CHARACTERIZATION OF *FBXO9* IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

The study of disease in model organisms is a fundamental and important stepping-stone in understanding and uncovering the mechanisms behind disease pathology in humans. The purpose of this work was to identify potential targets for the treatment and prevention of Parkinson disease using *Drosophila melanogaster*. Commonly known as the fruit fly, *D. melanogaster* is one of the important model organisms used extensively in biological research. Moreover, it has conserved developmental processes and mechanisms shared with human neurodegenerative disorders. Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by death of dopamine producing cells of the *substantia nigra* affects about 1% of people over 60 years old worldwide. In mammals, *Fbxo9* is a substrate recognition component of the SCF (SKP1-cullin-Fbox)-type E3 ubiquitin ligase complex. Some targets of *Fbxo9,* including an extensive array of proteins, are degraded via the ubiquitin-proteasome system. In this study, a potential *D. melanogaster* homologue of *Fbxo9*, *CG5961*, was identified. The *Fbxo9* homologue in *D. melanogaster* has been conserved through evolution and retains many of the functional domains. The main goal of this project was to determine if *Fbxo9* can be implicated in modeling PD in *D. melanogaster.* To investigate its role in neuronal survival, I over-expressed and down-regulated *Fbxo9* in neuron-rich eye and dopaminergic neurons. Through assessments of eye morphology, climbing ability and ageing analysis, it was found that loss-of-function of *Fbxo9* causes a PD like symptom. I expect that the knowledge obtained by determining the pathways involved in PD in *D. melanogaster* will help uncover potential new therapeutic approaches for research in human as well as other genes in both humans and flies.

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LIST OF ABBREVIATIONS

AD	autosomal dominant
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATP13A2	ATPase type 13A2
AR	autosomal recessive
BLAST	Basic Local Alignment Search Tool
BLASTp	Protein Basic Local Alignment Search Tool
C	carboxyl terminus
CI	confidence interval
cm	centimeter
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CO_2	carbon dioxide
DA	dopaminergic
Ddc	Dopa decarboxylase
ddH_2O	double distilled H_20
Dilp	Drosophila Insulin-like peptide
dLRRK	Drosophila leucine-rich repeat kinase
DNA	deoxynucleotide
dNTP	deoxynucleotide triphosphate
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-ligase
eIF3-f	eukaryotic initiation factor 3 - subunit 5
EOPD	early-onset parkinson disease
ERK	extra cellular signal-regulated kinase
FBP	F-box containing protein
FBXO9	F box-only protein 9
FBXO7	F box-only protein 7
FBXO32	F box-only protein 32
FBXL	F-box leucine rich repeat

FBXO	F-box other
FBXW	F-box WD40
Foxo	forkhead box other
FPD	familial forms of Parkinson disease
g	gram
GAK	cyclin G associated
GAL4	yeast transcriptional activator for galactase-inducible genes
GBA	glucocerebrosidase
GIGYF2	GRB10 interacting GYF protein 2
GMR	Glass Multiple Reporter
GOF	gain-of-function kinase
HECT	Homologous to the E6-AP Carboxyl Terminus
HLA-DRA	major histocompatibility complex, class II, DR alpha
HNHc	HNH nuclease family
HSP70	heat shock protein 70
HTRA2	HtrA serine peptidase 2
H_2O	water
IGF-1	Insulin-like Growth Factor 1
k	slope
kDa	kilo Daltons
L	litre
LBs	lewy bodies
LN	lewy neurite
LOPD	late-onset Parkinson disease
LRR	leucine rich repeat
LRRK2	leucine-rich repeat kinase 2
MAPKK	mitogen activated protein kinase kinase kinase
mg	milligram
MIT	microtubule interacting and trafficking domain
mL	mililitre
mRNA	messenger RNA
N/A	not applicable
NCBI	National Center for Biotechnology Information

NES	nuclear export sequence
NLS	nuclear localization sequence
ntc	nutcracker
ORF	open reading frame
PARK	Parkinson disease-associated gene locus
PD	Parkinson Disease
PI31	31 kDa proteasome inhibitor
P13K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PINK1	PTEN-induced putative kinase 1
PLA2G6	phospholipase A2, group VI
PRR	Proline rich region
PTEN	phosphatase and tension homologue
RING	really interesting new gene
RNAi	RNA-interference
RNA	Ribonucleic acid
ROS	reactive oxygen species
SCF	Skp1-Cullin1-Fbox
SEMs	scanning electron micrographs
SNc	*substntia nigra pars compacta*
SNCA	subthalamic nucleus
SOD	superoxide dismutase
SEM	standard error of the mean
TPR	tetracycline peptide repeat
TH	Tyrosine hydroxylase
UAS	upstream activator sequences
UbI	ubiquitin like-domain
UCH-L1	ubiquitin carboxyl-terminal hydrolase L1
UPS	ubiquitin proteasome system
WT	wild type
° C	degrees Celsius
µm	micrometre

α-synuclein	alpha-synuclein

INTRODUCTION

Parkinson Disease

Parkinson disease (PD) is the second most common progressive neurodegenerative disorder: affecting about 1% of people over 60 years old worldwide (Lew, 2007). It is associated with a movement disorder, which is characterized by tremor, rigidity, postural instability and bradykinesia. Although most symptoms are associated with motor disorder, non-motor symptoms such as cognitive, psychiatric and emotional problems are also seen in this disease (Tadaiesky *et al.,* 2008). The neuropathological distinctive characteristics showed by PD patients are Lewy Bodies (LB) and Lewy Neurites (LN) in surviving neurons. The ultimate dysfunction of these neurons is responsible for the symptoms and pathology of PD (Bekris *et al.*, 2010). PD is associated with the progressive degeneration of dopaminergic neurons in the *substantia nigra* of the midbrain region and the subsequent loss of dopamine (Dauer and Przedborski, 2003). The study of this disease in well-known genetically versatile model organism such as *Drosophila melanogaster* is a fundamental and important stepping stone in understanding and uncovering the mechanisms behind disease pathology in a human host.

Many genetic and environmental factors have been identified for the progression of PD. Most forms of PD are known to be sporadic with no known causes (Cauchi and Heuvel, 2006; Lu and Vogel, 2009). Several environmental factors have been well documented correlating with the onset of PD such as chemical exposure, brain trauma, obesity, age and diabetes (Vanitallie, 2008). Alternatively, some familial forms of PD have been found in association with different types of alteration in genes (Bereznai and Molnar, 2009). The discovery of the familial forms of PD-associated genes inherently provides us the opportunity to study both sporadic and familial PD in model organisms.

PD Gene Loci

There are 18 Parkinson-associated (*PARK*) gene loci identified to date through a combination of linkage, segregation and sequence analysis; though several of these gene loci require validation by independent studies (Table 1). The first of the genes found in association with the rare familial forms of PD (FPD) is *α-synuclein* (Polymeropoulos *et al.,* 1997; Kruger *et al.,* 1998). Among these gene loci, several have been cloned and include α-synuclein/*PARK1* (Polymeropoulos *et al.,* 1997), Parkin/*PARK2* (Kitada *et al.*, 1998), Ubiquitin C-terminal hydrolase1 (Uchl-1*)/PARK5* (Leroy *et al.,* 1998), Phosphatase and tensin homologue [PTEN] induced kinase (*Pink1*)/*PARK6* (Valente *et al.*, 2004), DJ-1/*PARK7* (Bonifati *et al.,* 2003) and leucine rich repeat kinase 2 (LRRK2)/*PARK8* (Zimprich *et al.*, 2004). Among the genes identified, α-synuclein/ *PARK1* and Leucine-rich repeat kinase 2 or LRRK2 are known as autosomal dominant alleles whereas the rest are autosomal recessive alleles (Staveley, 2012). The identification of the genes has helped to better understand the underlying pathological mechanism of FPD. This pathological mechanism also supports us in understanding sporadic causes of PD. A considerable number of studies prior to modeling PD in animal have been done and thus these studies proposed some pathogenic mechanisms like protein mis-folding, protein abnormal accumulation oxidative stress, mitochondrial dysfunction and caspase activation. The endeavors to identify the central reasons for PD significantly led us to determine the necessary tools through the use of animals in the experiments. The promising indications are found from the modeling of PD in animals including rats, monkeys, flies, mice and worms (Kuwahara *et al*., 2006). Among all model organism studies, the genetic studies in *Drosophila melanogaster* have advantages over other animal models.

Table 1: Gene loci implicated in Parkinson disease.

Locus	Gene	Chromosome	Inheritance	Clinical phenotype
PARK1/ PARK4	*SNCA*	4q21	AD	EOPD
PARK2	*Parkin*	6q25.2-q27	AR	Juvenile and EOPD
PARK3	*Unknown*	2p13	AD	LOPD
PARK5	*UCH-L1*	4p14	AD	LOPD
PARK6	*PINK1*	1p35-p36	AR	EOPD
PARK7	*DJ-1*	1p36	AR	EOPD
PARK8	*LRRK2*	12q12	AD	LOPD
PARK9	*ATPA13A2*	1p36	AR	Kufor-Rakeb syndrome
PARK10	Unknown	1p32	AD	Unclear
PARK11	*GIGYF2*	2q36-q37	AD	LOPD
PARK12	Unknown	Xq	X-linked	Unclear
PARK13	*HTRA2*	2p13	AD	Unclear
PARK14	*PLA2G6*	22q13.1	AR	Parkinsonian with additional features
PARK15	*FBXO7*	22q12-q13	AR	EOPD
PARK16	Unknown	1q32	Susceptibility locus	LOPD
PARK17	*GAK*	4p16	Susceptibility locus	LOPD
PARK18	*HLA-DRA*	6p21.3	Susceptibility locus	LOPD
Gaucher's locus	*GBA*	1q21	Information not available	Information not available

AD is autosomal dominant, AR is autosomal recessive, EOPD is early-onset Parkinson disease and LOPD is late-onset Parkinson disease (adapted from Kumar, 2012).

Identification of *Fbxo7* as *PARK15*

The *Drosophila melanogaster* homologue of the human *Fbxo7* gene has been identified as *PARK15* (Dolomount & Staveley, unpublished). The *D. melanogaster* homologue of *Fbxo7* was named as *nutcracker* for its involvement in terminal differentiation of male germ cells in (Bader *et al.*, 2010). *Fbxo7* in human and *D. melanogaster* has the highest amino acid similarity in their F-box domains.

Fbxo7 (*nutcracker, PARK15*) was originally studied for its involvement in the caspase activation during sperm differentiation, but later it was found that it is associated with the early onset of parkinsonian-pyramidal syndrome such as Babinski sign, hyper-reflexia, and spasticity with equinovarus deformity (Di Fonzo *et al.*, 2009). Mutation in *Fbxo7*/ *PARK15* gene has been found in association with early-onset parkinsonian-syndrome, which was described several decades ago, but the gene locus has been recently mapped (Davison, 1954; Di Fonzo *et al.*, 2009). The *nutcracker* protein has been found to act as an E3-ligase through interaction with the SCF-ubiquitin ligase complex. The F-box domain found in *nutcracker* binds and activates caspases required for differentiation of sperm. Loss-of-function in *nutcracker* has been found to decrease proteosomal activity while the number and distribution of proteasomes remain the same (Dolomount unpublished). This suggests that when *nutcracker* is dysfunctional, the proteolysis process is disrupted at the protein ubiquitination stage. This also suggests that *nutcracker*'s role as an E3-ligase in the ubiquitin proteolysis method that parallels to mutation process in *Fbxo7*.

***Drosophila melanogaster* as a model organism**

In order to investigate the different functions of human disease genes over one

hundred year, a wealthy number of experimental approaches have been applied. One approach that has drawn much attention for modeling neurodegenerative diseases involves human disease gene expression in the "common fruit fly" *Drosophila melanogaster. D. melanogaster* has been widely used as a model organism due to its small size, rapid generation time, and development of many tool-boxes of genetic techniques (Merzetti *et al.,* 2013). Meta-analysis of the genome of *D. melanogaster* showed that more than 75% of human disease genes are conserved between flies and mammals (Reiter *et al.,* 2001; Lloyd and Taylor, 2010). *D. melanogaster* was the first complex organism whose genome was sequenced (Adams *et al.,* 2000). The genome of *D. melanogaster* is simple compared to mammalian counterparts because it has lower genetic redundancies (Bier, 2005). Additionally, *Drosophila* is also used as a model organism for some other reasons (Celotto and Palladino, 2005). The high degree of amino acid conservation was discovered in *Drosophila* through different proteomic analyses. Moreover, the presence of a complex nervous system and the short life-time (approximately fifty days) has made *D. melanogaster* an effective model organism for studying different neurological diseases.

Drosophila is specifically advantageous in the area of neuroscience, where it has been used to study neural development, neural circuitry and neural disease (Venken *et al.,* 2011). The developing *D. melanogaster* eye is a suitable system for studying cellular mechanisms including cell fate specification, cell-cell communication and signalling methods (Thomas and Wassarman, 1999). The *D. melanogaster* brain has more than 300,000 neurons and is organized into different specialized areas that are used for learning, olfaction, vision and memory (Wolf and Herbelein, 2003; Cauchi and Heuvel, 2006; Hardaway, 2010). Flies like humans show complex behaviors including learning, memory and motor ability that decline with age (Mockett *et al.*, 2003; Simon *et al.*, 2006). The presence of homologous PD genes and a high degree of functional conservation contribute

to the use of *D. melanogaster* as a model organism for PD research. Previous work from different laboratories (Botella *et al.*, 2009) including in our lab, has shown that *D. melanogaster* is a useful model organism for PD research.

Drosophila in PD modeling

To produce loss-of-function and gain-of-function phenotypes that may recapitulate symptoms of a given disease, reverse genetics can be applied. Gain-of-function is a condition that confers new or enhanced activity upon a gene and the RNA-i dependent loss-of-function phenotypes, result in reduced or abolished gene function. To produce gain-of-function or the loss-of-function phenotypes, that results in reduced or abolished gene function, the bi-partite UAS/GAL4 (upstream activating sequence/ yeast transcriptional activator for galactose inducible genes) system has been extensively used for the ectopic expression of specific genes in Drosophila (Brand and Perrimon, 1993). This method allows the directed expression of target genes in different tissues including eyes, muscles, neurons or the whole body. To generate transgenic fly lines, Drosophila carrying the transgene under UAS control are crossed to flies expressing the yeast transcription factor GAL4 under the control of a specific cell- and tissue-specific promoter. The target gene is silent in the absence of GAL4. The UAS has binding sites for GAL4 proteins and is fused to the target genes. When these two lines are combined, GAL4 binds to the UAS and triggers the transcription of the gene of interest. This target gene expressed in the offspring of the controlled breeding experiments is subject to control of expression with regard to level, timing and tissue specificity. The GAL4 driver lines used in the PD disease models are dopaminergic neuron promoter *Ddc* (DOPA decarboxylase) and eye-specific promoters *GMR* (Glass Multiple Reporter) including many others (Gong and Golic, 2003).

Analyses of these progeny give the opportunity to approach an extensive range of basic biological investigations including the thorough modelling of human disease (Staveley, 2012). It should be noted that the use of the UAS/Gal4 system in Drosophila requires caution since there may be a Gal4 effect in some experiments including cell death in the neuron-rich compound eyes (Kramer and Staveley, 2003). This UAS/Gal4 system gives the study of PD in Drosophila an extensive and powerful method in identifying the mechanisms behind this disease in human subjects.

Another genetic method (that) has been developed in Drosophila couples RNA interference (RNAi) with the UAS/GAL4 system, which allows the study of transcriptional "knock down" effects (Dietzl *et al.,* 2007) or loss-of function phenotypes. Furthermore, UAS/GAL4 system is another genetic method (Feany and Bender, 2000) and this method was used for overexpression of both wild type and mutant human *α-synuclein* in Drosophila neurons.

Drosophila models of PD

The *α-synuclein* model

α-synuclein was the first gene in Drosophila that was found to have a link with the inheritable form of PD (Whitworth, 2011). The *α-synuclein* gene, the prime component of Lewy bodies (LB) in both sporadic and familial PD and its aggregation is believed to be the main cause of PD (Feany and Bender, 2000; Michno *et al.,* 2005). It has been reported that mutations that result in amino acid substitution in human *α-synuclein* (*PARK1/PARK4*) protein, including A30P, A53T and E46K produce autosomally dominant versions of PD (Polymeropoulos *et al.,* 1997; Kruger *et al.*, 1998; Zarranz *et al.,* 2004). An early onset of familial versions of PD results when a triplication occurs in the *α-synuclein* gene locus

(Singleton *et al.,* 2003). In the brains of PD patients, it has been found that phosphorylation occurs at Ser129 of *α-synuclein* (Fujiwara *et al.*, 2002). These results indicate the importance of phosphorylation of *α-synuclein* in the pathogenesis of PD.

Since Drosophila has been found to lack orthologues of *α-synuclein,* including other members of the synuclein family, the GAL4/UAS system was used to drive directed expression of both wild and mutant forms of the protein (A30P and A53T) to model PD in Drosophila (Botella *et al.*, 2009). It has been reported that when A30P, A53T and wild type *α-synuclein* are expressed, flies show the key features of PD such as dopaminergic (DA) neurons loss, retinal degeneration and locomotor dysfunction (Feany and Bender, 2000). These Drosophila phenotypes exhibiting PD provide the opportunity to study *α-synuclein* aggregation and toxicity and information about genetic interactions and pharmacological approaches. Using a Drosophila model of polyglutamine disease, it has been found that directed expression of the molecular chaperone HSP70 suppresses polyglutamine-induced neurodegeneration *in vivo* (Warrick *et al.,* 1999). When the *α-synuclein* expressing flies were fed with geldanamycin, a chaperone inductor, DA neurons were protected (Auluck *et al.,* 2005). This indicates that compounds that regulate the stress response are a promising approach to treating PD.

It has been found that when *α-synuclein* and *parkin* are co-expressed in the Drosophila developing eye, this decreases the retinal degeneration and improves the climbing ability and when they are co-expressed in the DA neurons, this slightly increases their lifespan (Haywood and Staveley, 2006). When *Rab1*, a guanosine triphosphate is co-expressed with *α-synuclein,* DA neuronal loss was rescued (Cooper *et al.,* 2006). When PTEN induced putative kinase 1 (*Pink1*) is overexpressed, premature loss of climbing ability, ommatidial array degeneration and eye development defects, induced by *α-*

synuclein, were found to be rescued (Todd and Staveley, 2008). When *α-synuclein* expressing flies are exposed to hyperoxia, neurotoxicity and DA degeneration result (Botella *et al.,* 2008). Pharmacological agents such as L-DOPA have been found to restore the PD phenotype in *α-synuclein* flies (M'Angale unpublished). Keep with same perspective, there is another study (Pendleton *et al.,* 2002) in which it has been found that atropine works to a lesser extent than other anti-Parkinson compounds.

To manipulate gene expression in this Drosophila model of PD, the ectopic expression method has been widely used. In a cross between *Ddc-Gal4* and *UAS-α-synuclein* transgenic flies, *α-synuclein* was expressed in the dopaminergic neurons (Haywood and Staveley, 2004). The resulting offspring showed loss of climbing ability and, impaired mobility control, which is characteristic of PD. This result supports the use of Drosophila as suitable model for PD. To determine the effect of over-expression and reduced expression of *Fbxo9* and *α-synuclein* in Drosophila, we have used dopaminergic neuron driver *Ddc-Gal4* to direct the expression of these genes in dopaminergic neurons. We hypothesized that $Fbxo9^{RNAi}$ in *α-synuclein* flies would result in the decreased loss of climbing ability and lifespan.

The *LRRK2/Lrrk* model

Leucine rich repeat kinase 2 (*LRRK2*) encodes a complex 2567 amino acid and contains a leucine rich repeat (LRR), Ras of complexes (ROC), a protein kinase domain of the MAPKKK family, putative serine/threonine kinase, GTPase domains and many WD40 protein–protein interactions domains (Zimprich *et al*., 2004; West *et al.,* 2005; Botella *et al.,* 2009). The physiological activity of this protein is unclear but its multiple domains indicate its involvement in different types of cellular processes (Paisan-Ruiz *et al.*, 2004;

Zimprich *et al.*, 2004; Banerjee *et al.,* 2009). It has been found that the *LRRK2/* MAPKKK domain is involved in PD, which indicates enzymatic phosphorylation alteration (Taylor *et al.*, 2006; Liu *et al.*, 2008). It has been found that there are 29 different mutations in the LRRK2 gene (Paisan-Ruiz *et al.,* 2004; Zimprich *et al.*, 2004) that is associated with the autosomal dominant or gain of function forms of PD.

Drosophila has a single orthologue (*dLRRK*) that is highly expressed in heads (DA neurons) and it is essential for DA neuron protection in flies (Lee *et al.,* 2007; Wang *et al.,* 2008; Imai *et al*., 2008). The pleomorphic structure of *LRRK2*-linked PD suggests that *LRRK2* is involved in the pathway of synthesis of other proteins implicated not only in PD, but also in other neurodegenerative diseases (Ross *et al.,* 2006; Taylor *et al*., 2006). It has been found in one study that *Lrrk* mutant flies show locomotor dysfunction and a decrease in immune-staining of tyrosine hydroxylase/TH in DA neurons (Lee *et al.*, 2007). While in another study, it has been found that *Lrrk* mutant flies were relatively normal indicating that *Lrrk* is not necessary for DA neuron survival (Wang *et al.*, 2008). Co-expression of mutant forms of human (G2019S) and Drosophila (12020T) of *LRRK2* resulted in DA neurons loss (Imai *et al.,* 2008; Liu *et al.*, 2008). The few studies of *Lrrk/LRRK2* in flies have provided information that is more likely to significantly help our understanding of PD.

The *parkin/PINK1* model

It has been found that the *parkin* gene acts as an E3 ubiquitin ligase that targets the mis-folded proteins for degradation via the ubiquitin proteasome pathway (Kitada *et al.*, 1998). The autosomal recessive form of early onset PD occurs due to loss of function of the *parkin* gene. This *parkin* protein has been found to be present in the mitochondria (Darios *et al.,* 2003) indicating its role for maintaining of this organelle.

The usefulness of Drosophila as a PD model has been recently found by reduction of neuronal-specific staining (either GFP or TH staining) or the dopaminergic neurons degeneration or cell death in *parkin* mutants (Greene *et al.*, 2003; Cha *et al.*, 2005; Whitworth *et al.,* 2005; Wang *et al.*, 2007). The similar findings have been found when *parkin* mutants are overexpressed, suggesting a harmful effect in Drosophila DA neurons (Sang *et al., 2007*). Mutant *parkin* flies have been found to show reduced lifespan, developmental delay, male sterility, and mobility dysfunction due to muscle degeneration (Green *et al*., 2003). When the human *parkin* mutant (R375W) is overexpressed in flies, it results in age-dependent DA neuron degeneration, locomotor dysfunction that increases with age and mitochondrial dysfunction in flight muscles (Wang *et al.,* 2007). These results indicate that *parkin* mutant (R375W) expression causes adverse outcomes. They also suggest the interesting possibility of selecting *parkin* mutations that may directly exert neurotoxicity.

The Human Phosphatase and Tension homologue (PTEN) induced kinase (*Pink1*) is a 581 amino acid protein with a highly conserved serine or threonine kinase domain of Ca^{2+} and mitochondrial targeting signal sequences (Thomas and Beal, 2007). *Pink1*, as observed for *parkin*, has been found to be expressed in heads and tastes of adult male flies and it also contains a targeting signal for mitochondria (Clark *et al.,* 2006; Park *et al*., 2006). It has been found that mutations in *PTEN-induced putative kinase 1 (Pink1)* are the main cause of autosomal recessive forms of PD (Kitada *et al.,* 1998; Valente *et al.*, 2004). In order to maintain the mitochondrial fission/fusion pathway, it has been identified that there is a genetic interaction between *parkin and* gene PTEN-induced putative kinase 1(*PINK1)* (Botella *et al.*, 2009). This involvement implicates *Pink1* as an important regulator of fission/fusion, acting upstream of the E3 ubiquitin ligase, and *parkin*, to maintain proper mitochondrial integrity and function (Clark *et al*., 2006 and Park *et al*.,

2006). This indicates that the *Pink1* and *parkin* act to regulate the mitochondrial fission/fusion pathway.

The human *Pink1* is homologous to *D. melanogaster Pink1* and they are functionally conserved (Clark *et al.*, 2006; Park *et al.*, 2006). Transgenic RNAi and transposon-mediated mutagenesis approaches were used to produce the Drosophila *Pink1* model. The resulting flies showed the same characteristics as *parkin* mutant, flies such as reduced lifespan, developmental delay, abnormal position of wings, ommatidial and DA neuron degeneration, male sterility, mobility dysfunction, and mitochondrial dysfunction in their flight muscle (Petit *et al.,* 2005; Clark *et al.*, 2006; Park *et al*., 2006; Yang *et al.*, 2006; Wang *et al*., 2006). It has been found that when human *Pink1*, Drosophila *Pink1* and *parkin* are expressed together, muscle integrity is restored (Clark *et al.*, 2006; Park *et al*., 2006; Yang *et al.*, 2006). It can be concluded that suppression of Drosophila *Pink1* may drive the age dependent muscle degeneration.

Skp-Cullin-F-box containing complex (SCF) Ubiquitin Ligases

Ubiquitin consists of 76 amino acids that were originally found to bind the chromosomal protein histone (Goldknopf and Busch, 1977). The ubiquitin-proteasome system (UPS) is a process by which intracellular proteins are degraded in a highly complex, temporally controlled and tightly regulated manner and plays an important role in different basic cellular processes (Ciechanover and Brundin, 2003). The UPS is essential for cell growth by controlling the mitotic cycle through degradation of cyclins, cyclin-dependent kinases, and cyclin dependent kinase inhibitors (King *et al.,* 1996; Hershko A, 1997). The ubiquitin-proteasome pathway drives cell cycle progression not only by regulating cyclin-dependent kinase activity but by directly influencing chromosome and spindle dynamics. Protein degradation via the ubiquitin-proteasome pathway is described

by two general steps: 1) tagging of the substrate via covalent bond formation with ubiquitin molecules and 2) degradation of the tagged protein by the 26S proteasome and release of free, reusable ubiquitin (Glickman and Ciechanover, 2002). In spite of intensive research, there are still unknown in areas in intracellular protein degradation and major questions have remained unexplored.

Ubiquitin ligases act as part of the UPS and consist of three major components. Initially, the ubiquitin-activating enzyme E1 activates ubiquitin in an ATP-dependent manner to form a high-energy thiol ester intermediate (Ciechanover and Brundin, 2003). The E2 ubiquitin-conjugating enzyme then forms a thioester linkage between ubiquitin and E2 (Scheffner, 1995). The ubiquitin protein ligase E3 acts to bind to the target protein and interacts with E2 to covalently bind to target protein (Adams, 2003). This process is repeated many times, creating a polyubiquitin chain, which gives a signal to the target protein for degradation by the 26S proteasome (Wilkinson, 1999; Pickart, 2001). There are two main classes of E3 enzymes. The first one is the Homologous to the E6-AP carboxyl terminus (HECT) domain E3s, which directly bind ubiquitin molecules via thio-ester linkages and function as intermediate proteins (Huibregtse *et al.,* 1995). The second class is the Really Interesting New Gene (RING) domain E3s, which use a Zn binding motifs to drive E2 towards the protein for ubiquitination instead of directly binding to ubiquitin molecules (Lorick *et al.,* 1999). F-box proteins are one essential component of RING domain E3 ubiquitin ligases and function as substrate specific targeting proteins for ubiquitination. The 26S proteasome recognizes lysine-48 linked polyubiquitin chains for destruction whereas lysine-63 linkages are involved in the modification of enzymatic activity (Li *et al*., 2007a).

One of the common groups of E3 ligases is SCF (Skp-Cul; F- box) complex and consists of four proteins: SKP1, Cul1, ROC1 and an F-box protein (Cardozo and Pagano,

2004). The cullin subunit Cul1 interacts with SKP1 that is bound to the F-box protein in the complex and ROC1 acts as a RING-finger protein that transfers the ubiquitin molecule from E2 to E3 via an interaction with Cul1 (Teixeira and Reed, 2013). The F-box proteins give the substrate specificity to the SCF complexes by binding to the target substrate through different domains when F-box binds to SKP-1 (Bai *et al.*, 1996). The link between these proteins and a number of important biological processes has cemented F-box proteins as essential members of cellular machinery.

In PD, loss-of-function mutations in enzymes essential for the ubiquitin proteasome system appear to play a major role in the build-up of proteinaceous inclusions and Lewy Bodies development (Layfield *et al.,* 2003). Investigating the relationship between the ubiquitin system and PD may provide us the opportunity to study the mechanisms of PD at the molecular and cellular level.

F-box proteins

The F-box proteins are characterized by approximately 50 amino acids conserved domains which function in mediating protein-protein interactions (Kipreos and Pagano, 2000). F-box proteins are named after Cyclin F, which has been characterized as the first member of the family and is involved in different processes in many organisms (Merzetti *et al.,* 2013). F-box proteins are divided into three classes: FBXW, FBXL and FBXO based on their protein interaction domains: WD40 repeat domain, leucine rich repeat (LRR) and 'other ', respectively (Jin *et al.,* 2004). The WD40 domain has been found to function as a scaffold for protein complex assembly and is essential in several cellular processes such as proteasome function (Neer *et al.,* 1994). LRRK2 consists of a series of amino acids and functions as a staging ground for other types of protein-protein interactions (Kobe and Deisenhofer, 1994). F-box proteins FBXO-, has other domains such

as PDZ (Post synaptic density protein, Drosophila disc large tumour suppressor, and Zonula occludens-1 protein), zinc-finger, CASH and proline-rich domains (Cardozo and Pagano, 2004). The diversity of these F-box proteins indicates that F-box proteins act as specific targeting adapters for SCF E3 ubiquitin ligases.

F-box proteins have been found to interact with the adaptor protein SKP1 (SkpA) in Drosophila (Bai *et al.,* 1996; Dui *et al.,* 2012). F-boxes function as the substrate recognition component in Skp1-Cullin-F-box (SCF) E3 ubiquitin ligases, where they are essential to target proteins for ubiquitylation and degradation by the 26S proteasome (Durr *et al.*, 2006). Substrate specificity of SCF complexes is the function of F-box proteins. F-box proteins also connect the ubiquitination machinery and other cellular processes by exerting controls over the stability of different substrate proteins (Ho *et al.*, 2006). The F-box proteins of the SCF ubiquitin ligase complex are responsible for recognizing different target substrates for ubiquitination. F-box proteins have also been found in an association with cellular functions such as signal transduction and cell cycle regulation (Craig and Tyers, 1999). Some F-box proteins may also function independently of the SCF complex and the 26S proteasome. Many F-box proteins have been found in gene networks widely regulated by microRNA-mediated gene silencing via RNA interference in plants (Jones-Rhoades *et al.*, 2006) and in many flowering plants the s-locus F-box has been found to inhibit self-fertilization (Qiao *et al.*, 2004). Two F-box proteins have been found to control mitochondrial fusion and tubule formation in *Saccharomyces cerevisiae* (Durr *et al.*, 2006). In both mammals and flies, F-box proteins have been found to be an essential factor in circadian rhythm (Merzetti *et al.,* 2013). They can also function in regulation of specific substrate such as cullins (Cope and Deshaies, 2003). Substrates for ubiquitination via F-box E3 activity act in regulating organ formation and hormone response in plants (Gray *et al.,* 1999), spermatogenesis in *Caenorhabditis elegans* (Clifford *et al.*, 2000), and as

regulators of cell cycle and cell progression in mammals (Merzetti *et al.,* 2013).

In flies and mammals, many conserved members of the F-box protein family have been identified that can be studied in flies to provide the potential relationship between various disorders and processes and their mammalian counterparts (Merzetti *et al.,* 2013). In mammals, 75 F-box proteins have been found (Jin *et al.,* 2004) compared with 45 in flies (Dui *et al.*, 2012). Among these identified proteins, 21 have been found to be present in both lineages (Merzetti *et al.,* 2013). In Drosophila, 12 F-box proteins have known substrates (Skaar *et al.,* 2009a, 2009b). The functions of these proteins are varied but mutations in their genes have been shown to lead to phenotypes of disease or defects in cell growth regulation in hosts (Merzetti *et al.,* 2013).

F-box only protein 7

F-box only protein 7 is a member of the F-box-containing protein (FBP) family containing 40 amino acid domains (F-boxes) and is encoded by the *Fbxo7* gene (Ho *et al.,* 2008). The F-box motif (329-375 residues) directs its interaction with E2-ubiquitin conjugating enzyme that contains many ubiquitin-binding domains (Kirk *et al.,* 2008). In the C-terminus region, *Fbxo7* contains a proline rich region (PRR). Substrates of *Fbxo7* destined for SCF ubiquitin proteolysis bind to the PRR and are then ubiquitinated and targeted for degradation. *Fbxo7* has been found to be expressed especially in cerebral cortex, *globus pallidum* and *substantia nigra* regions of the brain and less expressed in the hippocampus and cerebellum (Zhao *et al.,* 2011).

Fbxo7 has been found to function as the targeting component of the ubiquitin proteasome system and mutation of *Fbxo7* could lead to aggregation of protein build up and failure of protein recycling and repair (Merzetti *et al.*, 2013). Aberrant proteins can

cause impaired mitochondrial function as well as impede other important intracellular processes such as cell death. The *Fbxo7* gene has been found associated with the early-onset parkinsonian pyramidal syndrome, which was described a few decades ago but for which a gene locus only recently has been mapped (Davison, 1954; Di Fonzo *et al.,* 2009). The distinguishing features of this disease include progressive degeneration of pyramidal, and extrapyramidal regions in combination with the *substantia nigra pars compacta* in the brain (Di Fonzo *et al.,* 2009). Fibres expanding from the putamen to globus pallidus and ansa lenticularis are degenerated and motor neurons are reduced in the globus pallidus (Ross, 1955). This early-onset PD, which has been studied in European families, showed juvenile Parkinsonian traits with other symptoms such as increased tendon flexes spasticity and Babinski signs (Di Fonzo *et al.*, 2009). In a Dutch family, two affected siblings were found with two novel *Fbxo7* mutations: one is a splice-site mutation (IVS7 + IG/T) and another one is a single base substitution (p.Thr22Met). Mutations in F-box only protein 7 have been found to cause juvenile-onset Parkinsonism with many other characteristics, such as pyramidal signs, dementia and dystonia (Lai *et al.*, 2012). This most severe phenotype has been found in association with a homozygous truncation mutation (p. Arg498Stop) of *Fbxo7* in an Italian family. The severe phenotype was also associated with a homozygous similar mutation (p. Arg378Gly). The *Fbxo7* gene has recently been identified in an Iranian kindred who has shown the characteristics of young-onset PD with pyramidal signs, such as spastic weakness and Babinski signs (Shojaee *et al.,* 2008). So, loss-of-function mutation in may cause PD. The *Fbxo7* gene was hence termed *PARK15*.

Fbxo7 in both mammals and flies interacts with a 31 kDa proteasome inhibitor (PI31) to start the process of proteasome degradation of target substrates (Bader *et al.,* 2010). This proteasome inhibitor was named as it inhibits the 20S proteasome macropain (Chu-Ping *et al*., 1992). The Fbxo7 protein binds with PI31 via a shared N-terminal

domain named *Fbxo7*/PI31 (FP) (Kirk *et al.*, 2008). Evidently, PI31 acts as an essential mediator in the ubiquitin-proteasome system.

F-box only protein 9

F-box only protein 9 is, encoded in humans by the *Fbxo9* gene (Cenciarelli *et al.,* 1999; Winston *et al.,* 1999). The protein encoded by *Fbxo9* gene belongs to the Fbxo class. Alternative splicing of this gene generates at least 3 transcript variants diverging at the 5' terminus. The substrate specificity of *Fbxo9* is unknown.

F-box only protein 32

The *Fbxo32*/*atrogin,* a gene that is up-regulated in skeletal muscles during muscle wasting, is conserved between mammals and flies (Merzetti *et al.*, 2013). The *Fbxo32* has been found to be overexpressed in a significant amount of human diseases and through its ubiquitin ligase function as a part of the SCF ubiquitin complex it functions in muscle wasting (Russell, 2010). *Fbxo32* is a member of the highly conserved F-box protein family consisting of a PDZ domain instead of WD40 and LRR domains (Colleen Furlong [nee Connors], unpublished). It has been found that *atrogin* is overexpressed in amyotrophic lateral sclerosis (ALS) (Leger *et al.,* 2006). Also, *Fbxo32* has been found to be overexpressed in paraplegia, chronic obstructive pulmonary disease (COPD), injury of spinal cord and immobilization of limb (Doucet *et al.*, 2007; Ju and Chen, 2007; Russell, 2010; Urso *et al.*, 2007). The *D. melanogaster atrogin* homologue, *CG11658*, has been found to be over-expressed by 30% in the muscle-wasting mutant (Bulchand *et al.,* 2010). Conversely, when *atrogin* is under-expressed, it has been found that muscle loss decreases in Drosophila models (Colleen Furlong [nee Connors], unpublished). When *atrogin* is knocked out, there is a reduction in muscle wasting by 50 % in mice (Latres *et al.*, 2005). It

has been found that in mammals, atrogin targets proteins required for muscle synthesis including MyoD and eukaryotic initiation factor 3- subunit 5 (eIF3-f), degrades them and initiates cell death of muscle (Tintignac *et al.,* 2005; Lagirand-Cantaloube *et al.,* 2008). It can be concluded that *Fbxo32*/*atrogin* is essential for muscle degradation.

The forkhead box, subgroup "O" (*Foxo*) transcription factors were first discovered as proto-oncogenes, which were disrupted as a result of chromosomal translocations leading to acute myeloid leukemia and rabdomyosarcoma (Sublett and Shapiro, 1995 and Borkhardt *et al*., 1997). Studies in mammalian cell culture have shown that in the absence of Akt signaling, *Foxo* is able to activate gene transcription and cause cell death, cell cycle arrest, or cell senescence (Arden and Biggs, 2002 and Burgering and Kops, 2002). The *atrogin/ Fbxo32* is the target for forkhead box, sub-group "O" (*Foxo*) transcription factors and can trigger skeletal muscle atrophy when insulin or insulin-like growth factor-1 (IGF-1) is absent. In mammals, it has been found that when insulin receptor signalling pathway has decreased activity, muscle atrophy is increased (Bodine *et al.,* 2001b) and *atrogin* expression is also increased (Sacheck *et al.*, 2004). Conversely, it has been found that when the insulin signalling pathway is activated, muscle atrophy is suppressed (Rommel *et al.,* 2001). The expression of this gene in various human conditions makes it a potential candidate for further studies, in particular potential organismal modeling of this range of human conditions.

Thesis objectives

In my study, I have performed a bioinformatics analysis of *Fbxo9* and *Fbxo7/PARK15*. I have analyzed the speculated homologous relationship between these two F-box proteins to assess the possibility of using Drosophila as model for PD. Our research group has determined that *Fbxo9* is very similar to *Fbxo7*/*PARK15* (Colleen Furlong [nee Connors], unpublished). We hypothesized that *Fbxo9* loss-of-function would also cause a PD-like phenotype in *D. melanogaster.* The objectives of this thesis are to determine the effects of loss-of-function and gain-of-function of *Fbxo9* in Drosophila compound eye, climbing ability and lifespan. It will further determine the interaction between *Fbxo9* and *Foxo* in Drosophila compound eye. The effects of *Fbxo9* on the *α-synuclein* model will be further examined.

MATERIALS AND METHODS

Bioinformatics analysis

Identification of *Drosophila melanogaster* homologue of human *Fbxo9*

The amino acid sequence of human *Fbxo9* (accession number NP_258441.1) was obtained from the National Center for Biotechnology Information (NCBI) database (*http://www.ncbi.nlm.nih.gov/*). A translated nucleotide data base using protein query search (tBLASTn) was performed using the Basic Local Alignment Search Tool (BLAST) (*www.ncbi.blast.com*) to find the *D. melanogaster* homologue (accession number NP_650206.1) of human *Fbxo9*. The *D. melanogaster* homologue was identified as gene *CG5961*.

Identification of other homologues of *Drosophila melanogaster CG5961* and conserved domains

NCBI Homologene (*www.ncbi.nlm.nih.com*) and a nucleotide query search of the nucleotide database (BLASTn) were used to find homologues of *D. melanogaster CG5961*, by interrogating with the *D. melanogaster CG5961* was used to search the BLAST database. To find the similarity between sequences, ClustalW2 (*http://www.ebi.ac.uk/Tools/msa/clustalw2*) was used (Larkin *et al*., 2007). The NCBI Conserved Domain Search Tool (*http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi*) was used to find different conserved domains. To align (1) vertebrate and invertebrate versions of *Fbxo9* (2) *nutcracker* and *CG5961* and (3) *atrogin* and *Fbxo9,* ClustalW2 was used. The F-box domain, Tetracycline peptide repeat (TPR), HNH nuclease family (HNHc), (Jin *et al.,* 2004) and Microtubule Interacting and Trafficking molecule Domain (MIT) were identified using Pfam (pfam.xpfam.org) (Finn *et al*., 2014). Nuclear

localization Sequence (NLS) was identified using NLSmapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Kosugi *et al*., 2009). Cladograms were created using ClustalW2. The similarity scores correspond to the evolutionary relations between species.

Drosophila melanogaster

Media

D. melanogaster stocks were maintained on a standard cornmeal-yeast-molasses-agar medium (65 g/L cornmeal, 50 ml/L molasses, 10 g/L yeast, 5.5 g/L agar and 950 ml/L water) and those stocks were stored at room temperature. To prevent the growth of mold, the medium used was treated with 2.5 ml/L propionic acid and 5 mL of 10% methyl paraben in ethanol. Seven mL aliquots of the medium were poured into vials of plastic vials and allowed to solidify. The vials were then stored at 4° C to 6° C until they were used. *Drosophila* stocks were maintained on this medium for 2 to 3 weeks and were then transferred to new media. The medium was prepared by Dr. Brian E. Staveley approximately twice a month.

Stocks

Recombinant line *GMR-Gal4; UAS-Foxo*, prepared by Dr. Brian E. Staveley, was used in this study. *UAS-lacZ, GMR-Gal4* and *TH-Gal4* fly lines that were also used were obtained from Bloomington Drosophila Stock Centre at Indiana University. Moreover, *Ddc-Gal4* fly lines were provided *by* Dr. J. Hirsh (University of Virginia) and *UAS-α-synuclein* fly lines were provided by Dr. M Feany (Harvard Medical School) for this study. Fly lines of *Fbox9* ^{EP} stock number 30076 and *Fbox9*^{RNAi} stock number *31368* were obtained from Bloomington Drosophila Stock Centre. Additionally, *UAS-PI31* provided by

Dr. H. Steller of Rockefeller University was also used in this current study.

Ageing assay

To avoid crowding during development, crosses were made in several vials, each containing 2 to 4 females and 2 to 4 males of each genotype. Approximately 300 male flies were collected under gaseous carbon dioxide (CO_2) every 24 h-ours upon eclosion. These flies were then transferred to plastic vials containing fresh standard medium with 20 flies maximum in a single vial so that overcrowding could be avoided. Flies were observed, media replenished and scored for the presence of dead flies every second day starting two days after collection. Flies were considered dead when there was no movement during agitation (Staveley *et al*., 1990). The ageing assay was continued until all of the flies were dead. Data were analyzed using GraphPad Prism 5.03 software (Slade and Staveley, 2015). Survival curves were compared using a log-rank tests where a *p*-value less than or equal to 0.05 was considered significant.

Table 2. Genotypes of fly stocks used in this study.

Genotype	Abbreviation	Expression	Balancer	Reference
Control Lines				
w ; UAS-lacZ $^{4-1-2}$	*UAS-lacZ*			Brand *et al*., 1994
w; UAS-PI31	*UAS-PI31*			Bader *et al*., 2010
				H. Steller
Driver Lines				
w ; GMR-Gal4^{12}	*GMR-Gal4*	Eye		Freeman,1996
w ; Ddc-Gal4^{HL83D}	*Ddc-Gal4* ^{3D}	Dopaminergic neurons plus		Li *et al*., 2000
w ; Ddc-Gal4^{HL836}	*Ddc-Gal4* ^{36}	Dopaminergic neurons plus		Li *et al*., 2000
w ; pale-Gal43	*TH-Gal4*	Dopaminergic neurons		
Experimental Lines				
w; Fbxo9 ^{RNAiJFO1332}	*Fbxo9* ^{RNAi}			
w; Fbxo9 ^{EPCG5961}	*Fbxo9* ^{EP}			
Derivative Lines				
w; GMR-Gal4/CyO ; UAS-Foxo /TM3	*GMR-Gal4; UAS-Foxo*			Kramer *et al.,*2003
w; GMR-Gal4/CyO; UAS-PI31 /TM3	*GMR-Gal4; UAS-PI31*			Staveley construct
w; UAS-a-synuclein/CyO; Ddc-Gal4 /TM3	*a-synuclein;Ddc-Gal4*	Dopaminergic neurons	CyO, Curly; TM3, Stubble	Staveley construct
Compound Lines				
Ddc-Gal4^{HL83D}*/CyO; Ddc-Gal4*^{HL836}*/TM3*	*Ddc-Gal4* ^{3D}*;Ddc-Gal4* ^{36}			Staveley construct

Locomotion assay

Approximately 70 male flies were collected under gaseous CO_2 upon eclosion and scored for climbing ability as described by Todd and Staveley (2004). Flies were maintained at 25°C on standard cornmeal-yeast-molasses-agar medium. Flies were maintained in vials of fresh medium with a maximum of 10 flies per vial and transferred to new food twice in a week throughout the experiment. Flies were assayed for first climbing ability when they were 2 days old. Flies were then assayed every 7 days until all of the flies were dead. Climbing ability was determined using a climbing apparatus that of a 30 cm long glass tube with a 1.5 cm diameter. The tube was divided into five 2 cm sections along with a buffer zone. Transferred without anesthesia, each vial was assayed ten times and flies were given 10 seconds to see which sections they had reached. Flies were scored 10 times per trial. A climbing ability was determined using the climbing index $\Sigma(nm)/N$ where n is the number of flies at a given level, m is the level (1-5) and N is the total number of flies scored for the given trial (Todd and Staveley, 2004). Locomotion data analysis was done using GraphPad Prism 6.0 software. To compare locomotion ability, the climbing index was subtracted from 5 and climbing curves were fitted using non-linear regressions. Slopes of the climbing curve were compared using a 95% confidence interval, and a *p*-value less than or equal to 0.05 was considered significant.

Scanning electron microscopy of the compound eye

Several male flies were collected upon eclosion and were aged for 3 to 5 days on standard cornmeal-yeast-molasses-agar medium at 25° C. Flies were then stored at -80°C before being mounted on aluminum studs with the left eye facing upwards and desiccated overnight. Prepared flies were gold coated before photographs were taken using Hitachi S-570 Scanning Electron Microscope, located at Bruneau Centre for Innovation and Research

(IIC) at 150X magnification. At least 15 eye images per genotype were analyzed using NIH ImageJ software (Abromoff *et al.,* 2004) in order to determine the total number of ommatidia, total number of bristles and ommatidium area. Data were analyzed using GraphPad 6.0 Prism software. To compare the measured parameters, unpaired t-tests were carried out and *p* values less than or equal to 0.05 were considered significant.

RESULTS

Bioinformatics analysis

Identification of *Fbxo9* in *Drosophila melanogaster*

The amino acid sequence of the *Fbxo9* protein from *Homo sapiens* was obtained from Genbank (NP_258441.1). A tBLASTn search of the *D. melanogaster* genome was conducted and the *D. melanogaster* gene *CG5961* product was identified as the most similar protein sequence. The proteins share 150 identical, 99 highly conserved and 49 less conserved amino acids, and the overall similarity and identity between the proteins is 34% and 67%, respectively. The alignment shows that these proteins share the Tetracycline peptide repeat (TPR), Microtubule interacting and trafficking molecule (MIT), F box-only protein (F-box), Nuclear localization sequence (NLS), and HNH nuclease family (HNHc) (Figure 1).

Fbxo9 protein is conserved between vertebrates and invertebrates

A BLASTn search identified potentially homologous versions of vertebrate and invertebrate *Fbxo9*, including *Homo sapiens* (NP_258441.1), *Pan troglodytes* (NP_001153767.1), *Mus musculus* (NP_076094.2), *Drosophila melanogaster* (NP_650206.1), *Culex quinquefasciatus* (XP_001863207.1) and *Anopheles gambiae* (XP_308962). The alignment of vertebrate and invertebrate *Fbxo9* proteins show that the TPR domain, MIT domain, F-box domain, NLS domain, HNHc domain and Microtubule interacting and trafficking molecule domain are all highly conserved among the different proteins (Figure 2). The proteins share 112 identical, 91 highly conserved and 27 less conserved amino acids being 26% identical and 27% similar. The scores of similarity are

summarized in Table 3. This relative degree of similarity corresponds inversely to the evolutionary distances between vertebrate and invertebrate species of *Fbxo9*.

Relationship between *Fbxo9*, *Fbxo7* and *Fbxo32*

The Drosophila F-box protein nutcracker has been found to interact with *Drosophila* PI31 (Bader *et al.,* 2010). Human *Fbxo9* and Drosophila *Fbxo7* show some similarity in the PI31 and F-box region. They share 78 highly conserved, 51 conserved and 47 less conserved amino acids being 34% similar and 14% identical (Figure 3). Human *Fbxo9* and Drosophila *Fbxo32* show some similarity at the amino terminus and within the potential LZ, LCD and PDZ domains. They share 65 conserved, 65 highly conserved and 54 less conserved amino acids being 17% identical and 28 % similar (Figure 4). Bioinformatics analysis of the F-box proteins *Fbxo9* (*CG5961*), *Fbxo7* (*nutcracker*) and *Fbxo32* (*atrogin*) reveals that they are evolutionarily conserved between vertebrates and invertebrates (Figure 5). Within the sub-group, *Fbxo9* and *Fbxo7* are most similar, with Fbxo32 more distant. GenBank accession numbers for vertebrate and invertebrate versions of *Fbxo7/nutcracker*, *Fbxo9/CG5961* and *Fbxo32/atrogin* are shown in Table 4.

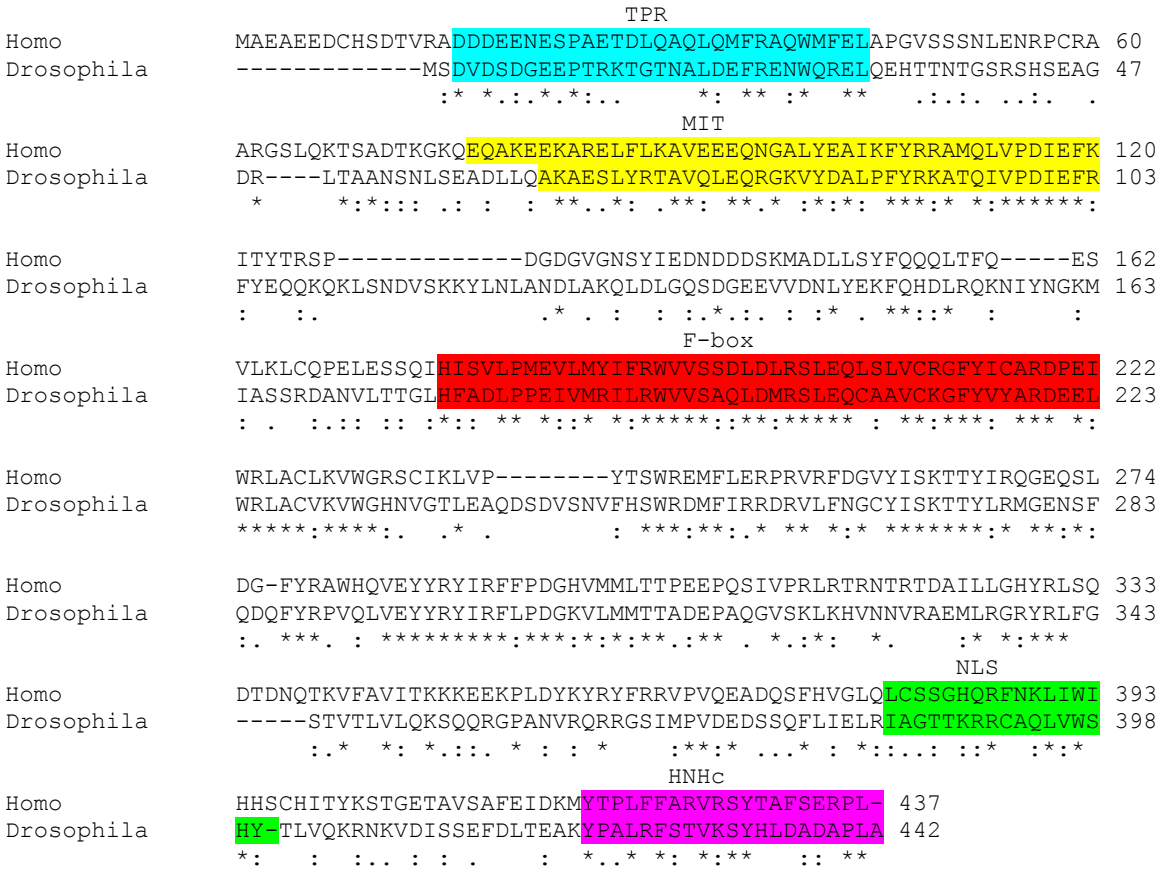
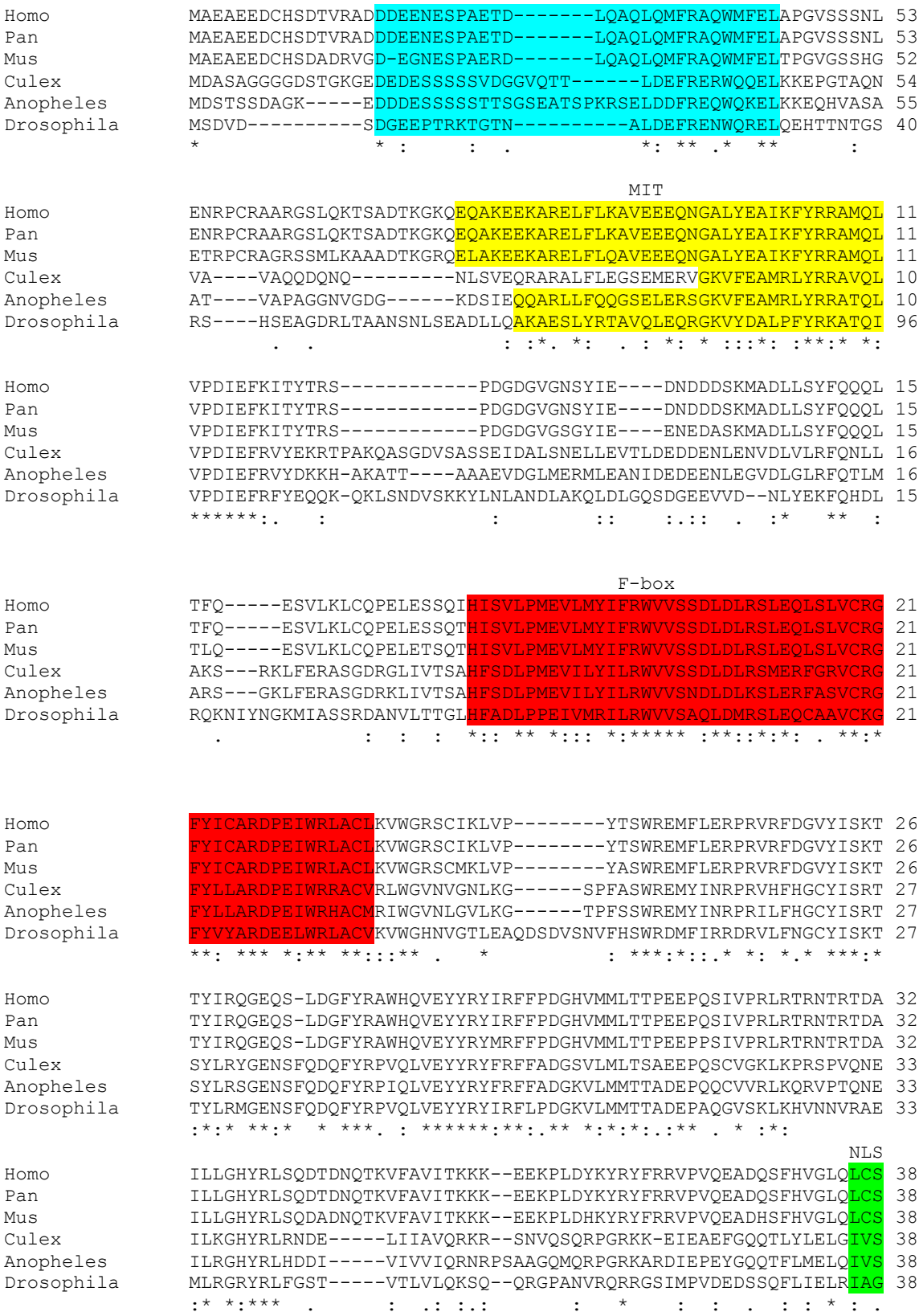


Figure 1: Alignment of human *Fbxo9* (NP_258441.1) protein with Drosophila *CG5961* (NP_650206.1). The highlighted turquoise region is the TPR domain, the yellow region is the MIT domain, the red region is the F-box domain, the green region is the NLS domain and the pink region is the HNHc domain. TPR is the Tetracycline peptide repeat, MIT is the Microtubule interacting and trafficking molecule domain, NLS is the Nuclear localization sequence and HNHc is the HNH nuclease family. "*" indicates fully conserved amino acid, ":"indicates conserved amino acid with less similar properties and "." indicates conserved amino acid with less similar properties.


MIT
F-box
NLS

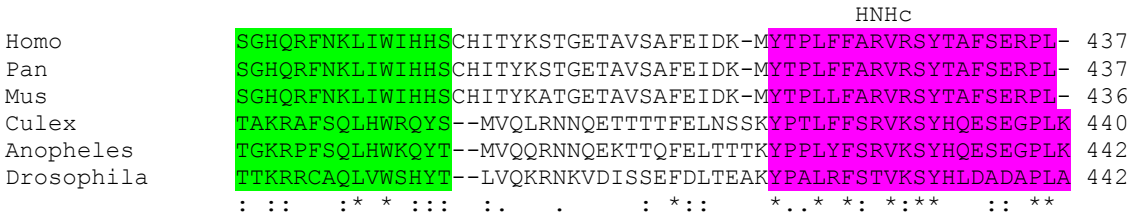


Figure 2: *Fbxo9* is well conserved in vertebrates and invertebrates. The highlighted turquoise region is the TPR domain, the yellow region is the MIT domain, the red region is the F-box domain, the green is the NLS domain and the pink region is the HNHc domain. TPR is the Tetracycline peptide repeat, MIT is the Microtubule interacting and trafficking molecule domain, NLS is the Nuclear localization sequence and HNHc is the HNH nuclease family. "*" indicates fully conserved amino acid, ":"indicates conserved amino acid with less similar properties and "." indicates conserved amino acid with less similar properties. The Genbank accession number for *Homo sapiens* is NP_258441.1, *Pan troglodytes* NP_001153767.1, *Mus musculus* NP_076094.2, *Drosophila melanogaster* NP_650206.1, *Culex quinquefasciatus* XP_001863207.1 and *Anopheles gambiae* XP_308962.

Table 3. Similarity scores of *Fbxo9* protein homologues from vertebrate and invertebrate species.

Species	**% Similarity to *Fbxo9* (*Homo sapiens*)**	**% Similarity to *CG5961* (*Drosophila melanogaster*)**
Pan troglodytes	99.77	34.22
Mus musculus	91.51	34.4
Anopheles gambiae	36.61	40.95
Culex quinquefasciatus	38.44	38.86
Drosophila melanogaster	34.22	…….
Homo sapiens	……	34.22

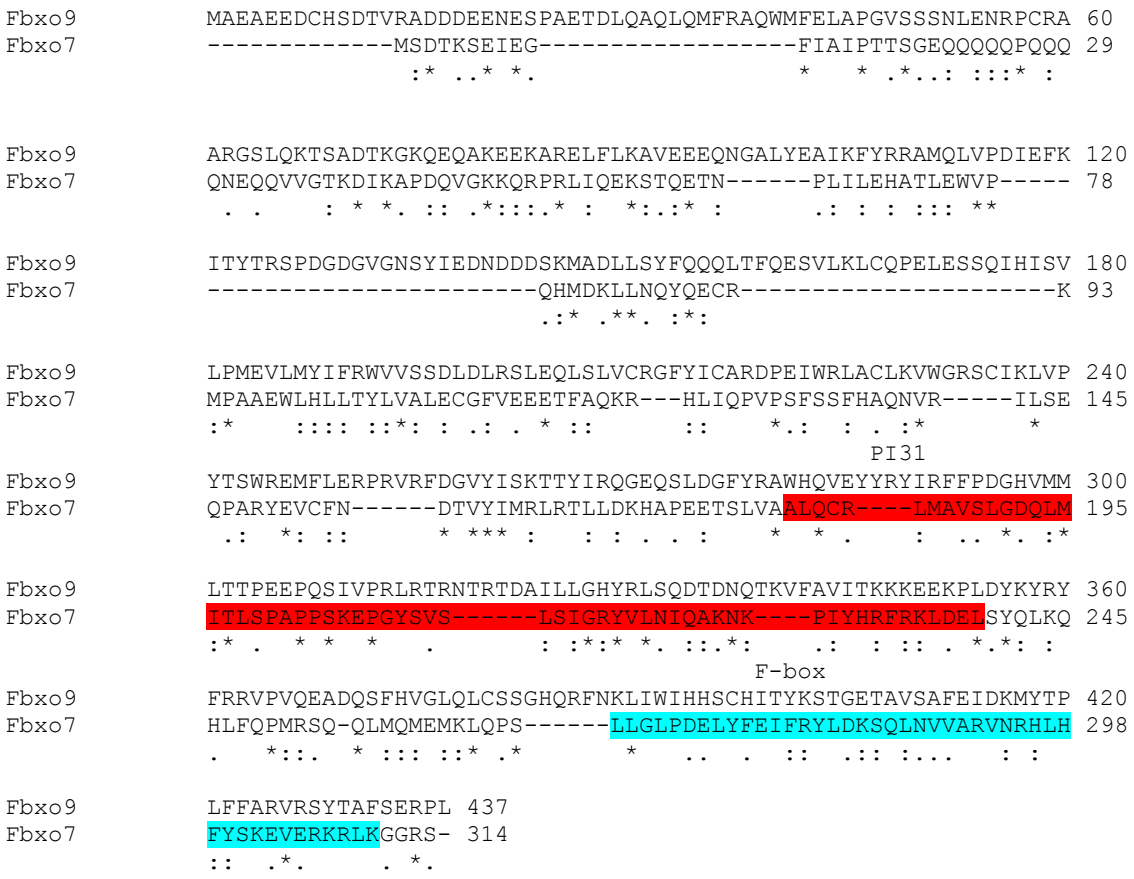


Figure 3: Human *Fbxo9* and Drosophila *Fbxo7* share some similarity. ClustalW2 multiple alignment of Human *Fbxo9* and Drosophila *Fbxo7*. The highlighted red region is the PI31 binding region and the turquoise region is the F-box domain. "*****" indicates fully conserved amino acid, ":"indicates conserved amino acid with less similar properties and "." indicates conserved amino acid with less similar properties.

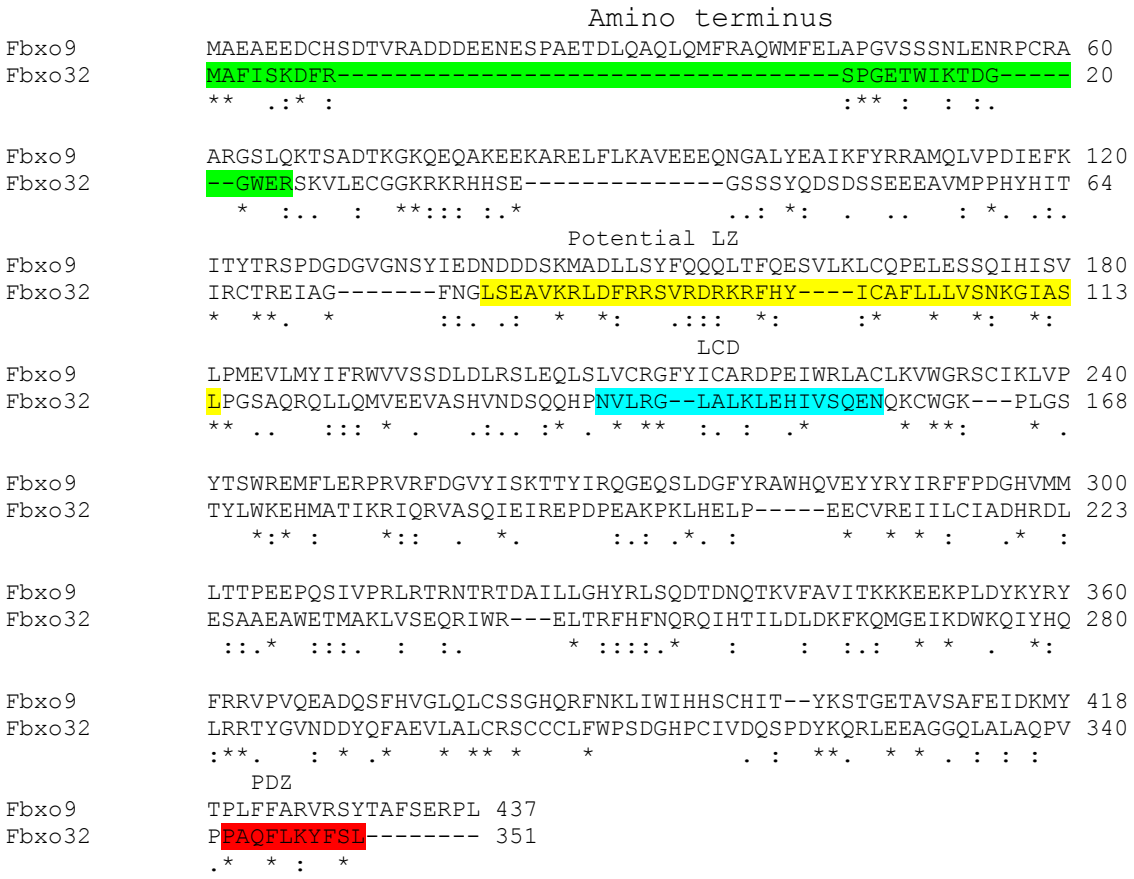


Figure 4: Similarity between Human *Fbxo9* and Drosophila *Fbxo32*. ClustalW2 multiple alignment of Human *Fbxo9* and Drosophila *Fbxo32.* The highlighted green region is the amino terminus, the yellow region is the potential LZ domain, the turquoise region is the LCD domain and the red region is the PDZ domain. "*" indicates fully conserved amino acid, ":" indicates conserved amino acid with less similar properties and "." indicates conserved amino acid with less similar properties.

Table 4. Protein names and GenBank accession numbers of vertebrate and invertebrate versions of *Fbxo7, Fbxo9* and *Fbxo32*.

Species	Accession number	Protein name
Homo sapiens	NP_258441.1	F-box only protein 9 isoform 2
Drosophila melanogaster	NP_650206.1	CG5961, isoform A
Homo sapiens	NP_036311.3	F-box only protein 7 isoform 1
Drosophila melanogaster	AAF47792.2	AAF47792.2
Homo sapiens	NP_478136.1	F-box only protein 32 isoform 1
Drosophila melanogaster	NP_648498.1	CG11658, isoform A

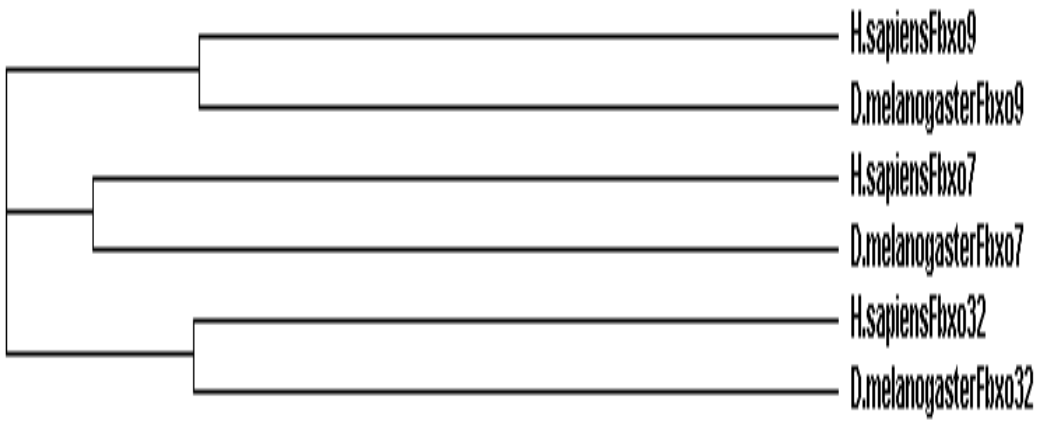


Figure 5: *Fbxo7*, *Fbxo9* and *Fbxo32* are conserved between Drosophila and Human. The GenBank accession number for *Homo sapiens Fbxo9 is* NP_258441.1, *Fbxo7* NP_036311.3, *Fbxo32* NP_478136.1; for *Drosophila melanogaster Fbxo9 is* NP_650206.1, *Fbxo7* is AAF47792.2 and *Fbxo32* NP_648498.1.

Eye analysis

Effects of directed overexpression and RNA interference of *Fbxo9*

The *D. melanogaster* compound eye development is very precise and regular. The development and organization of each ommatidium and its array is tightly controlled (Thomas and Wassarman, 1999). Each eye is composed of approximately 700-800 ommatidia under normal conditions. If any disruption occurs it may result changes in ommatidia number, bristle number and/ or ommatidia size. The eye is a photoreceptor organ and has within it neurons and other neuronally derived tissues such as glia, bristles and sockets. Under this precept, we investigated using biometric analysis whether overexpression or RNA-i dependent under-expression of *Fbxo9* has any effect on the development of specialized neurons in the eye. To determine the effects of gain-of-function and loss-of-function of *Fbxo9* in the Drosophila eye, the eye specific transgenic driver *GMR-Gal4* was used to express these transgenes. A summary of ommatidia number, bristle number and ommatidia area is shown in Table 5. Analysis of the scanning electron micrographs (Figure 6) showed that RNA-i of the *Fbxo9* RNAi gene significantly decreased ommatidia number, bristle number and size of each ommatidia . With expression of *Fbxo9* RNAi under the control of *GMR-Gal4* driver, the average number of ommatidia, bristle and ommatidium area per eye were 578.3 ± 1.745, 410.1 ± 3.952 and 166.3 ± 0.8489 μm^2, respectively. This is compared to the *UAS-lacZ* control where the average number of ommatidia, bristle and ommatidium area per eye were 678.3 ± 2.393, 575.4 ± 3.144 and 186.5 ± 1.410 μm^2, respectively (Table 5). Overexpression of *Fbxo9* significantly increased ommatidia number and bristle number but there was no significant difference in the ommatidium area. When *Fbxo9* was overexpressed using *GMR-Gal4* driver, the average number of ommatidia per eye was 699.4 ± 1.756, bristle number was 611.6 ± 1.693 compared to *UAS-lacZ* -expressing control where the average number of

ommatidia per eye was 678.3 ± 2.393 and bristle number was 575.4 ± 3.144. It can be concluded that loss-of-function of *Fbxo9* in the fly eyes through eye-specific expression of $Fbxo9^{RNAi}$ leads to a significant reduction in the number of ommatidia, bristles as well as in ommatidia area, whereas overexpression of *Fbxo9* leads to significantly increases.

Table 5. A summary of the biometric analysis of the directed overexpression and RNA interference of *Fbxo9* in the Drosophila compound eye.

A. Ommatidia number

Genotype	Number of eyes analyzed (N)	Mean ± SEM	*p*-value compared to control	Significant
GMR-Gal4/ UAS-lacZ	15	678.3 ± 2.393	N/A	N/A
GMR-Gal4/ Fbxo9^{RNAi}	15	578.3 ± 1.745	< 0.0001	Yes
GMR-Gal4/ Fbxo9^{EP}	15	699.4 ± 1.756	< 0.0001	Yes

B. Bristle number

Genotype	Number of eyes analyzed (N)	Mean ± SEM	*p*-value compared to control	Significant
GMR-Gal4/ UAS-lacZ	14	575.4 ± 3.144	N/A	N/A
GMR-Gal4/ Fbxo9^{RNAi}	14	410.1 ± 3.952	< 0.0001	Yes
GMR-Gal4/ Fbxo9^{EP}	14	611.6 ± 1.693	< 0.0001	Yes

C. Ommatidium area

Genotype	Number of eyes analyzed (N)	Mean ± SEM	*p*-value compared to control	Significant
GMR-Gal4/ UAS-lacZ	15	186.5 ± 1.410 μm^2	N/A	N/A
GMR-Gal4/ Fbxo9^{RNAi}	15	166.3 ± 0.8489 μm^2	< 0.0001	Yes
GMR-Gal4/ Fbxo9^{EP}	15	187.3 ± 1.236 μm^2	0.6988	No

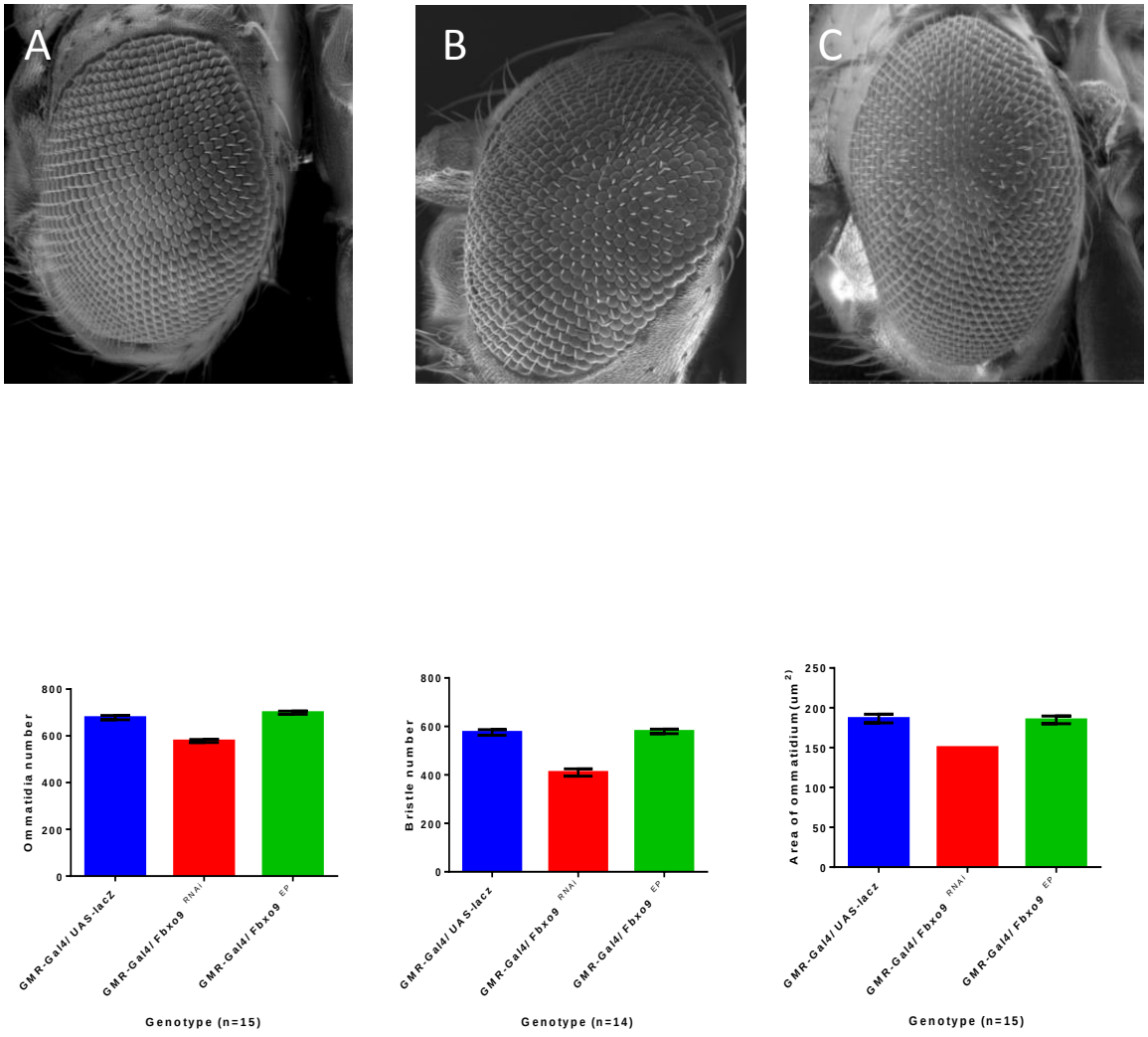


Figure 6: Biometric analysis of gain-of-function and loss-of-function of *Fbxo9* in the Drosophila compound eye. Genotypes are (A) *GMR-GAL4/ UAS-lacZ,* (B) *GMR-GAL4/ Fbxo9^{RNAi}* and (C) *GMR-GAL4/ Fbxo9^{EP}*. Loss-of-function of *Fbxo9* significantly decreases ommatidia number (D), bristle number (E) and ommatidium area (F). Overexpression of *Fbxo9* significantly increases ommatidia number (D) and bristle number (E) but there was no significant difference in the ommatidia area (F). Data was analyzed using one-way ANOVA and Dunnett's multiple comparison tests and $p < 0.05$ was used to indicate significant differences. Error bars represent standard error of the mean.

Investigation of *Fbxo9* and *Foxo* interactions in the compound eye

Next, the effects on the compound eye of co-overexpression of *Fbxo9* and *Foxo*, as well as overexpression of *Foxo* together with RNAi-dependent reduced expression of *Fbxo9*, were determined. *Fbxo9* was co-expressed with both a Drosophila and a mouse version (*Foxo1*) of *Foxo*, which was previously generated by another research group using *GMR-Gal4; UAS-Foxo* driver (Kramer *et al*., 2003). The elevated expression of *Foxo* can create an eye phenotype where a greater number of ommatidia and bristles are decreased (Kramer *et al.,* 2003). A summary of ommatidia number, bristle number and ommatidia area is shown in Table 6. Analysis of scanning electron micrographs of eyes (Figure 7) show that RNAi-dependent reduced expression of *Fbxo9* together with *Foxo* overexpression significantly decreased the ommatidia number, bristle number and ommatidium area compared to the *Foxo* overexpressing control fly *GMR-Gal4; UAS-Foxo/UAS-lacZ*. The average number of ommatidia decreased from 382.9 ± 3.344 to 183.1 ± 2, bristle number decreased from 7.467 ± 0.1333 to 3.600 ± 0.1309 and ommatidia area decreased from 197.9 ± 0.2794 μm^2 to 123.8 ± 0.3114 μm^2 (Table 6).

Over-expression of *Fbxo9* and *Foxo* together significantly increased the ommatidia number (Figure 7D) and, bristle number (Figure 7E) and ommatidium area (Figure 7F). The average number of ommatidia, bristles and ommatidium area in the *GMR-Gal4; UAS-Foxo/Fbxo9*^{EP} expressing flies was 432.7 ± 3.138, 11.60 ± 0.1309, and 205.9 ± 0.6252 μm^2 respectively, compared to the *Foxo* overexpressing control flies (*GMR-Gal4; UAS-Foxo/ UAS-lacZ*) where the average number of ommatidia, bristles and ommatidium area per eye was 382.9 ± 3.344, 7.467 ± 0.1333, 197.9 ± 0.2794 μm^2, respectively. It can be concluded that co-overexpression of *Fbxo9* and *Foxo* significantly increases ommatidia number and, bristle number and ommatidium area.

Table 6. A summary of the ommatidia number, bristle number and ommatidia area when *Foxo* is co-overexpressed with *lacZ*, *Fbxo9*^{RNAi} and *Fbxo9*^{EP} in the Drosophila compound eye.

A. Ommatidia number

Genotype	**Number of eyes analyzed (N)**	**Mean ± SEM**	***p*-value compared to control**	**Significant**
GMR-Gal4;UAS-Foxo/ UAS-lacZ	15	382.9 ± 3.344, n=15	N/A	N/A
GMR-Gal4;UAS-Foxo/ Fbxo9^{RNAi}	15	183.1 ± 2.918, n=15	< 0.0001	Yes
GMR-GAL4;UAS-Foxo/ Fbxo9^{EP}	15	432.7 ± 3.138, n=15	< 0.0001	Yes

B. Bristle number

Genotype	**Number of eyes analyzed (N)**	**Mean± SEM**	***p*-value compared to control**	**Significant**
GMR-Gal4;UAS-Foxo/ UAS-lacZ	15	7.467 ± 0.1333	N/A	N/A
GMR-Gal4;UAS-Foxo/ Fbxo9^{RNAi}	15	3.600 ± 0.1309	< 0.0001	Yes
GMR-GAL4;UAS-Foxo/ Fbxo9^{EP}	15	11.60 ± 0.1309	< 0.0001	Yes

C. Ommatidium area

Genotype	**Number of eyes analyzed (N)**	**Mean± SEM**	***p*-value compared to control**	**Significant**
GMR-Gal4;UAS-Foxo/ UAS-lacZ	15	197.9 ± 0.2794 μm^2	N/A	N/A
GMR-Gal4;UAS-Foxo/ Fbxo9 ^{RNAi}	15	123.8 ± 0.3114 μm^2	< 0.0001	Yes
GMR-GAL4;UAS-Foxo/ Fbxo9 ^{EP}	15	205.9 ± 0.6252 μm^2	< 0.0001	Yes

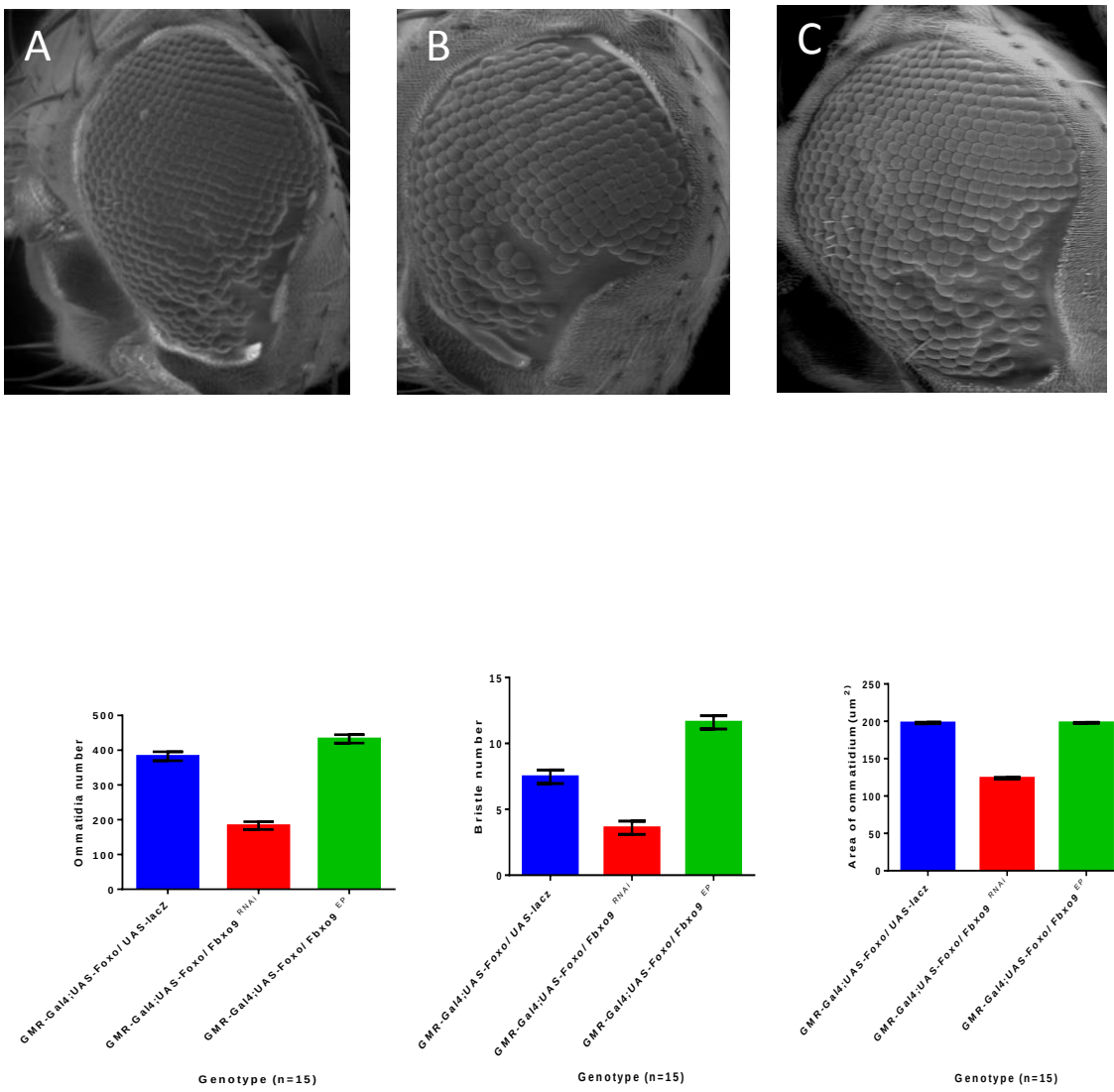


Figure 7: Biometric analysis of the Drosophila compound eye in flies overexpressing *Foxo* together with overexpression or loss-of-function of *Fbxo9*. Genotypes are (A) *GMR-Gal4; UAS-Foxo/UAS-lacZ*, (B) *GMR-Gal4; UAS-Foxo/Fbxo9*^{RNAi} and (C) *GMR-Gal4; UAS-Foxo/Fbxo9*^{EP}**.** Loss-of-function of *Fbxo9* together with overexpression of *Foxo* significantly decreases ommatidia number (D), bristle number (E) and ommatidium area (F). Overexpression of both *Foxo* and *Fbxo9* significantly increases ommatidia number (D) and bristle number (E) and ommatidia area (F). Data were analyzed using one-way ANOVA and Dunnett's multiple comparison tests and $p<0.05$ was used to indicate significant differences. Error bars represent the standard error of the mean.

Ageing analysis

The effects of loss-of-function and gain-of-function of *Fbxo9*

The main feature of PD is the age dependent degeneration of dopaminergic neurons. The selective death and degeneration of these dopaminergic neurons lead us to investigate the effects of *Fbxo9* on these neurons. To determine whether *Fbxo9* has any effects on the DA neurons, the gene was both overexpressed and silenced via RNA interference in the DA neurons. The ageing analysis was carried out in parallel with the climbing assay in order to determine the changes in the climbing ability as a result of premature senescence.

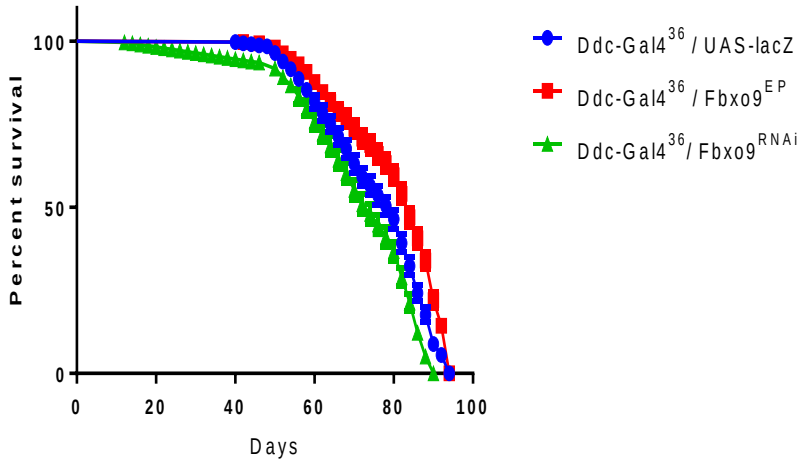
To investigate the effects of gain-of-function and loss-of-function of *Fbxo9* on ageing and lifespan, driver lines *Ddc-Gal4^{36} (II)*, *Ddc-Gal4^{3D} (III)*, *Ddc-Gal4^{3D}; DDC-Gal4^{36}* and *TH-Gal4* were used to express these transgenes in the fly dopaminergic neurons. Survival curves are illustrated in Figure 8. The results indicate that loss-of-function of *Fbxo9* using the *Ddc-Gal4^{36} (II), Ddc-Gal4^{3D} (III)*, *Ddc-Gal4^{3D}; Ddc-Gal4^{36}* and *TH-Gal4* drivers significantly decreased longevity compared to *lacZ*-expressing controls (Figure 8 A-D). The median lifespan for *Fbxo9^{RNAi}* flies using these four drivers was 74, 76, 72 and 70 days, respectively. This is compared to the control flies whose median lifespan was 80, 82, 78 and 75 days, respectively. The log-rank test showed that the *Fbxo9^{RNAi}* longevity curves are significantly different (p<0.0001) from the control curves.

Overexpression of *Fbxo9* using the *Ddc-Gal4^{36} (II)*, *Ddc-Gal4^{3D} (III)*, *Ddc-Gal4^{3D}; DDC-Gal4^{36}* and *TH-Gal4* drivers significantly increased longevity compared to *lacZ*-expressing controls (Figure 8 A-D). The median lifespan for flies overexpressing *Fbxo9* using these four drivers was 84, 86, 84 and 82, respectively. This is compared to the control flies whose median lifespan was 80, 82, 78 and 75, respectively. The log-rank test

showed that the longevity curves for the *Fbxo9* overexpression flies are significantly different (p<0.0001) from the control curves. Statistical analysis of longevity is summarized in Table 7. Loss-of-function of *Fbxo9* in fly dopaminergic neurons leads to a reduction in lifespan, which is characteristic of PD-like phenotype, whereas overexpression of *Fbxo9* significantly increases lifespan.

Table 7. The Log-Rank comparison of the survival curves for the directed overexpression and RNA interference of *Fbxo9* in the dopaminergic neurons. Chi-square values and *p*-values were calculated using *UAS-lacZ* controls.

Genotype	Number of Flies (Deaths)	Median Survival (Days)	Chi Square	*p*-value	Significant
Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ UAS-lacZ	300	78	125.6	N/A	N/A
Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ Fbxo9^{EP}	300	84	9.644	0.0019	Yes
Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ Fbxo9^{RNAi}	263	72	76.11	<0.0001	Yes
Ddc-Gal4^{36}/ UAS-lacZ	306	80	87.73	N/A	N/A
Ddc-Gal4^{36} / Fbxo9^{EP}	300	84	14.85	0.0001	Yes
Ddc-Gal4^{36} / Fbxo9^{RNAi}	277	74	59.55	<0.0001	Yes
Ddc-Gal4^{3D}/ UAS-lacZ	334	82	62.30	N/A	N/A
Ddc-Gal4^{3D}/ Fbxo9^{EP}	290	86	14.34	0.0002	Yes
Ddc-Gal4^{3D}/ Fbxo9^{RNAi}	300	76	47.59	<0.0001	Yes
TH-Gal4/ UAS-lacZ	274	75	82.27	N/A	N/A
TH-Gal4/ Fbxo9^{EP}	280	82	8.124	0.0042	Yes
TH-Gal4/ Fbxo9^{RNAi}	250	70	47.89	<0.0001	Yes


Percent survival
100
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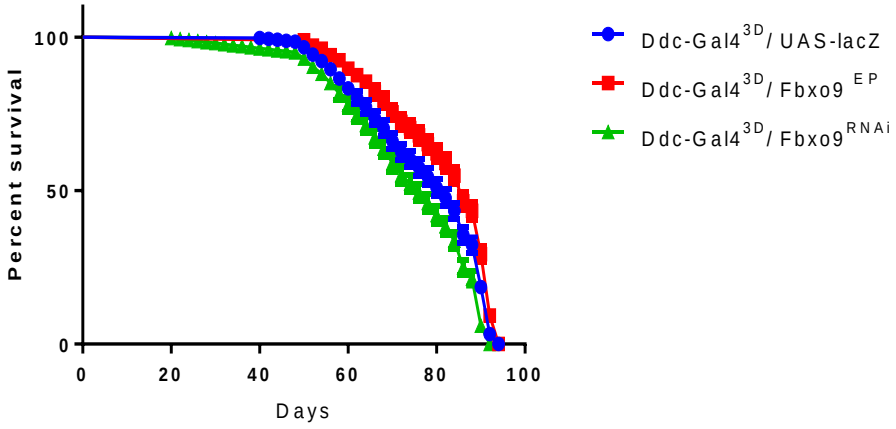
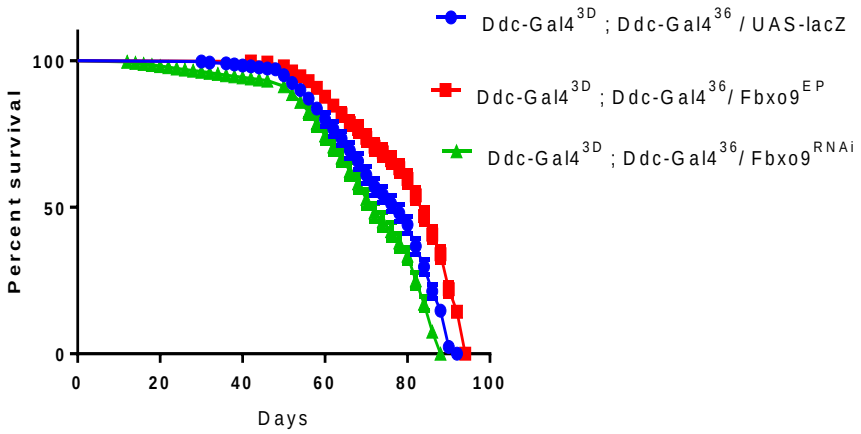
100
Days
Ddc-Gal4^36 / UAS-lacZ
Ddc-Gal4^36 / Fbxo9^EP
Ddc-Gal4^36/ Fbxo9^RNAi

A


Percent survival
100
50
0
0
20
40
60
80
100
Days
Ddc-Gal4^3D/ UAS-lacZ
Ddc-Gal4^3D/ Fbxo9 ^EP
Ddc-Gal4^3D/ Fbxo9^RNAi

B



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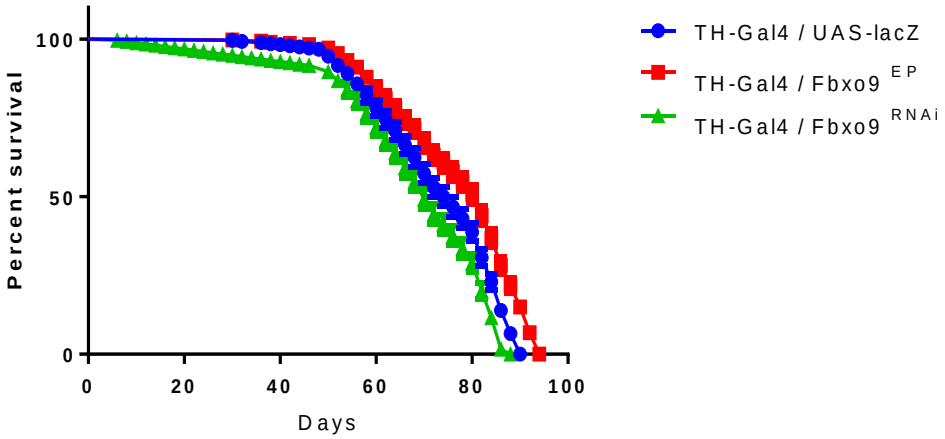

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Figure 8: Effect of loss-of-function and overexpression of *Fbxo9* on lifespan in Drosophila. Expression of *Fbxo9*^{RNAi} using the *Ddc-Gal4*^{36} (A), *Ddc-Gal4*^{3D} (B), *Ddc-Gal4*^{3D}*; Ddc-Gal4*^{36} (C), and *TH-Gal4 (D)* drivers significantly decreases longevity. Overexpression of *Fbxo9* using the same four drivers significantly increases longevity. Longevity is shown as the percent survival as a function of time. A *p*-value less than 0.01 is considered as significant by the log-rank test. Error bars represent the standard error of the mean.

Investigating the effects of *Fbxo9* on the *α-synuclein* model of PD

To determine the effects on Drosophila lifespan of *α-synuclein* overexpression together with *Fbxo9* overexpression or *Fbxo9* reduced expression due to RNA interference, the experimental lines and control line were co-expressed with *UAS-α-synuclein/CyO; Ddc-Gal4/TM3.* The resulting survival curve is illustrated in Figure 9. Loss-of-function of *Fbxo9* together with overexpression of *α-synuclein* significantly decreased lifespan compared to the *lacZ*-expressing controls. The median lifespan of $Fbxo9^{RNAi}$ / *UAS-α-synuclein* flies was 76 days as compared to 80 days for the control flies. Overexpression of both *Fbxo9* and *α-synuclein* significantly increased the lifespan compared to the *lacZ*-expressing controls. The median lifespan of the $Fbxo9^{RNAi}$ / *UAS-α-synuclein* flies was 82 days as compared to 80 days for the control flies. The statistical analysis of the longevity assay is summarized in Table 8.

Table 8. The Log-Rank survival curve comparison for the directed co-overexpression of *α-synuclein* and *Fbxo9* and or *Fbxo9*^{RNAi} in the dopaminergic neurons. Chi-square values and *p*-values were calculated using *UAS-lacZ* controls.

Genotype	Number of Flies (Deaths)	Median Survival (Days)	Chi Square	P value	Significant
UAS-αsyn/ UAS-lacZ	367	80	68.65	N/A	N/A
UAS-asyn/ Fbxo9 ^{EP}	347	82	45.52	<0.0001	Yes
UAS-asyn/ Fbxo9^{RNAi}	302	76	41.43	<0.0001	Yes

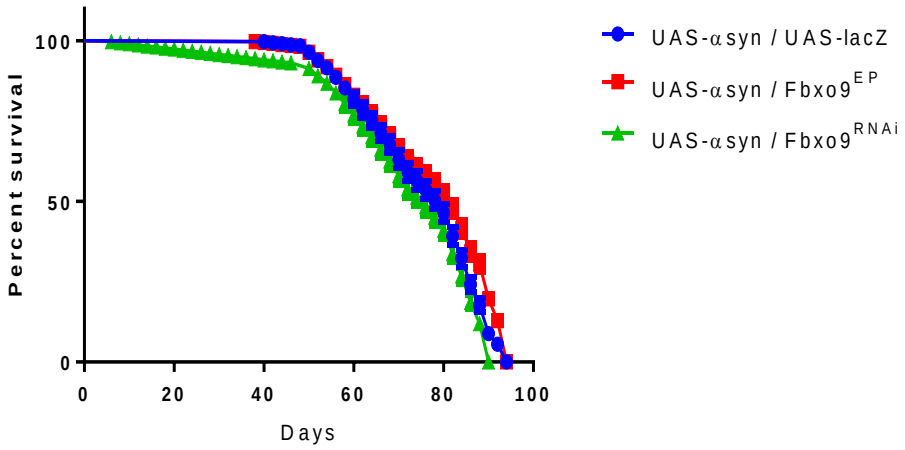


Figure 9: Effect of *α-synuclein* overexpression together with loss-of-function or overexpression of *Fbxo9* in dopaminergic neurons on lifespan in Drosophila. Loss-of-function of *Fbxo9* together with *α-synuclein* overexpression significantly decreases longevity while overexpression of both *Fbxo9* and *α-synuclein* significantly increases longevity. Ageing is shown as percent survival as a function of time. A *p*-value less than 0.01 was considered significant by the log-rank test. Error bars represent the standard error of the mean.

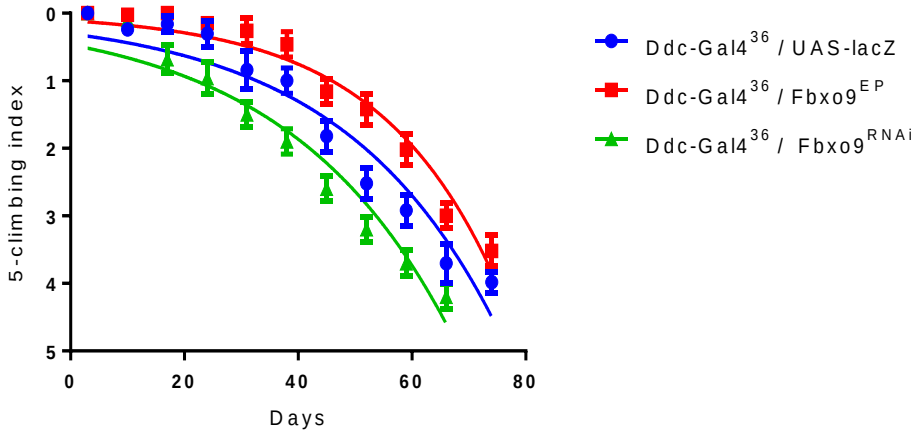
Climbing analysis

Effects of gain-of-function and loss-of-function of *Fbxo9*

The climbing analysis was carried out to investigate whether or not the overexpression and/or interference of expression of *Fbxo9* has any effect on mobility of Drosophila. The effects of gain-of-function and loss-of-function of *Fbxo9* on climbing ability were investigated using the, driver lines *Ddc-Gal4^{36}, Ddc-Gal4^{3D}*, *Ddc-Gal4^{3D}; Ddc-Gal4^{36}* and *TH-Gal4* in order to express the transgenes in the fly dopaminergic neurons. Loss-of-function of *Fbxo9* using *Ddc-Gal4^{36}, Ddc-Gal4^{3D}*, *Ddc-Gal4^{3D}; Ddc-Gal4^{36}* and *TH-Gal4* driver significantly decreased climbing ability compared to the *lacZ*-expressing control (Figure 10 A-D). The 95% confidence interval for *Ddc-Gal4^{36}/Fbxo9^{RNAi}* was 0.03111-0.03823, for *Ddc-Gal4^{3D}/ Fbxo9^{RNAi}* it was 0.02620-0.02977, for *Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ Fbxo9^{RNAi}* was 0.03127-0.04009, and for *TH-Gal4/ Fbxo9^{RNAi}* it was 0.02921-0.03464 and thus these climbing curves are significantly different from the control curves with 95% confidence intervals at 0.0324310-0.04035, 0.3102-0.03757, 0.04514-0.05483, and 0.03422-0.04296, respectively (Table 9). Overexpression of *Fbxo9* using *Ddc-Gal4^{36}*, *Ddc-Gal4^{3D}*, *Ddc-Gal4^{3D}; Ddc-Gal4^{36}* and *TH-Gal4* driver significantly increased climbing ability compared to the *lacZ*-expressing control (Figure 10 A-D).The 95% confidence interval for *Ddc-Gal4^{36}/Fbxo9 ^{EP}* was 0.0433210-0.05223, *Ddc-Gal4^{3D}/ Fbxo9 ^{EP}* was 0.03706-0.04485, *Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ Fbxo9^{EP}* was between 0.06194-0.07308, and *TH-Gal4/Fbxo9^{EP}* was 0.04498-0.05405 and thus these climbing curves are significantly different from the control curves with 95% confidence intervals at 0.0324310-0.04035, 0.3102-0.03757, 0.04514-0.05483, and 0.03422-0.04296, respectively. Loss-of-function of *Fbxo9* in fly dopaminergic neurons leads to a reduction in climbing ability, which is characteristic of PD-like phenotype, whereas overexpression of *Fbxo9* leads to an increase in climbing ability.

Table 9. Statistical analysis of climbing index curves for the directed overexpression and RNA interference of *Fbxo9* in dopaminergic neurons. The 95% confidence interval was compared to the *UAS-lacZ* control flies.

Genotype	Slope	Standard Error	95% Confidence Interval	R^2	Significant
Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ UAS-lacZ	0.04999	0.002415	0.04514-0.05483	0.9503	N/A
Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ Fbxo9^{EP}	0.06751	0.002777	0.06194-0.07308	0.9691	Yes
Ddc-Gal4^{3D}; Ddc-Gal4^{36}/ Fbxo9^{RNAi}	0.03568	0.002193	0.03127-0.04009	0.9032	Yes
Ddc-Gal4^{36}/ UAS-lacZ	0.03639	0.001974	0.0324310-0.04035	0.9238	N/A
Ddc-Gal4^{36} / Fbxo9^{EP}	0.04778	0.001771	0.0433210-0.05223	0.9509	Yes
Ddc-Gal4^{36} / Fbxo9^{RNAi}	0.03467	0.001771	0.03111-0.03823	0.9334	Yes
Ddc-Gal4^{3D}/ UAS-lacZ	0.03430	0.001632	0.3102-0.03757	0.9389	N/A
Ddc-Gal4^{3D}/ Fbxo9^{EP}	0.04096	0.001943	0.03706-0.04485	0.9448	Yes
Ddc-Gal4^{3D}/ Fbxo9^{RNAi}	0.02799	0.0008877	0.02620-0.02977	0.9673	Yes
TH-Gal4/ UAS-lacZ	0.03859	0.002174	0.03422-0.04296	0.9257	N/A
TH-Gal4/ Fbxo9^{EP}	0.04951	0.002255	0.04498-0.05405	0.9564	Yes
TH-Gal4/ Fbxo9^{RNAi}	0.03193	0.001344	0.02921-0.03464	0.9518	Yes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1
2
3
4
5
0
20
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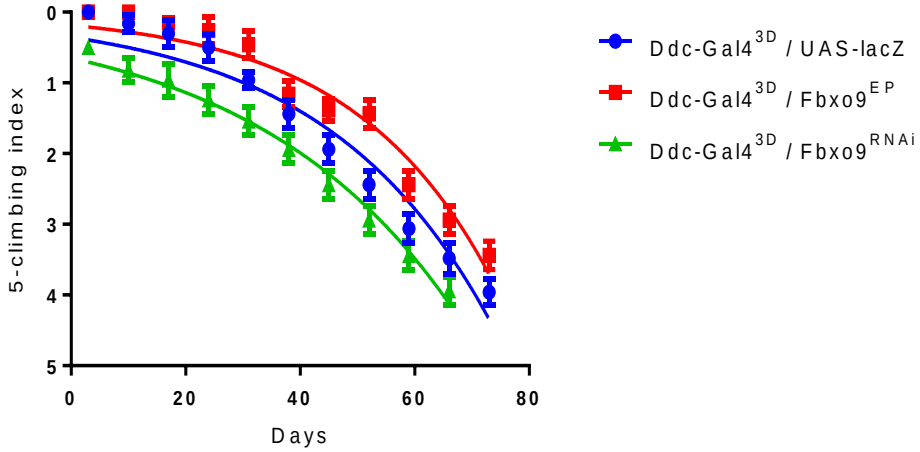
60
80
Days
Ddc-Gal4^36 / UAS-lacZ
Ddc-Gal4^36 / Fbxo9^EP
Ddc-Gal4^36 / Fbxo9^RNAi

A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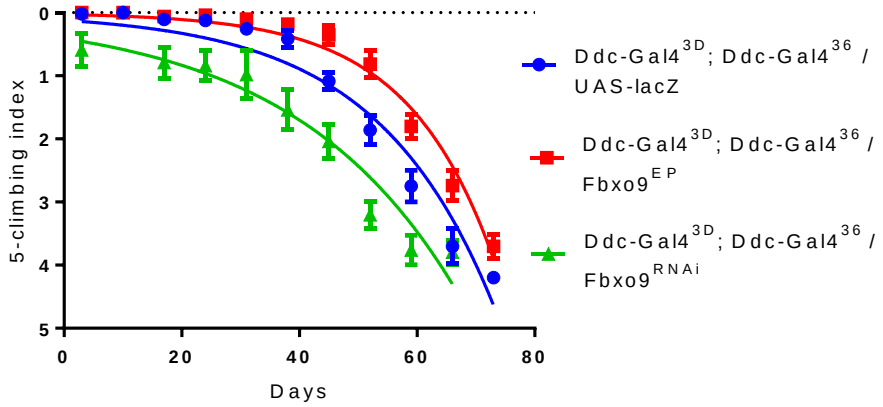
Ddc-Gal4^3D / UAS-lacZ
Ddc-Gal4^3D / Fbxo9^EP
Ddc-Gal4^3D / Fbxo9^RNAi

B



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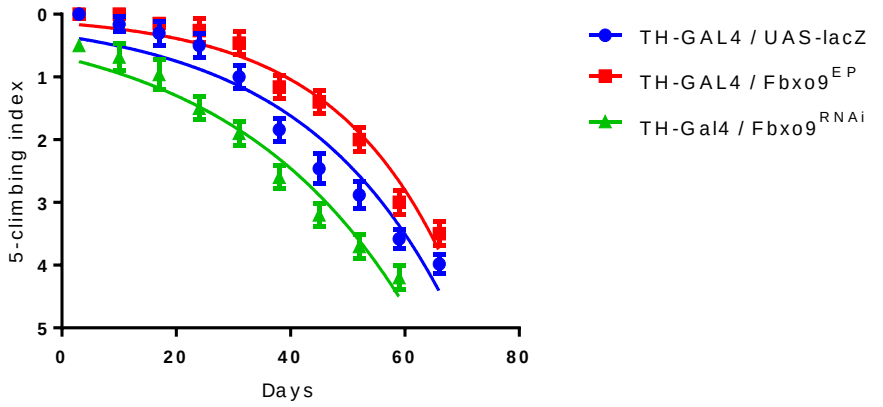

D

Figure 10: Effect of gain-of-function and loss-of-function of *Fbxo9* on climbing ability in Drosophila. Expression of *Fbxo9*^{RNAi} using *Ddc-Gal4*^{36} (A), *Ddc-Gal4*^{3D} (B), *Ddc-Gal4*^{3D}*; Ddc-Gal4*^{36} (C) and *TH-Gal4* (D) drivers significantly decreases climbing ability. Overexpression of *Fbxo9* using the same four drivers significantly increases climbing ability. The data were analyzed by non-linear fitting of the climbing curves with 95% confidence intervals to determine significance. Error bars represent the standard error of the mean.

Investigating the influence of altering *Fbxo9* expression upon the *α-synuclein* model

The most striking characteristic shown by the *α-synuclein* model of PD is the loss of climbing ability over time (Feany and Bender, 2000). This demonstrated that *D. melanogaster* could be used to model Parkinson disease.

The human *α-synuclein* gene was co-overexpressed with *Fbxo9 or* $Fbxo9^{RNAi}$ in the dopaminergic neurons to determine the effect of *Fbxo9* on climbing ability. The resulting climbing index curves for co-expression of *Fbxo9* and *α-synuclein* are shown in Figure 11 and shows that co-overexpression of $Fbxo9^{RNAi}$ with *α-synuclein* resulted in a significant reduction in climbing ability over time, with flies losing climbing ability earlier than control flies *UAS-αsynuclein/UAS-lacZ; Ddc-Gal4*). The 95% confidence interval for *UAS-α-syn/* $Fbxo9^{RNAi}$ was 0.02182-0.02547 compared to the *UAS-α-synuclein/UAS-lacZ* control, which was 0.03823-0.04503. Overexpression of $Fbxo9^{RNAi}$ with *α-synuclein* resulted in in a significant reduction in climbing ability compared to controls.

Figure 11 also shows that there was a significant increase in the climbing ability compared to controls when *Fbxo9* was overexpressed with *α-synuclein*. The $Fbxo9^{EP}$ flies climbed over an extended period of time compared to the control flies, which lost their climbing ability at an early-onset. The 95% confidence interval for *UAS- α-synuclein /* $Fbxo9^{EP}$ was 0.05472-0.06225 compared to the *UAS- synuclein/ UAS-lacZ* control which was 0.03823-0.04503. Statistical analysis of the climbing index curves is summarized in Table 10.

Table 10. Statistical analysis of climbing index curves for the co-overexpression of *α-synuclein* and *Fbxo9* or *Fbxo9^{RNAi}* in the dopaminergic neurons. The 95% confidence intervals were compared to the *UAS-lacZ* control flies.

Genotype	Slope	Standard Error	95% Confidence Intervals	R^2	Significant
UAS-asyn/ UAS-lacZ	0.04163	0.001690	0.03823-0.04503	0.9600	N/A
UAS-asyn/ Fbxo9^{EP}	0.05848	0.001872	0.05472-0.06225	0.9798	Yes
UAS-asyn/ Fbxo9^{RNAi}	0.02364	0.0009094	0.02182-0.02547	0.9504	Yes

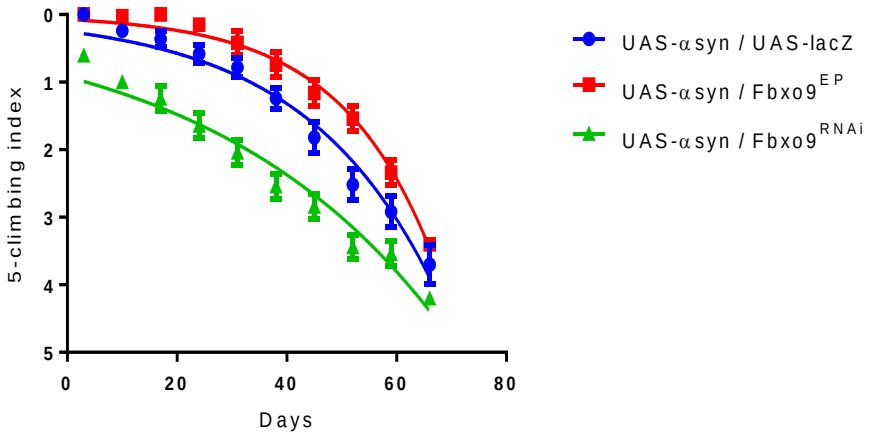

Figure 11: Analysis of climbing ability of Drosophila flies overexpressing *α-synuclein* together with *Fbxo9* or *Fbxo9^{RNAi}* in the dopaminergic neurons. RNAi dependent interference of *Fbxo9* expression together with *α-synuclein* overexpression significantly decreases climbing ability. Overexpression of both *Fbxo9* and *α-synuclein* significantly increases climbing ability. Data were analyzed by non-linear fitting of the climbing curves with 95% confidence intervals to determine significance. Error bars represent the standard error of the mean.

DISCUSSION

Drosophila *Fbxo9* is homologous to mammalian *Fbxo9*

Bioinformatics analysis was first performed on the *D. melanogaster* homologue in order to identify the similarity between *CG5961* and *Fbxo9* ,along with several other homologues from vertebrates and invertebrates. Furthermore, bioinformatics analysis was carried out to find out the relationship of *Fbxo9* with other FBXO proteins such as *Fbxo7* and *Fbxo32*. When a cladogram was created with these three FBXO proteins, *CG5961* clustered together with *Fbxo9*. This indicates that *CG5961* is most closely related to *Fbxo9*. As all of the genes from the different species clustered together, it may be concluded that Fbxo genes may exist in the common ancestor of the species. Alignment of human *Fbxo9* and Drosophila *CG5961* shows that the evidence of the conservation of the F-box proteins is 33% identical and 67% similar. The proteins are highly conserved in the F-box region that is essential for the SCF-ubiquitin ligase complexes. Alignment of human *Fbxo9* and Drosophila CG5961 with other vertebrate and invertebrate species further indicates the conservation of the F-box proteins being 26% identical and 27% similar and they further share the conserved domains such as TPR domain, MIT domain, F-box domain, SNHc and NLS. Due to the high level of conservation, it can be inferred that there are tight evolutionary constraints placed among the genes. Within the sub-group, it has been found that *Fbxo7* and *Fbxo9* are more similar than *Fbxo32*. Alignment of the Human *Fbxo9* and Drosophila *Fbxo7* showed that they are analogous, being 34% similar and 14% identical and they share structural similarities such as the PI31-binding region and the F-box region. Alignment of the human *Fbxo9* and Drosophila *Fbxo32* showed that they are analogous being 17% identical and 28 % similar and they additionally share structural similarities such as Foxo-binding region, amino terminus, LCD and PDZ domain.

In order to identify the similarity of *Fbxo9* with *Fbxo7* and *Fbxo32,* bioinformatics analysis was performed. Both the mammalian and fly form of *Fbxo7* bind PI31 to initiate successful proteasome-mediated degradation of a target substrate (Bader *et al*., 2010). FBXO7 was originally studied as a gene involved in caspase activation in sperm differentiation. However, it has been linked to early-onset parkinsonian-pyramidal syndrome. Since alignment of the Human *Fbxo9* and Drosophila *Fbxo7* showed that they are analogous being 14% identical and 34% similar and they share structural similarities such as the PI31-binding region and the F-box region, so it can be speculated that *Fbxo9* may also lead to PD-like symptom.

It has been found that when *Fbxo32,* also known as atrogin, is up-regulated in skeletal muscles, it results in muscle wasting. In mammals, atrogin targets proteins essential for muscle synthesis such as MyoD and eIF3-f (eukaryotic initiation factor 3 - subunit 5) and, consequently, it degrades them and induces muscle cell death (Tintignac *et al*., 2005 and Lagirand-Cantaloube *et al*., 2008). The *atrogin/ Fbxo32* is the target for *Foxo* and can trigger skeletal muscle atrophy when insulin or insulin-like growth factor-1 (IGF-1) is absent (Connors and Staveley, unpublished). Since alignment of the Human *Fbxo9* and Drosophila *Fbxo32* showed that they are analogous, being 17% identical and 28 % similar and they share structural similarities such as Foxo-binding region, amino terminus, LCD and PDZ domain, it can be speculated that expression of *Fbxo9* gene in various human conditions makes it a potential candidate for studies, in particular potential organismal modeling of human conditions.

Effects of loss-of-function of *Fbxo9* in the *Drosophila melanogaster* compound eye

Genetic expression studies using the Drosophila eye have been extensively used in the study of neurodegeneration because of the conservation of key signaling pathways

between humans and Drosophila and the ease of quantifying degeneration of photoreceptor neurons associated with each Drosophila ommatidium. In *D. melanogaster,* eye development is tightly controlled during the organization of the ommatidial array (Thomas and Wassarman, 1999). The eye also consists of specialized structures called sensory bristles that provide the opportunity for neurogenesis examination and for detection of even slight abnormalities (Baker, 2000). We expressed reduced levels of *Fbxo9* in the Drosophila eye using the transgenic driver GMR-Gal4. The characteristics can be studied through bioinformetric analysis to show the effects of altered gene expression, such as loss-of-function of *Fbxo9*.

In Drosophila, *Fbxo9* and *Fbxo7* are quite similar at the amino acid level. Therefore, *Fbxo9* was examined to investigate the possible redundancy in the function between *Fbxo9* and *Fbxo7*. Since *Fbxo7* is closely related to *Fbxo9*, so we hypothesized that suppressing of *Fbxo9* activity through RNA-interference would also lead to a PD-like phenotype in flies. We have shown that suppression of *Fbxo9* activity has a deleterious effect on the Drosophila eye morphology. As expected, loss-of-function of *Fbxo9* in the fly eyes through eye-specific expression of $Fbxo9^{RNAi}$ leads to a significant reduction in the number of ommatidia and, bristles as well as a reduction in ommatidia area, whereas overexpression of *Fbxo9* leads to a significant increase in the number of ommatidia and, bristles as well as a reduction in ommatidia area. No previous has been conducted on the effects of *Fbxo9* in Drosophila eyes. Therefore, the reason for this reduction in the number of ommatidia, bristle and ommatidia area is unclear. However, reduction in the number of ommatidia, bristle and ommatidia area suggests a reduction in cell number during eye development. Reduction of cell number can occur through either increased cell death, or decreased cell proliferation (Kramer *et al.,* 2003). This may be inferred that loss-of-function of *Fbxo9* causes inhibition of cell growth required for the normal eye

development.

Overexpression of *Foxo* under the control of the *GMR-Gal4* transgene results in a significant decrease in the number of ommatidia and bristles (Kramer *et al.,* 2003). Previous work in our lab showed that overexpression of the forkhead box, subgroup "O" (*Foxo*) transcription factors and F-box only protein 32/ *atrogin* decreases ommatidia number, bristle number and ommatidia area, that reflect cell number and cell size, which indicates its role in the control of body size through alterations in cell size and cell number. (Colleen Furlong [nee Connors], unpublished). The *atrogin/ Fbxo32* is the target for *Foxo* and can trigger skeletal muscle atrophy in the form of PD model when insulin or insulin-like growth factor-1 (IGF-1) is absent. Overexprssion of *Foxo* has been linked to neurotoxicity (Kanao *et al*., 2010). In our study, we have performed a bioinformatic analysis of *Fbxo9* and *atrogin/Fbxo32*. We have analyzed the speculated homologous relationship between these two F-box proteins to assess the possibility of using *Drosophila* as a disease model for PD. To determine the role of *Foxo* and *Fbxo9* in PD etiology, we have used the eye specific driver *GMR-Gal4* to drive the expression of these genes in the eyes. To determine the effect of loss-of-function of *Fbxo9*, we used RNA interference to decrease the expression of the genes to very low levels. In this study, we have found that the co-overexpression of $Fbxo9^{RNAi}$ and *Foxo* causes a reduction in the ommatidia number, bristle number and ommatidia area whereas the co-overexpression of *Fbxo9* and *Foxo* is causes an increase in the ommatidia number, bristle number and ommatidia area. Since no previous studies have been conducted in flies eyes associated with *Fbxo9* and *Foxo*, the reason for this reduction in the number of ommatidia, bristle and ommatidia area is unclear. However, reduction in the number of ommatidia, bristle and ommatidia area suggests a reduction in cell number during eye development. Reduction of cell number can occur through either increased cell death, or decreased of cell proliferation (Kramer *et al*., 2003).

This may be presumed that loss-of-function of *Fbxo9* produces inhibition of cell growth that is necessary for the normal eye development.

Effects of loss-of-function of *Fbxo9* on climbing ability and lifespan

We have shown that suppression of *Fbxo9* activity has a deleterious effect on Drosophila dopaminergic neurons. As loss-of-function mutations in human *Fbxo7* lead to parkinsonian-pyramidal syndrome, and *Fbxo7* is closely related to *Fbxo9*, we hypothesized that suppression of *Fbxo9* activity through RNA-interference would also lead to a PD-like phenotype in flies. A decrease in *nutcracker/Fbxo7* expression causes a decrease in lifespan (Merzetti and Staveley, unpublished). As expected, loss-of-function of *Fbxo9* in fly dopaminergic neurons leads to a reduction in climbing ability and lifespan, which is characteristic of PD-like phenotype, whereas overexpression of *Fbxo9* is favorable, compared to control flies. No previous studies have been conducted in climbing ability and lifespan associated with *Fbxo9,* therefore the reason for this reduction in lifespan and climbing ability is unclear. This may be due to an increase in apoptosis or a decrease in cell growth during development. This may be also due to selective apoptotic death of these DA neurons and decreased cellular protection and survival. Dopaminergic neurons may die as a result of apoptosis in PD (Lev *et al*., 2003). This process occurs may be due to the accumulation of endogenous toxic proteins or environmental toxins. Exploration of the role of cell survival signaling in the selective loss of dopaminergic neurons in Drosophila may provide further insight into the basis of PD. However, the *Fbxo7* gene has been found associated with the early-onset parkinsonian pyramidal syndrome and the distinguishing features of this disease include progressive degeneration of the pyramidal, and extrapyramidal regions in combination with the *substantia nigra pars compacta* in the brain (Davison, 1954; Di Fonzo *et al.,* 2009). Since *Fbxo9* and *Fbxo7* are closely related

and since *Fbxo7* leads to PD-like symptoms, it is not a surprise that *Fbxo9* also leads to a PD like phenotype.

The recapitulation of PD-like symptoms due to over-expression of a gene, especially the age-dependent loss of climbing ability in *D. melanogaster,* provides the potential model in the study of biological basis of the disease (Feany and Bender, 2000; Haywood and Staveley, 2004). Locomotion abnormality is one of the neurological characteristics of PD. In our study, loss-of-function of *Fbxo9* through RNA-interference, in the fly dopaminergic neurons, leads to a reduction in climbing ability compared to control flies (Figure 10). It is possible that this occurs due to inhibition of cell growth. Similar results have been reported before where the *α-synuclein* model resulted in an age-dependent loss of climbing ability where the mutant flies were unable to climb above the first section of the climbing apparatus during the last days of their lives (Feany and Bender, 2000). Additionally, it also has been reported that mutations in the TH-encoding gene *pale* cause an age dependent loss of climbing ability (Tempel *et al.*, 1984; Pendletont *et al.*, 2002). In another experiment it has been found that loss-of-function of *dLRRK* leads to a slight locomotor abnormality but has no effect on survival of DA neurons (Lee *et al.,* 2007; Tain *et al.,* 2009b). In one experiment it was found that when (*Pink1*) overexpression rescued the premature loss of climbing abilities induced by *α-synuclein* (Todd and Staveley, 2008). The similar result has been found in this experiment when *Fbxo9* is overexpressed within dopaminergic neurons, the flies climb longer and lose their climbing ability later than the control flies.

No previous ageing or mobility studies have been performed associated with the directed alteration of *Fbxo9* expression, so the reason for the observed results is not clear. This may be due to an increase in apoptosis or a decrease in cell growth during

development. This may be also due to selective apoptotic death of these DA neurons and decreased cellular protection and survival. It is possible that a reduction in *Fbxo9* expression increases the rate of apoptosis or inhibits cell growth. Similar results have been reported in a previous experiment where flies with loss-of-function of the *parkin* gene showed the characteristics of PD such as shortened lifespan, reduced climbing ability and degeneration of DA neurons (Greene *et al.*, 2003; Whitworth *et al.,* 2005). The mutant *parkin* flies were also found to have developmental delays and mobility dysfunction due to muscle degeneration. Additionally, the Drosophila *Pink1* fly model involving the gene shows the same characteristics such as reduced lifespan, developmental delay and DA neuron degeneration as the parkin mutant flies (Petit *et al.,* 2005; Clark *et al.*, 2006). It has been found that a *PINK1-RNAi* knockdown in flies resulted in loss of DA neurons even at 10 days (Wang *et al.,* 2006). In our study, when *Fbxo9* is overexpressed in the dopaminergic neurons, the flies live longer than the control flies, indicating that *Fbxo9* may play a protective role by decreasing the rate of apoptosis and thus possibly maintaining healthy neurons.

Effect of RNA-interference of *Fbxo9* in the *α-synuclein* model of PD

The *α-synuclein* model of PD in Drosophila did not result in any difference in lifespan between the control and wild type, A13T and A30P *α-synuclein* flies (Feany and Bender, 2000). Co-expression of $Fbxo7^{RNAi}$ with *α-synuclein* results in increased lifespan compared to control (Merzetti and Staveley, unpublished). In this study, overexpression of *Fbxo9* with *α-synuclein* also resulted in an increase in lifespan compared to controls. A similar result was found in an experiment where co-expression of *α-synuclein* and *parkin* slightly increased the lifespan of the flies (Haywood and Staveley, 2006). In our study, co-overexpression of *α-synuclein* and $Fbxo9^{RNAi}$ resulted in a decreased lifespan compared to

the control flies, and this may be due to inhibition of cell survival in the dopaminergic neurons.

Flies that express A30P, A53T and wild type versions of *α-synuclein* experience loss of DA neurons and age dependent loss of locomotor ability when *GAL4* drivers, including *Ddc-Gal4,* are used (Feany and Bender, 2000). In our study, co-overexpression of *α-synuclein* with $Fbxo9^{RNAi}$ under the control of the *Ddc-Gal4* driver, resulted in a significant difference in climbing ability compared to the control flies. $Fbxo9^{RNAi}$*/ UAS-α-syn* expressing flies lost their climbing ability earlier than the control flies (Figure 15). This may be because loss-of-function of *Fbxo9* causes inhibition of the survival of the dopaminergic neurons. Additionally in another experiment, it has been reported that *α-synuclein* flies show a less pronounced DA neuron degeneration as only a partial (50%) decrease was observed (Auluck *et al.,* 2002; Bayersdorfer *et al.*, 2010). In our study, an opposite effect was found when *α-synuclein* was co-overexpressed with *Fbxo9*. Overexpression of *Fbxo9* with *α-synuclein* resulted in an increase in climbing ability compared to controls. A similar result was found when *α-synuclein* and *parkin* were co-overexpressed in Drosophila DA neurons, and this resulted in a slight increase in climbing ability (Haywood and Staveley, 2006). So it can be concluded that when *α-synuclein* and *parkin* are co-overexpressed, they play a vital role in maintaining healthy dopaminergic neurons.

CONCLUSION

In this study, we have confirmed that gene *CG5961* of *Drosophila melanogaster* is the homologue of human *Fbxo9*. We have shown that *Fbxo9, Fbxo7* and *Fbxo32* are closely related but *Fbxo9* and *Fbxo7* are found more similar than *Fbxo9* and *Fbxo32*. We have shown suppression of *Fbxo9* through RNA interference in the dopaminergic neurons of Drosophila leading to parkinsonian-pyramidal syndrome. In this study, we have found that loss-of-function of *Fbxo9* through the eye specific driver *GMR-Gal4* leads to a significant reduction in the number of ommatidia, bristles as well as in ommatidia area, whereas overexpression of *Fbxo9* results in a significant increase in the number of ommatidia, bristles as well as in ommatidia area. We also have found that RNAi-dependent reduced expression of *Fbxo9* together with *Foxo* overexpression significantly decreased the ommatidia number, bristle number and ommatidium area compared to the *Foxo* overexpressing control fly *GMR-Gal4; UAS-Foxo/UAS-lacZ* whereas co-overexpression of *Fbxo9* and *Foxo* significantly increases the ommatidia number, bristle number and ommatidium area. We also found that, expression of $Fbxo9^{RNAi}$ using the $Ddc\text{-}Gal4^{3D}$ *(III)*, $Ddc\text{-}Gal4^{36}$ *(II)*, $Ddc\text{-}Gal4^{3D}$*;* $Ddc\text{-}Gal4^{36}$, and *TH-Gal4* drivers significantly decreases longevity and climbing ability whereas overexpression of *Fbxo9* using the same four drivers remarkably increases longevity and climbing ability.

In addition, co-overexpression of *α-synuclein* and $Fbxo9^{RNAi}$ results in a decreased longevity and climbing ability compared to control whereas overexpression of both Fbxo9 and *α-synuclein* is favorable compared to control. This demonstrates that the directed inhibition of expression of *Fbxo9* lead to the enhancement of PD-like symptoms in the *α-synuclein*-induced Drosophila model of PD. Further studies looking at the effect of *Fbxo9* on *Foxo* activity and *α-synuclein* activity may uncover underlying mechanisms that mediate a shift towards apoptosis. We have developed a new model of human Parkinson

disease that will provide further information in the disease etiology. We expect that the knowledge obtained by determining the pathways involved in Parkinson disease in Drosophila will help uncover potential new therapeutic approaches for human subjects.

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