme.
peroxidized lipids is performed by phospholipid hydroperoxide glutathione peroxidase (PHGPx) (Conrad et al., 2007). The two main superoxide dismutases in *Drosophila* are *Sod1* that is located in the cytosol and *Sod2* that is located in the mitochondrial matrix (Landis and Tower, 2005). Catalase, thioredoxin reductase and PHGpx are also conserved in *Drosophila* (Missirlis et al., 2001; Landis and Tower, 2005). Therefore, *Drosophila* is a good model system to study antioxidant function (Missirlis et al., 2001).

Complete loss or reduced functioning of antioxidant enzymes results in detrimental consequences to the organism. For example, complete loss of SOD2 activity in mice results in a number of pathological conditions including dilated cardiomyopathy and motor disturbances with neurodegeneration (in a mixed C57BL/6 and 129Sv background), and a very short life span (Li et al., 1995; Lebovitz et al., 1996). In *Drosophila*, knock-out or knockdown of *Sod2* throughout the body also severely shortens life span (Kirby et al., 2002; Duttaroy et al., 2003; Paul et al., 2007). Furthermore, *Sod2* deficient flies exhibit accelerated declines in locomotor behavior with age in addition to neurodegeneration and enhanced sensitivity to exogenous oxidative stress (Kirby et al., 2002; Bhandari et al., 2007; Paul et al., 2007). Decreased SOD1 activity in flies or mice also results in a shortened lifespan and detrimental phenotypes including sensitivity to exogenous oxidative stress and accelerated locomotor decline (Phillips et al., 1989; Muller et al., 2006; Martin et al., 2009a). In addition, decreased functioning of catalase, PHGpx or TrxR in flies or mice is also associated with a decreased lifespan (Mackay and Bewley, 1989; Missirlis et al., 2001; Missirlis et al., 2002; Imai et al., 2003; Ho et al., 2004; Jakupoglu et al., 2005). All of these studies underscore the importance of antioxidant enzymes in protecting cells and tissues from oxidative stress.
Endogenous oxidative stress is associated with a number of age-related diseases including Parkinson disease, Friedrich ataxia, type 2 diabetes, cancer and neurodegeneration (McFarland et al., 2002; Schapira, 2006; McFarland et al., 2007). For example, mutations in *Ataxin-3* cause Spinocerebellar Ataxia 3 (SCA3). Expression of the pathogenic form of *Ataxin-3* in cells resulted in decreased SOD and catalase activities (Yu et al., 2009). Furthermore, post mortem samples from patients’ brains with Huntington disease have mitochondria with increased oxidative damage (Polidori et al., 1999). Expression of the pathogenic form of huntingtin in cells also results in increased ROS production (Wyttenbach et al., 2002). Therefore, oxidative stress appears to be a component of many age-related diseases.

There are currently no reports on genetic manipulations that can compensate or alleviate the effects of decreased expression for any of the enzymes that function to remove ROS and reduce oxidative stress. *Drosophila Sod2* knockdown flies can serve as a model of endogenous oxidative stress and, therefore, can be used to identify genes that can reduce the negative consequences of endogenous oxidative stress. Studies in this model may lead to a better understanding of the genetic mechanisms that influence the effects of endogenous oxidative stress.

In this study we explored whether manipulation of a number of candidate genes could extend the short life span of *Sod2* knockdown flies with the goal of identifying mechanisms that control or influence the negative consequences of enhanced endogenous oxidative stress. Interestingly, we find that knockdown of *DJ-1β*, a fly ortholog of the Parkinson Disease-associated gene *DJ-1*, partially but reproducibly extends the life span of flies with ubiquitous knockdown of *Sod2*. 

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Additionally, we found that overexpression of human wild-type Ataxin-3 (46Q27) also reproducibly extends the short lifespan of flies with ubiquitous knockdown of Sod2. Hence, reduced expression of DJ-1β or expression of human wild-type Ataxin-3 might ameliorate some of the detrimental effects of endogenous oxidative stress due to loss of Sod2 in flies. Further study on how wild-type Ataxin-3 expression suppressed the short lifespan of Sod2 knockdown flies revealed that Ataxin-3 is protective only against some forms of exogenous oxidative stress. In addition, the ubiquitin-associated activity of Ataxin-3 is important for extending the short lifespan of Sod2 knockdown flies. We also discovered that Ataxin-3 expression also partially restores the short lifespan due to muscle knockdown of another antioxidant enzyme thioredoxin reductase. These studies support a protective role for reduced function of DJ-1β and expression of wild-type Ataxin-3 in ameliorating the short lifespan caused by endogenous oxidative stress due to loss of function of Sod2.

2. Results

2.1 Knockdown of DJ-1β and wild-type Ataxin-3 expression mitigates the short lifespan in Sod2 knockdown flies

Knockdown of Sod2 in Drosophila via RNA interference (RNAi) using GAL4 to ubiquitously express a UAS-Sod2-inverted repeat (UAS-Sod2-IR) transgene substantially reduces SOD2 enzymatic activity, shortens life span and results in sensitivity to the exogenous oxidative stressor paraquat (Kirby et al., 2002; Martin et al., 2009b). Understanding how Sod2 works in concert with other genes to protect organisms from oxidative stress is a major unresolved issue. We addressed this by screening candidate genetic manipulations as potential modifiers of the short life span of Sod2 knockdown flies. As a screening tool, we constructed via recombination a true-breeding stock of flies with Actin-Gal4 and Sod2-IR15 on the same chromosome (Actin-
Gal4::Sod2-IR15) and with da-Gal4 and Sod2-IR15 on the same chromosome (da-Gal4::Sod2-IR15). Actin-Gal4 and da-Gal4 drives expression ubiquitously (Wairkar et al., 2008). Ubiquitous knockdown of Sod2 via the da-Gal4 driver decreases median lifespan by 79% and ubiquitous knockdown of Sod2 via the Actin-Gal4 driver decreases median lifespan by 88%, respectively (Martin et al., 2009b). These stocks are maintained by constantly transferring progeny to new bottles every two weeks.

We initiated our screen by assessing life span in the F1 progeny of Actin-Gal4::Sod2-IR15 flies crossed to a series of candidate mutations or UAS transgenes (Table 10). Given the profound life span reduction resulting from loss of Sod2, we reasoned that manipulation of any single genetic modifier would likely have only partial effects on the life span of Sod2 knockdown flies. This candidate approach identified that expression of two independent UAS-DJ-1β inverted repeat (UAS-DJ-1β-IR) transgenes reproducibly extended the median life span of Sod2 knockdown flies by more than 50% (Figure 15). We performed a series of qRT-PCR analyses to address a number of questions related to the functionality of the UAS-DJ-1β-IR transgenes. Expression of DJ-1β-IR by Actin-Gal4 substantially knocked down endogenous DJ-1β expression in flies with a normal complement of Sod2 and in Sod2 knockdown flies (Figure 16A). Additionally, expression of DJ-1β-IR did not significantly alter the expression of the related gene DJ-1α (Figure 16B). Knockdown of Sod2 expression (Figure 16C) and SOD2 enzymatic activity (Figure 16D) by Sod2-IR were not significantly altered by concurrent expression of DJ-1β-IR. These studies indicate that DJ-1β-IR selectively knocks down DJ-1β and does not interfere with the ability of Sod2-IR to knockdown Sod2. Loss of function mutations in PARK7 which encodes DJ-1 causes a rare form of autosomal recessive early-onset Parkinson Disease (Bonifati et al., 2003).
Table 10: Representative candidate suppressors tested for the effect on the short lifespan of *Sod2* knockdown flies. A brief function (column 2) is listed for each of the candidate suppressors (column 1). A total of 76 candidate suppressors were assessed. Effect (column 3) refers to whether the candidate suppressors are gain or loss of function manipulations.

<table>
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<th>Function</th>
<th>Effect</th>
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<td>mitochondrial uncoupling protein</td>
<td>gain</td>
</tr>
<tr>
<td>porin f03616</td>
<td>mitochondrial VDAC</td>
<td>loss</td>
</tr>
<tr>
<td>porin K05123</td>
<td>mitochondrial VDAC</td>
<td>loss</td>
</tr>
<tr>
<td>UAS-sniffer</td>
<td>oxidative stress, carbonyl reductase</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-catalase</td>
<td>oxidative stress, catalase</td>
<td>gain</td>
</tr>
<tr>
<td>mt-catalase</td>
<td>oxidative stress, catalase (mito-targeted)</td>
<td>gain</td>
</tr>
<tr>
<td>mt-catalase</td>
<td>oxidative stress, catalase (mito-targeted)</td>
<td>gain</td>
</tr>
<tr>
<td>Cat-Sod1-TR-1</td>
<td>oxidative stress, catalase, Sod1, thioredoxin reductase</td>
<td>gain</td>
</tr>
<tr>
<td>Cat-Sod1-TR-2</td>
<td>oxidative stress, catalase, Sod1, thioredoxin reductase</td>
<td>gain</td>
</tr>
<tr>
<td>Cat-Sod1-TR-3</td>
<td>oxidative stress, catalase, Sod1, thioredoxin reductase</td>
<td>gain</td>
</tr>
<tr>
<td>Cat-Sod1-TR-4</td>
<td>oxidative stress, catalase, Sod1, thioredoxin reductase</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-DJ-1alpha IR</td>
<td>oxidative stress, DJ-1alpha</td>
<td>loss</td>
</tr>
<tr>
<td>UAS-DJ-1beta</td>
<td>oxidative stress, DJ-1beta</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-DJ-1beta IR-1</td>
<td>oxidative stress, DJ-1beta</td>
<td>loss</td>
</tr>
<tr>
<td>UAS-DJ-1beta IR-2</td>
<td>oxidative stress, DJ-1beta</td>
<td>loss</td>
</tr>
<tr>
<td>UAS-GClc-5</td>
<td>oxidative stress, glutamate-cysteine ligase (catalytic)</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-GClc-6</td>
<td>oxidative stress, glutamate-cysteine ligase (catalytic)</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-GClm-2</td>
<td>oxidative stress, glutamate-cysteine ligase (modulatory)</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-GClm-3</td>
<td>oxidative stress, glutamate-cysteine ligase (modulatory)</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-GSH Px</td>
<td>oxidative stress, glutathione peroxidase</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-MSRA(C)</td>
<td>oxidative stress, methionine sulfoxide reductase A</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-Sod1</td>
<td>oxidative stress, SOD1</td>
<td>gain</td>
</tr>
<tr>
<td>UAS-DTS-5-11</td>
<td>proteosome</td>
<td>loss</td>
</tr>
<tr>
<td>UAS-46Q27 (WT Ataxin-3)</td>
<td>ubiquitination</td>
<td>gain</td>
</tr>
</tbody>
</table>
Figure 15. *DJ-1β* knockdown partially restores life span in *Sod2* knockdown flies.
Expression of two independent UAS-*DJ-1βIR* transgenes (1 and 2) extended the life span of *Actin-Gal4::Sod2-IR15* flies (log-rank test, p<0.0001, n = 210-248 flies per genotype from 2 experiments). Median life span was increased by 57%. Experiments were performed by Ian Martin.
Figure 16. *DJ-1β* RNAi reduces *DJ-1β* expression and has no effect on *Sod2* expression or enzyme activity. Quantitative real-time PCR was performed as described in materials and methods using PCR primers listed in Table 4. (A) Ubiquitous expression of UAS-*DJ-1β*-IR transgenes in *Actin*-Gal4;*DJ-1β*-IR and *Actin*-Gal4::*Sod2*-IR15;*DJ-1β*-IR flies significantly reduced *DJ-1β* expression relative to controls containing *DJ-1β*-IR alone (one-way ANOVA, p < 0.0001, n = 3 groups of 25 flies per genotype, followed by Bonferroni’s post-test, *p* < 0.05). Bonferroni’s post-test also revealed no significant difference in the extent of *DJ-1β* knockdown in *Actin*-Gal4::*Sod2*-IR15;*DJ-1β*-IR relative to *Actin*-Gal4; *DJ-1β*-IR flies. *DJ-1β* RNAi had no significant effect on expression of *DJ-1α* (one-way ANOVA, not significant, *n* = 3 groups of 25 flies per genotype) (B) or *Sod2* (one-way ANOVA, not significant, *n* = 3 groups of 25 flies per genotype). (D) Activities of SOD2 or SOD1 were unaffected by expression of *DJ-1β*-IR (individual one-way ANOVAs, not significant, *n* = 3). SOD activity performed by Ian Martin.
Additionally, our candidate approach identified that ubiquitous expression of human wild-type *Ataxin-3* using the *Actin-Gal4* or *da-Gal4* driver also reproducibly extended the median life span of *Sod2* knockdown flies by more than 50% (Figure 17A and B). qRT-PCR analysis revealed that *Ataxin-3* was robustly expressed via the *da*-Gal4 driver in flies with a normal complement of *Sod2* and in *Sod2* knockdown flies (Figure 18A). Furthermore, *Ataxin-3* expression did not significantly alter knockdown of *Sod2* expression or SOD2 enzymatic activity (Figure 18B and C). These studies indicate that *Ataxin-3* is robustly expressed and does not interfere with the ability of *Sod2*-IR to knockdown *Sod2*. Mutations in the gene *Ataxin-3* result in Spinocerebellar Ataxia 3 a.k.a. Machado Joseph disease (MJD), a progressive neurodegenerative disorder (Takiyama et al., 1993). Given that wild-type *Ataxin-3* expression has demonstrated protective effects in neurodegenerative disease models in *Drosophila* (Warrick et al. 2005), we consequently decided to focus on the ability of *Ataxin-3* expression to suppress the short lifespan of *Sod2* knockdown flies.

2.2 *Ataxin-3* expression enhances \( \text{H}_2\text{O}_2 \) resistance of *Sod2* knockdown flies

To investigate how wild-type *Ataxin-3* expression suppresses the short lifespan of *Sod2* knockdown flies, we determined whether wild-type *Ataxin-3* expression could suppress the sensitivity of *Sod2* knockdown flies to various exogenous oxidative stresses (paraquat, hyperoxia and \( \text{H}_2\text{O}_2 \)). As expected, *Sod2* knockdown flies were sensitive to all three stressors compared to controls (Figure 19). Interestingly, wild-type *Ataxin-3* expression enhanced resistance of *Sod2* knockdown flies to \( \text{H}_2\text{O}_2 \) but not to paraquat and hyperoxia (Figure 19). We also determined that wild-type *Ataxin-3* expression (da-Gal4/ATX3) alone in *Drosophila* does not enhance resistance against any of these exogenous oxidative stressors, suggesting that expression of wild-type *Ataxin-3* has no protective effect on its own (Figure 19). These results suggest that wild-
Figure 17. *Ataxin-3* expression partially restores life span in *Sod2* knockdown flies. (A) Expression of the human wild-type *Ataxin-3* extends the life span of *Actin-Gal4::Sod2-IR15* flies (log-rank test, p<.0001 n = 100 flies per genotype from two experiments). Median life span was increased by 52%. (B) Expression of *Ataxin-3* extends the life span of *da-Gal4::Sod2-IR15* flies (log-rank test, p<.0001 n = 100 flies per genotype from two experiments). Median life span was increased by 100%. Experiments were performed by Devin Rhodenizer.
Figure 18. Expression of *Ataxin-3* does not alter *Sod2* expression or enzyme activity. (A) qRT-PCR analysis of *Ataxin-3* expression. *Ataxin-3* expression is not significantly altered by *Sod2* knockdown (one-way ANOVA, p<0.001, n=3; *Bonferroni’s multiple comparison test, p<0.05). Data are representative of 3 independent experiments. (B) qRT-PCR analysis of *Sod2* expression. Knockdown of *Sod2* mRNA via the *da-Gal4* driver is not altered by *Ataxin-3* expression (Compare last two columns, one-way ANOVA, p<0.001, n=3; *Bonferroni’s multiple comparison test, p<0.05). (C) Activities of SOD2 or SOD1 were unaffected by expression of *Ataxin-3* (individual one-way ANOVAs, not significant, n = 3). SOD activity performed by Devin Rhodenizer.
Figure 19. *Ataxin-3* expression enhances resistance of *Sod2* knockdown flies to H$_2$O$_2$ but not to paraquat and hyperoxia. Survival in the presence of paraquat (A), hyperoxia (B) and H$_2$O$_2$ (C). Survival in the presence of paraquat or hyperoxia was decreased in *Sod2* knockdown flies (*da-Gal4-Sod2IR/+*) and in *Sod2* knockdown flies with *Ataxin-3* (*da-Gal4-Sod2IR/ATX3*) expression compared to controls with normal levels of *Sod2* (*da-Gal4/+, da-Gal4/ATX3, Sod2IR/ATX3*) (one-way ANOVA p<0.001, n=3; *Bonferroni’s multiple comparison test, p>0.05). Survival in the presence of H$_2$O$_2$ was enhanced in *Sod2* knockdown flies with *Ataxin-3* expression compared to flies with *Sod2* knockdown alone (one-way ANOVA p<0.001, n=3; *Bonferroni’s multiple comparison test, p<0.001).
type *Ataxin-3* expression can enhance resistance to some but not all forms of exogenous oxidative stress in a *Sod2* deficient background.

2.3 The ubiquitin-associated function of *Ataxin-3* is important for extending the short lifespan of *Sod2* knockdown flies

One of the main functions of the proteasome is to remove oxidatively damaged proteins (Ciechanover, 2005). One of the main predicted functions of *Ataxin-3* is to support the transport of ubiquitinated proteins to the proteasome for degradation (Doss-Pepe et al., 2003) suggesting *Ataxin-3* facilitates in the degradation of oxidatively damaged proteins. *Ataxin-3* contains a ubiquitin protease domain and several ubiquitin interacting motifs located near the polyQ repeat (Thrower et al., 2000; Burnett et al., 2003). To investigate whether the ubiquitin-associated function of *Ataxin-3* is important for extending the short lifespan of *Sod2* knockdown flies we obtained *Drosophila* UAS-transgenes that have a mutation in the ubiquitin protease domain (UPD) or the ubiquitin interacting motifs (UIM) in *Ataxin-3*. These transgenes are not pathogenic but they do abolish ubiquitin protease activity and prevent ubiquitin binding, respectively (Warrick et al., 2005). We postulated that abolishing the ubiquitin-associated activities of *Ataxin-3* would prevent its ability to extend the short lifespan of *Sod2* knockdown flies. Therefore, we evaluated lifespan in *Sod2* knockdown flies and in *Sod2* knockdown flies with each *Ataxin-3* mutant using the ubiquitous da-Gal4 driver and the muscle 24B-Gal4 driver. Expression of *Ataxin-3* with the UPD mutated failed to extend the short lifespan of *Sod2* knockdown flies (Figure 20A and B). Expression of *Ataxin-3* with the UIM mutated failed to fully extend the short lifespan of *Sod2* knockdown flies. *Ataxin-3* to extend the short lifespan of
Figure 20. The ubiquitin-associated function of Ataxin-3 is important for extending the short lifespan of Sod2 knockdown flies. Survival under normal housing conditions using the ubiquitous da-Gal4 driver (A) and muscle 24B-Gal4 driver (B). Expression of Ataxin-3 (da-Gal4-Sod2IR/ATX3, 24B-Gal4-Sod2IR/ATX3) and Ataxin-3 with the ubiquitin interacting motif (UIM) mutated (da-Gal4-Sod2IR/UIM, 24B-Gal4-Sod2IR/UIM) partially extends the short lifespan of Sod2 knockdown flies. (log-rank test, p<.0001 n = 175 flies per genotype from one experiment). Expression of Ataxin-3 with the ubiquitin protease domain (UPD) mutated (da-Gal4-Sod2IR/UPD, 24B-Gal4-Sod2IR/UPD) does not extend the short lifespan of ubiquitous or muscle knockdown of Sod2. (C) Light microscope images of ATX3-TRQ78/+ (i), ATX3-TRQ78/ATX3 (ii), ATX3-TRQ78/UPD (iii), ATX3-TRQ78/UIM (iv). Ataxin-3 expression suppresses the degenerative eye phenotype of pathogenic ATX3-TRQ78 flies (compare ii with i). Ataxin-3 with the ubiquitin protease domain mutated fails to suppress the degenerative eye phenotype of pathogenic ATX3-TRQ78 flies (compare iii with i). Ataxin-3 with the ubiquitin
interacting motif mutated partially suppresses the degenerative eye phenotype of pathogenic ATX3-TRQ78 flies (compare iv with i).
Sod2 knockdown flies (Figure 20A and B). The effects of the UPD and UIM mutants on the short lifespan of Sod2 knockdown flies is similar to what was observed when these mutants were expressed in the eye for a pathogenic fly model of SCA3 (Figure 20C) (Warrick et al., 2005). Therefore, these results suggest that the ubiquitin-associated activities, especially the ubiquitin protease function of wild-type Ataxin-3 is important in extending the short lifespan of Sod2 knockdown flies.

2.4 Ataxin-3 expression mitigates the short lifespan in thioredoxin reductase knockdown flies

We showed that expression of wild-type Ataxin-3 suppresses (i.e. extends) the short lifespan of Sod2 knockdown flies (Figure 15). We extended these data by determining whether wild-type Ataxin-3 expression could also suppress the short lifespan due to knockdown of other antioxidant related genes in flies. To investigate this we obtained a number of RNAi transgenic strains from the Vienna Drosophila Research Center (VDRC) for a number of antioxidant related genes in Drosophila including thioredoxin reductase, catalase, PHGpx, and a DNA damage repair gene RpS3. We first investigated whether lifespan was altered in these RNAi transgenic strains when expressed ubiquitously via the da-Gal4 driver or in muscle via the mef2- Gal4 driver. We determined that flies with knockdown of RpS3 ubiquitously or in muscle were lethal (Table 11). Flies with ubiquitous knockdown of thioredoxin reductase were also lethal (Table 11). Additionally, flies with ubiquitous knockdown of Sod1, ubiquitous or muscle knockdown of PHGpx and flies with muscle knockdown of thioredoxin reductase were short-lived (Table 11). We consequently decided to focus on the RNAi strains that resulted in lethality or severely reduced lifespan when expressed ubiquitously or in muscle which included thioredoxin reductase, PHGpx, and RpS3.
Table 11: Median lifespan of antioxidant related and DNA repair RNAi strains. RNAi strains (column 1) and median lifespan via the ubiquitous da-Gal4 driver (column 2) and the muscle specific mef2-Gal4 driver (column 3). Median lifespan in a wild-type fly ~50 days.

<table>
<thead>
<tr>
<th>RNAi strain</th>
<th>da-Gal4</th>
<th>Mef2-Gal4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sod1 F103</td>
<td>15</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Sod1 31551</td>
<td>36</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Sod1 31552</td>
<td>12</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Trxr 47306</td>
<td>Lethal</td>
<td>12</td>
</tr>
<tr>
<td>Trxr 47307</td>
<td>Lethal</td>
<td>6</td>
</tr>
<tr>
<td>Trxr 47308</td>
<td>Lethal</td>
<td>6</td>
</tr>
<tr>
<td>Catalase 6283</td>
<td>30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Catalase 103591</td>
<td>&gt;60</td>
<td>&gt;30</td>
</tr>
<tr>
<td>PHGpx 100790</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>RpS3 37741</td>
<td>Lethal</td>
<td>Lethal</td>
</tr>
<tr>
<td>RpS3 37742</td>
<td>Lethal</td>
<td>Lethal</td>
</tr>
</tbody>
</table>
To determine whether expression of wild-type Ataxin-3 could extend the short lifespan or rescue the lethality in these knockdown strains we expressed wild-type Ataxin-3 in flies with concurrent ubiquitous or muscle knockdown of PHGpx, RpS3 and thioredoxin reductase. Expression of Ataxin-3 did not rescue the lethality caused by ubiquitous knockdown of thioredoxin reductase or the lethality caused by ubiquitous or muscle knockdown of RpS3. Additionally, expression of Ataxin-3 had no consistent effect on the short lifespan caused by ubiquitous or muscle knockdown of PHGpx (Figure 21A and B). Interestingly, Ataxin-3 expression reproducibly extended the short lifespan caused by muscle knockdown of thioredoxin reductase (Figure 21C). These results suggest that Ataxin-3 expression is protective against partial loss of function of thioredoxin reductase. Furthermore, these results support the possibility that Ataxin-3 might play a broader role in protecting against endogenous oxidative stress.

3. Discussion

Sod2 is a critical antioxidant localized to the mitochondria (Landis and Tower, 2005). Whole-body loss of Sod2 function through RNAi-mediated silencing or knock-out of the endogenous Sod2 gene, dramatically shortens life span and causes behavioral defects in flies (Kirby et al., 2002; Duttaroy et al., 2003; Bhandari et al., 2007; Paul et al., 2007), and mice (Li et al., 1995; Lebovitz et al., 1996). The identification of genes that work in concert with Sod2 is a critical step toward understanding mechanisms related to endogenous oxidative stress. We used Sod2 knockdown flies as a model of elevated endogenous oxidative stress to search for genetic manipulations that can modify the deleterious consequences of Sod2 knockdown on life span. Through a candidate approach, we found that RNAi-mediated knockdown of DJ-1β partially, but reproducibly, mitigated the effect of Sod2 knockdown on life span. Importantly, DJ-1β
Figure 21. Wild-type *Ataxin-3* expression only extends the short lifespan caused by knockdown of thioredoxin reductase in muscle. Ubiquitous (*da*-Gal4) or muscle (*mef2*-Gal4) knockdown of PHGpx results in a shortened lifespan (A and B). Expression of *Ataxin-3* fails to rescue the short lifespan in these animals (log-rank test, n.s. *n* = 175 flies per genotype from two experiments) (A and B). Knockdown of thioredoxin reductase in muscle results in a shortened lifespan (C). Expression of *Ataxin-3* extends the short lifespan caused by knockdown of thioredoxin reductase in muscle (C) (log-rank test, *p*<.0001 *n* = 175 flies per genotype from two experiments).
knockdown does not alter knockdown of Sod2 expression, the activity of SOD2 or SOD1, or
expression of the related gene DJ-1α. Loss of function mutants of DJ-1β results in a shortened
lifespan in Drosophila (Lavara-Culebras and Paricio, 2007) so the effect of extending the short
lifespan of Sod2 knockdown flies is specific to Sod2.

Loss of function mutations in DJ-1 cause autosomal recessive early-onset Parkinson Disease
(Bonifati et al., 2003). Although the biochemical role of DJ-1 has not been fully resolved, DJ-1
has been characterized as an atypical peroxiredoxin-like peroxidase (Andres-Mateos et al., 2007)
that also acts as a regulator of gene expression (van der Brug et al., 2008) and a molecular
chaperone (Zhou et al., 2006). Together, our studies point toward a surprisingly complex
relationship between Sod2, DJ-1 and oxidative stress. One possible explanation for this
relationship is that DJ-1β knockdown may cause compensatory changes in antioxidants or repair
enzymes that partially protect against mitochondrial oxidative stress related to the loss of Sod2.
Another possibility is that concomitant knockdown of Sod2 and DJ-1β together could result in
synergistic changes in the expression of antioxidants or repair enzymes that partially compensate
for the loss of Sod2. It is also possible that DJ-1β may be an oxidative stress signaling molecule
that triggers apoptosis and that partial loss of function of DJ-1β allows cells to survive, hence
extending the short lifespan of Sod2 knockdown flies. Evidence for a possible signaling role is
provided by evidence of a signaling event in two other genes associated with Parkinson disease.
Mutations in the genes Pink1 and Parkin cause recessive forms of familial Parkinson Disease.
There is evidence that these genes are involved in a mitochondrial turnover pathway. For
example, Pink1 accumulates on damaged mitochondria and Pink1 recruits Parkin to these
mitochondria (Narendra et al., 2009). Parkin then promotes the degradation of these
dysfunctional mitochondria (Narendra et al., 2009). Therefore, Pink1 and Parkin selectively promote the degradation of damaged mitochondria (Narendra et al., 2009). It is possible that DJ-1β could also be involved in this mitochondrial turnover pathway. Additionally, redox signaling might underlie the short life span of Sod2 knockdown flies. For example, if Sod2 knockdown flies are placed under hypoxia, their lifespan is fully restored (Wicks et al., 2009). Furthermore, placing Sod2 knockdown flies into a hypoxia environment after 50% mortality causes the remaining surviving flies to live as long as flies permanently placed under hypoxia (Wicks et al., 2009). This suggests that a redox signaling mechanism might be involved in the rapid reversal of mortality when flies are placed under a hypoxic environment. Additional studies will be important for determining whether DJ-1β is part of a signaling pathway that senses oxidative damage and leads to the removal of dysfunctional mitochondria. It will also be interesting to determine whether reduced DJ-1β expression can ameliorate additional negative consequences associated with loss of Sod2 including enhanced locomotor impairment and enhanced sensitivity to exogenous oxidative stress.

Our candidate approach also found that wild-type Ataxin-3 expression partially, but reproducibly, mitigated the effect of Sod2 knockdown on life span. Ataxin-3 expression did not alter knockdown of Sod2 expression, or the activity of SOD2 or SOD1. Wild-type Ataxin-3 expression is reported to have no effect on lifespan when expressed in Drosophila (Warrick et al., 2005) suggesting the extension of lifespan with Sod2 knockdown is specific to Sod2. Mutations in Ataxin-3 cause Spinocerebellar Ataxia 3, a polyglutamine repeat disease with more than 50 repeats needed for clinical representation of this disorder (Gusella and MacDonald,
An expanded polyQ repeat in *Ataxin-3* causes misfolding and aggregation resulting in a toxic gain of function property of the protein (La Spada et al., 1994).

*Ataxin-3* is a protease that is thought to remove ubiquitin from proteins (Burnett et al., 2003). *Ataxin-3* also assists in targeting proteins to the proteasome (Wang et al., 2006b). In addition to *Ataxin-3*, the human genome contains ~100 other deubiquitinating enzymes (Nijman et al., 2005). There is evidence that wild-type *Ataxin-3* may play a protective role against neurodegenerative disease. Interestingly, *Ataxin-3* was localized in the nuclear inclusions in patients with SCA3 and amyotrophic lateral sclerosis (Fujigasaki et al., 2000; Seilhean et al., 2004). The presence of nuclear inclusions are believed to represent protection or repair attempts of the cell and the presence of *Ataxin-3* in these nuclear inclusions supports the role of this protein in conferring protective properties (Arrasate et al., 2004). Additionally, expression of wild-type *Ataxin-3* in *Drosophila* suppresses the neurodegenerative eye phenotype for several neurodegenerative disease models including SCA3, SCA1 and Huntington disease (Warrick et al., 2005). The extension of the short lifespan of *Sod2* knockdown flies further suggests that *Ataxin-3* confers protective properties. To this end we investigated how *Ataxin-3* suppressed the short lifespan of *Sod2* knockdown flies.

Our studies demonstrated that although expression of *Ataxin-3* in a wild type genetic background did not enhance resistance to the exogenous oxidative stressors paraquat, hyperoxia and H$_2$O$_2$, it did enhance resistance to H$_2$O$_2$ stress in an elevated endogenous oxidative stress background via *Sod2* knockdown. The resistance to only one exogenous oxidative stressor could be due to each stressor having different effects on cells and the organism as a whole. For example, paraquat
generates reactive oxygen species (superoxide anion and hydroxyl radical) that can damage cells and also depletes cellular reducing equivalents including NADPH that are needed for normal cellular function (Bus and Gibson, 1984). In contrast, H$_2$O$_2$ can alter the overall redox potential of the cell and can stimulate growth arrest and cell death (Stone and Yang, 2006). We conclude that expression of Ataxin-3 does not provide an overall protection against exogenous oxidative stress.

We determined the ubiquitin-associated function of Ataxin-3 is important for suppressing the short lifespan of Sod2 knockdown flies. It is possible that the ubiquitin-associated function of Ataxin-3 enhances the activity of the proteasome to remove oxidatively damaged proteins. Increased and more efficient removal of oxidatively damaged proteins could have a protective effect that would allow the Sod2 knockdown flies to live longer. It would be interesting to determine whether expression of other genes involved in ubiquitin proteasome function including faf and other ubiquitin proteases (Wu et al., 1999; DiAntonio et al., 2001) or enhancement of ubiquitin dependent pathways could also extend the short lifespan of Sod2 knockdown flies. Further investigation into whether enhanced expression of other deubiquitinating enzymes in Drosophila extend the short lifespan of Sod2 knockdown flies will provide insight into a possible mechanism that may protect against endogenous oxidative stress.

Interestingly, we also determined that Ataxin-3 expression can partially rescue the short lifespan due to knockdown of another antioxidant enzyme thioredoxin reductase in the muscle. This suggests that Ataxin-3 expression may be protective against the short lifespan associated with endogenous oxidative stress due to reduction of two antioxidant related genes in flies. Further
study using the *Ataxin-3* UPD and UIM transgenes will provide insight into whether the ubiquitin-associated function of *Ataxin-3* is important for extending the short lifespan due to muscle knockdown of thioredoxin reductase. It would be interesting to determine whether expression of *Ataxin-3* in mice with *Sod2* knockdown improves survival in these mice due to loss of function of *Sod2*. It would also be interesting to determine whether enhanced expression of *Ataxin-3* can rescue the lethality in mice with inactivation of thioredoxin reductase. Additional studies to determine whether *Ataxin-3* can ameliorate other negative consequences associated with elevated endogenous oxidative stress will provide insight into whether *Ataxin-3* expression has protective effects across many physiological parameters. For example, *Ataxin-3* expression may forestall the accelerated ARLI that is observed in *Sod2* knockdown flies. *Ataxin-3* expression may also lower the amount of apoptosis present in *Sod2* knockdown flies and can be measured via a caspase assay. This has implications for the development of possible therapies that could either delay the onset or prevent the detrimental phenotypes associated with endogenous oxidative stress.
Chapter 5

Summary

This dissertation comprises research using Drosophila as a model to investigate the mechanisms associated with aging and age-related disease. Each independent project resulted in the elucidation of different mechanisms involved in non pathological and pathological aging. The following summarizes the findings from each project culminating with a discussion regarding future studies.

Age-related locomotor impairment (ARLI) is a significant challenge to the elderly and their caretakers. ARLI is associated with a decrease in mobility, increased fear of falling, increased risk of skeletal fractures and increased mortality. A better understanding of genes and genetic pathways that forestall ARLI has implications for the development of interventions that can lead to an improved quality of life for the elderly. Through a forward genetic screen the lab identified a partial loss of function mutation in PDK1 with delayed ARLI. Further study revealed that loss of function mutants in additional insulin signaling genes resulted in delayed ARLI. These results support the involvement of the insulin signaling pathway as a process that influences ARLI. This pathway is conserved across species thereby making any discoveries using model organisms likely applicable to humans. It would be interesting to determine whether loss of function mutations or polymorphisms within the insulin signaling pathway components in mice or humans also delay ARLI. Identification of key tissues where insulin signaling is important for...
regulating ARLI can provide insight into whether reduced insulin signaling in certain tissues is required for the beneficial effects on locomotor decline across age. For example, muscle and neuron Gal4 drivers can be used to express insulin signaling genes in these tissues and these manipulation can be evaluated for locomotor impairment across age. Additional studies to identify cellular changes or changes in patterns of gene expression across age via microarray analysis in these insulin signaling mutants will provide additional information into how reduced insulin signaling delays ARLI.

Wolfram Syndrome is a progressive neurodegenerative disease. The function of the more recently identified gene CISD2 has not been fully resolved. Additionally, the genes that interact with CISD2 or the pathway this gene resides in have yet to be identified. Drosophila contains one ortholog of CISD2 that we have named wfs2. We used RNAi to knockdown wfs2 to provide further insight into the function of this gene. A targeted genetic screen in the eye was performed to identify genes that interact with wfs2. Two genes were identified through this screen. We found that knockdown of wfs2 suppresses the black ommatidia phenotype caused by PPT1 overexpression. In contrast wfs2 knockdown enhances the small eye phenotype caused by overexpression of CLN3. Loss of function mutations in the genes PPT1 and CLN3 cause two forms of neuronal ceroid lipofuscinosis (NCL). The NCL diseases are pediatric neurodegenerative disorders and are classified as lysosomal storage diseases. We are the first to identify genes that interact with Drosophila wfs2. We believe that wfs2 and these lysosomal storage diseases may share common molecular-genetic mechanisms. We also believe wfs2 plays a role in the degenerative pathways associated with these lysosomal storage diseases. It will be imperative to demonstrate that PPT1 and CLN3 interact with wfs2 in mammalian systems to
confirm that these interactions are conserved in higher organisms. It will also be interesting to
determine whether knockdown of \textit{wfs2} interacts with other known lysosomal storage disease
genes in \textit{Drosophila} and if these potential interactions are observed in higher organisms.
Furthermore, studies to identify additional genes that interact with \textit{wfs2} will further elucidate
which pathway \textit{wfs2} resides in. A greater understanding of the function of \textit{wfs2} and what
pathway \textit{wfs2} resides in has implications for the management and treatment of patients with
Wolfram Syndrome.

Oxidative stress is associated with aging and age-related diseases. Antioxidant enzymes function
to decrease the amount of oxidative stress on the organism. Reduced functioning of antioxidant
enzymes is reported to decrease lifespan and causes other detrimental consequences. It is
currently unknown what genes or mechanisms protect from endogenous oxidative stress caused
by loss of function of antioxidant enzymes. Therefore, the lab performed a candidate suppressor
screen to identify genes that can serve a protective role against a model of endogenous oxidative
stress via \textit{Sod2} knockdown. Our screen identified that knockdown of \textit{DJ-1β} and expression of
human wild-type \textit{Ataxin-3} both partially extended the short lifespan due to ubiquitous
knockdown of \textit{Sod2}. The ability of \textit{DJ-1β} to mitigate the short lifespan of \textit{Sod2} knockdown flies
suggests that either \textit{DJ-1β} influences other antioxidant related enzymes or this gene could be
involved in signaling to the cell that oxidative damage is present. We further probed how
\textit{Ataxin-3} expression mitigated the short lifespan of \textit{Sod2} knockdown flies and determined that
the ubiquitin associated function of \textit{Ataxin-3} is important for extending the short lifespan.
Additionally, we determined that \textit{Ataxin-3} expression can also partially extend the short lifespan
caused by knockdown of thioredoxin reductase in muscle. \textit{Ataxin-3} is a deubiquitinating enzyme
and is involved in transporting ubiquitinated proteins to the proteasome for degradation. Therefore, expression of *Ataxin-3* in *Drosophila* may improve the removal of ubiquitin chains thereby allowing the proteasome to effectively degrade more oxidatively damaged proteins. These results suggest the function of deubiquitinating enzymes or genes involved in the ubiquitin-proteasome pathway may be very important in the regulation of removing oxidatively damaged proteins in diseases where oxidative stress is a component. Dysfunction of the ubiquitin-proteasome pathway is associated with a number of neurodegenerative diseases which further supports this hypothesis. It will be important to demonstrate that overexpression of *Ataxin-3* in mice deficient in *Sod2* or thioredoxin reductase can rescue the short lifespan or lethality observed in these animals. It would be interesting to determine if enhanced expression of deubiquitinating enzymes in *Drosophila* or genes involved in the ubiquitin proteasome pathway also extend the short lifespan of *Sod2* knockdown flies.

One possible connection with all three projects is with oxidative stress. Elevated endogenous oxidative stress has been implicated in ARLI and in neurodegenerative disease. Whether enhanced oxidative stress resistance is a requirement for delayed ARLI or whether oxidative stress could be a component to Wolfram Syndrome pathology is discussed below.

The lab determined that elevated endogenous oxidative stress influences ARLI. *Sod2* knockdown flies serve as a model of endogenous oxidative stress. Testing of the *Sod2* knockdown flies in the RING assay revealed that these flies have accelerated ARLI. Furthermore, oxidative stress is implicated in ARLI in rodents and may also be associated with ARLI in humans. We therefore investigated whether there was a connection between oxidative
stress resistance and a delay in ARLI. An increase in lifespan is correlated with an increased resistance to exogenous oxidative stress. Therefore an increase in exogenous oxidative stress resistance may also be correlated with delayed ARLI. This seemed feasible when the seven delayed ARLI mutants recovered from the forward genetic screen were all discovered to be resistant to the exogenous oxidative stressor paraquat. However, further analysis of additional insulin signaling mutants with delayed ARLI revealed that most of these strains were not resistant to paraquat. These results suggest that an enhanced resistance to exogenous oxidative stress is not required for delayed ARLI in *Drosophila*. Unlike lifespan, a delay in ARLI can be uncoupled from oxidative stress resistance. Therefore, mechanisms independent of enhanced resistance to exogenous oxidative stress contribute to the delayed ARLI observed in these insulin signaling mutants. Further analysis will reveal mechanisms that contribute to a delay in ARLI. For example, it would be interesting to determine whether enhanced immune function is important for delayed ARLI. Flies that are resistant to bacterial infection may have forestalled ARLI.

Oxidative stress is reported to be implicated in several neurodegenerative diseases including Parkinson, Alzheimer, and Huntington disease. A component of Wolfram Syndrome is progressive neurodegeneration. Many of the tissues involved in this disease have a high metabolic demand suggesting that mitochondrial dysfunction may be a component to Wolfram Syndrome. It is currently unclear whether oxidative stress contributes to the progressive neurodegeneration observed in Wolfram Syndrome patients. A knock-out mouse model of *CISD2* observed mitochondrial dysfunction in these mice. However, there was no significant difference in the levels of reactive oxygen species (ROS) in several cell lines including MEFs,
primary cortex, and primary hepatocytes in 12 week old Cisd2 knock-out mice compared to controls. Furthermore, no significant difference in the expression of enzymes that scavenge ROS including SOD1, SOD2, and catalase were identified in brain, heart, liver, and skeletal muscle tissue in 12 week old Cisd2 knock-out mice compared to controls. We also investigated whether oxidative stress could be a component to Wolfram Syndrome. Knockdown of wfs2 in flies did not result in sensitivity to a number of exogenous oxidative stresses compared to controls at 1 or 6 weeks of age. Ubiquitous knockdown of wfs2 also did not have a significant difference in Sod2 expression levels at either 1 or 6 weeks of age compared to controls. These results support the hypothesis that oxidative stress may not be a component of Wolfram Syndrome pathology. In contrast, knockdown of CISD2 in insulinoma cell lines increased the expression of the antioxidant enzymes SOD1 and SOD2. These cell lines also had an increase in nitrated tyrosines which is indicative of an oxidative stress response (Sami Amr, personal communication). These findings suggest oxidative stress may be a component of Wolfram Syndrome pathology. Therefore, it is still unclear as to whether oxidative stress is implicated in Wolfram Syndrome. However, it is possible that evaluation of ROS production or expression levels of the antioxidant enzymes in certain key tissues will reveal an oxidative stress component. Further study is required in cellular and animal models to identify whether oxidative stress contributes to the degenerative phenotypes associated with Wolfram Syndrome.

The mechanisms associated with non pathological and pathological aging appear to be different in ARLI, Wolfram Syndrome and in a model of endogenous oxidative stress. Drosophila is a very powerful model organism to identify mechanisms associated with both aging and age-related disease. A better understanding of which specific mechanisms are associated with a
delayed ARLI, further elucidation of which genes interact with wfs2 and the pathway this gene resides in, and the mechanisms involved for Ataxin-3 to suppress the short lifespan of Sod2 knockdown flies will provide insight into common or distinct genetic and cellular changes associated with non pathological and pathological aging. The discoveries in this dissertation research may provide a framework for further investigation that may aid in the development of interventions, management, therapy, or treatment options for humans afflicted with ARLI, Wolfram Syndrome, and in age-related diseases where oxidative stress is a component.
References


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Appendix:

Knock-down of \textit{wfs2} does not cause detrimental effects in \textit{Drosophila}

\begin{figure}
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\includegraphics[width=\textwidth]{figureA1}
\caption{Knockdown of \textit{wfs2} does not effect locomotor behavior across age. (A) Ubiquitous knockdown of \textit{wfs2} (Actin-Gal4/WFS2.1) did not alter locomotor performance (negative geotaxis) across age compared to controls (Actin-Gal4/+, WFS2.1/+) (two-way ANOVA, n.s.) (B) Muscle knockdown of \textit{wfs2} (mef2-Gal4/WFS2.1) did not alter locomotor performance (negative geotaxis) across age compared to controls (mef2-Gal4/+, WFS2.1/+) (C and D) Nervous system knockdown of \textit{wfs2} (188Y-Gal4/WFS2.1, appl-Gal4/WFS2.1) did not alter locomotor performance (negative geotaxis) across age compared to controls (188Y-Gal4/+, appl-Gal4/+, WFS2.1/+).}
\end{figure}
Figure A2. Knockdown of *wfs2* has no consistent effect on lifespan. (A) Survival under normal housing conditions in males was not altered by ubiquitous expression of WFS2.1 RNAi (actin-Gal4/WFS2.1) compared to controls (WFS2.1/+, actin-Gal4/+). (B) Survival in males was not altered with muscle expression of WFS2.1 RNAi (mef2-Gal4/WFS2.1) compared to controls (WFS2.1/+, mef2-Gal4/+). (C and D) Survival in males was not altered with nervous system expression of WFS2.1 RNAi (188Y-Gal4/WFS2.1 and apll-Gal4/WFS2.1) compared to controls (WFS2.1/+, 188Y-Gal4/+ , apll-Gal4/+).
Figure A3. Knockdown of wfs2 does not cause sensitivity to various stresses. (A and B) Ubiquitous knockdown of wfs2 in flies at week 1 (A) and week 6 (B) of age did not alter survival under desiccation stress compared to controls (da-Gal4/+, WFS2.1/+). (C and D) Ubiquitous knockdown of wfs2 in flies at week 1 (C) and week 6 (D) of age did not alter survival under starvation stress compared to controls (da-Gal4/+, WFS2.1/+). (E and F) Ubiquitous knockdown of wfs2 in flies at week 1 (E) and week 6 (F) of age did not alter survival under paraquat stress compared to controls (da-Gal4/+, WFS2.1/+). (G) Ubiquitous knockdown of wfs2 in flies at week 6 of age did not alter survival under thermal stress compared to controls (da-Gal4/+, WFS2.1/+).
Figure A4. wfs2 knockdown does not cause sensitivity to various exogenous stresses. (A and B) Ubiquitous (A) and nervous system (B) knockdown of wfs2 in flies at 1 week of age did not alter survival under FeCl3 stress compared to controls (da-Gal4/+, WFS2.1/+). (C and D) Ubiquitous (C) and nervous system (D) knockdown of wfs2 in flies at 1 week of age did not alter survival under H2O2 stress compared to controls (da-Gal4/+, WFS2.1/+). (E and F) Ubiquitous (E) and nervous system (F) knockdown of wfs2 in flies at 6 weeks of age did not alter survival under hyperoxia stress compared to controls (da-Gal4/+, WFS2.1/+).
Figure A5. *wfs2* knockdown does not cause sensitivity to ER stress at 1 week of age. (A and B) Survival in males was not altered by ubiquitous expression of WFS2.1 RNAi (actin-Gal4/WFS2.1, da-Gal4/WFS2.1) compared to controls (WFS2.1/+, actin-Gal4/+, da-Gal4/+). (C and D) Survival in males was not altered by nervous system expression of WFS2.1 RNAi (188Y-Gal4/WFS2.1, appl-Gal4/WFS2.1) compared to controls (WFS2.1/+, 188Y-Gal4/+, appl-Gal4/+). (E) Survival in males was not altered by muscle expression of WFS2.1 RNAi (mef2-Gal4/WFS2.1) compared to controls (WFS2.1/+, mef2-Gal4/+).
Figure A6. *wfs2* knockdown does not cause sensitivity to ER stress at 3 weeks of age. (A and B) Survival in males was not altered by ubiquitous expression of WFS2.1 RNAi (actin-Gal4/WFS2.1, da-Gal4/WFS2.1) compared to controls (WFS2.1/+, actin-Gal4/+, da-Gal4/+). (C and D) Survival in males was not altered by nervous system expression of WFS2.1 RNAi (188Y-Gal4/WFS2.1, appl-Gal4/WFS2.1) compared to controls (WFS2.1/+, 188Y-Gal4/+, appl-Gal4/+). (E) Survival in males was not altered by muscle expression of WFS2.1 RNAi (mef2-Gal4/WFS2.1) compared to controls (WFS2.1/+, mef2-Gal4/+).
Figure A7. *wfs2* knockdown does not cause sensitivity to ER stress at 6 weeks of age. (A and B) Survival in males was not altered by ubiquitous expression of WFS2.1 RNAi (actin-Gal4/WFS2.1, da-Gal4/WFS2.1) compared to controls (WFS2.1/+, actin-Gal4/+, da-Gal4/+). (C and D) Survival in males was not altered by nervous system expression of WFS2.1 RNAi (188Y-Gal4/WFS2.1, appl-Gal4/WFS2.1) compared to controls (WFS2.1/+, 188Y-Gal4/+, appl-Gal4/+). (E) Survival in males was not altered by muscle expression of WFS2.1 RNAi (mef2-Gal4/WFS2.1) compared to controls (WFS2.1/+, mef2-Gal4/+).
Melanie Ann Jones was born on October 21st, 1980 in Portland, Oregon, and is an American citizen. She graduated from Marshall High School in Portland, Oregon in 1999. She received her Bachelor of Science in Biology from Warner Pacific College, Portland, Oregon in 2003 and subsequently worked at Oregon Health Sciences University from 2003-2005 as a lab technician. She received a Doctor of Philosophy in Human and Molecular Genetics from Virginia Commonwealth University in 2010.

**Publications**


Teaching Experience

Graduate Teaching Assistant (2008)
Department of Human and Molecular Genetics
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Duties: Attending all lectures and holding review sessions with students to answer questions on the course work. Grading tests and providing input on how the class can be improved.

Honors and Awards

Roscoe D. Hughes Fellowshop (VCU) (2010)
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Graduate Student Association Travel Award (VCU) (2008)
Graduate School Travel Award (VCU) (2008)
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Graduate School Travel Award (VCU) (2007)
Science and Mathematics Service Award, Warner Pacific College (2003)