Characterizing the *spargel, nutcracker, PI31* and *FBXO9* homologues as models of

Parkinson Disease in *Drosophila melanogaster*

by

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ABSTRACT

Parkinson Disease is a progressive neurodegenerative disorder resulting from the premature destruction or improper function of dopamine producing neurons in the striatum of the brain. Symptoms include resting tremor, bradykinesia, rigidity, postural instability, gait abnormality and additional severe cognitive impairment. Although Parkinson Disease has historically been thought of as a disease with sporadic origin, there are a number of genetic links and specific gene mutations found conserved across patients. These mutations are typically found in genes responsible for the proper functioning of proteasome activity or intracellular organelle homeostasis. The upkeep and repair of mitochondria involves a number of components including *Pink1*, *Parkin*, and the Peroxisome-proliferator-activated receptor coactivator (PGC) family of genes. The PGC family of genes have a single homologue in *Drosophila melanogaster* known as *spargel*. In Chapter Two, I characterized this gene in neuronal tissues and found that altered gene activity in dopaminergic neurons leads to a decrease in longevity and locomotor ability over time, indicative of a Parkinson Disease like phenotype. In *Homo sapiens* the PGC family genes are regulated through the activity of an intermediate protein, PARIS. In Chapter Five I identified three potential homologues of the *PARIS* gene in *D. melanogaster* and compared altered expression of them in neuronal tissues resulting in the identification of a strong *PARIS* candidate and two novel genes involved in neuronal development. The proteasome complex acts upon and destroys proteins targeted for destruction by addition of a ubiquitin moiety. This process is undertaken by ubiquitin ligase complexes. The target specific component of many of these complexes

are the F-box genes. Chapters Three and Four characterize two F-box genes implicated in

H. sapiens PD and identifies putative *D. melanogaster* gene homologs.   The results of

this thesis provide expanded knowledge of both confirmed and putative *D. melanogaster*

homologues of *H. sapiens* disease related genes and new model systems with which

future study of disease mechanisms may be carried out.

ACKNOWLEDGEMENTS

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List of Symbols, Nomenclature or Abbreviations

AD   Alzheimer Disease

ALS   Amyotrophic Laterial Sclerosis

ATP   Adenosine Triphosphate

ATP13A2   ATPase Type 13A2

CASH   Carbohydrate binding proteins and sugar hydrolases

cMyBP-C   Cardiac Myosin-Binding Protein C

DA   Dopaminergic

eIF3-F   Eukaryotic Translation Initiation Factor 3 subunit F

EIF4G1   Eukaryotic translation Initiation Factor 4 Gamma 1

GFY   Golgi-associated, olfactory signaling regulator

GRB10   Growth factor Receptor Bound protein 10

GIGFY2   GIB10 Interacting GFY protein 2

HD   Huntington Disease

HECT   Homologous to the E6-AP Carboxyl Terminus

HTRA2   High Temperature Requirement A serine peptidase 2

HURP   Hepatoma Up-Regulated Protein

KAP-1   Kruppel Associated Protein-1

KRAB   Kruppel Associated Box

LBs   Lewy Bodies

LRR   Leucine Rich Repeat

LRRK2   Leucine Rich Repeat Kinase 2

Mfn2	<u>M</u>ito<u>f</u>usi<u>n</u>2
MIT	<u>M</u>icrotubule <u>I</u>nteracting and <u>T</u>rafficking Domain
MS	<u>M</u>ultiple <u>S</u>clerosis
mtDNA	<u>mit</u>ochondrial DNA
MyoD	<u>My</u>osin <u>D</u>
NLS	<u>N</u>uclear <u>L</u>ocalization <u>S</u>equence
NRF-1	<u>N</u>uclear <u>R</u>espiratory <u>F</u>actor-<u>1</u>
NRF-2	<u>N</u>uclear <u>R</u>espiratory <u>F</u>actor-<u>2</u>
ntc	nutcracker
PD	<u>P</u>arkinson <u>D</u>isease
PDZ	<u>P</u>ost synaptic density <u>*Drosophila*</u> disc large tumor suppressor and <u>Z</u>onula occludens-1
PGC	<u>P</u>eroxisome proliferation activated <u>C</u>o-receptor <u>G</u>amma
PI31	<u>P</u>roteasome <u>I</u>nhibitor <u>31</u>
PINK1	<u>PTEN</u> <u>I</u>nduced <u>P</u>utative <u>K</u>inase <u>1</u>
PLA2G6	<u>P</u>hospho<u>li</u>pase <u>A</u>2, group <u>6</u>
PPAR-γ	<u>P</u>eroxisome <u>P</u>roliferator-<u>A</u>ctivated <u>R</u>eceptor <u>G</u>amma
PPS	<u>P</u>arkinson <u>P</u>yramidal <u>S</u>yndrome
PRR	<u>P</u>roline <u>R</u>ich <u>R</u>egion
PTEN	<u>P</u>hosphatase and <u>Ten</u>sin homologue
RING	<u>R</u>eally <u>I</u>nteresting <u>N</u>ew <u>G</u>ene
RNF11	<u>R</u>ing <u>F</u>inger Protein <u>11</u>

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ROS	Reactive Oxygen Species
SCF	Skip-Cullin-F-box
s-IMB	sporadic Inclusion Body Myositis
SNCA	Synuclein Alpha
TRS	Tetratricopeptide Repeat Sequence
UAS	Upstream Activating Sequence
UCHL1	Ubiquitin Carboxyl-terminal Hydrolase isozyme L1
UPS	Ubiquitin Proteasome System
VPS35	Vacuolar Protein Sorting 35
WD40	Tryptophan-Aspartic acid 40
ZAD	Zinc Associated Domain

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Appendix 5 – Additional Published Works

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Merzetti, Brian E. Staveley. Neuroscience Discovery 2013. DOI:

http://dx.doi.org/10.7243/2052-6946-1-8

S5.2. Thinking Inside the Box: *Drosophila* F-Box Protein Models of Human Disease.

Eric M. Merzetti, Colleen B. Connors, Brian E. Staveley. Journal of Biology 2013.

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Chapter 1 – Introduction and Overview

1.1. Mediating Cell Survival and Cell Death

Whole organism survival and longevity depends largely on the ability of individual cells to adapt and respond to stressful conditions. When stress is placed upon a cell, either from external trauma or the impaired function of internal components, pro-survival pathways are activated. Pro-survival pathways seek to restore normal cell functionality by identifying and relieving cellular stressors and include anti-oxidant compounds, heat shock proteins, proteasomal machinery and elements such as the unfolded protein response of the endoplasmic reticulum (Jager *et al.*, 2012; Samali & Cotter, 1996; Trachootham *et al.*, 2008). If a stress is severe enough and repair is not possible, cell death pathways will be activated. Cell death can occur in a variety of ways through programmed modes of cell death, such as apoptosis and autophagic cell death to necrosis (Ashkenazi & Salvesen, 2014). This can affect not just the cell in question but also surrounding cells, and the final decision to undergo cell death can come from internal or external sources.

When the pro-survival mechanisms of a cell are unable to alleviate a stressful condition and cell death signals are activated there is a negative consequence on the whole organism as a result of premature cell death such as in the case of mitochondrial mediate apoptotic cascades (Estaquier *et al.*, 2012). Despite this, it is more favourable for a single cell to be sacrificed than for neighbouring cells to be subjected to the initial cause of stress in a process known as cell altruism (Allsopp & Fazakerley, 2000). The

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continued existence of dysfunctional cells can have more severe consequences. Mutation to a number of genes involved in these stress response pathways have been linked to disease pathogenesis. Failure to properly mediate the pathways of cell survival and cell death can lead to a number of undesired consequences including whole organism mortality. Understanding and characterizing the genes responsible for the upkeep and proper function of these mechanisms may lead to preventative treatment and new therapeutic options for existing conditions related to the dysfunction of survival and death pathways.

1.1.1. Premature Cell Death and Degenerative Disease

Degenerative diseases are progressive chronic conditions that may cause a deterioration of a tissue or group of cells over time. This accumulated premature loss of cells can be broken down into two specific branches: neurodegenerative and muscular degenerative. Common neurodegenerative diseases include Parkinson Disease (PD), Alzheimer Disease (AD), Huntington Disease (HD), and Multiple Sclerosis (MS), while the most common muscular degenerative diseases are Sporadic Inclusion-Body Myositis (s-IBM) Spinal Muscle Atrophy (SMA) and Amyotrophic Lateral Sclerosis (ALS) (Merzetti & Staveley, 2013; Sendtner, 2014). The progressive nature of these diseases has resulted in the development of therapeutic agents. Unfortunately, most of these agents aim to reduce symptom severity and do not address the underlying cause of disease.

Originally, the cause of degenerative disease was linked primarily to environmental factors (Chin-Chan *et al.*, 2015). New advances in whole genome

sequencing technologies and genetic approaches have shown that many common genetic

risk loci are found in patients with these diseases and that many of them are directly

inheritable. To better understand the initial cause of disease, it is essential to determine

what genes or metabolic pathways are commonly affected in inherited variants. Improper

function of cellular pathways including mitochondrial maintenance and upkeep,

autophagy and proteasome function caused by specific gene mutations have been

implicated in disease, (Bajic *et al.*, 2012; Lionaki *et al.*, 2015; Menzies *et al.*, 2015).

Determining the genes and pathways related to these processes that contribute to

degenerative disease may lead to new options for treatment and prevention.

1.1.2. Parkinson Disease

PD is a common and progressive neurodegenerative condition that is estimated to

afflict 1% of all individuals over the age of 60 worldwide (Lew, 2007). The initial cause

of sporadic PD dysfunction is unknown. Clinical symptoms include resting tremor,

bradykinesia, rigidity and postural instability. In addition to these classical symptoms,

recent discoveries have shown that additional effects may include loss of memory and

depression as the number of areas in the brain affected by degeneration increase

(Williams-Gray *et al.*, 2006; Wirdefeldt *et al.*, 2011). These symptoms are caused by a

decrease in the amount of the neurotransmitter dopamine available in the striatum area of

the brain and characterized by an accumulation of harmful protein aggregates known as

Lewy bodies in the neurons of the *substantia nigra pars compacta* and destruction of

neurons in this region (Olanow & McNaught, 2011). It is believed that the eventual

dysfunction and breakdown of these neurons is responsible for the symptoms and

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pathology of PD (Bekris *et al.*, 2010). The pathways leading to this neuronal breakdown are not yet fully elucidated and, as such, the exact cause of PD is not currently understood.

Pathogenesis of PD occurs by disrupting the neurotransmitter mediated signal cascade from the prefrontal cortex to the motor and emotional control areas found in the midbrain. Destruction of neuronal tissues in the *substantia nigra pars compacta* leads to a decreased amount of dopamine being present across the striatum (Obeso *et al.*, 2008). Decreased dopamine availability leads to diminished cellular response and eventual loss of motor control. As disease progression continues, additional areas of the brain involved in processes such as emotional control and memory are also affected by this gradual loss of neurons, resulting in the non-motor symptoms common in late stage PD (Narayanan *et al.*, 2013). Although dopamine replacement therapy is the most common approach to treating PD, this therapy does not stop disease progression and the early mortality associated with PD remains.

It was initially believed that PD was caused by a number of environmental factors, including chemical exposure, brain trauma, obesity, diabetes and age (Vanitallie, 2008). Although these stressors may lead to sporadic forms of PD, there is also a great deal of evidence to show that many forms of PD are caused by genetic factors (Bereznai & Molnar, 2009). Since the sequencing of the human genome, efforts have been made to determine what genetic risk factors may make a patient more susceptible to disease onset in response to environmental factors. A comparison of whole genomes of PD patients has revealed a number of genetic risk variants not found in control groups (Labbe & Ross,

2014). The current belief is that PD may be caused solely by inherited or environmental means, but the majority of cases likely stem from a combination of genetic risk factors and environmental exposure.

At a molecular level, the loss of dopaminergic neurons (DA) may be directly or indirectly caused by a number of factors, including, but not limited to cellular stress and oxidation, impaired cellular dynamics, signal cascade dysfunction, or improper functioning organelles (Choi *et al.*, 2011; Levy *et al.*, 2009; Pedrosa & Soares-da-Silva, 2002). Genes responsible for a number of diverse yet important cellular functions, such as ubiquitination, cellular dynamics, proteasomal activity and biogenesis of new organelles, have been implicated in PD; Table 1 shows a list of these genes and their "Park" family designation (a nomenclature used to denote their putative link to PD). Although these genes are diverse in nature, they share a number of similar functions including ubiquitin ligase activity, serine protease activity, protein transport, neuroprotective effects, tyrosine kinase signaling and initiation of translation (Anderson *et al.*, 2007; Kinghorn *et al.*, 2015; Labbe & Ross, 2014; Orenstein *et al.*, 2013; Park *et al.*, 2014; Ragland *et al.*, 2009; Ross *et al.*, 2015; Ruiz-Martinez *et al.*, 2015; Vande Walle *et al.*, 2008; Xiu *et al.*, 2013; Zondler *et al.*, 2014). Identifying the functions of orthologous genes in a model organism may lead to new therapeutic targets and better understanding of PD.

Table 1: Common Parkinson Disease genes and functions in *Homo sapiens*.

Name	Inheritance	Gene	Function
PARK1	Autosomal Dominant	SNCA	Synaptic vesicle supply, forms Lewy Bodies
PARK2	Autosomal Recessive	Parkin	E3 ubiquitin ligase
PARK3	Risk-Factor	FBXO48	E3 ubiquitin ligase
PARK4	Autosomal Dominant	SNCA	Synonym for *PARK1*
PARK5	Autosomal Dominant	UCHL1	C-terminal hydrolase deubiquitinating enzyme
PARK6	Autosomal Recessive	PINK1	Serine / threonine-protein kinase, recruits
PARK7	Autosomal Recessive	DJ-1	Neuroprotective role, prevents *α-synuclein*
PARK8	Autosomal Dominant	LRRK2	Macroautophagy, chaperone-mediated
PARK9	Autosomal Recessive	ATP13A2	Zinc homeostasis
PARK1	Risk-Factor	RNF11	E3 ubiquitin ligase (unconfirmed candidate)
PARK1	Autosomal Dominant	GIGYF2	Tyrosine kinase receptor signaling
PARK1	Risk-Factor	unknown	Unknown
PARK1	Autosomal Dominant	HTRA2	Mitochondrial serine protease
PARK1	Autosomal Recessive	PLA2G6	Mitochondrial lipid balance
PARK1	Autosomal Recessive	FBXO7	E3 ubiquitin ligase
PARK1	Risk-Factor	unknown	Unknown
PARK1	Autosomal Dominant	VPS35	Protein transport
PARK1	Autosomal Dominant	EIF4G1	Translation initiation factor

1.1.3. The Role of Mitochondria in Parkinson Disease

Mitochondria are important and necessary components of nearly all eukaryotic cells. They are often referred to as the "power plant" of the cell due to their function in the creation of adenosine triphosphate (ATP) for use as chemical energy. Mitochondria also have additional important functions in cellular growth and death signal control, cellular signaling, and differentiation (Campbell, 2006). With mitochondrial involvement in such diverse and important cellular functions, it is not surprising that the breakdown or dysfunction of mitochondria may result in a number of disorders, including, but not limited to neuro-, and musculo degenerative diseases (Wallace & Fan, 2009). Many of these disorders are caused by defective removal of damaged or non-functional mitochondria and a subsequent lack in *de novo* synthesis of replacement organelles (Huang & Manton, 2004). Thus, characterizing and understanding the function of the genes that regulate and monitor the health of these mitochondria is an essential step in determining potential disease prevention strategies.

Mitochondria have a number of characteristics that make them key intermediaries in the pathogenesis of many forms of degenerative disease. They are the site of chemical energy production through the activity of five protein complexes located within the inner mitochondrial membrane. This process often results in electron leakage which, in turn, leads to the formation of toxic reactive oxygen species (ROS) (Rigoulet *et al.*, 2011). These ROS are produced in the mitochondrial matrix. Mitochondrial DNA (mtDNA) is also found in the matrix and the close proximity of these ROS and easily damaged mtDNA can lead to organelle dysfunction and breakdown. In order to combat this

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potential destruction, mitochondria are equipped with intra-organellular pathways that lead to apoptosis when severe dysfunction or ROS buildup is detected (Sinha *et al.*, 2013). Although these apoptotic pathways are in place to prevent the spread of harmful material to neighbouring cells, they also result in premature cell death which in many tissues ultimately causes degenerative disease phenotypes.

There are a number of gene variants that have been identified in *Homo sapiens* cases of PD that show importance in mitochondrial function. One of the most studied pathways relating to mitochondrial upkeep and turnover is the *Pink1/Parkin* pathway (Figure 1) (Eiyama & Okamoto, 2015). PARKIN (PARK2) is a component of a multi-protein E3 ubiquitin ligase that leads to the ubiquitination and subsequent destruction of cellular components (Narendra *et al.*, 2008). Mutations to the *Parkin* gene result in the degeneration of DA neurons, proposed to be due to the aggregation of multiple dysfunctional mitochondria that eventually lead to apoptotic cell death (Greene *et al.*, 2003). *Phosphatase and tensin homologue induced putative kinase 1* (*PINK1*, *PARK6*) is a serine / threonine-protein kinase that acts by recruiting PARKIN to damaged mitochondria (Koh & Chung, 2011). Like *Parkin*, mutations to *PINK1* lead to degeneration and dysfunction of DA neurons (Yang *et al.*, 2006). A third player in this pathway, *Mitofusin2* (*Mfn2*), acts as the mediator between the PARKIN and PINK1 proteins (Pallanck, 2013). MFN2 is an outer membrane protein that functions in the process of mitochondrial fusion (Santel & Fuller, 2001). PARKIN recruitment is dependent upon the phosphorylation of MFN2 by PINK1 (Y. Chen & Dorn, 2013). These proteins act together in concert to remove any damaged or dysfunctional

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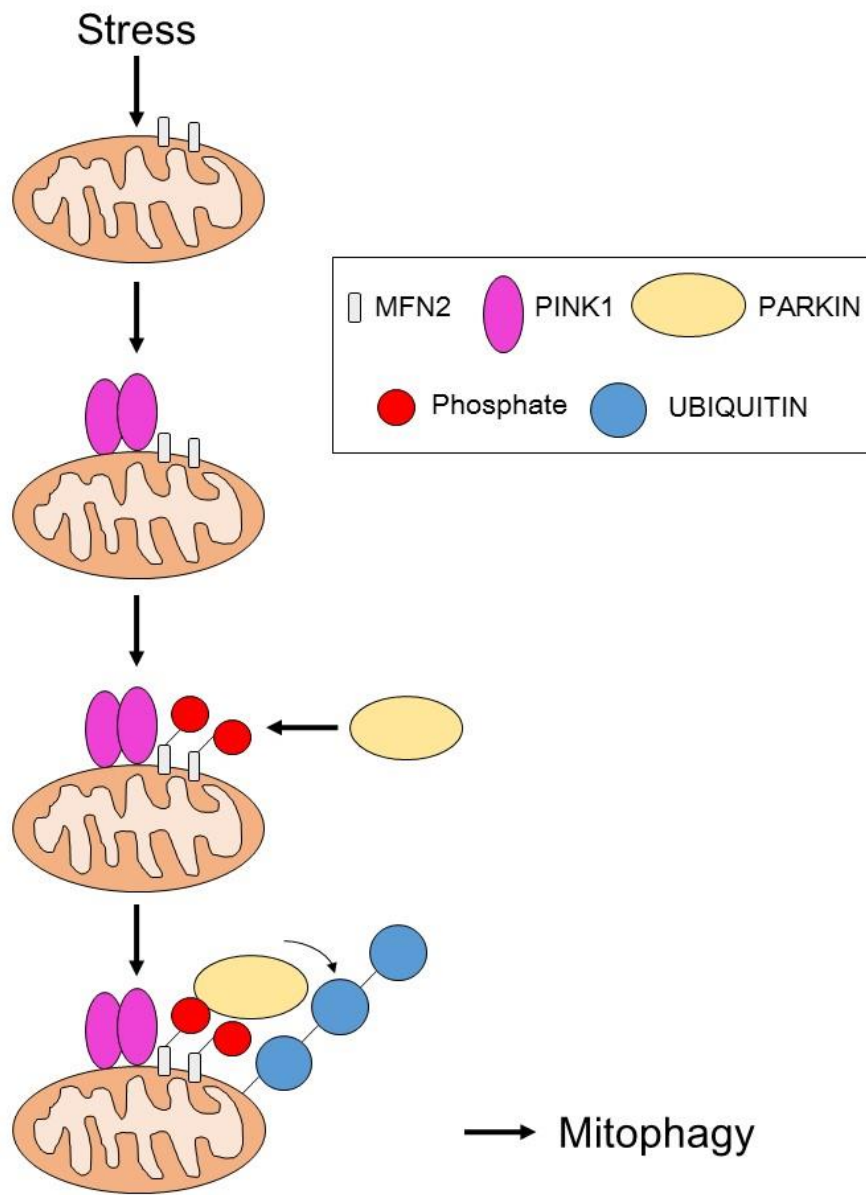
Figure 1: The *Parkin / Pink1 / Mfn2* pathway of mitophagy. Under conditions of stress mitochondria undergo a change in polarization causing an accumulation of PINK1 at the outer membrane. PINK1 phosphorylates MFN2 which in turn attracts PARKIN. PARKIN, through activity as an E3 ubiquitin ligase, marks the damaged mitochondria for destruction. This process results in mitophagy.

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mitochondria that may be present in a process known as mitophagy. Mutation in either of these genes negates the cell's ability to target and remove dysfunctional mitochondria which in turn leads to cell death.

1.1.4. The Ubiquitin Proteasome System in Parkinson Disease

The ability to respond and adapt to traumatic events within a cell is an essential and necessary part of homeostasis. Damaged cellular components must be either repaired or removed to prevent the premature destruction of the cell as a whole. There are specific components of the cell responsible for marking abnormal or impaired proteins for destruction. One such marker is addition of the protein ubiquitin. Ubiquitin is a 76 amino acid peptide which contains 7 lysine residues, allowing for the addition of single or multiple ubiquitin residues to a protein of interest (Wilkinson, 2005). The addition of ubiquitin moieties is mediated by a complex known as the Ubiquitin Proteasome System (UPS), while the process of protein destruction is undertaken by the 26S proteasome (Atkin & Paulson, 2014). Impairment or failure of either the UPS or 26S proteasome leads to a buildup of dysfunctional proteins within the cell and may eventually cause death.

The UPS works through the action of three enzymes named E1, E2 and E3. E1, known as the activating enzyme, creates a thioester bond between a cysteine moiety of the E1 enzyme and the ubiquitin protein via ATP hydrolysis. This ubiquitin protein is then transferred to E2, known as the conjugating enzyme, once again at an active site cysteine. The final step sees the E3 ligating enzyme catalyze attachment of the ubiquitin to a lysine of the target protein (Wilkinson, 2005).   In this system, the E3 ligating
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enzymes are in highest abundance as they are responsible for targeting the protein of interest. They may be broken down into two main classes: Homologous to the E6-AP Carboxyl Terminus (HECT) and Really Interesting New Gene (RING) domain E3s (Metzger *et al.*, 2012). The main difference between HECT and RING E3 ubiquitin ligases lies in the mode of ubiquitin transfer. While HECT domain E3s will directly bind the ubiquitin moiety via thioester linkage, the RING family E3s catalyze the addition of ubiquitin by directing the E2 enzyme toward the protein to be targeted via a zinc binding motif (Huibregtse *et al.*, 1995; Lorick *et al.*, 1999). Mutation to any of the components in this process leads to a buildup of protein aggregates due to a reduced efficiency of proteolysis.

The accumulation of harmful protein aggregates is a common hallmark of neurodegenerative disease and a number of inherited forms of PD have been linked to defects in UPS function. Additionally, it has been found that impaired UPS activity causes a loss of synaptic connections in a dose dependent manner, causing symptoms prior to actual tissue loss (Bajic *et al.*, 2012; Ehlers, 2003). Of the 18 *PARK* genes, 5 have functions related to the UPS, with 4 of those being E3 ubiquitin ligases (Table 1). *Pink1* and *Parkin* are two of these genes and have been discussed in detail above, while the other two are *FBXO7* and *FBXO48*. F-box proteins function as part of the multi-protein Skp1-Cullin1-F-box (SCF) ubiquitin ligase complex (Cardozo & Pagano, 2004). This specific sub-family of UPS ligases will be discussed at length in section 1.3. The fifth *PARK* gene related to UPS function is *Synuclein alpha* (*SNCA*).

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Mutation to the *SNCA* gene (Park 1/4), which codes for the α-SYNUCLEIN protein, leads

to autosomal dominant forms of PD and aggregate protein clusters (Deng & Yuan, 2014).

These intracellular proteinaceous inclusions have been named Lewy Bodies (LBs)

(Takeda *et al.*, 2008). LBs are protein aggregates consisting mainly of α-SYNUCLEIN

protein polymers bound to ubiquitin molecules (Baba *et al.*, 1998). α-SYNUCLEIN is a

small soluble protein capable of binding to many intracellular components, including the

microtubule stabilizing molecule TAU, due to its small amphipathic structure (Payton *et

al.*, 2001). In addition to being present in the neurons of many PD affected individuals,

mutation of the *α-synuclein* gene has been shown to directly lead to dominant early onset

PD (Polymeropoulos *et al.*, 1997). The role of aggregate α-SYNUCLEIN and how

accumulation of these LBs leads to improper cell function and eventual neuronal death is

currently under investigation.

LBs may contribute to the overall instability of internal cellular components

leading to increased cellular oxidative stress, decreased organelle movement and

disrupted protein function (Jenner & Olanow, 1998). Neuronal cells are especially

vulnerable to reduction in energy due to their high energetic requirements and disruption

of mitochondrial dynamics due to these protein clusters may be a significant sign of

impending cell destruction (Detmer & Chan, 2007). It is of particular interest that recent

evidence has been found supporting the theory that these LBs may be able to move

between neurons, perhaps presenting a prion-like mode of pathology in disease

progression (George *et al.*, 2013). Although cells are equipped to deal with abnormal

protein aggregates by means of proteosomal machinery, these LBs seem to remain intact

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in models of disease, suggesting that improper function of intracellular proteasomal components may be one of the first steps in disease progression.

1.1.5. *Drosophila melanogaster* as a Model of Parkinson Disease

The study of human degenerative disease is complicated by many factors, including long term disease development, gene redundancy and ethical constraints. As a result, many of the current treatments for degenerative disorders focus on alleviation of symptoms and do not address the underlying cause of disease. Studying these complex diseases in an organism such as *D. melanogaster* alleviate many of these constraints and offer a model of disease to identify and characterize genetic pathways conserved between organisms. Functional homologues of approximately 75% of human disease genes have been characterized in *D. melanogaster* and it is possible that uncharacterized disease homologues may still be discovered (Pandey & Nichols, 2011). *D. melanogaster* are small and easy to culture with a generation time of 10 days at 25°C, which allows quick generation of desired genotypes as well as a relatively small window to investigate differences in lifespan. Insertion of foreign genes into the genome of *Drosophila* via the Gal4-UAS transgene system is particularly useful (Brand & Perrimon, 1993) (Figure 2). The Gal4-UAS system uses the yeast transcription factor target, Upstream Activating Sequence (UAS), to drive expression of a gene of interest under control of the yeast transcription factor Gal4, which is under the control of a tissue specific enhancer. The Gal4-UAS system can be used to ectopically express genes of interest or to determine the effect of a transcript knockdown via RNAi expression. This is a powerful tool that allows characterization of altered gene function, not just in a whole organism but in individual
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tissues associated with disease by the expression of targeted, double stranded RNA that is converted into an active form by the enzyme DICER and introduced to the RNA induced silencing complex where it is converted into an active inhibitor form(Clemens *et al.*, 2000).

Although the nervous system of *D. melanogaster* is, as a whole, less complex then that of *H. sapiens*, a number of important pathways in cancer, immunity and development are conserved (Pires-daSilva & Sommer, 2003). Furthermore, the ectopic expression of mutant gene products that cause inherited *H. sapiens* diseases such as polyglutamine repeats have been modelled in *D. melanogaster* (Krench & Littleton, 2013). Thus, *D. melanogaster* has been used as an efficient and inexpensive model of other neurodegenerative diseases including PD (Baba *et al.*, 1998). DA neuron production of dopamine causes similar downstream effects in both *D. melanogaster* and *H. sapiens* including wakefulness, sleep, arousal, learning behaviors and motor coordination (Yamamoto & Seto, 2014). A decrease in locomotor ability and longevity has been associated with a decrease in activity of dopamine precursors in *D. melanogaster*, providing a link between observed *H. sapiens* phenotypes and fly consequences of DA expression loss (Wills *et al.*, 2010). Taken together, these previous findings make the DA neurons of *D. melanogaster* an ideal system to assay *H. sapiens* neurodegenerative disease and expected phenotypes of disease causing genes would be a decrease in longevity, impaired locomotor ability and neuronal destruction.

In addition to DA neurons which may be assayed for degenerative phenotypes based on similarities between species, the neuron-rich *Drosophila* eye is a highly

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sensitive system to determine the effects of gene alteration on neuronal cells (Tsachaki &

Sprecher, 2012). Two well defined tissues formed from neuronal precursors compose the

outer layer of the eye: ommatidia and bristles (Cook *et al.*, 2011). Standard *D.*

melanogaster compound eyes consist of between 750-800 ommatidia and 575-625 inter-

ommatidia bristles (Ready *et al.*, 1976). Ommatidia contain pre-determined array of

photoreceptor cells that have been shown to be susceptible to neurodegenerative effects

(Colley, 2012). Thus, it is possible to assess the effect of mutations in genes related to *H.*

sapiens neurodegenerative disease in *D. melanogaster* by eye specific expression. This

technique offers two distinct advantages: the first is that the phenotypes of destruction

associated with neurodegeneration in the eye are readily observable with a scanning

electron microscope, the second is that the fly develops normally in all other aspects as

the eye is a dispensable organ for survival (Sang & Jackson, 2005). Finally, multiple

Drosophila genomes have been sequenced, this allows the use of bioinformatics analyses

to determine regions of fly proteins that correspond to known human proteins (A. G.

Clark *et al.*, 2007). Taken together, these advantages make *D. melanogaster* an excellent

organism to study complex human degenerative disease such as PD.

Although *D. melanogaster* provides a number of advantages as a model organism

for modeling neurodegenerative disease it also comes with a number of disadvantages to

performing assays in human cell culture or PD patients. One important consideration is

that there is no readily quantifiable measure of complex behavior in flies and so the

neurodegenerative phenotypes associated with gene dysfunction can only infer that there

is an effect, not how that effect comes about (Prussing *et al.*, 2013). Models of *H. sapiens*

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disease in lower organisms also often results in therapeutic effects not conserved across

species, however, this effect is also found in *H. sapiens* cell culture.
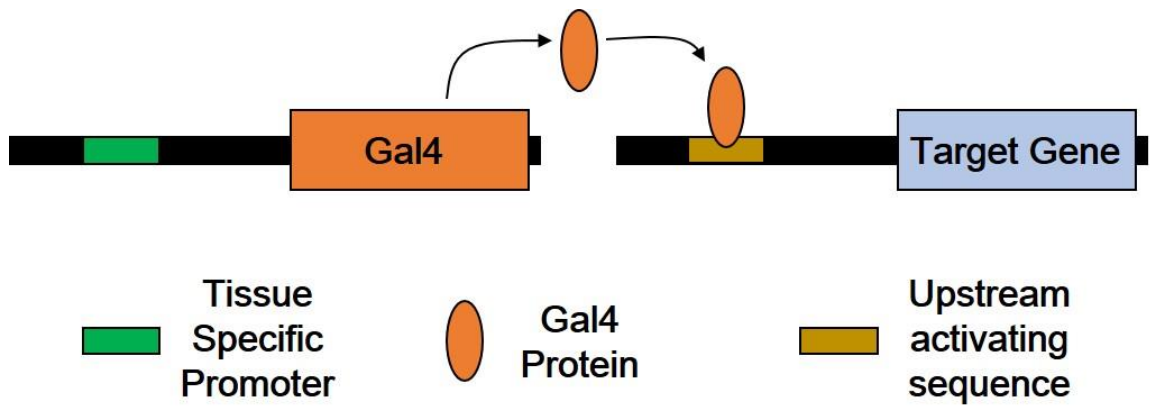
Figure 2: The Gal4-UAS transgene system of *Drosophila*. The insertion of two *Saccharomyces cerevisiae* genes into *Drosophila* allows specific gene expression. The transcription factor, Gal4, causes an increase in transcription of any gene inserted downstream of the target sequence UAS. Insertion of the Gal4 gene downstream of a promoter of interest and the UAS upstream of a gene of interest allows the promoter specific expression of that gene.

1.2. The PGC Family of Proteins

The peroxisome proliferation activated co-receptor gamma (PCG) family of genes have been characterized as regulatory transcription factors in pathways of mitochondrial biogenesis (Krempler *et al.*, 2000). Functionally, they interact with the nuclear peroxisome proliferator-activated receptor gamma (PPAR-γ) and act as docking platforms for additional proteins to remodel chromatin and initiate the assembly of basal transcription machinery leading to increased gene expression (Baar, 2004). Mutant PGC proteins have been implicated as causative factors in a number of diseases including AD, HD, PD, and stroke (Chen *et al.*, 2012; Johri *et al.*, 2013). The PGC family contains three genes with partial functional redundancy: *PGC-1α*, *PGC-1β* and *PRC* (also known as *PGC-1γ*). While both *PGC-1α* and *PGC-1β* are expressed predominantly in tissues with high energy requirements, *PRC* is expressed equally across various tissues (Andersson & Scarpulla, 2001). Although *PGC-1α* and *PGC-1β* show similar expression patterns, *PGC-1α* is upregulated under conditions of stress, such as endurance exercise, while *PGC-1β* is not (Meirhaeghe *et al.*, 2003; Mortensen *et al.*, 2007). This suggests that *PGC-1β* has a role in the basal level of gene expression in tissues with high energetic requirements, while *PGC-1α* expression is likely more dependent upon stress related activation.

1.2.1. *PGC-1α*, the Stress Responsive PGC Family Member

Of the three PGC proteins, *PGC-1α* is the most likely to be altered in models of disease based on its upregulation in conditions of stress. This effect has been documented

in a number of tissues; it is upregulated in response to adrenergic stimulation in cold

exposure to initiate a shivering response, induced in muscle fibers during endurance

exercise, and dramatically up-regulated in conditions of fasting (Baar *et al.*, 2002;

Puigserver *et al.*, 1998; Yoon *et al.*, 2001). *PGC-1α* has also been found to be involved

in pathways of mitochondrial biogenesis in various tissues including the liver and brain

(Uldry *et al.*, 2006). Medical data have shown that certain *PGC-1α* polymorphisms in

human populations are linked to a higher risk or earlier age of onset of PD symptoms

(Clark *et al.*, 2011). The *PGC-1α* mediated mitochondrial biogenesis pathway involves

the activity of 5 genes. *PGC-1α* initiates activity of a downstream transcription factor,

Nuclear Respiratory Factor-1 (*NRF-1*) (DeSimone *et al.*, 1996). A second mitochondrial

transcription factor, *Nuclear Respiratory Factor-2* (*NRF-2*), has been found to operate in

a parallel pathway to *PGC-1α,* also leading to mitochondrial growth and biogenesis

(Tiefenbock *et al.*, 2009).

Transcriptional regulation of *PGC-1α* is controlled by a negative regulator, *Parkin*

Interacting Substrate (*PARIS*). When *PARIS* is present, it binds to the upstream region of

the *PGC-1α* gene and prevents transcriptional activity from occurring. This prevents

PGC-1α transcription and reduces the amount of mitochondrial biogenesis of the cell

(Castillo-Quan, 2011). The PARIS protein consists of two distinct motifs, an N-terminal

Kruppel Associated Box (KRAB) domain and a C-terminal C2HC/C2H2 zinc-finger

binding domain (Shin *et al.*, 2011). Proteins containing KRAB domains have commonly

been found to act as transcriptional repressors via interaction with Kruppel Association

Protein-1 (KAP-1) (Witzgall *et al.*, 1994). Work by Shin *et al*. (2011) found the

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consensus DNA binding sequence target of PARIS to be the insulin response element

TATTTT(T/G), which is most often found upstream of genes involved in energy

metabolism including *PGC-1α* (Mounier & Posner, 2006). The PARIS protein was

originally isolated by a yeast two-hybrid screen using PARKIN as bait. Further analysis

of the interaction between these two proteins found that PARKIN is responsible for the

ubiquitin dependent control of PARIS levels in the cell (Shin *et al.*, 2011). This newly

discovered role in mitochondrial biogenesis in tandem with its activity as a mediator of

mitochondrial upkeep and turnover shows *Parkin* as a master regulator of overall

mitochondrial concentration in the cell and a necessary component of the *PGC-1α*

pathway.

1.2.2. The *Drosophila melanogaster* PGC Family Homologue, *spargel*

The three members of the PGC family in mammals are transcription factors that

stimulate the same response elements. Furthermore, PRC is expressed at a basal level in

almost all tissues while *PGC-1β* and *PGC-1α* are expressed at a basal level in all tissues

with high energy requirements (Andersson & Scarpulla, 2001; Kamei *et al.*, 2003). This

overlapping pattern of expression makes the study of altered PGC family function

difficult. Although studies in mice have focused on identifying the differences between

these genes by using a double-knockout gene approach, elimination of all three genes is

lethal (Lai *et al.*, 2008). There is only a single PGC family homologue found in

Drosophila melanogaster, *spargel* (*srl*). *spargel* mutant flies have a "lean" phenotype

that is typical of mutations that affect growth and proliferation, and have been shown to

affect mitochondrial fitness *in vivo* (Tiefenbock *et al.*, 2009). Similar to *PGC-1α*, *srl*

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activity has been linked to pathways of mitochondrial biogenesis and insulin signaling, exercise endurance and has been shown to interact with the *D. melanogaster* homologues of *Parkin* and *Pink1* (Mukherjee & Duttaroy, 2013; Tiefenbock *et al.*, 2010; Tinkerhess *et al.*, 2012). Characterizing the consequence of altered gene function of *srl* in the neurons of *D. melanogaster* will provide additional insight into the mechanisms and common pathways shared with the *Homo sapiens PGC-1α* homologue.

1.3. SCF Ubiquitin Ligase Complexes and F-box Proteins

Skp1-Cullin1-F-box (SCF) ubiquitin ligase complexes are the most common class of RING E3 ubiquitin ligases and are formed by the binding of CULLIN1 to ROC1, SKP1 and an F-box protein (Lyapina *et al.*, 1998). The activity of this multi-protein complex can be increased or decreased by the binding of co-factors to the C-terminal of the CULLIN1 protein, which provides an important role in regulating function (Duda *et al.*, 2008). Of all the components of this complex, perhaps the most important are the F-box proteins, which convey substrate specificity for ubiquitination and through the activity of stabilizing co-factors respond to changes in cellular landscape and various stimuli (Lee & Diehl, 2014). Mutation to components of SCF ubiquitin ligase complexes has been implicated in a number of disease models due to the breakdown and decrease in cellular proteasomal activity associated with a lack of ubiquitination (Skaar *et al.*, 2013). Understanding how specific proteins function within this complex, specifically the targeting F-box proteins, is an important step in determining how to combat disease causing mutations.

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F-box proteins are named after Cyclin F, the first F-box gene to be discovered, and are characterized by a specific and highly conserved C-terminal F-box domain approximately 50 amino acids in length (Bai *et al.*, 1994). Classification of F-box proteins is based upon the substrate interacting domain present in each protein. There are three classes of these domains: the WD40 repeat domain, the leucine rich repeat domain (LRR) and a third class consisting of all domains that do not fit into the first two termed the F-box other or "O" domain. F-box WD40 repeat domains contain a short motif of approximately 40 amino acids ending with a tryptophan and aspartic acid residue, and are referred to as FBXW proteins (Neer *et al.*, 1994). LRR domains are characterized by a repeat of leucine residues, which functions as an important staging ground for protein-protein interactions, and are designated FBXL proteins (Kobe & Deisenhofer, 1994). FBXO proteins contain variable interacting regions, including carbohydrate binding proteins and sugar hydrolase (CASH), post synaptic density *Drosophila* disc large tumor suppressor and Zonula occludens-1 protein (PDZ), proline-rich and zinc finger domains (Cardozo & Pagano, 2004). Although they differ widely in the composition of interacting domains, all F-box proteins serve the same targeting function in SCF ubiquitin ligase complexes.

1.3.1. The FBXO7, FBXO9 and FBXO32 Sub-family of F-box Proteins

A number of F-box genes have been implicated in human disease. Almost all have links to growth pathways that when mutated can cause abnormal growth and cancerous phenotypes but one specific sub-set of the FBXO family has been implicated specifically in degenerative conditions (Merzetti *et al.*, 2013). This sub-family contains
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three genes: *FBXO7*, *FBXO9* and *FBXO32*, however, to date only two of them have been

directly linked to degenerative phenotypes (Cenciarelli *et al.*, 1999). Mutation to the

FBXO7 (also known as *PARK15*) gene has been linked to a severe early onset form of PD

known as Parkinson Pyramidal Syndrome (PPS) (Di Fonzo *et al.*, 2009). Mutation to the

FBXO32 (also known as *Atrogin-1*) gene causes impaired function of cardiac myosin-

binding protein C (cMyBP-C), leading to autosomal dominant forms of hypertrophic

cardiomyopathy (Mearini *et al.*, 2010). Although not much information is currently

available on the *FBXO9* gene and no ubiquitination targets have been uncovered, it has

been implicated in growth factor signaling in multiple myeloma (Fernandez-Saiz *et al.*,

2013). It is clear that genes in this FBXO sub-family are active in pathways of disease

and further study into the mechanisms by which they target and degrade proteins is

required.

  FBXO7 was initially identified as a ubiquitin ligase targeting the Hepatoma Up-

Regulated Protein (HURP) (Hsu *et al.*, 2004). *FBXO7* has also been implicated in

regulation of the cell cycle through interaction with cyclin proteins (Laman *et al.*, 2005).

As a ubiquitin ligase, the activity of the FBXO7 protein is not possible without the

stabilizing activity of a co-factor, *Proteasome Inhibitor 31* (*PI31*) (Kirk *et al.*, 2008).

Although *PI31* is an essential component of the SCFBOX7 ubiquitin ligase construct, it

also has an independent function as a binding inhibitor of the proteasome complex (Zaiss

et al., 2002). Thus, *PI31* expression is a key regulator of proteasome activity: too little

will prevent the proper targeting of the SCF complex while too much will prevent the

response of proteasomal machinery to ubiquitinated targets (Li *et al.*, 2014). *FBXO32* is

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a muscle-specific ubiquitin ligase which is also known as *Atrogin-1* (Bodine & Baehr, 2014). *FBXO32* is upregulated in muscle wasting conditions in skeletal muscle and targets proteins essential in muscle synthesis, including Myogenic Differentiation 1 (MyoD) and Eukaryotic translation initiation factor 3 subunit F (eIF3-F) (Lagirand-Cantaloube *et al.*, 2008; Tintignac *et al.*, 2005). *FBXO7* and *FBXO32* are essential ubiquitin ligases involved in a variety of pathways necessary for neuronal and muscular health.

1.3.2. Potential *FBXO7* and *FBXO9* Homologues in *Drosophila melanogaster*

Many F-box genes have functional homologues found conserved between *H. sapiens* and *D. melanogaster*. Of the 75 identified *H. sapiens* F-box proteins, 21 have a known *D. melanogaster* homologue (Dui *et al.*, 2012; Jin *et al.*, 2004) (Table 2). These 21 genes share function in various physiological and biochemical pathways, including circadian rhythm, growth regulation, proteasomal degradation and muscle wasting (Merzetti *et al.*, 2013). Of these 21 genes, the three FBXO sub-family proteins, *FBXO7*, *FBXO9* and *FBXO32,* all have putative homologues found in *D. melanogaster*. The putative *D. melanogaster* homologue of *FBXO7* is *nutcracker* (*ntc*), originally named for its role in the terminal differentiation of sperm cells and mutational phenotype of male sterility (Bader *et al.*, 2010). The putative *D. melanogaster* homologue and *H. sapiens* *FBXO7* gene share a conserved function as ubiquitin ligase targeting components with a similar involvement in terminal differentiation of sperm cells. Both *ntc* and *FBXO7* have been implicated as potential factors in PD pathogenesis (Burchell *et al.*, 2013). *D. melanogaster FBXO7* has also been associated with a homologue of *PI31*, showing that

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stabilization of the SCF ubiquitin ligase complex is necessary between organisms (Bader *et al.*, 2011). In contrast *FBXO9* has not been well characterized in *D. melanogaster,* but a putative homologue, *CG5961*, has been identified. Studying the putative homologues of *FBXO7* and *FBXO9* in the simplified *D. melanogaster* system may lead to a better understanding of the pathways these genes control in disease models.

Table 2: F-box genes conserved between *Drosophila melanogaster* and *Homo sapiens*. Many F-box genes share similar functions across species even if they are not necessarily homologous proteins (*jetlag* and *overtime* for example). Table taken from (E. Merzetti *et al.*, 2013).

Gene Name (D. melanogaster)	Identified Function (D. melanogaster)	Gene Name (H. sapiens)	Identified Function (H. sapiens)
FBXW			
FBXW1 / slimb	circadian rhythm	FBXW1	β-catenin degradation
FBXW4 / ebi	ARM pathway signaling	FBXW4	β-catenin degradation
FBXW5	TSC1/2 degradation	FBXW5	TSC1/2 degradation
FBXW7 / ago	growth regulation	FBXW7	growth regulation
FBXL			
FBXL1 / Skp2	growth promoter	FBXL1 / Skp2	mitotic spindle formation
CG9003	unknown	FBXL2	cyclin D2 degradation
CG2010	unknown	FBXL3 / overtime	circadian rhythm
CG1839	unknown	FBXL4	lysine demethylase
CG4221	unknown	FBXL7	mitosis regulation
FBXL14 / ppa	centromere mediation	FBXL14	SNAIL 1 degradation
FBXL15 / jetlag	circadian rhythm	FBXL15	Smurf1 regulator
CG32085	unknown	FBXL16	tumor suppressor
FBXL19 / kdm2	histone demethylase	FBXL19	IL-33 degradation

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FBXO			
FBXO1 / pallbearer	phagocytosis	FBXO28	phagosome maturation
CG4911	unknown	FBXO4	cyclin D1 regulation
FBXO5 / rca	mitosis regulation	FBXO5	unknown
CG6758	unknown	FBXO6	Chk1 regulation
FBXO7 / nutcracker	sperm differentiation	FBXO7 / PARK15	sperm differentiation / PPS
FBXO9	unknown	FBXO9	growth signaling
CG9461	unknown	FBXO11	β-catenin degradation
FBXO32	unknown	FBXO32 / Atrogin-1	muscle wasting

1.4. Research Goals

Previous research has found that genes which lead to PD like symptoms when mutated have similar biological functions in both *Homo sapiens* and *Drosophila melanogaster*. The main goal of this research project was to identify models of PD in *D. melanogaster* that could be used as tools to expand knowledge of the pathways related to disease pathogenesis and onset. The single *D. melanogaster* homologue of the well-studied *H. sapiens* PGC gene family was determined to be a good target for disease modeling as it removes the complexity associated with analyzing three genes with overlapping expression patterns. It has been documented that *spargel* mutants show severe growth defects and lean phenotypes in whole organisms, but the result of altered *srl* activity in specific tissues has been found to lead to variable effects. To date, no study of the consequences of altered *srl* expression has been undertaken in dopaminergic neurons. Thus, the initial goal of this research project was to assay *srl* mutant phenotypes in dopaminergic neurons and to determine if *srl* dysfunction leads to a PD phenotype. When it was determined that *srl* mutation had a profound effect on dopaminergic neurons and lead to a PD-like phenotype, a new goal to identify a potential *D. melanogaster* homologue of the transcriptional regulator *PARIS* was established. Identification of a *PARIS* homologue in *D. melanogaster* would confirm the conservation of the mitochondrial overturn and biogenesis system between species.

In a parallel experiment, the consequence of altered expression of the highly conserved FBXO sub-family gene members *FBXO7* and *FBXO9* was determined. The goal of this project was to once again characterize the putative homologues of genes

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related to human disease phenotypes in *D. melanogaster*. The putative *FBXO7* homologue *nutcracker* has been identified as a component of an SCF ubiquitin ligase complex in *D. melanogaster*. Additionally, a *D. melanogaster* homologue of the stabilizing co-factor of *FBXO7*, *PI31* has been identified in flies. These proteins interact in a similar fashion in flies as they do in humans. At the onset of this project no study had looked at the consequence of altered expression of *ntc* or *PI31* in the dopaminergic neurons of flies. The goal of this project was to determine if altered expression of either *ntc* or *PI31* was sufficient to induce a PD like phenotype in *D. melanogaster*. These genes were also assayed in an existing model of PD in which ectopic expression of *α-synuclein* is driven in dopaminergic neurons. To our knowledge this was the first time that these genes have been assayed together in a model of PD. Finally, the putative *D. melanogaster* homologue of *FBXO9* was assayed in neuronal tissue to determine if altered expression caused a disease-like phenotype. As *FBXO9* is not yet characterized in *Homo sapiens* or *D. melanogaster* but related sub-family members *FBXO7* and *FBXO32* both show the capacity to cause disease, the goal of this project was to simply provide the first study into the function of this gene in flies. The hypothesis of this work is that *H. sapiens* disease genes related to PD are conserved in *D. melanogaster* and the function of the protein these genes express is conserved among these organisms. If this is true, I expect to be able to model neurodegenerative phenotypes in *D. melanogaster* which may then be used as a basic framework for future experiments to understanding the pathways associated with PD genes both in *D. melanogaster* and *H. sapiens*.

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1.5. References

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Chapter 2 – *spargel*, the *PGC-1α* homologue, in models of Parkinson Disease in *Drosophila melanogaster*

2.1. Introduction

Parkinson disease (PD) is a common and progressive neurological disease that is estimated to afflict 1% of all individuals over the age of 60 years worldwide (Lew, 2007). Although the direct cause of PD is as of yet unknown, the clinical symptoms include resting tremor, bradykinesia, rigidity and postural instability. In addition to these classical symptoms recent discoveries have shown that effects may include cognitive impairment such as loss of memory and depression (Wirdefeldt *et al.*, 2011). These symptoms are caused by a decrease in the amount of dopamine available in the striatum area of the brain and in many cases characterized by an accumulation of harmful protein aggregates known as Lewy bodies in the neurons of the *substantia nigra pars compacta* (Olanow & McNaught, 2011). The eventual dysfunction and breakdown of these neurons is responsible for the symptoms and pathology of PD (Bekris *et al.*, 2010). Both genetic and environmental factors have been found to contribute to PD, with many forms of the disease being attributed to a combination of the two.   Environmental factors include chemical exposure, brain trauma, obesity, diabetes and age (Vanitallie, 2008). Alternatively, there have been a number of familial cases of PD identified, which suggests a genetic link to certain forms of this disease (Bereznai & Molnar, 2009). This link is especially strong in the case of early onset PD which shows a bias towards genetic

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causes, while environmental conditions seem to more often than not be linked to late onset PD (Wirdefeldt *et al.*, 2011). Identifying and analyzing the genes responsible for these inherited forms of PD may give rise to mechanisms of disease progression that are not presently understood. Impaired neuronal activity has been shown to contribute to the lack of dopamine commonly associated with PD etiology. Identifying the cause of this neuronal impairment may help lead to new, effective, strategies to combat PD.

As important and necessary components of eukaryotic cells, mitochondria are integral components in the creation of adenosine triphosphate (ATP) for use as chemical energy and are involved in other important pathways such as the control of cellular growth and death, cell signaling and differentiation (Campbell, 2006). As the mitochondria are involved in such diverse and important cellular functions, it is not surprising that the breakdown or dysfunction of mitochondria may result in a number of disorders or diseases including, but not limited to, movement disorders such as PD (Greene *et al.*, 2003). Many of these disorders are caused by defective removal of damaged or non-functional mitochondria and a subsequent lack of *de novo* synthesis of new mitochondria to replace the aforementioned damaged organelles (Exner *et al.*, 2012; Winklhofer & Haass, 2010). Thus, the study of genes that regulate and monitor the health of mitochondria is a reasonable next step for determining potential disease prevention strategies.

The peroxisome proliferation activated co-receptor gamma (PCG) family of genes have been linked to mitochondrial biogenesis (Krempler *et al.*, 2000). *PGC-1α* has been found to be involved in *de novo* mitochondrial synthesis in various tissues, including the

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liver and brain (Uldry *et al.*, 2006). Genetic data has shown that polymorphisms in *PGC-1α* have been commonly found in patients with early onset and severe PD (Clark *et al.*, 2011). Other members of this gene family, *PGC-1β* (Kamei *et al.*, 2003) and *PRC* (PGC-1-related-cofactor) (Andersson & Scarpulla, 2001), have been shown to maintain some functional homology with *PGC-1α*. A comparison of the proteins translated from these genes shows close similarity and conservation of active sites (Gershman *et al.*, 2007), making it difficult to completely study the loss of function in human cells.

PGC-1α shares a functional pathway with two previously characterized PD genes: *PINK1* and *Parkin* (Ventura-Clapier *et al.*, 2008). *Parkin* is a component of a multi-protein E3 ubiquitin ligase that leads to the ubiquitination and subsequent destruction of cellular proteins (Narendra *et al.*, 2008). Mutations to the *Parkin* gene result in the degeneration of dopaminergic (DA) neurons, most likely by allowing the aggregation of multiple dysfunctional mitochondria that eventually lead to overall cell death (Greene *et al.*, 2003). *PINK1* (PTEN induced putative kinase 1) is a serine / threonine-protein kinase that acts by recruiting *Parkin* to damaged mitochondria (Koh & Chung, 2011). Similar to mutations in *Parkin*, mutations in *PINK1* lead to degeneration and dysfunction of dopaminergic neurons (Yang *et al.*, 2006). *Parkin* and *PINK1* act together in concert along with *mitofusin 2* (*Mfn2*) to remove any damaged or dysfunctional mitochondria that may be present, while *PGC-1α* activity regulates the creation of new mitochondria to replace damaged or removed organelles (Chen & Dorn, 2013). Mutation to any of the components of this pathway can lead to impaired mitochondria, decreased cellular fitness and eventual cell death.

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A single PGC family gene homologue, *spargel* (*srl*), has been identified in *Drosophila melanogaster*. The SRL protein has been characterized as a downstream component of the insulin signaling TOR pathway, *srl* mutant flies have a "lean" phenotype, typical of mutations that affect growth and proliferation and reduced mitochondrial fitness (Tiefenbock *et al.*, 2009). Although ubiquitous overexpression of *srl* has been found to negatively impact organism survival, tissue specific *srl* expression has been found to provide beneficial effects. Overexpression of *srl* has been shown to be sufficient to increase mitochondrial activity and mediate tissue specific lifespan extension in the digestive tract and intestine (Rera *et al.*, 2011). Altered expression of *srl* in the heart has been shown to increase capacity for exercise based endurance improvement, while decreased *srl* in cardiac muscle decreases the locomotor and endurance ability of flies (Tinkerhess *et al.*, 2012). The lack of gene redundancy present in *D. melanogaster* makes it an ideal model system to determine the effects of reduced or increase levels of *srl* expression on whole organism and neuronal longevity, leading to a new model of PD for use in future therapeutic studies.

2.2. Materials and Methods

2.2.1. *Drosophila* Media

The standard cornmeal-yeast-molasses-agar medium is made with 65 g/L cornmeal, 10 g/L nutritional yeast and 5.5 g/L agar supplemented with 50 ml/L Crosby's fancy grade molasses and 5 ml of 0.1 g/ml methyl 4-hydroxybenzoate in 95% ethanol and 2.5 ml of

propionic acid in standard plastic shell vials. The medium is stored at 4 to 6°C and warmed to room temperature for use.

2.2.2. *Drosophila* Transgenic Lines

To express transgenes in a subset of cells, including the dopaminergic neurons, the *Ddc-Gal4$^{HL4.3D}$* line was the generous gift of Dr. Jay Hirsh (University of Virginia) (Lin *et al.*, 2000). The following lines were obtained from the Bloomington Drosophila Stock Center (at Indiana University-Bloomington): 1) to drive expression behind the morphogenetic furrow in the developing eye disc *glass <u>m</u>ultiple <u>r</u>eporter-Gal4*[12] (*GMR-Gal4*; (Freeman, 1996)); 2) to act as a benign control for the ectopic expression of transgenes *UAS-lacZ^{4-2-1}* (*UAS-lacZ*; (Brand & Perrimon, 1993)) ; 3) to express in the presence of Gal4 the endogenous *srl* gene product a line bearing an *EPgy2* insertion in the 5 prime flanking region of *srl: y w; P{EPgy2^{srlEY05931}* (*UAS- srl^{EY05931}*) and 4) to, in the presence of Gal4, express a dsRNA for RNA inhibition (RNAi) of *srl*: *y sc v; P{TRiP.HMS00857}attP2* (*UAS-srl^{HMS00857}*) and *y sc v; P{TRiP.HMS00858}attP2* (*UAS-srl^{HMS00858}*).

2.2.3. Scanning Electron Microscopy of *Drosophila melanogaster* Eye

Female virgins of the *GMR-Gal4* were mated with *UAS-lacZ*, *UAS- srl^{EY05931}*, *UAS-srl^{HMS00857}* and *UAS-srl^{HMS00858}* males. Male progeny of each cross were collected, aged for 3 to 5 days and frozen at -80ºC. Flies were mounted under a dissecting microscope, and desiccated overnight. The eyes of mounted flies were imaged via scanning electron micrograph at 130 times magnification with a Mineral Liberation Analyzer 650F

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scanning electron microscope. Total bristle count, and total ommatidia count were obtained using ImageJ (Schneider *et al.*, 2012).

2.2.4. Ageing Analysis

Female virgins of the *Ddc-Gal4* line were mated with *UAS-lacZ*, *UAS- srl*[EY05931] and *UAS-srl*[HMS00858] males. Male progeny of each cross were collected, maintained in cohorts of no more than 20 to avoid crowding, and were placed on new medium every two or four days for the duration of the experiment. Flies were scored for viability every two days until all flies in all genotypes perished. Survival curves were compared by the log-rank (Mantel Cox) test.

2.2.5. Locomotor Analysis

From each of the crosses described above, 50 male progeny were collected and maintained in vials of 10 flies, and transferred to new medium twice weekly throughout the duration of the experiment. One week (seven days) after collection, and in 7-day intervals, five cohorts of flies for each genotype were assessed for climbing ability as previously described (Todd & Staveley, 2008) . Flies were scored every 7 days for their ability to climb within a glass tube of 1.5 cm diameter. Ten trials of each cohort of ten or less flies were scored based upon 2 cm intervals of height reached. A climbing index was calculated for each vial by the equation: Climbing Index $= \sum nm / N$. Where n is the number of flies at a given level, m is the score for that level (1-5), and N is the total number of flies climbed for that trial. A nonlinear regression curve of 95% confidence intervals was used to analyze graphs of 5 – climbing index as a function of time in days

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for each genotype. The rate (k) and Y-intercept (Y°) of each non-linear regression curve were calculated, where slope represents the rate of decline in climbing ability, and the Y-intercept represents the initial climbing ability (in the form of 5 – climbing index). As neither the slope nor the Y-intercept remained constant across all groups, it was necessary that both parameters were incorporated into statistical analysis to determine differences in climbing ability. A comparison of fits concluded whether or not curves differed between groups.

2.3. Results

A multiple alignment of the SRL protein with the three mammalian homologues; PGC-1α, PGC-1β and PRC, provides evidence of evolutionarily conserved protein structure between the human and *D. melanogaster* forms of this gene (Figure 3). These proteins differ in length from 1664 amino acids in the case of PRC, to 798 amino acids for PGC-1α. However, functional domains remain consistent across all four. Each contains an N terminal proline rich domain, a bipartite nuclear localization signal, C terminal serine rich region and a highly conserved RNA recognition motif as well as an arginine rich region in all except *PGC-1β* (Figure 3). Each of the mammalian proteins contain at least one leucine rich motif (LXXLL) that can interact with nuclear receptors (Matsuda *et al.*, 2004). This motif is not found in *srl*, however, an alternative leucine rich motif (FEALLL) is present which has been shown to also interact with nuclear receptors and serve the same function in *D. melanogaster* (Wang *et al.*, 2007). The similarities between *D. melanogaster* and mammalian proteins indicate that SRL is an ideal candidate

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A

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           A                                                      FEALLL
     SRL   ▭▭▭▭▭▭▭▭▭▭░░▭▭▭▭▭▭▭▭▭▭▭■▨▨▭□▭▥▥▭▭▭   1067

           LXXLL
   PGC-1α   ▭▭▭▭▭▭▭░░▭▭▭▭▭▭▨▨▭□▭▥▥▭▭   798

          LXXLL      LXXLL
   PGC-1β   ▭▭▭░▭▭▭▭▭▭▭■▭▭▭▭▭▭▭▨▨▭▥▥▭▭   1023

         LXXLL                              LXXLL
     PRC   ▭▭▭░░▭▭▭▭▭▭▭▭▭░░░░░░▭▭▭▭▭▭▭▭▭▨▨■▨▨▭▥▥▭   1664
```

| ▥ - RNA recognition | ▨ - Serine rich | □ - Arginine rich | ░ - Proline rich |

LXXLL / FEALLL - Leucine rich motif   ■ – Nuclear localization signal

B

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SRL      915  RIVYVGRIEQETTKEILRRKFLPYGSIKQITIHYKEN-GMKYGFVTYERAQDAFTAIDTS  974
PGC-1α   676  RVIYVGKIRPDTTRTELRDRFEVFGEIEECTVNLRDD-GDSYGFITYRYTCDAFAALENG  735
PGC-1β   901  RVVYIQNLSSDMSSRELKRRFEVFGEIEECEVLTRNRRGEKYGFITYRCSEHAALSLTKG  961
PRC     1542  RVVFIGKIPGRMTRSELKQRFSVFGEIEECTIHFRVQ-GDNYGFVTYRYAEEAFAAIESG  1601
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Figure 3: The PGC mammalian family and the *Drosophila melanogaster* protein SRL share conserved protein domains. A) Aligned sequences show the position of each domain in one *D. melanogaster* and three human PGC family protein sequences. Light upward diagonal indicates proline rich region, wide downward diagonal indicates serine rich region, dark vertical indicates RNA recognition region, solid black box indicates nuclear localization sequence, black frame indicates arginine rich region, LXXLL and FEALLL indicate leucine rich nuclear recognition motifs.   B) A multiple alignment between PGC-1α, PGC-1β, PRC and SRL shows a high degree of sequence conservation within the RNA recognition motif found at the carboxyl terminal of each protein. Domains were identified using ScanProsite (De Castro, 2006), alignment was done using ClustalW2 (Larkin, 2007). Protein sequences obtained from UniProt, accession numbers [Uniprot NP_037393 (PGC-1α)], [Uniprot NP_573570 (PGC-1β)], [Uniprot NP_055877 (PRC)] and [Uniprot NP_730835 (SRL)]. Elements of this figure were adapted from Scarpulla *et. al.* (Scarpulla, 2011).

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to study PGC family activity while avoiding the functional redundancy found in other systems.

In order to assay the effect of altered *srl* activity in neurons, we induced expression of three constructs previously used by Mukherjee and Duttaroy (Mukherjee & Duttaroy, 2013): *srl-RNAi* (*UAS-srl^{HMS00857}*), *srl-RNAi* (*UAS-srl^{HMS00858}*) and a *srl-EY* (*UAS- srl^{EY05931}*) in the neuron rich *D. melanogaster* eye. Eyes develop two separate yet equally important tissues which can be assayed for neuronal loss during development: ommatidia and bristles. At 25°C and 29°C (the latter for increased expression) under the direction of *GMR-Gal4*, expression of both *srl-RNAi* transgenes decreases the number of ommatidia and bristles present in the eye (Figure 4). Tissue specific expression of *srl-EY* results in a slight decrease in number of ommatidia and bristles at 25°C and an increase in number of ommatidia and bristles at 29°C. Similarly, *UAS-lacZ* under the control of *GMR-Gal4* shows a slight rough eye phenotype at 29°C (Whitworth, 2011) which is not produced by the expression of *srl-EY*.
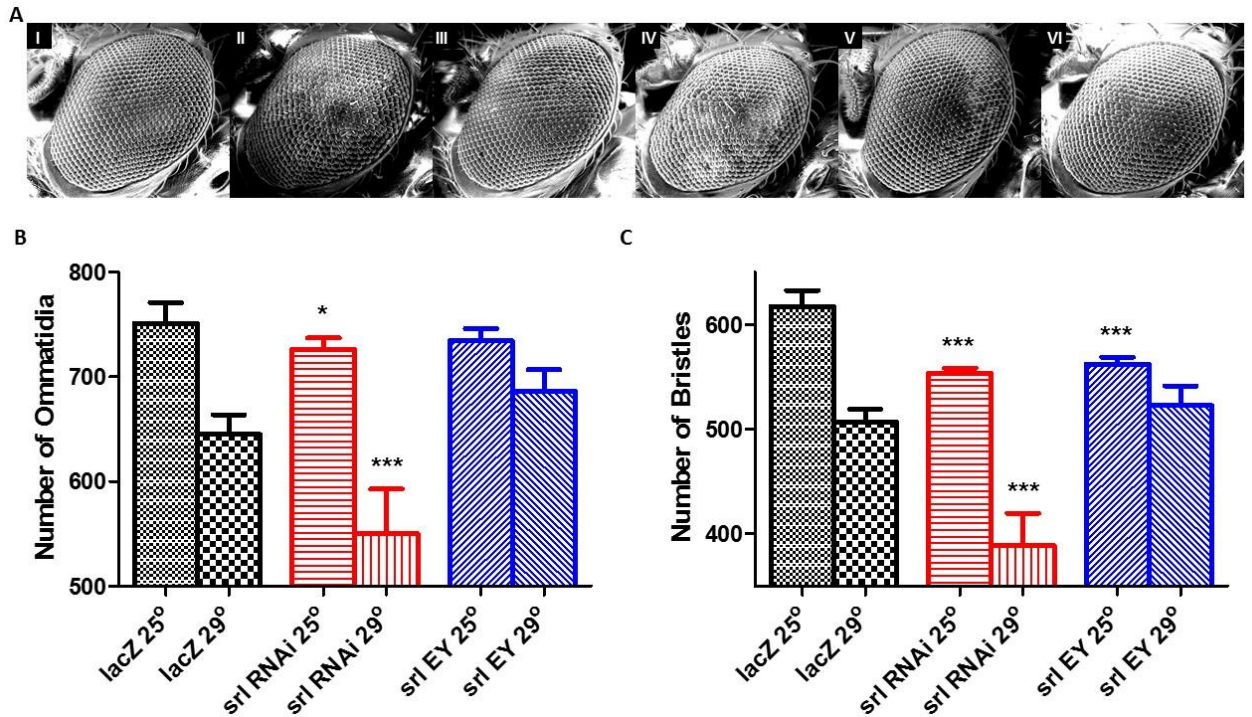
Figure 4: Tissue specific *srl* expression in the *Drosophila melanogaster* eye results in a reduction in both ommatidia and bristle number. A) Scanning electron micrographs of *D. melanogaster* eyes taken at a horizontal field width of 500 µm. Genotypes are as follows: I) *GMR-Gal4 / UAS-lacZ* 25°C, II) *GMR-Gal4 / srl-RNAi 1* (*UAS-srl^{HMS00857}*) 25°C, III) *GMR-Gal4 / srl-RNAi 2* (*UAS-srl^{HMS00858}*) 25°C, IV) *GMR–Gal4 / srl-EY* (*UAS- srl^{EY05931}*) 25°C, V) *GMR-Gal4 / UAS-lacZ* 29°C, VI) *GMR-Gal4 / srl-RNAi 1* (*UAS-srl^{HMS00857}*) 29°C. Images were taken with a FEI MLA 650. B) Flies show a decrease in the mean number of ommatidia present when *srl-RNAi 1* (*UAS-srl^{HMS00857}*) and *srl-RNAi 2* (*UAS-srl^{HMS00858}*) are driven with the *GMR-Gal4* driver in both standard conditions (25°C) and at a higher temperature (29°C). Flies show a slight but not significant decrease in ommatidia number when *srl-EY* is expressed in a tissue specific manner (*UAS- srl^{EY05931}*) in both standard conditions (25°C) and at a higher temperature (29°C). C) Flies show a strong decrease in bristle number in standard conditions (25°C) when *srl-RNAi 1* (*UAS-srl^{HMS00857}*), *srl-RNAi 2* (*UAS-srl^{HMS00858}*) and *srl-EY* (*UAS- srl^{EY05931}*) are expressed in *D. melanogaster* eyes. At a higher temperature (29°C) *srl-EY* causes an increase in number of bristles formed compared to *lacZ* controls, however, this is not statistically significant (*UAS-lacZ*). Comparisons between LacZ and altered gene conditions were measured using a one-way ANOVA and significance was tested using a Tukey post-hoc test, n=10 flies per genotype. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Statistical values may be found in supplemental table S1.1.

To determine if the phenotype caused by altered *srl* expression found in the eye is conserved in all neurons we next looked at tissue specific expression of *srl* in the DA neurons. To assay the effect of altered *srl* activity in DA neurons, we conditionally expressed a *srl-RNAi* transgene (*UAS-srl^{HMS00857}*) and a tissue specific *srl-EY* construct under the control of the *Ddc-Gal4* driver. *srl-RNAi* expression leads to a significant increase in median lifespan with a trending premature loss of climbing ability over time when compared to the *UAS-lacZ* controls (Figure 5). Tissue specific expression of *srl-EY* lead to both a decrease in lifespan and locomotor climbing. The data found in the eye and DA neuron of *D. melanogaster* were not identical and indicates that *srl* expression may be very tissue dependent and small changes in certain tissues may lead to differential effects.
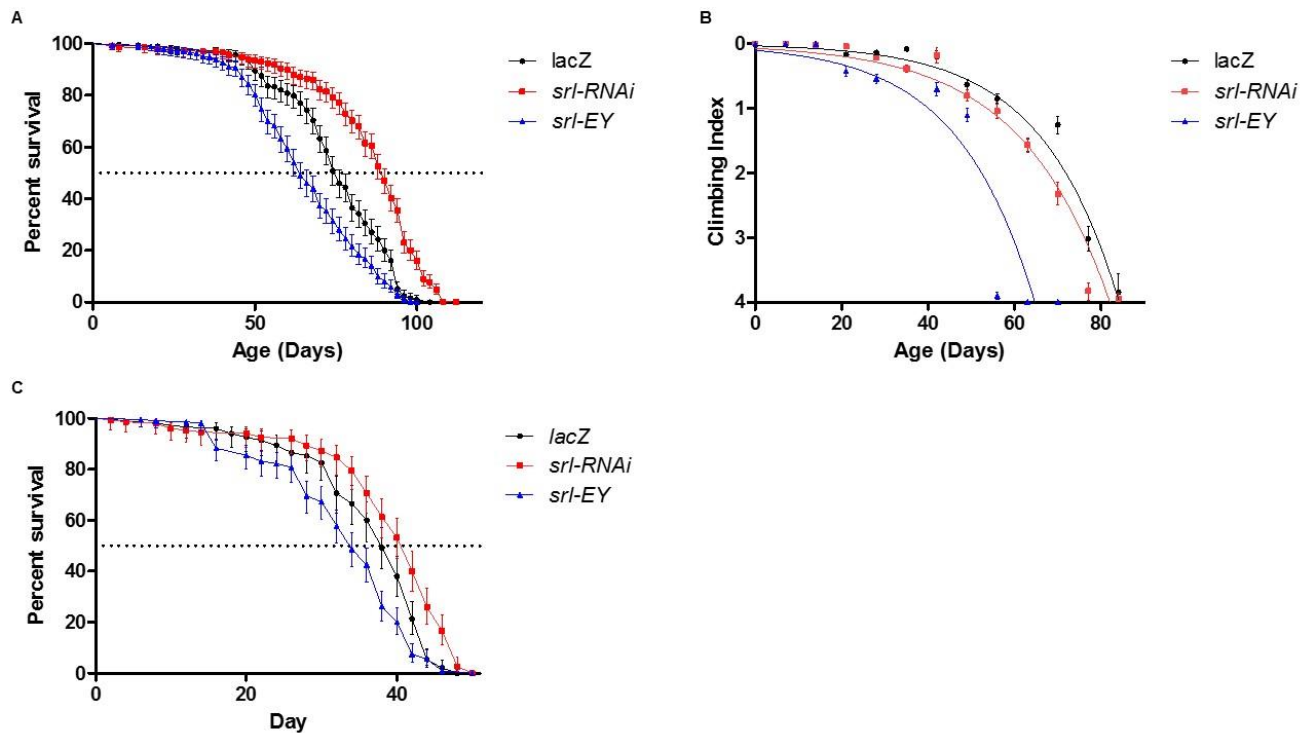
Figure 5: Tissue specific altered *srl* expression in dopaminergic neurons can lead to a model of Parkinson disease *Drosophila melanogaster*. A) Expression of *srl-RNAi* (*UAS-srl^{HMS00858}*) driven by *Ddc-Gal4* results in an increase in lifespan compared to *UAS-lacZ* controls. Dopaminergic *srl-EY* (*UAS-srl^{EY05931}*) expression resulted in a decrease in lifespan compared to *UAS-lacZ* controls. Longevity is shown as a percent survival (P<0.05 as determined by the Mantel-Cox Log Rank test) N= >200). B) Expression of *srl-RNAi* (*UAS-srl^{HMS00858}*) and *srl-EY* (*UAS- srl^{EY05931}*) driven by *Ddc-Gal4* cause a decrease in climbing ability over time. Dopaminergic specific expression of *srl-EY* causes a more severe decrease, however, *srl-RNAi* expressing flies live longer and climb slightly worse than *UAS-lacZ* controls indicating a potential decrease in climbing activity compared to lifespan. Climbing ability was determined via nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean, n = 50. C) Expression of *srl-RNAi* (*UAS-srl^{HMS00858}*) driven by *Ddc-Gal4*^{HL4.3D} at 29°C results in an increase in lifespan while *srl-EY* (*UAS- srl^{EY05931}*) expression at 29°C resulted in a decrease in lifespan compared to *UAS-lacZ* controls. Longevity is shown as a percent survival (P<0.0001 as determined by the Mantel-Cox Log Rank test) N=>150). Statistical values may be found in supplemental table S1.2. (aging) and S1.3. (climbing).

2.4. Discussion

PGC-1α has been identified as a modulator of mitochondrial biogenesis, energy metabolism (Liang & Ward, 2006), insulin signaling (Pagel-Langenickel *et al.*, 2008) and is believed to be implicated in the pathogenesis of human ailments including Alzheimer (Qin *et al.*, 2009), Huntington (Cui *et al.*, 2006) and Parkinson diseases (Zheng *et al.*, 2010). The study of human *PGC-1α* is complicated by the partial functional redundancy of the other PGC family members, *PGC-1β* and *PRC* (PGC-1-related-cofactor). The single Drosophila PGC family homologue, *srl*, has been linked to mitochondrial biogenesis and insulin signaling (Mukherjee & Duttaroy, 2013). We seek to model PGC related disease in *D. melanogaster* through an analysis of *srl* function.

The *D. melanogaster* eye has been used to study potential genes involved in neurodegenerative disease due to the neuron rich nature of the developing tissues located there as well as the ease of access for phenotypic analysis. When two *srl-RNAi* constructs are expressed in this tissue, there is a significant reduction in the number of ommatidia and bristles formed. This effect is exacerbated when flies are raised at 29°C, most likely caused by an increase in the activity of the Gal4 expression system, although stress responses connected to the fly being at higher than ideal temperatures could also cause some of the observed differences. Alternatively, expression of a previously characterized *srl-EY* transgene seems to have no significant effect on ommatidial viability under normal physiological conditions (25°C). At 29°C, the amount of ommatidial degeneration increases significantly across all RNAi genotypes. Interestingly, overexpression of *srl-EY* at 29°C does not cause significant degeneration of ommatidia or bristles as found in the

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UAS-lacZ control at higher temperatures. This could indicate that the ommatidial and bristle destruction found when lacZ is expressed at 29°C could be rescued by an overexpression of *srl*. Experiments by Mukherjee and Duttaroy have shown that *srl* overexpression can rescue FoxO mediated eye destruction (Mukherjee & Duttaroy, 2013). It is possible that tissue specific expression of *srl* in the eye may be sufficient to prevent physiological stressors from causing aberrant cell formation under these conditions.

Surprisingly, expression of *srl-RNAi* in DA neurons under the control of the *Ddc-Gal4* driver caused a significant increase in the mean lifespan of flies. Despite the increased longevity of these flies, they lose their climbing ability slightly earlier than controls. When considering the increase in lifespan, this indicates that although they live longer, they may have severe locomotor defects during the latter part of their life. Alternatively, DA expression of the *srl-EY* transgene under the control of the *Ddc-Gal4* driver showed a significant decrease in lifespan compared to *UAS-lacZ* controls. The locomotor and climbing ability of these flies was also decreased. Taken together, the premature mortality and decreased climbing ability displayed in flies expressing *srl-EY* in dopamine decarboxylase neurons appears to give a new, previously uncharacterized, model of PD in *D. melanogaster*.

Altered *srl* expression has been found to cause various phenotypes in a tissue specific manner. Ubiquitous overexpression of *srl* has been shown to moderately reduce mean lifespan while overexpression in intestinal stem cells and cells of the digestive tract caused increased lifespan (Rera *et al.*, 2011). Similarly, increased *srl* expression in cardiac muscle has been linked to exercise based endurance improvement and

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cardiovascular performance (Tinkerhess *et al.*, 2012). These findings lead us to believe that altered *srl* expression does not react the same way in the eye model of neurodegeneration as in DA neurons.

Although an increase in lifespan caused by the expression of *srl-RNAi* was an unexpected result, we hypothesize that a strong decrease in *srl* expression causes an amount of mitochondrial stress sufficient to activate a protein stress response which has been shown to increase lifespan (Houtkooper *et al.*, 2013). The exact mechanism of this increase is a topic of much debate with the most popular hypothesis involving an activation of the unfolded protein response (UTP) (Haynes & Ron, 2010). However, it has recently been found that in *Caenorhabditis elegans,* many of the genes involved in the UTP are non-essential for this increase in longevity, suggesting another mechanism for the increase in organismal longevity (Bennett *et al.*, 2014). Alternatively, this increase in longevity may involve the concept of stress causing the formation of reactive oxygen species (ROS) at a low level which provoke cellular anti-oxidants, causing a stronger response to future ROS exposure (Lopez-Torres & Barja, 2008). The most commonly used term for this is mitochondrial hormesis (or mitohormesis) (Ristow & Zarse, 2010). It is quite plausible that stress causes increased longevity through a combination of factors involving ideas from both of the aforementioned explanations. The use of a model organism in identifying and characterizing the pathways of disease progression is a fundamental step in creating new and novel treatment options. Studying the consequences of altered *srl* expression in *D. melanogaster* allows us to study the complex mammalian PGC gene family in a system containing only a single homologue.

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Identifying the role of *srl* will lead to an understanding of the associations and pathways related to proper function of this gene and subsequently the consequences of improper gene function. Currently, there is no preventative treatment available for PD, and only limited options are available to combat advanced symptoms. Identification of this new model of PD provides a framework for more advanced studies into complex gene interactions. Connecting cellular processes and characterizing genetic pathways of disease progression may eventually allow for the preventative treatment of genetic forms of PD and novel therapeutic options.

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Chapter 3 – The *FBXO7* homologue *nutcracker* and binding partner *PI31* in *Drosophila melanogaster* models of Parkinson Disease

3.1. Introduction

Parkinson Disease (PD) is a progressive neurodegenerative disorder caused by decline of function and eventual destruction of dopamine-producing neurons in the brain. Symptoms include resting tremor, rigidity, postural instability, bradykinesia and general loss of muscle control, as well as cognitive impairment at later stages of disease progression (Williams-Gray *et al.*, 2006; Wirdefeldt *et al.*, 2011). Dopamine is an essential neurotransmitter and destruction of neurons that produce dopamine in the *substantia nigra pars compacta* leads to a decreased amount of dopamine being present across the striatum of the brain and a subsequent decrease in dopamine-dependent signal transduction (Obeso *et al.*, 2008). This neuronal degeneration may be caused by a number of different factors, including dysfunction of molecular machinery relating to the removal and overturn of damaged or impaired proteins, increased mitochondrial stress resulting in a release of reactive oxygen species, and impaired cellular movement and kinetics (Hauser & Hastings, 2013; Imai & Lu, 2011; Shastry, 2003). The most successful pharmacological approaches to PD seek to slow progression of the disease or abrogate symptoms by replacing available dopamine in the brain. To date, no treatment seeking to abrogate disease symptoms has been successful.

For the most part, Parkinson Disease has been viewed as an age-related sporadic condition, but severe early onset cases of PD have been identified and found to be linked

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to mutations in specific genes. Initially, it was believed that these inherited forms of disease were independent anomalies, but many of these cases share similar methods of disease progression (Chung *et al.*, 2001). One of the most common underlying causes for cellular destruction in PD is a breakdown or partial loss of function in proteasomal machinery (Matsuda & Tanaka, 2010; McNaught *et al.*, 2006). Proteasomal systems within cells are responsible for protein turnover and removal of potentially harmful unwanted cellular components. Ubiquitin mediated proteolysis is an ATP-dependent, multi-step process by which this protein turnover occurs. The first step tags the target protein with ubiquitin acting as a marker for termination. Next, the 26S proteasome degrades the targeted protein. Finally, the components of this proteasome system and the short peptide fragments of the targeted protein are recycled (Lecker *et al.*, 2006). The coordinated effort of three enzymes allows this system to properly function. The first, E1 or activating enzyme, functions as a ubiquitin carrier. The second, E2 or conjugating enzyme, accepts this ubiquitin molecule from the E1 enzyme and presents it to the final enzyme, E3. This ligating enzyme is responsible for specific attachment of the ubiquitin molecule to the target of interest (Neutzner & Neutzner, 2012). Of the three enzymes, the E3 ligating enzymes are the most diverse. This allows for a high degree of target specificity and a cascade of activity stemming from a few general E1 activators to dozens of E2 conjugators and hundreds of E3 ligators (Pickart & Rose, 1985). Improper function of any component in this system may lead to harmful consequences and a potentially deadly build-up of dysfunctional proteins.

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Evidence of a severe form of inherited PD has been found that results in cellular

dysfunction and breakdown not just in the dopaminergic neurons but also in neurons

located in other areas of the brain. This condition has been termed Parkinsonian-

pyramidal syndrome (PPS) and has been described to result in a severe and precocious

form of disease (Rajendran *et al.*, 2000). Like traditional models of PD, PPS acts by

damaging neurons in specific areas of the mid-brain to cause movement dysfunction and,

eventually, cognitive decline. Unlike traditional models of PD, PPS affects neurons in the

pyramidal area of the brain, leading to direct loss of muscle control and symptoms that

present earlier than other forms of PD (Lai *et al.*, 2012). Mutation of the *FBXO7* gene

has been implicated as a causative factor in the pathology of this extreme form of PD (Di

Fonzo *et al.*, 2009). Although specific mutations have been isolated that lead to this

disease phenotype, the mechanisms behind this system and why these mutations lead to a

parkinsonian phenotype have yet to be determined.

  FBXO7 encodes the <u>F</u>-<u>box</u> <u>o</u>nly protein <u>7</u> (also known as *Park15*), a component of

Skp-Cullin1-Fbox (SCF) E3 ligases (Hsu *et al.*, 2004). These complexes mediate the

attachment of ubiquitin to proteins in the cell either for modified function in the case of

single attachment at lysine-63 (Zeng *et al.*, 2009), or to mark targets for degradation by

the 26S proteasomal machinery via polyubiquitination of lysine-48 (Chau *et al.*, 1989).

Within the SCF complex, F-box proteins provide target specificity, allowing the

regulation of essential cellular processes such as differentiation, DNA quality control,

circadian rhythm and neuronal synapse formation (Merzetti *et al.*, 2013). Evidence of

intracellular proteinaceous inclusions in *FBXO7* forms of PPS suggest that breakdown of

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the proteasomal machinery may be a direct result of mutation in this gene (Zhao *et al.*, 2013). A decrease in the concentration of FBXO7 protein in neuronal cells correlates with an onset of PD phenotypes through a mechanism with *Parkin* (Burchell et al., 2013). Structurally, FBXO7 contains an F-box motif (residues 329-375) which interacts with Skp1 (Cenciarelli *et al.*, 1999). Interaction with the E2-ubiquitin conjugating enzyme occurs at the N-terminal ubiquitin-like domain. The specificity of this protein comes from a proline-rich region (PRR) at the C-terminal. Substrates of the SCF complex bind to this C-terminal PRR to hasten ubiquitination (Chang *et al.*, 2006). FBXO7 is differentially expressed throughout the brain and recent evidence has shown that FBXO7 interacts with PARKIN and PINK1 *in vivo* to mediate proper mitochondrial function (Burchell *et al.*, 2013). Furthermore, FBXO7 is highly expressed in regions of the brain associated with PD, including the cerebral cortex, *globus pallidus* and *substantia nigra* (Nelson *et al.*, 2013). The function of FBXO7 relies upon stabilization by a binding partner. Originally named for its inhibition of the 20S proteasome (Chu-Ping *et al.*, 1992), proteasomal-inhibitor-31 (PI31) interacts with FBXO7 through a shared N-terminal region aptly named the FBXO7/PI31 (FP) domain (Kirk *et al.*, 2008). Despite its original identification as a proteasomal inhibitor, it appears as if PI31 acts as an activator of the 26S proteasome through stabilization of the SCF complex.

D. melanogaster has proven to be a convenient model organism for the study of human disease *in vivo*. An abundance of genetic tools, access to the complete genome and a quick generation time make flies an ideal system to study specific disease linked genes. Furthermore, *D. melanogaster* has been extensively used as a model neurological system

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due to similar complexity of neurons and neurotransmitter systems compared to higher organisms (Bilen & Bonini, 2005). A putative *D. melanogaster* homologue of *FBXO7* has been identified by Bader *et al* (2010). This gene, termed *nutcracker* (*ntc*), shares sequence similarity to *FBXO7* within the F-box domain and has been shown to function in pathways of proteolysis (Bader *et al.*, 2011). Initially, *ntc* was implicated to play a role in the terminal differentiation of male germ cells, a process that relies heavily upon proteasomal machinery (Bader *et al.*, 2010). Disruption of *ntc* directly leads to a reduction in proteasome activity without reducing proteasomal machinery. This indicates that disruption of the proteolytic cascade likely occurs at protein ubiquitination when *ntc* is dysfunctional, however, protein degradation catalase enzymes could also be accountable (Cao *et al.*, 2003). Thus, *ntc* seems to play the same role as an E3 ubiquitin ligase in *D. melanogaster* as *FBXO7* does in mammals.

A *D. melanogaster* homologue of PI31 has been studied as a regulator of ubiquitin mediated proteolysis. In cells where a mutation of *PI31* is present, there is a marked accumulation of poly-ubiquitinylated proteins, indicating a defective proteasome (Bader *et al.*, 2011). This phenotype can be rescued by moderate overexpression of the wild type PI31 protein but a high level of expression or a complete loss of function in *PI31* leads to lethal phenotypes. This indicates that *PI31* is tightly regulated and important for proper cellular function. Furthermore, cells expressing a mutation in the FP binding domain show similar phenotypes to *PI31* null mutants (Bader *et al.*, 2011). Taken together, it is clear that *PI31* and *ntc* are necessary regulators of the 26S proteasome in *D. melanogaster*. In this paper, we investigate the role of both *ntc* and *PI31* in *D.*

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melanogaster and determine what role these genes may play in neuron function in this model system.

3.2. Materials and Methods

3.2.1. Fly Stocks

The *UAS-HA-ntc* (*UAS-ntc*), *UAS-PI31^{RNAi28858}* (*PI31-RNAi*), and *UAS-HA-PI31* (*UAS-PI31*) stocks were generously provided by Dr. H. Steller of Rockefeller University (Bader *et al.*, 2010; Bader *et al.*, 2011). *Ddc-Gal4^{HL4.3D}* and the *Ddc-Gal4^{HL4.36}* were provided by Dr. J. Hirsh (University of Virginia). *UAS-α-synuclein* was provided by Dr. M. Feany (Harvard Medical School). The *GMR-Gal4^{12 ,} UAS-lacZ and UAS-ntc^{RNAi}* (*ntc-RNAi*) stock were obtained from the Bloomington Drosophila Stock Center at Indiana University. All fly stocks were maintained on standard cornmeal-yeast-molasses-agar at 25°C.

3.2.2. Scanning Electron Microscopy of *Drosophila melanogaster* Eye

Female virgins of the *GMR-Gal4^{12}* were mated with *UAS-lacZ, ntc-RNAi,UAS-ntc, PI31-RNAi and UAS-PI31* males. Male progeny of each cross were collected, aged for 3 to 5 days and frozen at -80°C. Flies were mounted under a dissecting microscope, and desiccated overnight. The eyes of mounted flies were imaged via scanning electron micrography at 130 times magnification with a Mineral Liberation Analyzer 650F scanning electron microscope. Ommatidia and bristle counts were performed and annotated using ImageJ (Schneider *et al.*, 2012).

3.2.3. Ageing Analysis

Female virgins of *Ddc-Gal4*[HL4.3D,] *Ddc-Gal4*[HL4.36] and *UAS-asyn;Ddc-Gal4*[HL4.36]
genotypes were collected every 12 hours for several days. These virgins were placed in
vials with males of genotypes *UAS-lacZ*, *UAS-ntc*, *UAS-PI31*, *ntc-RNAi* and *PI31-RNAi*.
Male progeny of each cross were collected over 18 days until at least 250 flies for each
genotype were obtained. Progeny were maintained in vials of 20 flies maximum and were
scored for viability every second day beginning two days after collection. Flies were
placed on new food twice weekly and after a death event for the duration of the
experiment. Ageing analysis continued every second day until all flies in all genotypes
were deceased. Survival curves were compared by the log-rank (Mantel Cox) test
performed by Graph Pad Prism Statistical Software.

3.2.4. Locomotor Analysis

Female virgins of *Ddc-Gal4*[HL4.3D,] *Ddc-Gal4*[HL4.36] and *UAS-asyn;Ddc-Gal4*[HL4.36]
genotypes were collected every 12 hours for several days. These virgins were placed in
vials with males of genotypes *UAS-lacZ*, *UAS-ntc*, *UAS-PI31*, *ntc-RNAi* and *PI31-RNAi*.
Fifty male progeny of each cross were collected and maintained in vials of 10 flies, and
were placed on new food twice weekly throughout the duration of the experiment. One
weeks after collection and each week thereafter, flies were assessed for climbing ability.
Flies were scored for their ability to climb a 30 cm glass tube of 1.5 cm diameter. Ten
flies were given 10 seconds to climb and were scored based on the height reached. The
outside of this glass vial was labelled with 5 lines, each 2 cm apart with each space

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between lines acting as a score from 1 (under 2cm) to 5 (over 8cm). 10 trials were performed for each vial of 10 flies. A climbing index was calculated for each vial by the equation:

$$\text{Climbing Index} = \sum nm \,/\, N$$

where n is the number of flies at a given level, m is the score for that level (1-5), and N is the total number of flies climbed for that trial. A non-linear regression curve of 95% confidence intervals was used to analyze graphs of 5 – climbing index as a function of time in days for each genotype. The rate (k) and Y-intercept (Y^0) of each non-linear regression curve were calculated, where slope represents the rate of decline in climbing ability, and the Y-intercept represents the initial climbing ability (in the form of 5 – climbing index). As neither the slope nor the Y-intercept remained constant across all groups, it was necessary that both parameters were incorporated into statistical analysis to determine differences in climbing ability. A comparison of fits concluded whether there was a significant difference between data groups. This assay and subsequent statistical analysis has been developed previously by Stavely and Haywood (2004).

3.3. Results

A National Centre for Biotechnology Information basis local alignment search tool (BLAST) search using the *Homo sapiens FBXO7* protein as a query returns *ntc* as a match at 27% identical. Closer analysis of the specific protein domains in both the FBXO7 and NTC proteins shows a much higher degree of conservation in the active regions of the proteins (Figure 6).   Although NTC is smaller than FBXO7 (314 versus 522 amino acids in length), both proteins retain PI31 and F-box binding regions (Figure
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6A). Retention of these domains indicates similar evolutionarily conserved functions for both proteins in distantly related organisms. The *H. sapiens* and *D. melanogaster* forms of the PI31 protein are 30% identical, similar in size (271 versus 270 amino acids), and they retain the C-terminal proteasome binding region and N-terminal F-box binding domains found to be functionally important in human cell culture (Figure 6B) (Kirk *et al.*, 2008; Li *et al.*, 2014). The high degree of similarity and conserved domain structure found between *H. sapiens* and *D. melanogaster* proteins allows the possibility that NTC and PI31 may be *D. melanogaster* homologues of their prospective human proteins. To determine the consequence of altered *ntc* expression in neuronal tissue, we directed the expression of the transgenes *UAS-ntc* and *ntc-RNAi* in the *D. melanogaster* eye. Eyes develop two distinct yet equally important neuronal tissues: ommatidia and inter-ommatidial bristles. Assaying developmental defects in these tissues gives a good indication of the consequences in other neurons affected by similar alteration in gene activity. To manipulate gene activity, we used a *GMR-Gal4*[12] transgene in combination with the responder transgenes and carried out this experiment at both $25°C$ and $29°C$; the latter is typically utilized to produce a greater level of activity due to the *Gal4-UAS* system's increased performance at higher temperatures.

Tissue specific expression of the *UAS-ntc* transgene caused a similar and significant decrease in the number of ommatidia and bristles at $25°C$. Interestingly, no significant difference in tissue number was found in either bristles or ommatidia at $29°C$ (Figure 7A). It is possible that altered *ntc* expression has a negative effect on the cell up until a certain threshold is reached and then is no longer able to elicit a response.

67
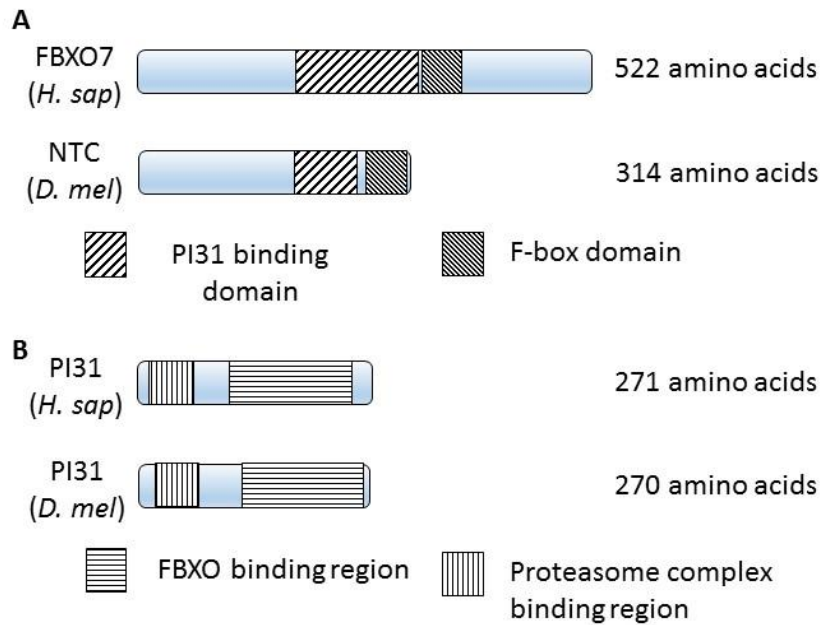
Figure 6: The mammalian FBXO7 and PI31 proteins share conserved protein domains with putative *Drosophila melanogaster* **homologues** *nutcracker* **and** *PI31*. A) Aligned protein sequences show the domains conserved between FBXO7 and NTC. Wide upward diagonal indicates PI31 binding domain, dark downward diagonal indicates F-box domain. B) Human and *D. melanogaster* PI31 show highly conserved protein size and domains. Light horizontal indicates the C-terminal proteasome complex binding region; light vertical indicates N-terminal FBXO binding region. Protein sequences obtained from UniProt, accession numbers [Uniprot NP_036311 (FBXO7)], [Uniprot NP_ 647829.1 (ntc)], [Uniprot NP_848693.2 (PI31)] and [Uniprot NP_ (*D. melanogaster* PI31)]. Alignment may be found in supplemental figure S2.1.
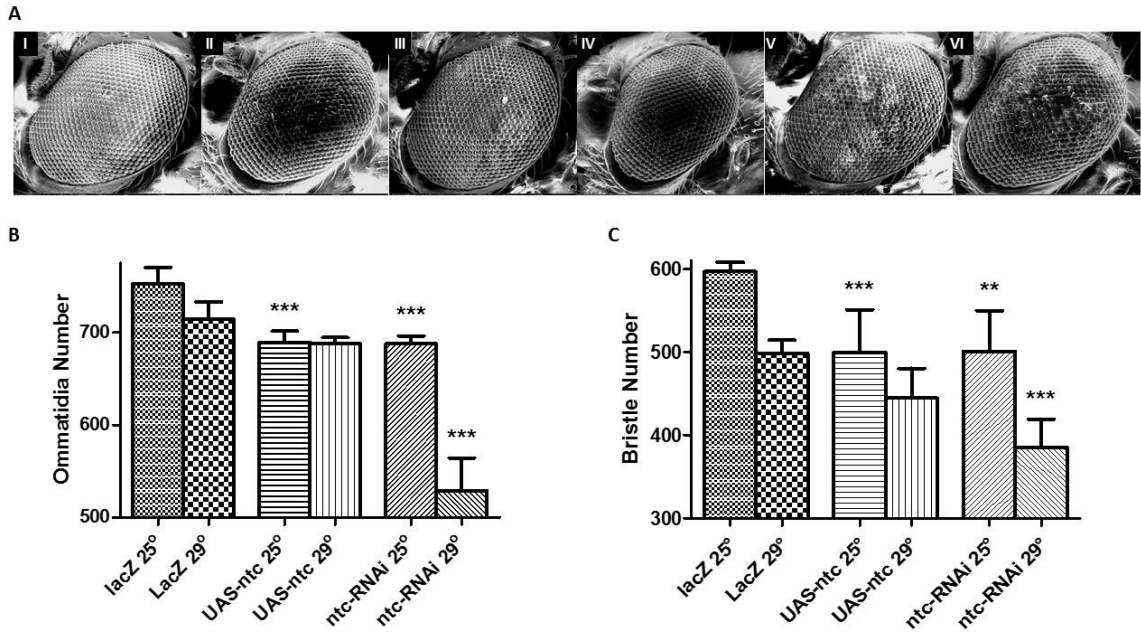
Figure 7: Tissue specific expression of *ntc* in the *Drosophila melanogaster* eye leads to a decrease in ommatidia and bristle numbers. A) Scanning electron micrographs of *D. melanogaster* eyes taken at a horizontal field width of 500 µm. Genotypes are as follows: I) *GMR-Gal4[12] / UAS-lacZ* (25°C), II) *GMR–Gal4[12] / UAS-lacZ* (29°C), III) *GMR-Gal4[12] / UAS-ntc* (25°C), IV) *GMR-Gal4[12] / UAS-ntc* (29°C), V) *GMR–Gal4[12] / ntc-RNAi* (25°C), VI) *GMR–Gal4[12] / ntc-RNAi* (29°C). B) Flies show a decrease in the overall number of ommatidia when *ntc* and *ntc-RNAi* transgenes are directed by *GMR-Gal4[12]*. Expression of the *ntc* transgene appears to have a similar effect on tissue formation at both 25°C and 29°C while the *ntc-RNAi* transgene results in a severe decrease in ommatidia number at 29°C. C) Expression of *ntc* and *ntc-RNAi* transgenes causes a similar significant decrease in bristle formation when directed by *GMR-Gal4* at 25°C. When the expression is enhanced at 29°C, both transgenes show an increase in tissue disruption compared to *lacZ* controls. Comparisons were between 25°C controls and experimental flues and 29°C controls and experimental flies and measured using a one-way ANOVA and significance was tested using a Tukey post-hoc test, n=10. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Statistical values may be found in supplemental table S2.2.

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Alternatively, it is possible that the destruction caused by the increase of stress and transgene expression system at 29°C is more disruptive then the addition of *ntc*. Tissue specific expression of *ntc-RNAi* caused a significant decrease in both ommatidia and bristle numbers at 25°C and a similar, yet more severe, rough eye phenotype at 29°C. Expression of *PI31* in the *D. melanogaster* eye caused a significant decrease in the number of ommatidia formed compared to *lacZ* controls at 29°C (Figure 8A). This effect was not found at 25°C. The expression of *ntc-RNAi* caused a decrease in bristle number at both 25°C and a significant more severe decrease at 29°C. This effect is found at 29°C in ommatidia but once again not a consequence of expression at 25°C in this tissue. This may indicate that *PI31* is more tightly regulated in the *D. melanogaster* eye, especially at higher levels of expression, than *ntc* but ommatidia seem to be less susceptible to altered expression levels until high levels of expression exist.
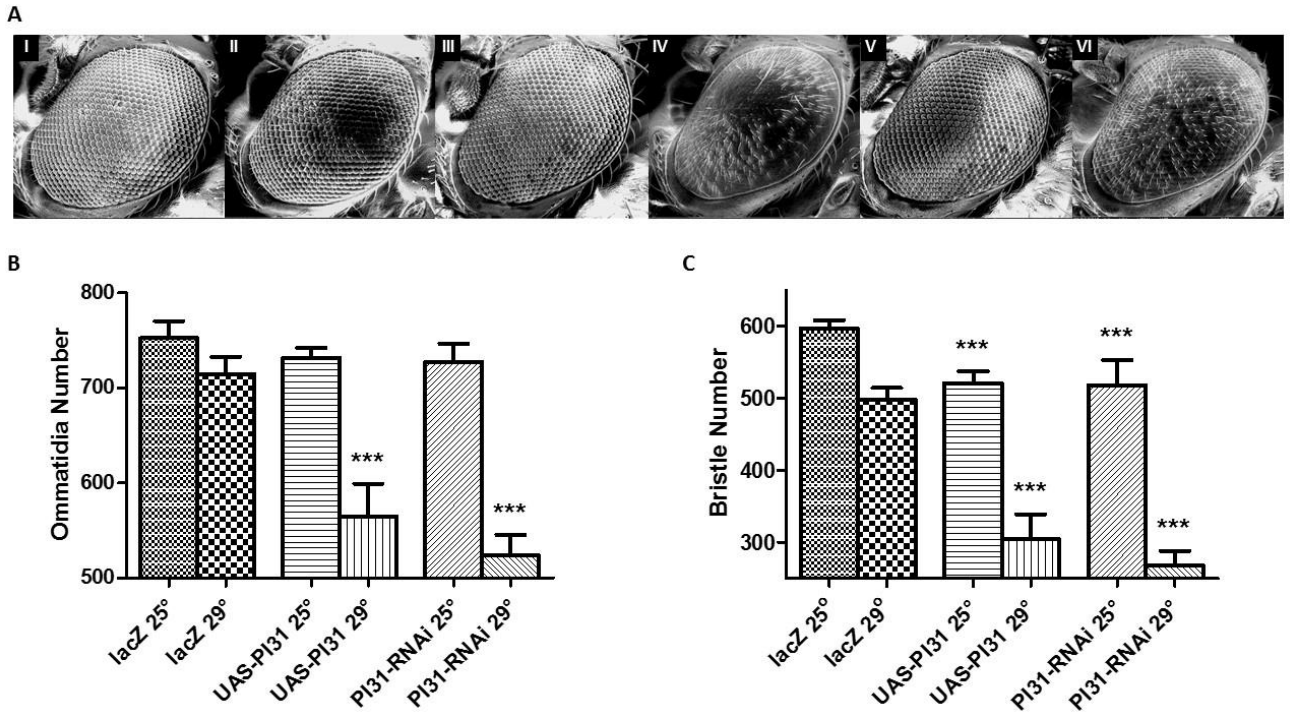
Figure 8: Altered *PI31* expression can lead to a rough eye phenotype in *Drosophila melanogaster*. A)
Scanning electron micrographs of *D. melanogaster* eyes taken at a horizontal field width of 500 µm.
Genotypes are as follows: I) *GMR-Gal4^{12} / UAS-lacZ* (25°C), II) *GMR–Gal4^{12} / UAS-lacZ* (29°C), III)
GMR-Gal4^{12} / UAS-PI31 (25°C), IV) *GMR-Gal4^{12} / UAS-PI31* (29°C), V) *GMR–Gal4^{12} / PI31-RNAi* (25°C),
VI) *GMR–Gal4^{12} / PI31-RNAi* (29°C). B) Tissue specific expression of *PI31* and *PI31-RNAi* transgenes in
the *D. melanogaster* eye cause a small yet significant decrease in ommatidia number at 25°C and a severe
decrease in ommatidia with increased *GMR-Gal4^{12}* activity at 29°C. C) Bristle formation is severely
disrupted when *PI31* and *PI31-RNAi* transgenes are directed by *Gal4* at both 25°C and 29°C, with the
expression of *PI31-RNAi* at 29°C resulting in a severe rough eye phenotype. Comparisons were between
25°C controls and experimental flues and 29°C controls and experimental flies and measured using a one-
way ANOVA and significance was tested using a Tukey post-hoc test, n=10. *$P<0.05$, **$P<0.01$,
***$P<0.001$. Statistical values may be found in supplemental table S2.2.

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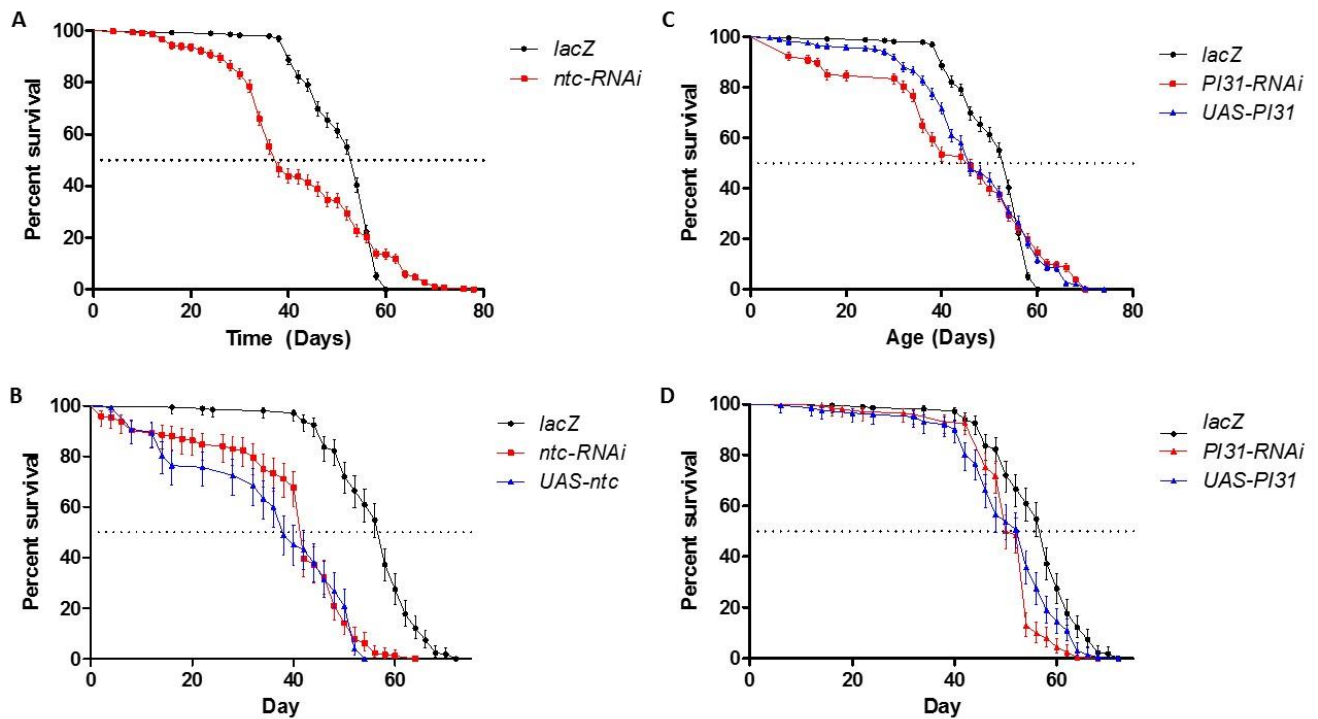
Figure 9: Expression of *ntc*, *ntc-RNAi*, *PI31* and *PI31-RNAi* in the dopaminergic neurons of *Drosophila melanogaster* reduces mean lifespan. A) Tissue specific expression of *ntc-RNAi* and *ntc* cause a significant decrease in mean lifespan when driven in the dopaminergic neurons by *Ddc-Gal4*[HL4.3D]. Expression of a *UAS-ntc* transgene causes a more severe decrease in mean lifespan compared to wild type *UAS-lacZ* controls. B) These trends are also observed when a separate driver, *Ddc-Gal4*[HL4.36], is used to provide dopaminergic specific expression. C, D) *PI31* and *PI31-RNAi* expression in dopaminergic neurons causes a slight yet significant decrease in mean lifespan compared to *lacZ* controls with both *Ddc-Gal4*[HL4.3D] and *Ddc-Gal4*[HL4.36] drivers. Longevity is shown as a percent survival (P<0.05 as determined by the Mantel-Cox Log Rank test), N=>200. Statistical values may be found in supplemental table S2.3.

To determine if the negative effects associated with altered *ntc* and *PI31* expression in the eye are conserved in other neuronal tissues, we next altered gene activity in the dopaminergic neurons. Dopaminergic expression of *ntc-RNAi* under the control of the transgenic insertions *Ddc-Gal4*[HL4.36] and *Ddc-Gal4*[HL4.3D] led to significant decreases in lifespan compared to the *lacZ* controls (Figure 9A and 9B). Expression of *UAS-ntc* under the control of *Ddc-Gal4*[HL4.36] lead to a similar significant decrease in mean lifespan compared to *lacZ* controls (Figure 4B). Altered *ntc* activity in the dopaminergic neurons has a severe negative effect on the mean lifespan of *D. melanogaster*.

Expression of either *PI31* or *PI31-RNAi* in the dopaminergic neurons under the control of *Ddc-Gal4*[HL4.36] or *Ddc-Gal4*[HL4.3D] resulted in a significant decrease in the mean lifespan of flies compared to *lacZ* controls (Figure 9C and 9D). Although this decrease in lifespan was not as severe as witnessed in the case of altered *ntc* expression, flies with altered *PI31* expression still failed to live as long as the control group. This indicates that *PI31* may be tightly regulated in dopaminergic neurons and a change in expression leads to decreased lifespan in *D. melanogaster*.

Ectopic expression of human *α-synuclein* in *D. melanogaster* has been shown to produce disease-modelling phenotypes, including impaired locomotor function and tissue disruption (Feany & Bender, 2000). Altered activity of *ntc* or *PI31*, along with *α-synuclein* in the dopaminergic neurons, was assayed to investigate the potential to influence these phenotypes.

Dopaminergic expression of a *ntc-RNAi* construct significantly increased median lifespan compared to a *lacZ* control in an *α-synuclein* background (Figure 10A). This

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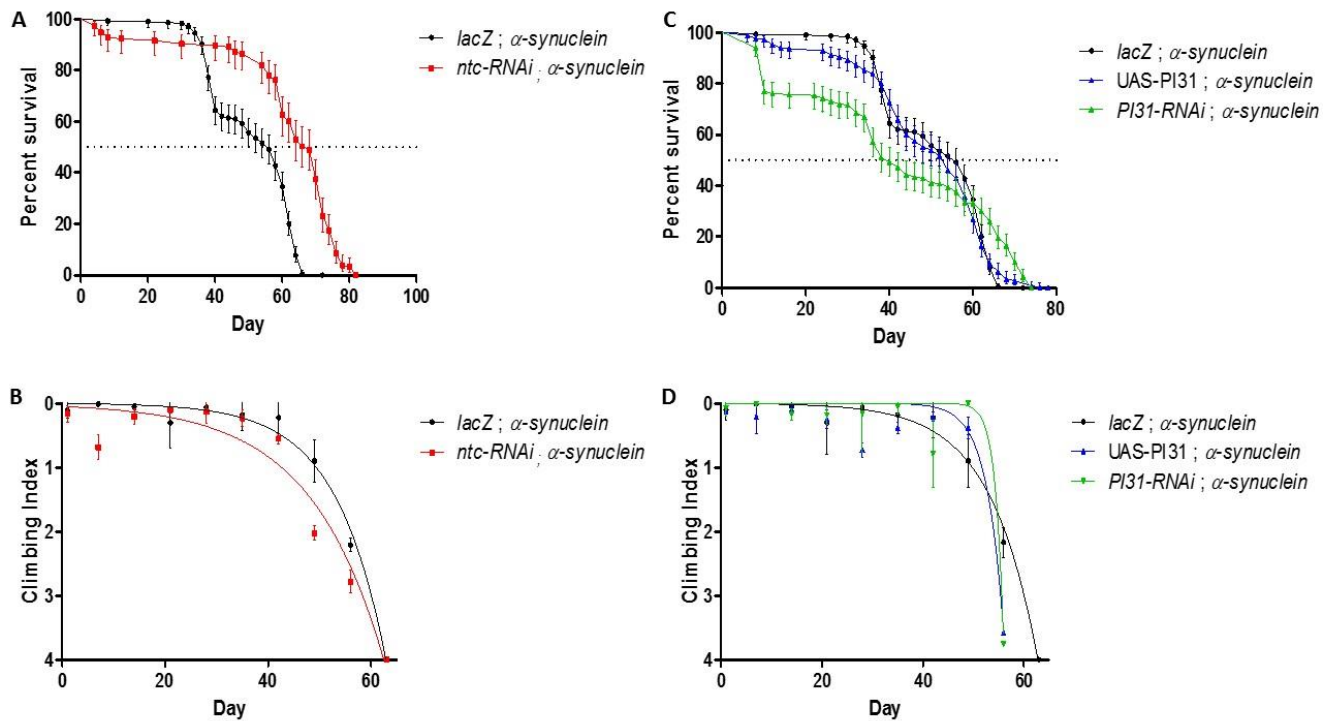
Figure 10: Expression of *ntc-RNAi* in an *α–synuclein* background rescues the observed decrease in median lifespan associated with a Parkinson Disease model. A) Tissue specific expression of *ntc-RNAi* in an *α–synuclein* background increases the mean lifespan of flies compared to *UAS-lacZ;α -synuclein* controls. B) Despite this increase in lifespan, the locomotor activity of flies expressing the *ntc-RNAi* construct is decreased compared to controls. C) *UAS-PI31* expression had no significant effect on the mean lifespan of flies in an *α–synuclein* background but *PI31-RNAi* construct expression causes a severe and significant decrease in mean lifespan compared to *UAS-lacZ;α–synuclein* controls. D) The locomotor ability of *UAS-PI31* expressing flies is not significantly different from controls but *PI31-RNAi* expressing flies climb slightly better than *UAS-lacZ;α –synuclein* controls. Longevity is shown as a percent survival ($P<0.05$ as determined by the Mantel-Cox Log Rank test), N=>200. Climbing ability was determined via nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean, n = 50. Statistical values may be found in supplemental table S2.4. (Aging) and S2.5. (Climbing).

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indicates that expression of *ntc-RNAi* in an *α-synuclein* background has a positive effect

in dopaminergic neurons. Despite this increase in mean lifespan, however, locomotor

climbing assays indicate that there is no significant difference in time between when *ntc-*

RNAi; α-synuclein and *lacZ ; α-synuclein* flies lose their climbing ability (Figure 10B).

This indicates that although the mean lifespan of *ntc-RNAi* flies is longer in a PD

background, the motor ability of these organisms may be compromised earlier in overall

lifespan than in *lacZ* controls.

Dopaminergic *PI31-RNAi* expression in an *α-synuclein* background led to a severe

and significant decrease in longevity compared to *lacZ* controls (Figure 10C). Flies

expressing both *α-synuclein* and *PI31-RNAi* lived only about 60% as long as *α-synuclein*

and *lacZ* expressing controls. Expression of *PI31* caused no significant difference in

mean lifespan compared to a *lacZ* control. Additionally, there was no significant

difference in the locomotor climbing ability of flies expressing both *PI31* and *PI31-RNAi*

in an *α-synuclein* background (Figure 10D).

3.4. Discussion

Mammalian *FBXO7* functions as an E3 ubiquitin ligase, which is the targeting

sub-unit of a multi-component proteasomal system responsible for recognizing and

destroying damaged or dysfunctional proteins in the cell. Mutations in *FBXO7* have been

identified as the cause of a particularly severe form of PD (Lai *et al.*, 2012). A putative

homologue of *FBXO7*, *nutcracker*, has been identified in *D. melanogaster*. We have

attempted to characterize the consequences of altered expression of this putative

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homologue in the neuron rich eye and in dopaminergic neurons on organism development and locomotor control.

Expression of a *UAS-ntc* transgene in the *D. melanogaster* eye led to a significant decrease in the number of ommatidia and bristles compared to *lacZ* controls at 25°C. Although the number of bristles continued to decrease significantly between 25°C and 29°C, the number of ommatidia remained constant across both temperatures. The largest discrepancy in this data is that no significant different in ommatidia or bristles was observed when *ntc* was overexpressed at 29°C. This indicates that *ntc* may be an important regulator of neuronal function but after a certain amount of this protein is absent the cell is able to adapt and prevent further destruction by alternative means. The E3 ubiquitin ligase proteins MuRF1 and FBXO32 (ATROGIN in *D. melanogaster*) have been shown to be rate limiting factors in cardiac muscle (Baumgarten *et al.*, 2013; Kedar *et al.*, 2004), thus it is possible that increased *ntc* expression has a negative effect on neuronal tissue formation until a certain threshold of expression is reached upon which time *ntc* it no longer the rate limiting factor in proteasome activity. It is also possible that the effect of *ntc* overexpression on the eye is less severe than the destruction associated with general transgene expression in the eye at this temperature (29°C).

Eye specific expression of *ntc-RNAi* leads to a significant decrease in both the number of ommatidia and bristles in the *D. melanogaster* eye compared to *lacZ* controls. Unlike the *ntc* overexpression phenotypes, this tissue disruption resulted in significantly less ommatidia and bristles at 29°C and 25°C. This indicates that expression of *ntc-RNAi* in the eye directly influences the viability of the eye tissues and causes more severe

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defects. Given that *ntc* is a necessary component for proper proteasome function, altered gene activity by the expression of an RNAi likely results in more aberrant protein accumulation within the cell and eventually cell death, leading to the observed phenotype of eye disruption. The increased severity of this phenotype with increased expression in the 29°C eye supports this observation. Altered expression of ntc both by *UAS-ntc* and *ntc-RNAi* lead to similar phenotypes, indicating that proper SCF proteasome complex function is imperative to overall cell health. An increase of *ntc* may lead to over stimulation of this complex, causing off target ubiquitin mediated destruction while a decrease in ntc expression may lead to harmful protein accumulations within the cell, eventually leading to premature cell mortality.

Altered dopaminergic expression of *ntc* caused by both *UAS-ntc* and *ntc-RNAi* led to a decrease in mean lifespan compared to *lacZ* controls. This decrease was significant and caused flies to live approximately 66% as long as the control group (mean lifespan of 40 days versus 60 days). It would be expected that expression of *ntc-RNAi* would decrease the mean lifespan of flies in relation to a control, since reduced *FBXO7* expression has been shown to reduce lifespan in vertebrate models (Randle *et al.*, 2015). Decreased longevity associated with expression of *UAS-ntc* may be due to an increase in proteasome activity, potentially causing non-specific degradation of target proteins. Direct interaction of FBXO7 and PARKIN has been found in human cells; this interaction has been shown to be important in the process of mitophagy (Burchell *et al.*, 2013). *FBXO7* and *ntc* may share a similar role in mammals and *D. melanogaster,* respectively, with regard to neuronal cell function and development.

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Altered activity of *PI31* caused a negative phenotype on the *D. melanogaster* eye. Expression of either a *UAS-PI31* or a *PI31-RNAi* transgene led to developmental defects in both the ommatidia and bristles of the eye and reduced longevity when expressed directly in dopaminergic neurons. This effect has been previously characterized in the *D. melanogaster* eye by Bader *et. al.* (Bader *et al.*, 2011), however, we are the first to look at the effect of altered *PI31* expression in dopaminergic neurons. Although PI31 acts as a stabilizing protein in the SCF ubiquitin ligase complex, it has also been characterized as a direct binding inhibitor of the 20S proteasome complex (Li *et al.*, 2014). Two separate areas of the PI31 protein are responsible for this activity with the C-terminal region binding to FBXO7 and the N-terminal region binding to the 20S proteasome (Kirk *et al.*, 2008). It is possible that a decrease in the amount of PI31 reduces the efficiency of the SCF proteasome complex due to de-stabilization while an increase in PI31 leads to inhibition of proteasome machinery via direct binding of extra PI31 to the proteasome itself.

Mutation of the *SNCA* gene leads to the accumulation of harmful protein aggregates of misfolded *α-synuclein* known as Lewy bodies (Spillantini *et al.*, 1998). Although no *D. melanogaster* homologue of this gene exists, transgene constructs expressing *α-synuclein* in flies have been characterized as effective models of human disease pathways (Feany & Bender, 2000). Ectopic expression of *α-synuclein* in neurons causes tissue defects and premature loss of locomotor ability. Over expression of *parkin* or *Pink1* with ectopic dopaminergic expression of *α-synuclein* results in a rescue phenotype, restoring locomotor ability to wild type levels (Haywood & Staveley, 2004;

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Todd & Staveley, 2008).   We co-expressed *α-synuclein* with either *ntc* or *PI31* in dopaminergic neurons to determine if the negative phenotype associated with ectopic *α-synuclein* expression is rescued or made worse in each case.

Co-expression of *ntc-RNAi* and *α-synuclein* in dopaminergic neurons led to a significant increase in lifespan compared to controls. Despite benefitting from increased longevity, flies expressing both *ntc-RNAi* and *α-synuclein* lose their climbing ability at the same time as *α-synuclein*-expressing control flies, indicating that although they live longer, they suffer from impaired locomotor control. Decreased *ntc* activity by the expression of *ntc-RNAi* may lead to lowered activity of the SCF ubiquitin proteasome. It is possible that this diminished proteasome activity could reduce the amount of cell death occurring, leaving unhealthy neurons containing intracellular inclusions that could in turn cause locomotor defects. Unhealthy neurons continue to function until a buildup of dysfunctional components causes whole cell death but are not eliminated immediately in this case which could explain why longevity is increased but climbing ability is not significantly different from controls.   Alternatively, a decrease in *ntc* coupled with the presence of intracellular inclusions caused by ectopic *α-synuclein* expression could lead to a protein stress response which has been linked to increased lifespan in flies (Houtkooper *et al.*, 2013). The exact mechanism of this protein response is yet unknown, however, it may be triggered by the low level presence of reactive oxygen species, which in turn trigger activity of cellular anti-oxidants (Lopez-Torres & Barja, 2008). The increased longevity induced by expression of *ntc-RNAi* in an *α-synuclein* background is likely caused by a combination of these factors.

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Co-expression of *PI31-RNAi* and *α-synuclein* led to a decrease in mean lifespan, while co-expression of *PI31* and *α-synuclein* had no significant effect on the mean lifespan of flies when expressed in dopaminergic neurons. The expression of *α-synuclein* caused a decrease in locomotor activity when expressed alone (Todd & Staveley, 2008), though we observed no significant difference in the climbing ability of flies when either *PI31* or *PI31-RNAi* were co-expressed.

Studying the homologues of known genes in a model organism such as *D. melanogaster* is a fundamental step in understanding and characterizing disease onset and progression. *FBXO7* has been implicated in a rare and severe form of disease known as PPS. In this paper, we show that expression of the putative *D. melanogaster* homologue of *FBXO7*, *nutcracker*, leads to reduced mean lifespan when altered in neuronal cells. We have shown that the putative *D. melanogaster* homologue of the tightly controlled mammalian *PI31* regulator leads to severe defects when expression is altered in neurons. Additionally, we have shown that altered expression of either *ntc* or *PI31* in an *α-synuclein* model of PD leads to a different outcome than in a control background. These models of *H. sapiens* PD in *Drosophila melanogaster* may be further studied to determine pathways of onset which may eventually lead to novel therapeutic treatments for combating disease progression.

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Chapter 4 – Altered expression of a putative *FBXO9* homologue models Parkinson Disease in *Drosophila melanogaster*

4.1. Introduction

Progressive and continuous loss of function of neurons in specific areas of the brain can lead to neurodegenerative disorders such as Parkinson Disease (PD) and Alzheimer Disease (AD) (Xie *et al.*, 2014). Neuronal dysfunction can be caused by environmental factors, such as exposure to pesticide and neurotoxic metals, or by the inheritance of disease causing alleles of specific genes (Chin-Chan *et al.*, 2015; Johnson, 1991). Although either environmental or genetic influences can lead to disease independently, in many cases, it is a combination of the genetic risk factors and exposure to toxins that can determine the onset of disease (Kalia & Lang, 2015). The identification and characterization of genes responsible for inherited forms of neurodegenerative disease may lead to new therapeutic approaches and novel treatments to slow and potentially prevent disease progression, regardless of the underlying cause.

PD and AD, the two most prevalent human neurodegenerative disorders, may both result from the accumulation of proteins into proteinaceous inclusions. A hallmark of PD is the presence of Lewy bodies, which are intracellular cytoplasmic inclusions composed of mis-folded aggregate clusters of α–synuclein (α–SYN), produced by altered expression of the *SNCA* gene (Olanow & McNaught, 2011). In the conventional model of AD, the presence of TAU inclusions, hyper-phosphorylated deposits of the microtubule stabilizing protein TAU, and an over-abundance of the amyloid precursor protein cause premature

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neuronal death and disease symptoms (Goedert *et al.*, 1995; Wright *et al.*, 1991).

Although these proteins were originally implicated in the pathogenesis of either PD or

AD, aberrant activity of *α–SYN* and *Tau* products has been determined to be present in

both conditions (Moussaud *et al.*, 2014). *α–SYN* has been implicated in the pathogenesis

of AD by playing a role in abnormal synapse formation, while high levels of TAU in

cerebral spinal fluid has been used as a powerful diagnostic tool in pre-screening for PD

(Kang *et al.*, 2013; Kim *et al.*, 2004). This suggests that a mechanism of neuronal

destruction and disease may be somewhat conserved between PD and AD. Aberrant

protein clusters, caused by mutant forms of α–SYN and TAU, prevent normal cellular

function and increase the oxidative stress level of the cell, eventually leading to premature

mortality (Atkin & Paulson, 2014). Although the cell is equipped with mechanisms to

prevent permanent damage associated with aberrant protein function, defects in

intracellular protein turnover have been associated with neurodegenerative disease.

The ubiquitin proteasome system is one of the basic cellular mechanisms devoted

to the degradation of intracellular components in a tightly regulated and highly specific

manner (Glickman & Ciechanover, 2002). This complex selectively targets proteins

within the cell and adds a single or multiple ubiquitin moieties, which in turn either alters

the function of the protein or marks it for future destruction by the 26S proteasome (Chau

et al., 1989; Zeng *et al.*, 2009). This system works through the activity of three enzymes:

1) the E1, activating enzyme; 2) the E2 conjugating enzyme; and 3) the E3 ligating

enzyme (Ciechanover *et al.*, 2000). Mutation of E3 enzymes has been implicated as a

cause of disease pathology (Bielskiene *et al.*, 2015). A sub-class of E3 enzymes contain

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unique proteins known as F-box proteins. These proteins contain a conserved F-box domain used to bind to the Skp-Cullin complex, creating the Skp-Cullin-F-box (SCF) ubiquitin ligase complex (Cardozo & Pagano, 2004). The SCF ubiquitin ligase complex is integral to proper cellular health and protein turnover, with impaired function leading to premature cell death and disease phenotypes (Genschik *et al.*, 2013). Current research into new therapeutics has focused on emulating or mimicking the activity of the E3 components of these complexes to stimulate the removal of harmful proteins in the cell (Bulatov & Ciulli, 2015). The targeting function of the F-box proteins is an essential aspect of the SCF ubiquitin ligase complex.

In particular, the FBXO group of proteins is of interest to the study of disease, as a number of these are involved in essential biological processes and have been implicated as causative factors in disease onset and progression. Two of these FBXO genes have been directly implicated in degenerative disorders: *FBXO7*, also known as *PARK15*, has been shown to be mutated in a severe early onset form of PD known as Parkinsonian-pyramidal syndrome (Di Fonzo *et al.*, 2009); while *FBXO32*, or *atrogin*, has been linked to muscle wasting phenotypes (de Palma *et al.*, 2008). Both *FBXO7* and *FBXO32* belong to the same evolutionary sub-family along with another gene, *FBXO9*, which is poorly characterized (Cenciarelli *et al.*, 1999). Understanding the role that *FBXO9* plays in pathways of ubiquitination may lead to a better understanding of various disease phenotypes.

The putative *D. melanogaster* homologue of *FBXO7*/*nutcracker* (*ntc*) was originally identified as a factor in the terminal differentiation of sperm with *ntc* mutations

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causing male sterility (Bader *et al.*, 2010). The highly conserved F-box binding domain found in *FBXO7* is conserved in *ntc*. Altered expression of *ntc* in both the neuron rich *D. melanogaster* eye and dopaminergic neurons has been found to lead to degenerative, PD-like phenotypes, mimicking the symptoms observed with altered *FBXO7* function in *Homo sapiens* (See Chapter 3). The high degree of functional conservation between *FBXO7* in humans and flies suggests that *FBXO9* may also have a functional homologue in *D. melanogaster*. We have attempted to identify and characterize the putative fly homologue of *FBXO9* and we have determined that altered expression can influence development of the eye and reduce longevity when expressed in the dopaminergic neurons.

4.2. Materials and Methods

4.2.1. Drosophila Media

The standard cornmeal-yeast-molasses-agar medium is made with 65 g/L cornmeal, 10 g/L nutritional yeast and 5.5 g/L agar supplemented with 50 ml/L fancy grade molasses and 5 ml of 0.1 g/ml methyl 4-hydroxybenzoate in 95% ethanol and 2.5 ml of propionic acid in standard plastic vials. Media is stored at $4°C$ but warmed to room temperature before use.

4.2.2. Drosophila Transgenic Lines

The *Ddc-Gal4$^{HL4.3D}$* (*Ddc-Gal4*) line was the generous gift of Dr. Jay Hirsh (University of Virginia) (Lin *et al.*, 2000). The following lines were obtained from the Bloomington Drosophila Stock Center at Indiana University-Bloomington: 1) to drive expression

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behind the morphogenetic furrow in the developing eye disc *Glass Multiple Reporter-Gal4*[12] (*GMR-Gal4*; (Freeman, 1996)); 2) to act as a control for the ectopic expression of transgenes *UAS-lacZ*[4-2-1] (*UAS-lacZ*; (Brand & Perrimon, 1993)) ; 3) To alter expression of *CG5961, P{EP}CG5961*[G4347] (*CG5961*[EP]) and *P{TRiP.JF01332}attP2 [CG5961-JF01332]*(*CG5961-RNAi*).

4.2.3. Scanning Electron Microscopy of *Drosophila Melanogaster* Eyes

Female virgins of the *GMR-Gal4* were mated with *UAS-lacZ, CG5961*[EP] and *CG5961-RNAi* males. Male progeny of each cross were collected, aged for 3 to 5 days and frozen at -80°C. Flies were mounted to metal stubs and desiccated for 24 hours pre-imaging. The eyes of mounted flies were imaged via scanning electron micrography at 130 times magnification with a Mineral Liberation Analyzer 650F scanning electron microscope. Total bristle count and ommatidia count were obtained using ImageJ (Schneider *et al.*, 2012).

4.2.4. Ageing Analysis

Female virgins of the *Ddc-Gal4* line were mated with *UAS-lacZ, CG5961*[EP] and *CG5961-RNAi* males. Male progeny of each cross were collected each day and placed into separate vials of no more than 20 flies. Vials were scored for survival every 2 days and media was changed after each death event or every 3 days. Survival curves were compared by the log-rank (Mantel Cox) test, graphs were created using GraphPad Prism software.

4.3. Results

FBXO9 belongs to a sub-family of genes responsible for target specificity in ubiquitin ligase complexes along with the well-established Parkinson gene *FBXO7/PARK15*. A phylogenetic analysis shows that evolution between the FBXO orthologues is consistent among species (Figure 11A). Despite divergent evolution, functional domains and conserved residues are well-maintained between both *FBXO9* and *FBXO7*, suggesting important functions for both genes with regard to organism survival. A bioinformatics search (tBLASTn) using human *FBXO9* as a query identified a putative *D. melanogaster* homologue, *CG5961*. The overall similarity between the two proteins is 33%, however, the level of conservation within protein functional domains is greater. The human FBXO9 protein is 437 amino acids in length, while the putative Drosophila protein is 442 amino acids. Each of the two proteins shares 4 distinct domains: a microtubule interacting and trafficking domain (MIT), a tetratricopeptide repeat sequence (TRS), an F-box domain and a c-terminal nuclear localization sequence (NLS) (Figure 12B). Of these domains, the highest degree of similarity is in the F-box domain that spans 51 amino acids in length with 33 absolutely conserved amino acid residues and 12 well-conserved functionally similar amino acid residues (Figure 12C). However, human FBXO9 contains an HNH nuclease family domain (Jin *et al.*, 2004) that we were unable to identify in the *D. melanogaster* protein. The high degree of similarity between conserved active domains suggests that *CG5961* is the *D. melanogaster* homologue of *FBXO9*.

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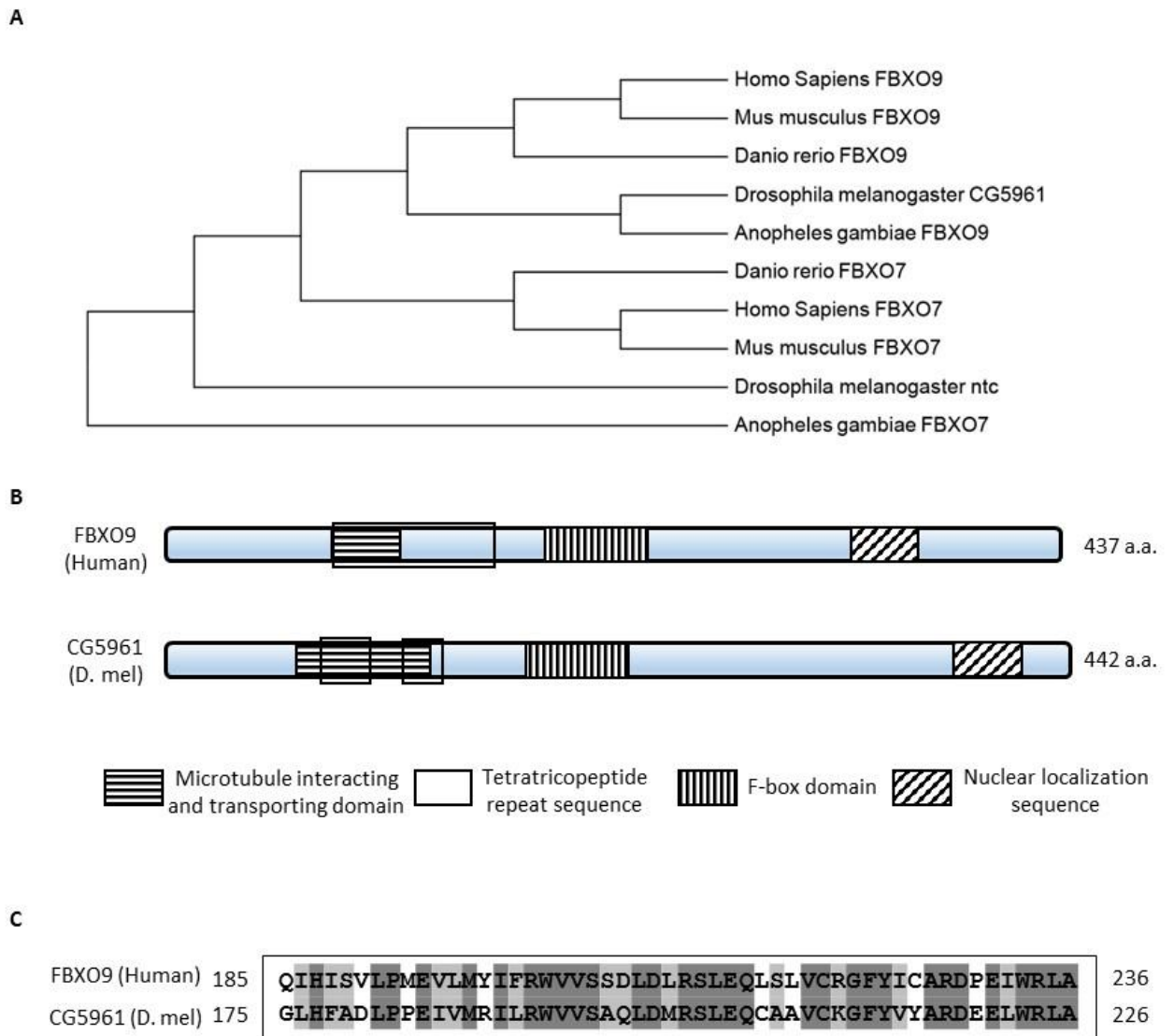
Figure 11: Alignment of the human FBXO9 and *Drosophila melanogaster* CG5961 proteins shows a high degree of conservation. A) FBXO7 and FBXO9 are highly conserved among vertebrates and invertebrates. B) Both human and *D. melanogaster* FBXO9 proteins contain similar microtubule interacting and trafficking domains(dark horizontal), tetratricopeptide repeat (no fill), and F-box domains (dark vertical) and have a similar protein length of 437 amino acids and 442 amino acids respectively. Each protein also contains a nuclear localization sequence (wide upward diagonal) at the carboxyl-terminal end. C) Alignment of the protein sequence of the F-box domain between *D. melanogaster* and human FBXO9 shows a high degree of similarity. Dark grey indicates a perfect match; light grey indicates substitution of an amino acid with similar function. Domains were identified using ScanProsite (De Castro E, 2006), Pfam (Finn *et al.*, 2014) and NLS mapper (Kosugi *et al.*, 2009) alignment generated by ClustalW2 (Larkin MA,

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2007), cladogram created using maximum parsimony method and bootstrapped 5000 times using MEGA6 (Tamura *et al.*, 2013). Protein sequences were obtained from UniProt, ascension numbers [NP_258441] *Homo sapiens* FBXO9, [NP_036311] *Homo sapiens* FBXO7, [NP_001074959] *Mus musculus* FBXO9, [NP_694875] *Mus musculus* FBXO7, [NP_956012] *Danio rerio* FBXO9, [NP_001020670] *Danio rerio* FBXO7, [NP_650206] *Drosophila melanogaster* CG5961, [AAF47792] *Drosophila melanogaster* ntc, [XP_308962.3] *Anopheles gambiae* FBXO9, [XP_565383] *Anopheles gambiae* FBXO7. Alignment may be found in supplemental figure 3.1.
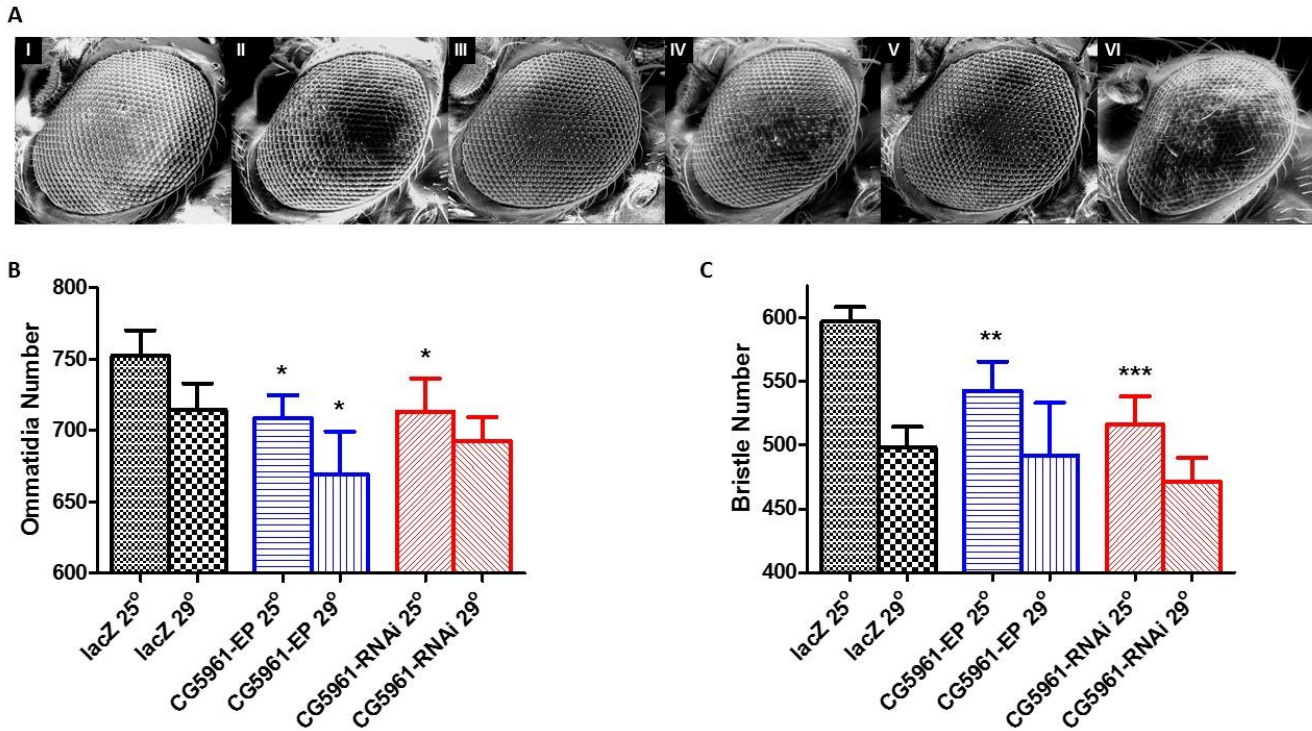
Figure 12: Altered tissue specific expression of *CG5961* in the *Drosophila melanogaster* eye causes a decrease in ommatidia and bristle number. A) Scanning electron micrographs of *D. melanogaster* eyes taken at a horizontal field width of 500 µm. Genotypes are as follows: I) *GMR-Gal4 / UAS-lacZ* 25°C; II) *GMR–Gal4 / UAS-lacZ* 29°C; III) *GMR-Gal4 / CG5961^{EP}* 25°C; IV) *GMR-Gal4 / CG5961^{EP}* 29°C; V) *GMR–Gal4 / CG5961-RNAi* 25°C; VI) *GMR–Gal4 / CG5961-RNAi* 29°C. Images were taken with a FEI MLA 650. B) The *D. melanogaster* eye shows a decrease in number of ommatidia and bristles when either *CG5961^{EP}* or a *CG5961-RNAi* transgene is under the control of *GMR-Gal4*. This decrease in tissue formation is more severe at the elevated temperature of 29°C likely due to the increased efficiency of the *Gal4-UAS* system. Comparisons were made between 25°C control and experimental flies and 29°C control and experimental flies and measured using a one-way ANOVA and significance was tested using a Tukey post-hoc test, n=10. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Statistical values may be found in supplemental table S3.2.

The *D. melanogaster* eye has been used to determine the neurodevelopmental effects of gene alteration since both ommatidia and bristles, the two main tissues composing the eye, are of neuronal origin (Cook *et al.*, 2011). To determine the broad scale neuronal implications of altered *CG5961* activity, we directed the expression of *CG5961* (via *CG5961^{EP}*) and the inhibition of *CG5961* (via a *CG5961-RNAi* transgene), using the *GMR-Gal4* transgene in the developing *D. melanogaster* eye. These experiments were conducted at both at 25°C and 29°C, the former acting as the standard physiological condition and the latter to elevate the activity of *Gal4-UAS* system expression. Tissue specific expression of *CG5961* caused a significant decrease in both ommatidia and bristle number compared to *lacZ* controls at 25°C (Figure 12). Directed inhibition through the expression of the *CG5961-RNAi* construct caused a similar loss in ommatidia and bristle number as expression of *CG5961* did compared to *lacZ* controls at 25°C but this effect was not seen at 29°C. It would appear as though *CG5961* has a purpose in the neurons composing the *D. melanogaster* eye and changes to expression have a significant negative effect at 25°C, however, this effect is not observed at 29°C and no significant effect on eye formation is observed.

To determine the effect of altered *CG5961* expression in neuronal cells directly linked to neurodegenerative disease, both *CG5961^{EP}* and *CG5961-RNAi* transgenes were expressed in the dopaminergic neurons under the control of the *Ddc-Gal4* transgene. Dopaminergic specific expression of *CG5961^{EP}* caused a significant decrease in mean lifespan compared to *lacZ* controls (Figure 3). Fifty percent of the *CG5961^{EP}* flies died by day 48, while the control *lacZ* flies did not reach 50 percent survival until day 60.

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This is a significant decrease in lifespan of approximately twenty percent. Expression of

CG5961-RNAi in dopaminergic neurons caused a decrease in longevity (44 days)

compared to *lacZ* controls of approximately twenty-five percent (Figure 13). This

decrease was slightly more severe than seen with the expression of *CG5961^{EP}* and

indicates that in dopaminergic neurons, much like the *D. melanogaster* eye, *CG5961* is

tightly regulated and altered expression of this gene causes severe negative consequences.
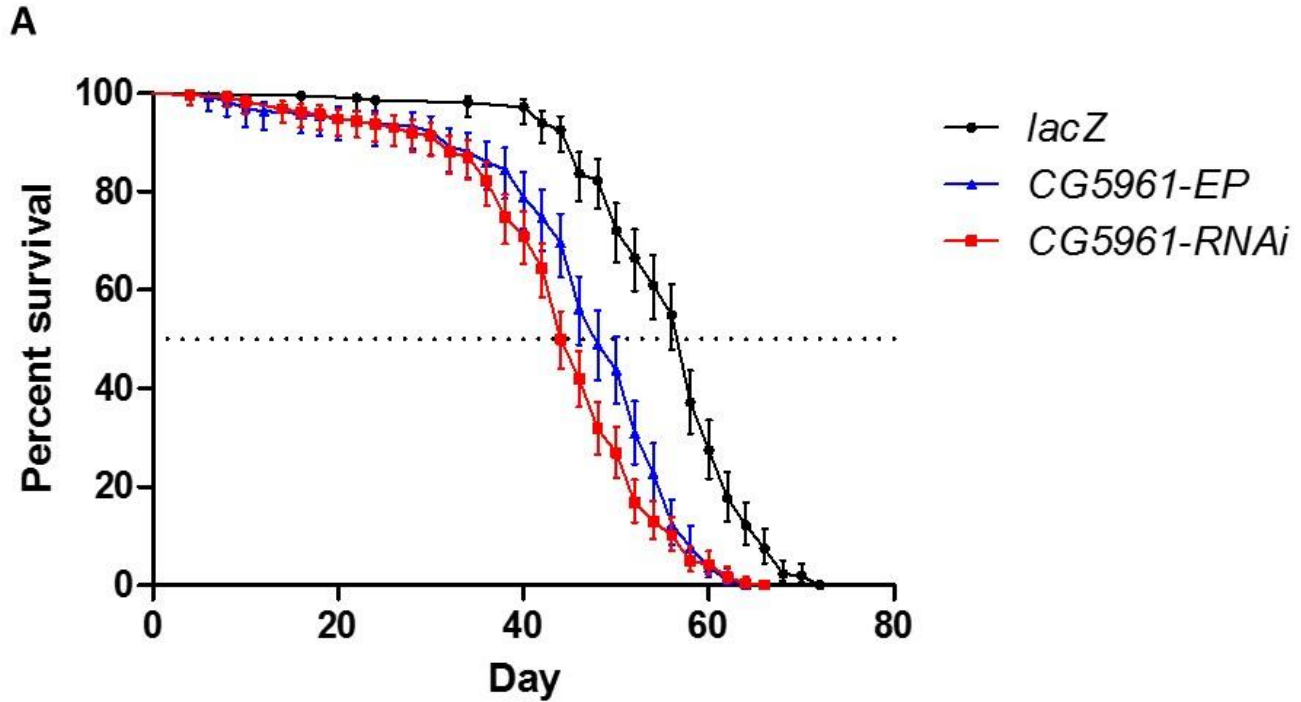
Figure 13: Tissue specific expression of both *CG5961* and *CG5961-RNAi* causes a decrease in longevity similar to established models of Parkinson Disease in *Drosophila melanogaster*. Expression of *CG5961* under the control of *Ddc-Gal4* causes a significant decrease in lifespan (*Ddc-Gal4*; *CG5961^EP*: median 48 days) compared to *lacZ* controls (*Ddc-Gal4*; *UAS-lacZ*: median 60 days). Similarly, expression of a *CG5961-RNAi* transgene under the control of *Ddc-Gal4* causes a greater loss in longevity (*Ddc-Gal4*; *CG5961-RNAi*: median 44 days). (P<0.0001 as determined by the Mantel-Cox Log Rank test) N=>200). Statistical values may be found in supplemental table S3.3.

4.4. Discussion

The four protein domains conserved between human *FBXO9* and the putative *D. melanogaster* homologue provide some insight into the function of this gene. The highly conserved F-box domain is found in each of the *D. melanogaster* homologues of the *FBXO* family of F-box proteins including *FBXO7* and *FBXO32* and has been determined to be responsible for mediating the protein-protein interaction between the F-box protein and the SCF ubiquitin ligase complex (Cardozo & Pagano, 2004). The microtubule interacting and trafficking (MIT) domain has been implicated in endosomal trafficking and microtubule movement (Ciccarelli *et al.*, 2003). Current knowledge of MIT domains focuses on the endosomal trafficking ATPase Vps4, which becomes activated by the direct binding of an additional protein termed the MIT-interacting motif (Hurley & Yang, 2008). The tetratricopeptide repeat (TPR) domain is a 34 amino acid sequence motif found in a number of diverse proteins responsible for forming scaffolds for protein-protein interactions (Blatch & Lassle, 1999). The structure of the TPR closely resembles that of the MIT and both suggest a role in protein-protein interaction within the cell, a role suited to the targeting function associated with other FBXO proteins. The nuclear localization sequence tags a protein for import into the nucleus and suggests that the activity of *FBXO9* may be localized to that location (Marfori *et al.*, 2011). Taken together, the presence of these conserved domains indicates that human *FBXO9* and the putative homologue *CG5961* likely both function as components of a multi-component SCF ubiquitin ligase complex.

The ommatidia and bristles of the *D. melanogaster* eye are formed from neuronal precursors and allow for highly sensitive characterization of mutations that alter neural developmental function (Sang & Jackson, 2005). Specific altered expression of *CG5961* in the neuron rich *D. melanogaster* eye leads to the disruption of tissue formation and a decrease in both ommatidia and bristle number. Expression of *CG5961* appears to be highly regulated in the neurons of the eye and deviation of this expression leads to severe phenotypes. Although there has been no previous investigation into the effects of altered *CG5961* expression in the *D. melanogaster* eye, *CG5961* appears to be essential and necessary for proper neuronal cell development.

Dopaminergic neurons produce dopamine, a neurotransmitter necessary for motor control. Impaired dopamine production through the improper functioning of these neurons leads to severe defects and premature mortality. To determine if the observed negative phenotype associated with driven expression of *CG5961* in the eye was conserved in neuronal tissues, we evaluated the effects of altered expression in dopaminergic neurons to determine what, if any, consequence it would have on overall lifespan in *D. melanogaster*. Directed dopaminergic expression of either *CG5961* or *CG5961-RNAi* led to a significant decrease in overall lifespan compared to *lacZ* controls. This decrease in lifespan indicates that *CG5961* is essential in the proper function of dopaminergic neurons. Furthermore, it appears as if *CG5961* may be regulated in a highly controlled manner, however, the nature of this regulation remains unknown. Mutation of both *FBXO7* and *FBXO32* have been linked to human disease phenotypes, with altered expression of *FBXO7* causing a PD like phenotype in *D. melanogaster* (See

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Chapter 3). The decrease in longevity found when *CG5961* is altered in dopaminergic neurons suggests the possibility of a link between the human *FBXO9* gene and human degenerative disease.

The FBXO family of genes have been implicated in a number of inherited genetic disorders. Although *FBXO7* and *FBXO32* have been identified to participate in the processes of neurodegeneration and muscular degeneration respectively, a closely related gene, *FBXO9*, has not been well characterized. We show the *D. melanogaster CG5961* gene has a high degree of similarity to *Homo sapiens FBXO9* and that altered expression of this gene leads to degenerative phenotypes in both the eye and dopaminergic neurons of flies. This is the first evidence of a potential link between *FBXO9* and degenerative disease phenotypes and shows that the putative *D. melanogaster* homologue of *FBXO9* may be an important gene in neuronal development and function. Further research into the pathways and interacting proteins involved in pathways of *FBXO9* mediated proteolytic activity may lead to new targets for disease prevention and therapeutic strategy.

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Chapter 5 – Identification and evaluation of potential PARIS homologues in *Drosophila melanogaster*

5.1. Introduction

Parkinson Disease (PD) is the second most common neurodegenerative disorder, affecting more than 1.6% of individuals over 65 years of age, with an associated annual healthcare cost of an estimated $25 billion US dollars (as reported in 2011) (Beitz, 2014). PD patients present with symptoms related to impaired motor control including bradykinesia, postural rigidity and resting tremor plus, in later stages, diminished cognitive function including dementia and memory loss (Klockgether, 2004). The former symptoms are well known to occur as a direct result of lost neurons in the *substantia nigra pars compacta* region of the brain, leading to a decrease in the available neurotransmitter dopamine. Efforts to combat PD have focused on symptom alleviation and fail to prevent progression or stem the initial onset of disease. PD may be caused by inherited or environmental factors and in many cases a combination of genetic risk factors, and exposure to harsh environmental conditions such as toxins combine to lead to symptoms of disease (Mitsui, 2013). Many genes implicated in PD onset have function either related to or directly linked to the proper maintenance and upkeep of mitochondria. Mitochondria are the site of oxidative phosphorylation and produce much of the energy for a given cell; impaired function of these organelles in neurons with high energy requirements may be the origin of premature mortality and disease onset found in PD (Ryan *et al.*, 2015). The intracellular balance of mitochondria is an essential part of

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proper cell function and changes to this balance can result in a number of negative

consequences. In the event of mitochondrial damage, impaired organelles are repaired or

removed and recycled into usable components. If removal is necessary, a separate

process initiates the formation of new mitochondria to restore the energetic capacity of

the cell (Palikaras & Tavernarakis, 2014). Mutation to genes involved in either of these

processes can cause a loss of cellular energy and eventual cell mortality. Identifying and

characterizing the genes responsible for mitochondrial impairment in PD may lead to new

therapeutic targets and novel options in disease management.

Parkin and *PTEN-induced putative kinase 1* (*Pink1*) are two genes that function in

the normal turnover of damaged or dysfunctional mitochondria and prevent the

accumulation of potentially toxic impaired organelles (Eiyama & Okamoto, 2015).

Parkin encodes an E3 ubiquitin ligase that leads to the ubiquitination and subsequent

destruction of cellular proteins (Narendra *et al.*, 2008). Mutations to the *Parkin* gene

result in the degeneration of dopaminergic (DA) neurons, most likely by allowing the

aggregation of multiple dysfunctional mitochondria that eventually lead to overall cell

death (Greene *et al.*, 2003). PINK1 is a serine / threonine-protein kinase that acts by

recruiting PARKIN to damaged mitochondria (Koh & Chung, 2011). Similarly,

mutations to PINK1 lead to degeneration and dysfunction of DA neurons (Yang *et al.*,

2006). Under normal physiological conditions, mitochondria prevent the outer membrane

accumulation of the PINK1 protein. When the mitochondria becomes depolarized this no

longer occurs, allowing a build-up of PINK1 at the mitochondria which then recruits the

Parkin gene product (PARKIN) and begins the cascade of ubiquitin mediated organelle

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destruction (Eiyama & Okamoto, 2015). Mutations in *Pink1* or *Parkin* have been directly linked to early onset forms of human PD.

The *Parkin* mediated removal of impaired mitochondria leads to an overall decrease in number of cell organelles, this mitochondrial deficit is corrected by a parallel pathway involving the peroxisome proliferation activated co-receptor (*PGC*) family of genes (Scarpulla, 2011). The three members of this gene family, *PGC-1α*, *PGC-1β* and *PGC-1 related cofactor* (PRC) all share similar and partially redundant function in mitochondrial biogenesis. *PRC* has been implicated to be present mostly in proliferating cells while *PGC-1α* and *PGC-1β* are both found in tissues with high energetic requirements such as skeletal muscle (Gleyzer & Scarpulla, 2011; Rowe *et al.*, 2012; Russell *et al.*, 2005). Although *PGC-1α* and *PGC-1β* are expressed in the same tissues and have been linked to the regulation of exercise endurance, *PGC-1α* has specifically been found to be induced by stimuli including stress, exercise and fasting (Lehman *et al.*, 2000; Watson *et al.*, 2007). Under normal physiological conditions, the *PGC-1α* gene is repressed by the negative regulatory protein PARIS (Parkin Interacting Substrate) (Shin *et al.*, 2011). The PARIS protein in *Homo sapiens* is coded for by the *ZNF746* gene (herein referred to simply as *PARIS*) and has been shown to be localized to neurons, including those of the *substantial nigra pars compacta* (Shin *et al.*, 2011). Overexpression of *PARIS* in a mouse model resulted in destruction of dopaminergic neurons which could be rescued with the overexpression of either *Parkin* or *PGC-1α* (Shin *et al.*, 2011). It appears that *Parkin* may act as a master regulator keeping the balance between mitochondrial removal and biogenesis and ensuring the cell receives an

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appropriate amount of energy for normal function. Subsequently, altered expression of

downstream components, including *PARIS* and *PGC-1α*, lead to disease phenotypes in

otherwise healthy organisms.

       Drosophila melanogaster has been used as a model organism of human disease

research with great success. Similarities between the nervous system of *Drosophila* and

Homo sapiens allow for complex study of neurodegenerative disorders (Sang & Jackson,

2005; West *et al.*, 2015). The neuron rich *Drosophila* eye is an ideal system to study the

broad effect of genetic mutation on neuronal tissue, providing a highly sensitive array of

ommatidia and bristle tissues that respond and are altered by any negative change to

neuron cell architecture (Mishra & Knust, 2013). Many genes, including *Parkin* and

Pink1, implicated in PD pathology have functional *D. melanogaster* homologues and

show severe neuronal dysfunction when expression is altered (Clark *et al.*, 2006;

Haywood & Staveley, 2004). Unlike the three PGC family members present in humans,

D. melanogaster have only a single *PGC-1α* homologue, *spargel* (*srl*) (Tiefenbock *et al.*,

2010). Previous research has indicated that altered *srl* expression may lead to

parkinsonian phenotypes in *D. melanogaster*, providing a powerful model to study the

complex interactions behind disease progression (Merzetti & Staveley, 2015). The

conserved activity of gene homologues for *Parkin*, *PGC-1α* and *Pink1* shows that

pathways of mitochondrial upkeep and biogenesis are highly conserved across organisms.

Despite this, the only *H. sapiens* gene found to act upon the *Parkin/Pink1* pathway

without a putative *D. melanogaster* homologue is *PARIS*. We seek to identify potential

PARIS candidates in *D. melanogaster* and to characterize these candidates in neuron tissues to determine their potential role in pathways of disease.

5.2. Materials and Methods

5.2.1. Drosophila Culture

The *Ddc-Gal4$^{HL4.3D}$* (*Ddc-Gal4*) line was the gift of Dr. Jay Hirsh (University of Virginia) (Lin *et al.*, 2000). The following lines were obtained from the Bloomington Drosophila Stock Center at Indiana University-Bloomington: 1) to drive expression behind the morphogenetic furrow in the developing eye disc *glass multiple reporter-Gal4*[12] (*GMR-Gal4*; (Freeman, 1996)); 2) to act as a control for the ectopic expression of transgenes *UAS-lacZ^{4-2-1}* (*UAS-lacZ*; (Brand & Perrimon, 1993)); 3) to express *spargel (srl): y w; P{EPgy2^{srlEY05931}}5.2.2(srl-EY)* and 4) to inhibit the expression of *srl*: *y sc v; P{TRiP.HMS00858}attP2* (*UAS-srl^{HMS00858}*) (*UAS-srl-RNAi*). RNAi transgenes for putative *PARIS* homologues were obtained from the Vienna Drosophila Resource Center stock numbers 7779 *UAS-Crol-RNAi*, 43655 (*UAS-CG15269-RNAi*) and 39986 (*UAS-CG15436-RNAi*) (Dietzl *et al.*, 2007).

The standard cornmeal-yeast-molasses-agar medium is made with 65 g/L cornmeal, 10 g/L nutritional yeast and 5.5 g/L agar supplemented with 50 ml/L fancy grade molasses and 5 ml of 0.1 g/ml methyl 4-hydroxybenzoate in 95% ethanol and 2.5 ml of propionic acid in standard plastic vials. All media was stored at 4°C and warmed to room temperature for use as required.

5.2.2. Scanning Electron Microscopy of the *Drosophila melanogaster* Eye

Female virgins of the *GMR-Gal4* line were mated with *UAS-lacZ*, *UAS-Crol-RNAi*, *UAS-CG15269-RNAi*, *UAS-CG15436-RNAi*, *UAS-srl-EY* and *UAS-srl-RNAi* males. Male progeny of each cross were collected, stored in vials of no more than 20 flies, aged 3-5 days and frozen at

-80°C. Flies were mounted to metal stubs and desiccated for 24-48 hours pre-imaging. The eyes of mounted flies were imaged via scanning electron micrograph at 130 times magnification with a Mineral Liberation Analyzer 650F scanning electron microscope. Total bristle count, and ommatidia count were obtained using ImageJ (Schneider *et al.*, 2012).

5.2.3. Ageing Analysis

Female virgins of the *Ddc-Gal4* line were mated with *UAS-lacZ*, *UAS-Crol-RNAi*, *UAS-CG15269-RNAi* and *UAS-CG15436-RNAi* males. Male progeny of each cross were collected in vials of no more than 20 flies. Vials were scored for survival every 2 days and media was changed after each death event or every 3 days. Survival curves were compared by the log-rank (Mantel Cox) test, graphs were created using GraphPad Prism software.

5.3. Results

The *H. sapiens PARIS* gene encodes a protein of a predicted 645 amino acids with identified homologues in chimpanzee, rhesus monkey, cow, mouse and rat. A detailed bioinformatics analysis of PARIS shows the presence of an N-terminal Kruppel

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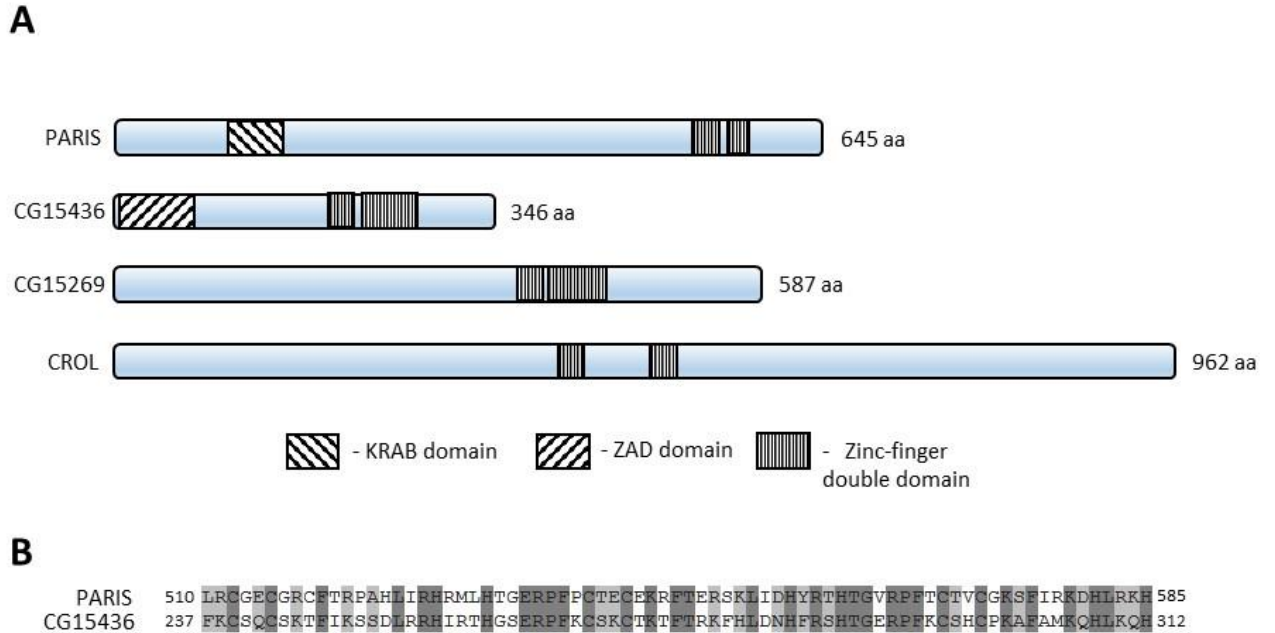
Figure 14: The *Homo sapiens* PARIS protein shares conserved protein domains with putative *Drosophila melanogaster* homologues *Crol*, *CG15269* and *CG15436*. A) Aligned protein sequences show the position of protein domains in three putative *D. melanogaster* homologues of *PARIS*. Wide downward diagonal indicates KRAB domain, wide upward diagonal indicated ZAD domain and narrow vertical box indicates zinc-finger double binding domains. B) Alignment of the zinc-finger double binding domain in the *Homo sapiens* PARIS and *D. melanogaster* CG15436 proteins shows a high degree of amino acid conservation. Dark grey indicates same amino acid; light grey indicates an amino acids of similar function. Domains were identified using ScanProsite (De Castro E, 2006), and Pfam (Finn *et al.*, 2014) alignment generated by ClustalW2 (Larkin MA, 2007). Protein sequences were obtained from UniProt, ascension numbers NP_001156946.1 (PARIS), NP_477243.1 (CROL), NP_609739.2 (CG15269 and NP_608840.1 (CG15436). Alignment and annotation of proteins may be found in supplementary figure S4.1.

Associated Box (KRAB) domain and two C-terminal zinc-finger double domains (Figure

1). KRAB domains are transcriptional repressors found in C2H2 zinc finger proteins.

These functional modules comprise the largest group of transcriptional regulators in

mammals and are limited to the tetrapod superclass of organisms (Urrutia, 2003). Despite

the significance of this gene in regulation of mitochondrial biogenesis and cellular energy

production, currently there are no known *PARIS* homologues identified in non-

mammalian lineages. The PARIS protein was used as a query to identify potential *D.*

melanogaster homologues using NCBI Blast (Altschul *et al.*, 1990). Of the proteins, the

three top identified candidates were crooked legs (CROL) with 35% identity, CG15436

with 33% identity and CG15269 with 29% identity to the PARIS *H. sapiens* protein. The

overall protein structure of these putative homologues were compared to the PARIS

protein to determine potential conserved domains (Figure 14). CG15436, CG15269 and

CROL all contain C-terminal zinc-finger double domains similar to those found in the

PARIS protein (Figure 14A). CROL is a protein composed of 962 amino acids and has

activity as a negative regulatory factor involved in proper limb development. CG15269

encodes a predicted protein of 587 amino acids with no identified function. The activity

of CROL and presence of zinc finger motifs in CG15269 would suggest that it may also

function as a negative transcriptional repressor. In addition to the zinc finger motifs

found in all three putative homologues, CG15436 contains a specialized N-terminal zinc-

finger domain known as a zinc associated domain (ZAD). ZAD domains have been

theorized to act in a similar fashion to KRAB domains and are thought to be the *D.*

melanogaster equivalent of this domain (Jauch *et al.*, 2003). Alignment of the N-terminal

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KRAB domain of PARIS and ZAD domain of CG15436 shows a high degree of protein

sequence similarity with 51 amino acids, either identical or of similar function over a 75

amino acid span. CG15436 is a 346 amino acid long protein that has not yet been

characterized. Due to the presence of multiple zinc-finger domains, it is believed that the

activity of this protein is related to transcriptional regulation. Although little is known

about this protein, a single homologue has been identified in *Xenopus tropicalis(Altschul*

et al., 1990). Based on the presence of both N-terminal C2H2 zinc-finger domains and

the C-terminal ZAD domain, CG15436 appears to be the most PARIS-like gene identified

by our bioinformatics analysis.

The adult *D. melanogaster* eye is composed of two separate yet equally important

tissues: ommatidia and bristles. The formation of these tissues stems from neuronal

precursors, and they can easily be imaged and quantified using a scanning electron

microscope, making the eye a highly sensitive system for determining the effect of altered

gene expression in neurons. We expressed RNAi transgene lines of the three putative

PARIS candidates driven by GMR-Gal4 to determine the consequence of reduced gene

function (Figure 15). Eye specific expression of the CG15269-RNAi construct resulted in

a slight, yet significant, loss of ommatidia at 25°C. This effect was exacerbated at 29°C,

a temperature used to increase the amount of driven transgene expression as

higher temperatures increase the efficiency of the Gal4-UAS system, indicating that

further expression of *CG15269-RNAi* caused further tissue destruction. Expression of the

Crol-RNAi construct caused a severe decrease in both ommatidia and bristle formation at
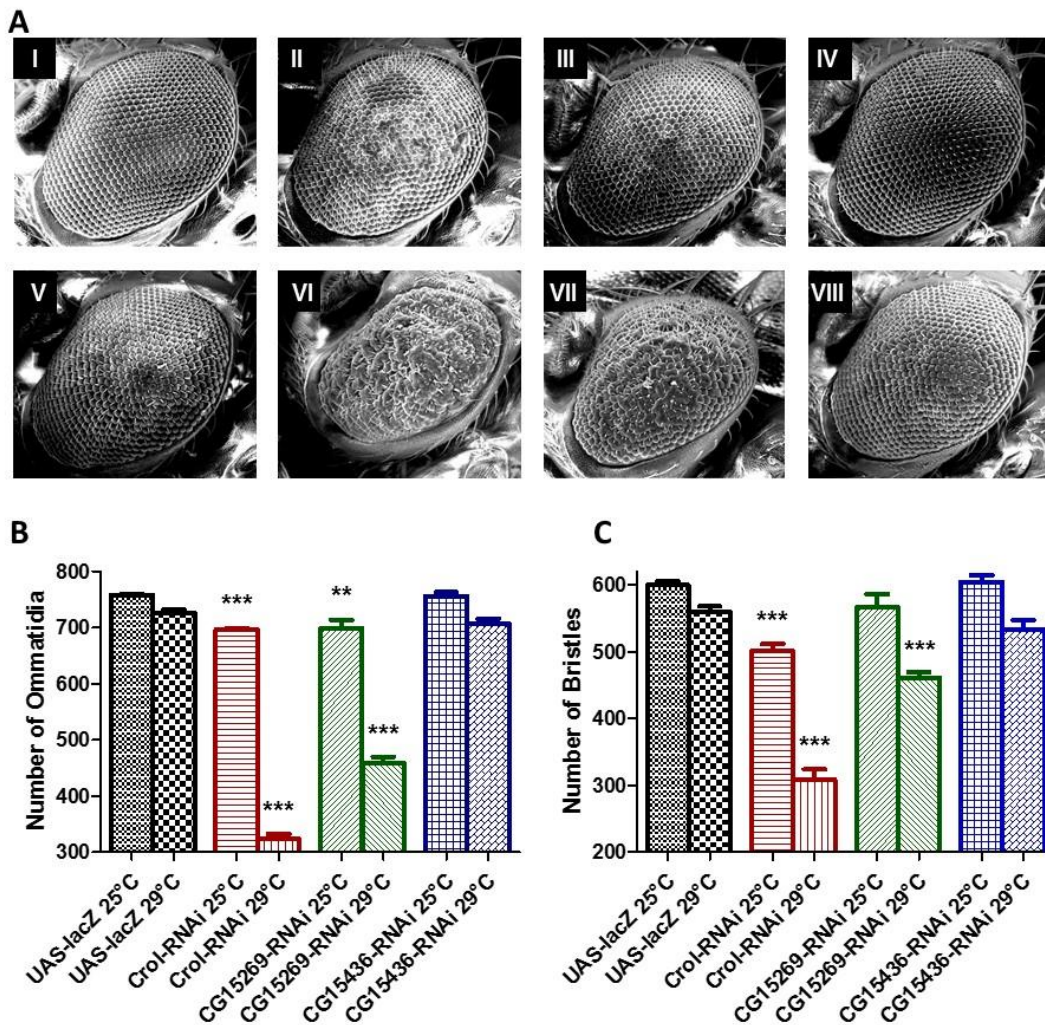
Figure 15: Expression of *Crol-RNAi* and *CG15269-RNAi* transgenes causes a decrease in both ommatidia and bristle count in the *Drosophila melanogaster* eye. A) Scanning electron micrographs of *D. melanogaster* eyes taken at a horizontal field width of 500µm. Genotypes are as follows: I) *GMR-Gal4 / UAS-lacZ* 25^{o}C, II) *GMR-Gal4 / Crol-RNAi* 25^{o}C, III) *GMR-Gal4 / CG15269-RNAi* 25^{o}C, IV) *GMR-Gal4 / CG15436-RNAi* 25^{o}C, V) *GMR–Gal4 / UAS-lacZ* 29^{o}C VI) *GMR–Gal4 / Crol-RNAi* 29^{o}C, VII) *GMR-Gal4 / CG15269-RNAi* 29^{o}C, VIII) *GMR-Gal4 / CG15436-RNAi* 29^{o}C. Flies show a decrease in the number of ommatidia and bristles present when *Crol-RNAi* and *CG15269-RNAi* transgenes are expressed at 25^{o}C. This effect intensifies at 29^{o}C causing a further decrease in tissue number for both transgenes with *Crol-RNAi* being more severe in both cases. Expression of a *CG15436-RNAi* has no effect on ommatidia and bristle number at 25^{o}C and only a shows only a slight decrease in tissue number at 29^{o}C. All counts compared to a *UAS-lacZ* control of the same temperature, comparisons were measured using a one-way ANOVA and significance was tested using a Tukey post-hoc test, n=10. Images were taken with a FEI MLA 650 Scanning Electron Microscope. *P<0.05, **P<0.01, ***P<0.001. Statistical values may be found in supplemental table S4.2.

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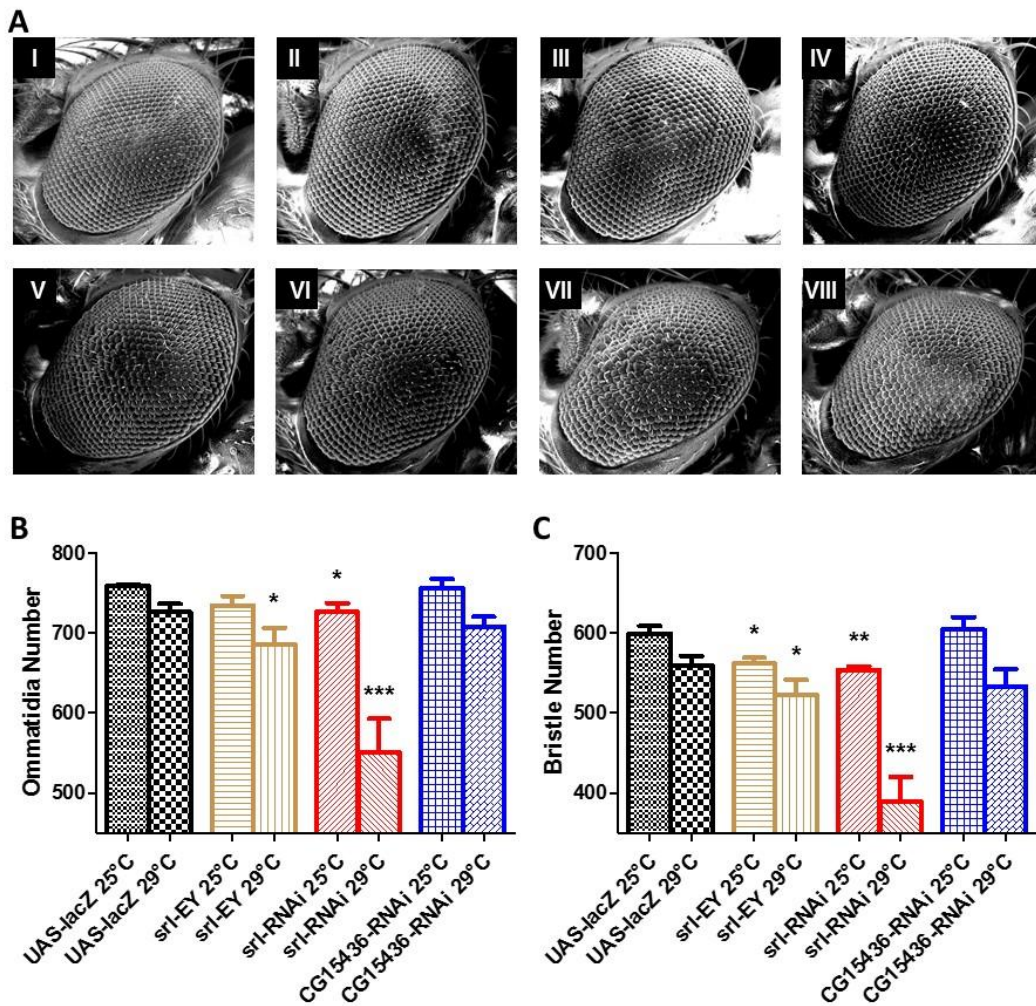
Figure 16: Expression of *srl-EY* and *CG15436* transgenes show a similar phenotype while expression of a *srl-RNAi* line causes a severe decrease in ommatidia and bristle number in the *Drosophila melanogaster* eye. A) Scanning electron micrographs of *D. melanogaster* eyes taken at a horizontal field width of 500µm. Genotypes are as follows: I) *GMR-Gal4 / UAS-lacZ* 25°C, II) *GMR-Gal4 / Crol-RNAi 25*°C, III) *GMR-Gal4 / CG15269-RNAi* 25°C, IV) *GMR-Gal4 / CG15436-RNAi 25*°C, V) *GMR–Gal4 / UAS-lacZ* 29°C VI) *GMR–Gal4 / Crol-RNAi* 29°C, VII) *GMR-Gal4 / CG15269-RNAi 29*°C, VIII) *GMR-Gal4 / CG15436-RNAi 29*°C. Flies show a decrease in both ommatidia and bristle count with a *srl-RNAi* transgene is expressed at both 25°C and 29°C. Expression of *srl-EY* causes no effect on ommatidia count and a slight decrease in bristle count at 25°C and a slight decrease in both ommatidia and bristle count at 29°C. Similarly, there is no effect on the eye when a *CG15436* transgene is expressed at 25°C and a slight decrease in both ommatidia and bristle count at 29°C. All counts compared to a *UAS-lacZ* control of the same temperature, comparisons were measured using a one-way ANOVA and significance was tested using a Tukey post-hoc test, n=10. Images were taken with a FEI MLA 650 Scanning Electron Microscope. *P<0.05, **P<0.01, ***P<0.001. Statistical values may be found in supplemental table S4.4

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25°C compared to a *UAS-lacZ* control, and a severe loss of both bristles and ommatidia at 29°C.

To determine if one of the three candidate PARIS genes is acting in pathways related to *srl* function, we looked at the effect of altered *srl* expression in the *D. melanogaster* eye. Driven expression of a *srl-EY* transgene construct causes a slight decrease in ommatidia number at both 25°C and 29°C, and a slight decrease in bristle count at 29°C compared to a UAS-*lacZ* control (Figure 16). There was no decrease in bristle number at 25°C. Expression of a *srl-RNAi* transgene caused a sharp and severe decrease in both ommatidia and bristle number at both 25°C and 29°C. In comparison, expression of a *CG15436-RNAi* transgene caused no change in ommatidia and bristle number at 25°C or 29°C. Expression of both a *srl-EY* and *CG15436-RNAi* transgene seem to cause similar effects when driven by a *GMR-Gal4* driver in the *D. melanogaster* eye.

Dopaminergic neurons are essential producers of dopamine and altered expression of genes implicated in neurodegenerative disease in these neurons leads to a decrease in mean lifespan compared to controls. This results in an ideal system to identify potential causative disease genes by assaying the tissue specific consequences of altered gene expression in these dopaminergic neurons. To determine the effect of altered putative *PARIS* homologue expression in dopaminergic neurons, the *Ddc-Gal4* transgene was used to direct the inhibition of the three candidate genes via RNAi. Tissue specific expression of *Crol-RNAi* in dopaminergic neurons resulted in a significant decrease in mean lifespan (56 days) compared to *UAS-lacZ* controls (60 days) while the directed expression of a

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CG15436-RNAi transgene resulted in a significant increase in mean lifespan (64 days)

(Figure 17). Expression of a *CG15269* transgene did not result in a significant difference

in mean lifespan (62 days) compared to the *UAS-lacZ* controls.
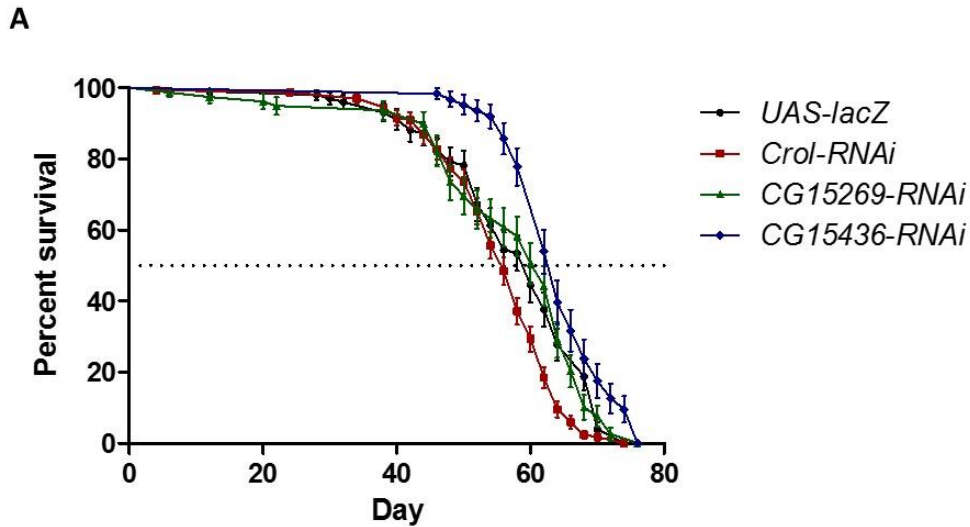
Figure 17: Dopaminergic expression of a *CG15436-RNAi* transgene increases while expression of a *Crol-RNAi* transgene decreases mean lifespan in *Drosophila melanogaster*. Altered expression of *Crol* in the dopaminergic neurons by expression of a *Crol-RNAi* transgene causes a significant decrease in mean longevity compared to *UAS-lacZ* controls. *CG15436* seems to have an opposite effect as expression of a *CG15436-RNAi* transgene causes a significant increase in lifespan compared to *UAS-lacZ* controls. *CG15269-RNAi* expression has no significant effect on mean lifespan when expressed in dopaminergic neurons. All transgene lines driven by the *Ddc(II)-Gal4* driver, longevity is shown as a percent survival (P<0.05 as determined by the Mantel-Cox Log Rank test) N= >150). Statistical values may be found in supplemental table S4.4.

5.4. Discussion

Beginning with the sequence of the *H. sapiens* PARIS protein, bioinformatics analysis has led to the identification of three similar genes with the potential to be the *D. melanogaster* homologue(s). Two of these genes, *CG15269* and *CG15436*, have not been investigated to any great extent except that they have been characterized as encoding zinc-finger containing proteins. The third gene, *crooked-legs* (*Crol)*, has been identified to encode a transcription factor involved in leg morphogenesis and as a mediator of cell cycle progression that has been studied as a potential interacting factor in models of Huntington Disease (D'Avino & Thummel, 1998; Kaltenbach *et al.*, 2007; Mitchell *et al.*, 2008). Although *Crol* has been implicated in models of human disease, little to no analysis of the effect upon a range of neuronal tissues has been performed. Interestingly, the KRAB motif, as found in the *H. sapiens PARIS* gene product, is a highly conserved motif found in over one third of all mammalian zinc-finger transcription factors (Urrutia, 2003). The KRAB domain is not well conserved in *D. melanogaster* zinc-finger transcription factors, although a domain with similar function and composition exists: ZAD (Chung *et al.*, 2002). The presence of this domain in only one of the three putative *D. melanogaster* homologues suggests that *CG15436* may be the most likely *PARIS* candidate. In addition to this N-terminal ZAD domain, CG15436 contains two C-terminal zinc-finger double domains, similar to that found in the human *PARIS* gene product. This domain is important in protein-protein binding interactions and, although some KRAB domain proteins exist without the C-terminal zing-finger domains, these proteins have been found to function in the sequestration of KRAB activating proteins

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and not in the activity of the domain (Chung *et al.*, 2007). ZAD containing proteins may require the C-terminal zinc-finger double domains for proper co-factor binding (Jauch *et al.*, 2003). The activity of KRAB domain proteins is dependent upon the presence of a co-activator identified as the <u>K</u>ruppel <u>A</u>ssociate <u>P</u>rotein-<u>1</u> (KAP-1) (Friedman *et al.*, 1996). KAP-1 is a member of the multi-component <u>T</u>ranscriptional <u>I</u>ntermediary <u>F</u>actor <u>1</u> (TIF1) family, which in humans contains four structurally and functionally similar proteins (Khetchoumian *et al.*, 2004; Le Douarin *et al.*, 1995; Venturini *et al.*, 1999). Although no direct binding factor of ZAD has currently been identified, there is a single TIF1 protein family member identified in *D. melanogaster*, *Bonus* (*Bon*) (Beckstead et al., 2001). The presence of conserved activation and binding domains between the *Homo sapiens* PARIS and *D. melanogaster* CG15436 proteins provide a large base of evidence for homologous function across species.

PARIS is a directly binding inhibitor of the PGC-1α protein in *Homo sapiens* (Castillo-Quan, 2011); lowered expression would be predicted to result in a reduction of inhibition and thus an increase in mitochondrial biogenesis through elevated *PGC-1α* activity. As the *D. melanogaster* eye provides an ideal tissue for the study of gene involvement in pathways of neurodevelopment as both ommatidia and bristle number can be easily quantified and allow for the distinction of subtle changes based on gene alteration, we expressed RNAi transgene constructs for our three putative *PARIS* homologues to determine the consequence they have on the formation of the eye tissues.

D. melanogaster eye specific expression of a *Crol-RNAi* transgene causes a severe disruption in the formation of ommatidia and bristles in the *D. melanogaster* eye. The

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CROL protein was most similar to PARIS (35% similarity) and has been implicated to play a role in leg morphogenesis. Expression of *Crol* occurs in the imaginal discs of *D. melanogaster* eyes, suggesting that it may play a similar role in eye development (D'Avino & Thummel, 1998). This is the first time altered *Crol* expression has been assessed and characterized in the *D. melanogaster* eye. *D. melanogaster* eye specific expression of a *CG15436-RNAi* transgene resulted in no significant change in ommatidia or bristle number at 25°C or 29°C. The CG15436 protein shares 33% similarity with the *Homo sapiens* PARIS protein and no role of the CG15436 protein has not yet been identified, however, it may act as a DNA binding transcription factor based on the presence of zinc finger motifs. The third putative homologue, *CG15269* shows a slight yet significant decrease in ommatidia number at 25°C and a severe decrease in both tissues at 29°C compared to *UAS-lacZ* controls. This gene currently has no identified function but, like *PARIS* (which it shares 29% protein similarity with) and our other putative homologues, contains the zinc finger motifs associated with transcriptional repression. We show the first evidence that *CG15269* may play a role in the development of *D. melanogaster* eye tissue and that altered expression of this gene may lead to developmental defects in these tissues. These eye specific developmental phenotypes associated with the loss of function of the three putative *PARIS* homologues *CG15436*, *CG15269* and *Crol* have not been previously identified and could show involvement of all three genes in neuronal development.

With the *Ddc-Gal4* transgene, the *PARIS* candidate genes were inhibited in the dopaminergic neurons to determine the consequence of improper gene function and

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potential links to disease. Altered expression of *CG15269* showed no effect on mean

lifespan in DA neurons. This result differs from the data obtained in the *D. melanogaster*

eye, however, the eye is a more sensitive system allowing for the detection of subtler

changes compared to aging assays. Thus, it is possible that *CG15269* does play a role in

neuronal formation but does not have a strong enough effect when altered to cause

premature organism mortality. Expression of a *Crol-RNAi* construct caused a significant

decrease in the mean longevity compared to control flies. This supports the evidence

found in the *D. melanogaster* eye that *Crol* is involved in neuronal tissues and altered

expression of this gene leads to tissue disruption and in this case reduced mortality.

Finally, altered expression of *CG15436* caused a significant increase in mean lifespan

compared to controls. This result would be expected from the *PARIS* homologue as

RNAi inhibition of that gene would cause an increase in mitochondrial biogenesis

through the *PGC-1α* pathway. Of the three putative *PARIS* homologues we identified and

characterized, *CG15436* is the best candidate and may be the *D. melanogaster* homologue

of *PARIS*.

In this study, we identified and evaluated potential *D. melanogaster* functional

homologues of the recently discovered human gene *PARIS*. Finding a functional

homologue of *PARIS* would provide a better *D. melanogaster* model of the *srl*-mediated

pathway of mitochondrial biogenesis initiated by the PD gene *Parkin*. Through this

process, we have found that three uncharacterized genes having a neuronal function: *Crol*,

CG15436 and *CG15269* are important in the proper function of both dopaminergic and

precursor eye tissue neurons. Of these three genes, we found that *CG15436* is the most

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PARIS-like in both structure and function. Fully characterizing the *D. melanogaster* pathway of mitochondrial biogenesis will allow for a more precise model to study human disease and potential preventative treatments for neurodegenerative disease.

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Chapter 6 - Summary

6.1. Summary

The work presented in this thesis expands the current working knowledge of

Parkinson Disease model biology in *D. melanogaster*. In recent years there has been a

shift from the existing belief that PD onset is solely sporadic in origin. Advances in the

fields of molecular biology and genetics have allowed for specific identification of

mutations leading to PD via single patient genome comparison and large scale screening.

How gene dysfunction and mutation contribute to the onset and clinical symptoms of PD

is a complex and difficult to answer question. Although monogenic forms of inherited

PD do make up approximately 30% of cases (Klein & Westenberger, 2012), most variants

involve multiple environmental, epigenetic and genetic factors that eventually result in a

parkinsonian phenotype (Labbe & Ross, 2014). Many characterized gene mutations

leading to disease phenotypes inhibit the proper function of the intracellular proteasome

complex or organelle function (Lim, 2007; Ryan *et al.*, 2015). I have looked at two

separate yet equally important pathways that have been linked to the onset of PD when

protein activity is altered. The first involves the processes governing proper

mitochondrial upkeep and repair. The second involves ubiquitin ligase attachment and

the subsequent proteasomal activity leading to proper destruction and recycling of

unwanted and potentially dangerous cellular components. Determining why these

complex processes breakdown and what genes are essential and imperative to proper

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pathway function may lead to new therapeutic targets and future research goals aimed at not just alleviating symptoms but preventing PD altogether.

Chapter 2 of this thesis investigated the *D. melanogaster* homologue of the human PD causing gene *PGC-1α*. *PGC-1α* functions as a growth factor and is one of three genes in the PGC family responsible for the activation of signals leading to mitochondrial biogenesis (Jornayvaz & Shulman, 2010). *PGC-1α* itself is up-regulated under conditions of stress and fasting while the other members of this gene family are constituently expressed in tissues at a basal level, making it difficult to determine the effect of altered *PGC-1α* expression in mammalian models (Gleyzer & Scarpulla, 2011; Meirhaeghe *et al.*, 2003; Mortensen *et al.*, 2007). I investigated the sole *D. melanogaster* homologue of *PGC-1α*, *spargel*, to determine the effect of altered PGC pathway expression on the development of neurons. SRL was previously characterized as a growth factor and whole flies containing a hypomorph of this gene have stunted growth and small body size compared to wild type control flies (Tiefenbock *et al.*, 2010). After performing a bioinformatics analysis of the SRL protein and comparing the functional domains present with all three members of the PGC family, I believed that *srl* was the *D. melanogaster* homologue of *PGC-1α*. To determine if aberrant *srl* function could cause the neurodegeneration found in the brains of *H. sapiens* patients with abnormal *PGC-1α* activity, I expressed *srl-RNAi* and *srl*-EY transgene constructs in the *D. melanogaster* eye. As expected, decreasing *srl* expression caused a degenerative phenotype while increasing *srl* seemed to have no real effect on the development of the eye through

neuronal precursors with the exception of a slight decrease in ommatidia and bristles at 29°C.

Next, I expressed the *srl-RNAi* and *srl-EY* transgene constructs in dopaminergic neurons to attempt and mimic the neurodegeneration found in PD patients and produce a new model of disease. Dopaminergic expression of *srl-RNAi* resulted in a significant increase in lifespan while *srl-EY* expression decreased mean longevity compared to *lacZ* controls. This result was unexpected but remained consistent when the assay was repeated at 29°C. Altered *srl* expression leads to different phenotypes depending on the tissue in question. Overexpression of *srl* in tissues with high energy requirements, such as intestinal cells, digestive cells and cardiac muscle leads to an increased lifespan, while overexpression of *srl* in the whole organism decreases mean lifespan (Rera *et al.*, 2011; Tinkerhess *et al.*, 2012). Thus, is it not surprising that directed *srl* expression in the eye did not lead to the same phenotype as directed *srl* expression in a tissue with a much higher energy requirement such as dopaminergic neurons. Locomotor assays performed on the same genotypes showed that flies do not perform better in climbing tests despite living longer, suggesting that although flies are living longer they may still be experiencing neuronal dysfunction.

Two significant results were produced as a result of this research project. The first is the characterization of a new model of PD in *D. melanogaster,* obtained through the tissue specific expression of *srl-EY* in dopaminergic neurons. Expression of this construct results in a phenotype with decreased mean longevity and decreased locomotor ability compared to a control. The second is the result found through the dopaminergic

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expression of a *srl-RNAi* construct. Expression of this construct increases the mean

longevity of flies compared to controls, but does not increase the locomotor ability,

leading to flies that outlive control counterparts but are unable to perform basic locomotor

movements throughout that increase in lifespan. There are two potential explanations for

this result, both involving inducible stress pathways that result in increased organism

longevity. The first potential explanation involves the induction of the unfolded protein

response pathway, while the second involves the formation of low level reactive oxygen

species, which in turn provoke cellular anti-oxidants, causing a stronger response to

future stress exposure and is known as mitohormesis (Haynes & Ron, 2010; Ristow &

Schmeisser, 2014). It is my suggestion that future work on this project should aim to

determine what pathways are upregulated when *srl-RNAi* is expressed in dopaminergic

neurons via by searching for interactors in a modifier of phenotype screen. This

experiment should focus on known stress response genes, specifically those involved in

the unfolded protein response and reactive oxygen mitigation. Identification of a pathway

stimulated by *srl-RNAi* expression in dopaminergic neurons could lead to new therapeutic

targets for eventual disease prevention.

For Chapter 3 of this thesis, I investigated the potential homologue of the gene

responsible for a severe early onset form of PD, *FBXO7*. *FBXO7* functions as the

targeting component of the SCF ubiquitin ligase complex and point mutations in this gene

have been directly linked to parkinsonian pyramidal syndrome (Di Fonzo *et al.*, 2009;

Gunduz *et al.*, 2014). A putative homologue of *FBXO7*, *ntc*, has been identified in *D.

melanogaster* but only characterized as a mediator of terminal sperm differentiation

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(Bader *et al.*, 2010). A second gene of interest, *PI31*, functions as a stabilizing component of the SCF ubiquitin complex and must bind directly to *FBXO7* for proper complex function (Kirk *et al.*, 2008). *PI31* has also been characterized as an inhibitor of proteasome activity when it is not bound and freely available within the cell (Li *et al.*, 2014). This project aimed to confirm *ntc* was the *D. melanogaster* homologue of *FBXO7* through bioinformatics analysis, followed by determining if aberrant function of *ntc* in neuronal tissues could lead to the degenerative phenotypes associated with the *Homo sapiens FBXO7* gene. Additionally, we aimed to evaluate the *D. melanogaster* homologue of *PI31* and determine if it was necessary for proper proteasome activity in neuronal tissues, mimicking the complex found in *Homo sapiens*.

I compared the protein sequences and domains of FBXO7 and PI31 from *H. sapiens* with the putative *D. melanogaster* homologues and found a high degree of similarity. Both FBXO7 and NTC contain a PI31 binding domain and an F-Box domain, indicating that they may provide a similar function in both organisms. To confirm this, I also compared the protein sequence and domains of *PI31* between *H. sapiens* and *D. melanogaster* and found a strong similarity, the sequences differing in length by only a single amino acid and containing the same proteasome binding and FBXO domains. Since it appeared the *D. melanogaster* homologues *ntc* and *PI31* were structurally similar to their *Homo sapiens* counterparts, I next wanted to determine if altered expression of these genes could lead to neurodegenerative phenotypes. I began by directing expression of four transgene constructs in the *D. melanogaster* eye: *ntc-RNAi*, *ntc-EY*, *PI31-RNAi* and *PI31-EY*. Altered expression of both *ntc* and *PI31* was sufficient to cause a

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degenerative phenotype. This phenotype was most severe with *PI31*. This result was replicated when the transgene constructs were expressed directly in dopaminergic neurons, suggesting that *ntc* and *PI31* are tightly regulated in both the eye and dopaminergic neurons of *D. melanogaster*. However, in dopaminergic neurons, *ntc* did show a more severe decrease in longevity compared to controls than *PI31,* suggesting perhaps a greater role for this gene specifically in tissues with high energetic requirements due to the increased response found in the dopaminergic neurons versus the *D. melanogaster* eye.

The classical *D. melanogaster* model of PD involves the ectopic expression of *H. sapiens α-synuclein* to induce a PD like phenotype of locomotor disruption and destruction in dopaminergic neurons (Feany & Bender, 2000). This phenotype mimics the *H. sapiens* model of PD in which harmful *α-synuclein* aggregates are created in dopaminergic neurons eventually leading to cell death (Baba *et al.*, 1998). This phenotype has been shown with pan neuronal expression by use of an *elav-Gal4* driver but I was unable to replicate the longevity reducing phenotype in our flies. This putative model involves the accumulation of harmful proteinaceous inclusions that lead to cell death when cellular machinery is no longer able to negate their formation (Navarro *et al.*, 2014). One such method of removing harmful components is the ubiquitin mediated proteasome complex. Since PI31 and NTC are components of a ubiquitin ligase complex responsible for the targeting of the proteasome, I assayed the potential cumulative effect of mutation in *ntc* and *PI31* along with ectopic expression of *α-synuclein* in the dopaminergic neurons of *D. melanogaster*. Co-expression of *α-synuclein* and *PI31-RNAi*

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resulted in a decrease in mean longevity, indicating that altered *PI31* expression coupled

with an abundance of aberrant protein inclusions significantly decreases lifespan

compared to controls. Alternatively, co-expression of *α-synuclein* and *PI31-EY* did not

significant impact longevity indicating that the decrease in longevity found when *PI31-EY* was expressed by itself may be abrogated by the presence of harmful protein clusters,

this is an unexpected result and requires further work to confirm. Interestingly, the co-

expression of *α-synuclein* and *ntc-RNAi* resulted in a significant increase in lifespan

compared to *α-synuclein* and *lacZ* expressing controls. However, as I found with the

expression of *srl-RNAi* in dopaminergic neurons, these flies do not differ significantly

from the control group in locomotor activity, indicating a decrease in mobility over time.

This experiment resulted in the identification and classification of two new *D. melanogaster* models of PD in which longevity is reduced and neuronal tissues are

disrupted by altered expression of these genes. Altered expression of both *ntc* and *PI31*

in the eye and dopaminergic neurons of *D. melanogaster* caused tissue destruction and

decreased longevity indicative of the neuronal dysfunction and destruction found in forms

of PD. This indicates not only that *ntc* and *PI31* are likely the *D. melanogaster*

homologues of *Homo sapiens FBXO7* and *PI31* but also that the stabilizing activity of

PI31 is conserved between species. Future experiments should focus on creating a double

mutant construct of both *ntc* and *PI31* and assaying the degenerative effect on the *D. melanogaster* eye. It may also be pertinent to determine which of these genes is the rate

limiting factor in the activity of this ubiquitin proteasome complex which may be done by

attempting a rescue of the degenerative phenotype caused by a decrease in one gene by overexpression of the other.

I found evidence that expression of *ntc-RNAi* in dopaminergic neurons also expressing *α-synuclein* increases the mean longevity compared to *α-synuclein* and *lacZ* expressing controls. This is an interesting result and it is my suggestion that follow up experiments focus on the cause of this increase and what additional survival mechanisms may be invoked within the organism. A simple starting point for this analysis would be to assess the level of gene expression between experimental and control flies in a micro-array and compare the genes with higher or lower expression to those implicated in stress response mechanisms.

F-box genes perform essential cellular functions, targeting specific intracellular components for destruction via ubiquitination. Given this information, it is not surprising that a sub-family of FBXO genes, including *FBXO7* and *FBXO32,* shows degenerative phenotypes in neurons and muscle tissue, respectively, when altered through mutation (Bodine & Baehr, 2014; Di Fonzo *et al.*, 2009). The third member of this gene sub-family, *FBXO9*, has not been well characterized in *H. sapiens*, though it was implicated as a possible growth factor involved in multiple myeloma (Fernandez-Saiz *et al.*, 2013). I looked at the putative *D. melanogaster* homologue of this gene, *CG5961*, to determine if altered expression in neuronal tissues could cause a degenerative phenotype found associated with other gene sub-family members.

A comparison of the *FBXO9* and *CG5961* proteins shows full conservation of all domains present and almost an identical length in terms of amino acid sequence. An

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alignment of these proteins shows the most similarity in the F-box binding domain, indicating that the original function of *FBXO9* is likely conserved across species. Altered expression of *CG5961* in the *D. melanogaster* eye caused a significant disruption of neuronal tissue formation. This effect was found for expression of both a *CG5961-RNAi* and *CG5961-EP* construct, but it was interesting to note that increased expression of either of these constructs via higher temperature growth did not seem to amplify the negative effect, suggesting that *CG5961* may not be sufficient to fully disrupt tissue formation alone. Having established a neuronal role of *CG5961* in the eye, I next expressed the *CG5961* constructs directly in the dopaminergic neurons of flies. Both constructs significantly lowered the mean longevity of flies compared to *lacZ* controls.

The main result of Chapter 4 is that altered expression of *CG5961* causes neurodegenerative phenotypes in both the eye and dopaminergic neurons of *D. melanogaster*. *CG5961* was previously uncharacterized and I present the first evidence of a function for this gene in neuronal tissues. It appears as though it is tightly regulated specifically in the dopaminergic neurons, as altered expression in either direction results in a significant loss in mean longevity, similar to the results found with *ntc*. Future work on this project should integrate *CG5961* into the larger project focused upon *ntc*, and *PI31*. The initial theory that all three members of this sub-family of FBXO genes have ties to degeneration appears to be correct and studies of the *Homo sapiens FBXO9* gene should also look into potential links between that gene and human disease.

The process of mitochondrial biogenesis is mediated through the PGC family of proteins, while the parallel process of mitochondrial upkeep and turnover is mediated

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through the *Pink1* / *Parkin* pathway (Eiyama & Okamoto, 2015; Jornayvaz & Shulman, 2010). Between these pathways is a regulatory protein known as PARIS. PARIS is a transcriptional repressor of *PGC-1α* that is susceptible to destruction by the ubiquitin ligase activity of *Parkin* (Shin *et al.*, 2011). Since PARIS is the direct link between the processes of mitochondrial upkeep / turnover and biogenesis, I wanted to query the genome of *D. melanogaster* for a potential *PARIS* gene homologue. A BLAST search using *Homo sapiens* PARIS as a query resulted in three potential matches in the *D. melanogaster* genome: CROL, CG15436 and CG15269. A detailed analysis of PARIS revealed a KRAB zinc finger domain and a zinc double finger domain. Each of the candidate *D. melanogaster* proteins contained the zinc double finger domain, but only CG15436 contained a domain homologous to the KRAB domain of PARIS. A direct comparison of the amino acid sequence of the KRAB domain with the homologous ZAD domain revealed a high degree of similarity, suggesting CG15436 was the most likely homologue of the three candidates.

To determine if any of the three candidate genes were involved in pathways of neuronal development, I directed expression of transgene constructs expressing an RNAi for each gene in the *D. melanogaster* eye. Surprisingly, altered expression of both *Crol* and *CG15269* resulted in a degenerative phenotype compared to *lacZ* controls while *CG15436* did not significantly change the development of the tissues of the eye. A comparison of the eye phenotype generated from expression of *CG15436-RNAi* with the phenotypes generated by the altered expression of *srl* showed that the *srl-EY* construct was most similar in terms of effect on ommatidia and bristles. After finding that both

Crol and *CG15269* had negative consequences on neuronal development, I directed expression of all three constructs in dopaminergic neurons. Expression of *Crol-RNAi* caused a significant decrease in lifespan while *CG15436-RNAi* caused a significant increase.

This line of experiments resulted in three significant findings. The first is the unexpected neuronal destruction caused by the altered expression of *Crol* in both the eye and dopaminergic neurons of *D. melanogaster*. This is the first experiment we are aware of, suggesting a neuronal function for *Crol* which is characterized as a negative regulatory factor in limb development (D'Avino & Thummel, 1998). The second is the neuronal destruction imparted by the altered expression of *CG15269*, a gene previously not characterized and with no known function. Both *Crol* and *CG15269* should be further evaluated in neuronal cells. It is my suggestion that constructs overexpressing these genes are used in a follow up study to further identify their role in neuronal development in both the eye and dopaminergic neurons. Furthermore, the role of these proteins may be determined through modifier screening. The third is the similar phenotype produced by the directed eye expression of both *CG15436-RNAi* and *srl-EY*. Coupled with the increase in life witnessed with the dopaminergic expression of *CG15436-RNAi*, it would appear that *CG15436* is the best preliminary candidate for a *D. melanogaster* homologue of *PARIS*. Additional experiments are necessary to confirm that *CG15436* is the *D. melanogaster* homologue of *PARIS*. *CG15436* appears to be able to extend lifespan when removed in the dopaminergic neurons of *D. melanogaster* indicating that it may be a repressor of a necessary neuronal factor such as *srl*. Experiments in *Homo sapiens* have

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found that *Parkin* loss leads to declines in mitochondria number and size, indicative of a

loss in mitochondrial biogenesis due to the increased inhibition of *PGC-1α* that results

from an overabundance of *PARIS* (Stevens *et al.*, 2015). If a *D. melanogaster* line

combining the expression of *Parkin-RNAi* and *CG15436-RNAi* shows a rescue phenotype

versus a line expressing only *Parkin-RNAi*, it would confirm the identity of *CG15436* as

the *PARIS* homologue. Alternatively, a western blot could be performed on *Parkin*

mutant flies versus wild type flies to compare the amount of CG15436 protein. A

decrease in CG15436 in a *Parkin* mutant would infer that the E3 ubiquitin ligase activity

of PARKIN is responsible for the destruction of this gene as is found in the *H. sapiens*

system with *Parkin* and *PARIS*.

6.2. Limitations in Interpretation

There are a number of limitations in interpretation that is present in this work due

both to technical reasons and flaws in experimental design. To determine the effect of

putative homologues of *H. sapiens* neurodegenerative causative genes, we decreased

expression through RNAi and increased expression by means of the Gal4-UAS transgene

system with the targeting eye driver GMR-Gal4. This provides a visual model of

destruction that has been traditionally correlated with degeneration, however, we have not

actually quantified or tested these tissues for a degenerative effect. No molecular analysis

of the components of these eye tissues was undertaken. This limitation could be

overcome by assaying for proteins related to apoptotic or autophagic processes within the

cells of these eyes.

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The transgenes employed have been previously characterized by other groups, however, I did not assay the expression of these transgenes personally. It is possible that the assays completed by other groups were not correct and that these transgenes were guilty of providing off target effects or alternative expression then indicated by the genotype. This could be rectified by performing a western blot of the proteins targeted by these transgenes to confirm expression of the resulting protein is either lowered or increased in tissues of interest. Without this analysis it is not possible to say with certainty that no confounding effects were introduced into our study. I did utilize more than a single RNAi for both the *srl* and *ntc* RNAi expression analysis which helps alleviate this effect in these datasets. In a number of instances data revealed that both the RNAi and UAS transgenes had a similar effect on development of the *D. melanogaster* eye. I have attempted to explain this effect by inferring that the gene in question is necessary in a specific amount for neurons to develop appropriately. It is possible that the expression of any non-native protein in these tissues causes a negative developmental response and that the added stress of raising flies at 29°C increases this response. Molecular work to assay the amount of protein expression in these cases would help to explain some of the effects witnessed in these assays. If a gene is highly regulated and altered expression in any direction causes a severe degenerative phenotype additional experiments aiming to exacerbate this phenotype could be undertaken (expression of two UAS constructs together or additional RNAi).

To determine the effect of dopaminergic expression of the potential gene homologues we expressed RNAi and UAS lines of these homologues in the dopaminergic

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neurons by use of a dopamine decarboxylase (Ddc)-Gal4 driver. I choose this driver because there is currently a discussion in the literature with regards to the efficacy of tyrosine hydroxylase (Th)-Gal4 as a driver for reproducible dopaminergic expression and also because this driver in my hands was not consistent in result (Whitworth, 2011). Ddc is expressed in dopaminergic and serotonergic neurons and as a result it is possible that the results obtained are not entirely due to dopaminergic neuron expression of the potential gene homologues. Serotonergic neurons have recently been implicated to play a role in *H. sapiens* forms of PD and, as such, expression of our putative homologue genes in these tissues could be giving our results a stronger effect then would be found in dopaminergic neurons alone (Zhang *et al.*, 2008). Further confounding our analysis of altered gene expression in dopaminergic neurons is the knowledge that dopamine is also expressed in the cuticle and involved in melanin production (Yamamoto & Seto, 2014). It has been shown that a full knockout of dopaminergic neurons leads to larval lethality (Riemensperger *et al.*, 2011). This dopaminergic neuron production of melanin is not conserved in higher animals such as *H. sapiens*, making the *D. melanogaster* model of dopamine related neurodegeneration more complex. Contrary to this point *D. melanogaster* are simple organisms compared to *H. sapiens* and lack the high complexity required to fully develop models of neurodegenerative disease. This has been discussed in Chapter 1 as a strength and weakness of this model. Finally, given the information obtained for gene homologues through comparison of protein domains and an NCBI BLAST comparing proteins to the *D. melanogaster* whole genome it is not possible for me to make a true homology call between *PARIS*, *FBXO7*, or *FBXO9* and a fly

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homologue. Additional assays need to be undertaken to confirm that any of these three genes are the direct *D. melanogaster* homologue of the *H. sapien* disease causative gene. A simple rescue assay introducing the *H. sapiens* protein in tandem with a knock down of the *D. melanogaster* protein could be an efficient step in proving this homology.

6.3. Conclusion

The work I have documented in this thesis provides a number of new models of Parkinson Disease in *Drosophila melanogaster* that may be expanded upon and studied to further current knowledge of disease onset and progression in *Homo sapiens*. Originally, it was known that the *Homo sapiens* genes *PGC-1α*, *FBXO7*, *PI31* and *PARIS* were implicated as causative factors in Parkinson Disease etiology. I have provided evidence that each of these genes have functional homologues in *D. melanogaster* and that altered expression of these homologues leads to neurodegenerative phenotypes in two separate neuronal tissues: the eye and dopaminergic neurons. Additionally, I have shown that *Crol*, *CG15269* and *CG5961*, genes previously not linked to neuronal development, cause a degenerative phenotype when expression is altered in neuronal tissues.

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Appendix 1 – Supplemental Material for Chapter 2

S1.1. Statistical breakdown and comparison of ommatidia and bristle number counts between

lacZ control and *srl* transgene constructs in the *Drosophila melanogaster* eye

A. Ommatidia Number 25°C, control mean 751.20 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	27.5431
GMR-Gal4;UAS-srl[RNAi1]	10	25.00	<0.05	15.23009
GMR-Gal4;UAS-srl[RNAi2]	10	4.80	>0.05	16.61459
GMR-Gal4;UAS-srl	10	16.80	>0.05	16.34489

B. Ommatidia Number 29°C, control mean 644.90 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	26.69353
GMR-Gal4;UAS-srl[RNAi1]	10	94.70	<0.0001	59.60015
GMR-Gal4;UAS-srl[RNAi2]	10	70.10	<0.0001	34.66603
GMR-Gal4;UAS-srl	10	-41.30	>0.05	28.81666

C. Bristle Number 25°C, control mean 617.30 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	21.55639
GMR-Gal4;UAS-srl^{RNAi1}	10	63.70	<0.0001	6.834553
GMR-Gal4;UAS-srl^{RNAi2}	10	64.20	<0.0001	18.00278
GMR-Gal4;UAS-srl	10	55.30	<0.0001	9.614803

D. Bristle Number 29°C, control mean 507.10 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	16.79583
GMR-Gal4;UAS-srl^{RNAi1}	10	118.40	<0.0001	43.40264
GMR-Gal4;UAS-srl^{RNAi2}	10	63.20	<0.0001	10.89801
GMR-Gal4;UAS-srl	10	-15.60	>0.05	26.20454

Table S1.2. Log-rank (mantel cox) survival analysis comparison of altered *srl* transgene constructs and *lacZ* control

A. Longevity at 25°C

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4;UAS-lacZ	324	76	N/A	N/A
Ddc-Gal4;UAS-srl^{RNAi}	400	90	<0.0001	Yes
Ddc-Gal4;UAS-srl	373	64	<0.0001	Yes

B. Longevity at 29°C

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4;UAS-lacZ	158	38	N/A	N/A
Ddc-Gal4;UAS-srl^{RNAi}	151	42	<0.0001	Yes
Ddc-Gal4;UAS-srl	214	34	<0.0001	Yes

Table S1.3. Statistical comparison of climbing index curves between *lacZ* control and *srl* transgene constructs

Genotype	Rate (k)	Standard error	95% Confidence interval	R^2	P value
Ddc-Gal4;UAS-lacZ	0.05600	0.002425	0.05114 to 0.06087	0.9572	N/A
Ddc-Gal4;UAS-srl^{RNAi}	0.04856	0.002107	0.04434 to 0.05278	0.9561	0.7598
Ddc-Gal4;UAS-srl	0.05897	0.005450	0.04795 to 0.06998	0.8333	0.6163

Appendix 2 – Supplemental Material for Chapter 3

S2.1. Protein sequence alignment of *Homo sapiens* FBXO7 and *Drosophila melanogaster* NTC.

Alignment performed in ClustalW2. Purple = F-Box domain.

```
ntc      --MSDTKSEIEGFIAIPTTSGEQQQQQPQQQQ------------------NEQQVVGTK 39
FBXO7    MRLRVRLLKRTWPLEVPETEPTLGHLRSHLRQSLLCTWGYSSNTRFTITLNYKDPLTGDE 60
            :       :    : :* *.     : :.: :*                  :: :.* :

ntc      DIKAPDQVGKKQRPRLIQEKSTQETNPLILEHATLEWVPQHMDKLLNQYQECRKMPAAEW 99
FBXO7    ETLASYGIVSGDLICLILQDDIPAPNIPSSTDSEHSSLQNNEQPSLATSSNQTSMQDEQP 120
            :   *.  : . :    ** :..   .*      .:  . : :: :  *   .:  .*   :

ntc      LHLLTYLVALECGFVEEETFAQKRHLIQPVPSFSSFHAQNVRILSEQPARYEVCFN---- 155
FBXO7    SDSFQGQAAQSGVWNDDSMLGPSQNFEAESIQDNAHMAEGTGFYPSEPMLCSESVEGQVP 180
            . :    .* .  : ::. :. .:::     . .:. *:.. :  ..:*    . ..:

ntc      ----------------DTVYIMRLRTLLDKHAPEETS-------------------- 176
FBXO7    HSLETLYQSADCSDANDALIVLIHLLMLESGYIPQGTEAKALSMPEKWKLSGVYKLQYMH 240
                            : :::*  *  . : *: *.

ntc      -LVAALQCRLMAVSLGDQLMITLSPAPPSKEPGYSVSLSIGRYVLNIQAKNKPIYHRFRK 235
FBXO7    PLCEGSSATLTCVPLGNLIVVNATLKINNEIRSVKRLQLLPESFICKEKLGENVANIYKD 300
            *   . .. * .*.**: :::. :     .:  . .     :  . .:  :  .: : : ::.

ntc      LDELSYQLKQHLFQPMRSQQLMQMEMKLQPSLLGLPDELYFEIFRYLDKSQLNVVARVNR 295
FBXO7    LQKLSRLFKDQLVYPLLAFTRQALNLPDVFGLVVLPLELKLRIFRLLDVRSVLSLSAVCR 360
            *::**   :*::*. *: :     :::    .*: ** ** :.*** **   .:   :: * *

ntc      HLHFYS------------------------------KEVERKRLKGGRS--------- 314
FBXO7    DLFTASNDPLLWRFLYLRDFRDNTVRVQDTDWKELYRKRHIQRKESPKGRFVMLLPSSTH 420
            .*.   *                               :.::**.    **

ntc      ------------------------------------------------------------
FBXO7    TIPFYPNPLHPRPFPSSRLPPGIIGGEYDQRPTLPYVGDPISSLIPGPGETPSQFPPLRP 480

ntc      ----------------------------------------
FBXO7    RFDPVGPLPGPNPILPGRGGPNDRFPFRPSRGRPTDGRLSFM 522
```

S2.2. Statistical breakdown and comparison of ommatidia and bristle number counts between

lacZ control, *ntc*, and *PI31* transgene constructs in the *Drosophila melanogaster* eye

A. Ommatidia Number 25°C, control mean 752.4 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	25.012
GMR-Gal4;UAS-ntc[RNAi]	10	64.60	<0.0001	25.012
GMR-Gal4;UAS-ntc	10	63.20	<0.0001	16.91679
GMR-Gal4;UAS-PI31[RNAi]	10	24.90	>0.05	26.86696
GMR-Gal4;UAS-PI31	10	20.90	>0.05	26.85765

B. Ommatidia Number 29°C, control mean 714.40 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	25.85085
GMR-Gal4;UAS-ntc[RNAi]	10	328.70	<0.0001	34.75054
GMR-Gal4;UAS-ntc	10	26.70	>0.05	9.381424
GMR-Gal4;UAS-PI31[RNAi]	10	190.90	<0.0001	31.08858
GMR-Gal4;UAS-PI31	10	149.90	<0.0001	21.32708

C. Bristle Number 25°C, control mean 596.90 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	15.97533
GMR-Gal4;UAS-ntc[RNAi]	10	95.80	<0.001	7.81807
GMR-Gal4;UAS-ntc	10	97.30	<0.0001	71.79013
GMR-Gal4;UAS-PI31[RNAi]	10	78.90	<0.0001	49.0374
GMR-Gal4;UAS-PI31	10	75.90	<0.0001	23.04585

D. Bristle Number 29°C, control mean 498.20 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	22.77816
GMR-Gal4;UAS-ntc[RNAi]	10	112.50	<0.0001	40.61144
GMR-Gal4;UAS-ntc	10	53.60	>0.05	50.2664
GMR-Gal4;UAS-PI31[RNAi]	10	229.9	<0.0001	28.42944
GMR-Gal4;UAS-PI31	10	193.7	<0.0001	36.47221

S2.3. Log-rank (mantel cox) survival analysis comparison of altered *ntc*, *PI31*, and *FBXO9* transgene constructs with *lacZ* control

A. Longevity of *ntc* and *PI31* trial 1 of 2

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4;UAS-lacZ	292	54	N/A	N/A
Ddc-Gal4;UAS-ntc^{RNAi}	317	38	<0.0001	Yes
Ddc-Gal4;UAS-PI31^{RNAi}	314	46	<0.0001	Yes
Ddc-Gal4;UAS-PI31	361	46	<0.0001	Yes

B. Longevity of *ntc* and *PI31* trial 2 of 2

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4;UAS-lacZ	215	58	N/A	N/A
Ddc-Gal4;UAS-ntc^{RNAi}	208	42	<0.0001	Yes
Ddc-Gal4;UAS-ntc	214	38	<0.0001	Yes
Ddc-Gal4;UAS-PI31^{RNAi}	202	51	<0.0001	Yes
Ddc-Gal4;UAS-PI31	199	52	<0.0001	Yes

S2.4. Log-rank (mantel cox) survival analysis comparison of altered *ntc* and *PI31* transgene constructs in an *α-synuclein* background using *UAS-lacZ* as a control

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4/UAS-α-syn;UAS-lacZ	295	56	N/A	N/A
Ddc-Gal4/UAS-α-syn;UAS-ntc^{RNAi}	275	68	<0.0001	Yes
Ddc-Gal4/UAS-α-syn;UAS-PI31^{RNAi}	296	40	<0.001	Yes
Ddc-Gal4/UAS-αsyn;UAS-PI31	252	54	>0.05	No

S2.5. Statistical comparison of climbing index curves between *LacZ* control, *ntc* and *PI31* transgene constructs

Genotype	Rate (k)	Standard error	95% Confidence interval	R^2	Significant
Ddc-Gal4/UAS-α-syn;UAS-lacZ	0.1026	0.005079	0.09243 to 0.1129	0.9658	N/A
Ddc-Gal4/UAS-α-syn;UAS-ntc^{RNAi}	0.06986	0.004319	0.06117 to 0.07855	0.9314	No
Ddc-Gal4/UAS-α-syn;UAS-PI31^{RNAi}	0.6317	0.6638	N/A	0.8176	No
Ddc-Gal4/UAS-αsyn;UAS-PI31	0.2968	0.05522	0.1847 to 0.4089	0.6505	No

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Appendix 3 – Supplemental Material for Chapter 4

S3.1. Alignment and annotation of *Homo sapiens* FBXO9 and the *Drosophila melanogaster* putative FBXO9 homologue. Alignment done with ClustalW2. Bold = microtubule interacting and transporting domain, Yellow = Tetratricopeptide repeat, Purple = F-box domain, Underline = HNHc domain, Green = nuclear localization sequence.

```
FBXO9h    MAEAEEDCHSDTVRADDDEENESPAETDLQAQLQMFRAQWMFELAPGVSSSNLENRPCRA 60
CG5961    ------------MSDVDSDGEEPTRKTGTNALDEFRENWQRELQEHTTNTGSRSHSEAG 47
               :* *.:.*.*.*:..    *: ** :* **    .:.:. ..:.  .

FBXO9h    ARGSLQKTSADTKGKQEQAKEEKARELFLKAVEEEQNGALYEAIKFYRRAMQLVPDIEFK 120
CG5961    DR----LTAANSNLSEADLLQAKAESLYRTAVQLEQRGKVYDALPFYRKATQIVPDIEFR 103
           *      *:*::: .: :  : **..*: .**: **.* :*:*: ***:* *:******:

FBXO9h    ITYTRSP-------------DGDGVGNSYIEDNDDDSKMADLLSYFQQQLTFQ-----ES 162
CG5961    FYEQQKQKLSNDVSKKYLNLANDLAKQLDLGQSDGEEVVDNLYEKFQHDLRQKNIYNGKM 163
           :    :.              .* . :  : :.*.:. : :* . **:** :     :

FBXO9h    VLKLCQPELESSQIHISVLPMEVLMYIFRWVVSSDLDLRSLEQLSLVCRGFYICARDPEI 222
CG5961    IASSRDANVLTTGLHFADLPPEIVMRILRWVVSAQLDMRSLEQCAAVCKGFYVYARDEEL 223
           : .  :.:: :: :*:: ** *::* *:*****::**:***** : **:***: *** *:

FBXO92    WRLACLKVWGRSCIKLVP--------YTSWREMFLERPRVRFDGVYISKTTYIRQGEQSL 274
CG5961    WRLACVKVWGHNVGTLEAQDSDVSNVFHSWRDMFIRRDRVLFNGCYISKTTYLRMGENSF 283
           *****:****:.   .* .       : ***:**:.* ** *:* *******:* **:*:

FBXO9h    DG-FYRAWHQVEYYRYIRFFPDGHVMMLTTPEEPQSIVPRLRTRNTRTDAILLGHYRLSQ 333
CG5961    QDQFYRPVQLVEYYRYIRFLPDGKVLMMTTADEPAQGVSKLKHVNNVRAEMLRGRYRLFG 343
           :. ***. : *********:***:*:*:**.:** . *.:*: *.   :* *:***

FBXO9h    DTDNQTKVFAVITKKKEEKPLDYKYRYFRRVPVQEADQSFHVGLQLCSSGHQRFNKLIWI 393
CG5961    -----STVTLVLQKSQQRGPANVRQRRGSIMPVDEDSSQFLIELRIAGTTKRRCAQLVWS 398
                :.*  *: *.::. * : : *   :**:* ...* : *::..: ::*  :*:*

FBXO9h    HHSCHITYKSTGETAVSAFEIDKMYTPLFFARVRSYTAFSERPL- 437
CG5961    HY-TLVQKRNKVDISSEFDLTEAKYPALRFSTVKSYHLDADAPLA 442
          *:   :  :..: :  .    :  *..* *: *:**   :: **
```

S3.2. Statistical breakdown and comparison of ommatidia and bristle number counts between

lacZ control and *FBXO9* transgene constructs in the *Drosophila melanogaster* eye

A. Ommatidia Number 25°C, control mean 752.4 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	25.012
GMR-Gal4;UAS-FBXO9^{RNAi}	10	39.40	<0.05	32.93765
GMR-Gal4;UAS-FBXO9	10	43.60	<0.05	22.40932

B. Ommatidia Number 29°C, control mean 714.40 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	25.85085
GMR-Gal4;UAS-FBXO9^{RNAi}	10	21.90	>0.05	23.77323
GMR-Gal4;UAS-FBXO9	10	45.40	<0.05	42.54148

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C. Bristle Number 25°C, control mean 596.90 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	15.97533
GMR-Gal4;UAS-FBXO9^{RNAi}	10	81.00	<0.0001	31.29945
GMR-Gal4;UAS-FBXO9	10	54.10	<0.001	31.68526

D. Bristle Number 29°C, control mean 498.20 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	22.77816
GMR-Gal4;UAS-FBXO9^{RNAi}	10	27.10	>0.05	26.91324
GMR-Gal4;UAS-FBXO9	10	6.30	>0.05	71.58592

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S3.3. Log-rank (mantel cox) survival analysis comparison of altered *FBXO9* transgene constructs
with *lacZ* control

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4;UAS-lacZ	292	54	N/A	N/A
Ddc-Gal4;UAS-FBXO9^{RNAi}	286	45	<0.0001	Yes
Ddc-Gal4;UAS-FBXO9	194	48	<0.0001	Yes

Appendix 4 – Supplemental Material for Chapter 5

S4.1. Alignment and annotation of *Homo sapiens* ZNF746 protein (PARIS) with *Drosophila melanogaster* CG15436. Alignment done with ClustalW, Yellow highlight = zinc finger domain, green highlight = KRAB / ZAD domain

```
ZNF746    MAEA--VAAPISPWTMAATIQAMERKIESQAARLLSLEGRTGMAEKKLADCEKTAVEFGN 58
CG15436   MAEICRVCMDIS-GKLVNIFDARRRTRVSIA------------------------EMIA 34
          ***    *.  **   .:.   ::* .*.  * *                       *:

ZNF746    QLEGKWAVLGTLLQEYGLLQRRLENVENLLRNRNFWILRLPPGSKGESPKEWGKLEDWQK 118
CG15436   QCTGFEVKRGDLFSEMICPQ--------------------------------------- 54
          *  *   .  * * *:.*      *

ZNF746    ELYKHVMRGNYETLVSLDYAISKPEVLSQIEQGKEPCNWRRPGPKIPDVPVDPSPGSGPP 178
CG15436   ---------CYEDVKSAYGIRQTCEESHQFY-----CRVR-------------------- 80
                   ** : *     .. *   *:       *. *

ZNF746    VPAPDLLMQIKQEGELQLQEQQALGVEAWAAGQPDIGEEPWGLSQLDSGAGDISTDATSG 238
CG15436   --------------------------------DEGIEDALCALLEEEDWEISEDEDAR 106
                                          * * *    : *:.   :** *  :

ZNF746    VHSNFSTTIPPTSWQTDLPPHHPSSACSDGTLKLNTAASTEADVKIVIKTEVQEEEVVAT 298
CG15436   IDS-----------------ASAADDDG--KS-------DSKKVAFECR--ECHKKY 135
          :.*                 .*:* .**  *       * * *         *

ZNF746    PVHPTDLEAHGTLFGPGQATRFFPSPAQEGAWESQGSSFPSQDPVLGLREPARPERDMGE 358
CG15436   QRKGTFLRHMRTHMD-------------------GQSFPCPYCKR------------- 161
           : * *.   * :.                    *.***.

ZNF746    LSPAVAQEETPPGDWLFGGVRWGWNFRCKPPVGLNPRTGPEGLPYSSPDNGEAILDPSQA 418
CG15436   -----------------------NFRLRVTLKAHMKTHNAAKPYECS------------ 185
                                 *** : .: : :*   . **...

ZNF746    PRPFNEPCKYPGRTKGFGHKPGLKKHPAAPPGGRPFTCATCGKSFQLQVSLSAHQRSCGA 478
CG15436   -----------HCAKTFAQQSTLQSHERTHTGERPFKCSQCSKTFIKSSDLRRHIR---- 230
                     :* *.::. *:.*   : .* ***.*: *.*:*  . .*  * *

ZNF746    PDGSGPGTGGGGSGSGGGGGGSGGGSARDGSALRCGECGRCFTRPAHLIRHRMLHTGERP 538
CG15436   --------------------THGSERP---FKCSKCTKTFTRKFHLDNHFRSHTGERP 265
                              ** *   ::*.:* : *** ** .*  ******

ZNF746    FPCTECEKRFTERSKLIDHYRTHTGVRPFTCTVCGKSFIRKDHLRKHQRNHAAGAKTPAR 598
CG15436   FKCSHCPKAFAMKQHLKQHSRLHLPDRPFRCSHCPKTFRLSSTLKEHKLVHNAER----- 320
          * *:.* * *: :.:* :* * *   *** *: * *:*   .. *::*:   * *

ZNF746    GQPLPTPPAPPDPFKSP--ASKGP----LASTDLVTDWTCGLSVLGPTDGGDM 645
CG15436   -----------TFKCPHCASFYKQRKTLARHILEIHK-------------- 346
                     .**.*  **        **   *  .
```

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S4.2. Statistical breakdown and comparison of ommatidia and bristle number between *PARIS* candidate RNAi transgene constructs and *lacZ* control.

A. Ommatidia Number 25°C, control mean 758.6 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	3.130495
GMR-Gal4;UAS-CROL^RNAi	10	62.20	<0.0001	6.503845
GMR-Gal4;UAS-CG15269^RNAi	10	60.00	<0.001	35.57808
GMR-Gal4;UAS-CG15436^RNAi	10	2.800	>0.05	17.12308

B. Ommatidia Number 29°C, control mean 726.2 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	*p*-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	14.77159
GMR-Gal4;UAS-CROL^RNAi	10	402.20	<0.0001	19.55761
GMR-Gal4;UAS-CG15269^RNAi	10	268.00	<0.0001	25.73325
GMR-Gal4;UAS-CG15436^RNAi	10	18.40	>0.05	18.33576

C. Bristle Number 25°C, control mean 599.6 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	13.2212
GMR-Gal4;UAS-CROL^{RNAi}	10	98.60	<0.0001	23.90607
GMR-Gal4;UAS-CG15269^{RNAi}	10	33.40	>0.05	44.7236
GMR-Gal4;UAS-CG15436^{RNAi}	10	-4.60	>0.05	23.12358

D. Bristle Number 29°C, control mean 559.4 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	18.17416
GMR-Gal4;UAS-CROL^{RNAi}	10	251.20	<0.0001	35.20937
GMR-Gal4;UAS-CG15269^{RNAi}	10	98.20	<0.0001	19.07092
GMR-Gal4;UAS-CG15436^{RNAi}	10	25.80	>0.05	31.18974

S4.3. Statistical breakdown and comparison of ommatidia and bristle number between *PARIS* candidate RNAi transgene construct *CG15436*, *srl* constructs and *lacZ* control.

A. Ommatidia Number 25°C, control mean 758.6 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	2.951459
GMR-Gal4;UAS-srl	10	24.20	>0.05	16.34489
GMR-Gal4;UAS-srl^RNAi	10	32.40	>0.05	59.60015
GMR-Gal4;UAS-CG15436^RNAi	10	2.800	>0.05	17.28712

B. Ommatidia Number 29°C, control mean 726.2 ommatidia per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	13.92679
GMR-Gal4;UAS-srl	10	40.00	<0.05	28.81666
GMR-Gal4;UAS-srl^RNAi	10	176.00	<0.0001	52.82161
GMR-Gal4;UAS-CG15436^RNAi	10	18.40	>0.05	16.1438

C. Bristle Number 25°C, control mean 599.6 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	12.46506
GMR-Gal4;UAS-srl	10	37.60	<0.05	9.614803
GMR-Gal4;UAS-srl^{RNAi}	10	46.00	<0.001	6.834553
GMR-Gal4;UAS-CG15436^{RNAi}	10	-4.600	>0.05	21.80112

D. Bristle Number 29°C, control mean 559.4 bristles per eye

Genotype	Number of eyes (N)	Mean difference in number vs control	p-value compared to control	Standard Deviation
GMR-Gal4;UAS-lacZ	10	N/A	N/A	17.13476
GMR-Gal4;UAS-srl	10	36.70	<0.05	26.20454
GMR-Gal4;UAS-srl^{RNAi}	10	170.70	<0.0001	43.40264
GMR-Gal4;UAS-CG15436^{RNAi}	10	25.80	>0.05	29.40597

S4.4. Log-rank (mantel cox) survival analysis comparison of *PARIS* homologue candidate RNAi transgene constructs with *lacZ* control.

Genotype	Number of Flies (N)	Median Survival (Days)	*p*-value compared to control	Significant
Ddc-Gal4;UAS-lacZ	201	60	N/A	N/A
Ddc-Gal4;UAS-CROL[RNAi]	267	56	0.0192	Yes
Ddc-Gal4;UAS-CG15269[RNAi]	179	62	0.9885	No
Ddc-Gal4;UAS-CG15436[RNAi]	163	64	0.0005	Yes

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