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Inhibition of *Atg6* and *Pi3K59F* autophagy genes in neurons decreases lifespan and locomotor ability in *Drosophila melanogaster*

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ABSTRACT. Autophagy is a cellular mechanism implicated in the pathology of Parkinson's disease. The proteins Atg6 (Beclin 1) and Pi3K59F are involved in autophagosome formation, a key step in the initiation of autophagy. We first used the *GMR-Gal4* driver to determine the effect of reducing the expression of the genes encoding these proteins on the developing *Drosophila melanogaster* eye. Subsequently, we inhibited their expression in *D. melanogaster* neurons under the direction of a Dopa decarboxylase (*Ddc*) transgene, and examined the effects on longevity and motor function. Decreased longevity coupled with an age-dependent loss of climbing ability was observed. In addition, we investigated the roles of these genes in the well-studied *α-synuclein*-induced *Drosophila* model of Parkinson's disease. In this context, lowered expression of *Atg6* or *Pi3K59F* in *Ddc-Gal4*-expressing neurons results in decreased longevity and associated age-dependent loss of locomotor ability. Inhibition of *Atg6*

or *Pi3K59F* together with overexpression of the sole pro-survival *Bcl-2 Drosophila* homolog *Buffy* in *Ddc-Gal4*-expressing neurons resulted in further decrease in the survival and climbing ability of *Atg6-RNAi* flies, whereas these measures were ameliorated in *Pi3K59F-RNAi* flies.

Key words: Atg6; Beclin 1; Pi3K59F; Buffy; α-synuclein; Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is the second most common human neurodegenerative condition, and is characterized by progressive degeneration and loss of dopamine-producing neurons in the centrally located substantia nigra pars compacta region of the brain. PD is marked by severe locomotor dysfunction, as well as non-motor symptoms such as autonomic, cognitive, and psychiatric deficiencies (Forno, 1996). Although the majority of PD cases are sporadic, familial forms with a genetic component have been identified and studied extensively in model organisms (Staveley, 2014). The first gene to be associated with familial PD encodes α-synuclein, a small soluble protein predominantly found in neural tissues (Polymeropoulos et al., 1997; Dehay et al., 2016). The association of α-synuclein with mitochondrial components is thought to lead to oxidative stress, apoptosis, autophagy, and ultimately, neurodegeneration (Chinta et al., 2010; Esteves et al., 2011). A link between autophagic dysfunction and aberrant α-synuclein has been established in the pathogenesis of both sporadic and familial forms of PD (Cuervo et al., 2004). PD pathogenesis must therefore involve the failure of several cellular mechanisms, with the disruption of autophagic homeostasis contributing to the development of this neurodegenerative disorder.

Autophagy is a tightly regulated catabolic mechanism in eukaryotic cells that degrades long-lived proteins and organelles. It comprises a multi-step pathway involving key regulatory factors such as TOR kinase, AMP-activated protein kinase, Bcl-2/Bcl-X_L inhibition of the Beclin 1/class III PI3K complex, and p53 tumor suppressor protein, among others, that respond to energy levels and the absence or presence of nutrients and growth factors (Levine and Kroemer, 2008; Xilouri and Stefanis, 2011). The initiation of autophagy is marked by phagophore formation, in which the ULK1-Atg13-FIP200 complex is pivotal. The nucleation phase of early phagophore formation requires interaction between the ULK1 complex and the Atg6-interacting complex, composed of Atg6, class III PI3K, Vps15, and Atg14L. Stimulation of this complex generates phosphatidylinositol-3-phosphate, which promotes autophagosomal membrane nucleation (Levine and Kroemer, 2008). This is followed by elongation and maturation of the autophagosome, before it finally fuses with an acidic lysosome.

The Atg6-interacting complex acts in a regulatory step, with autophagy being promoted by Atg6-binding proteins such as AMBRA1, UVRAG, and Bif-1, and inhibited by Rubicon and Bcl-2/Bcl-X_L. Inhibition of Atg6-dependent autophagy by anti-apoptotic Bcl-2 proteins implies a regulatory role for this important protein family (Pattingre et al., 2005). One mechanism thought to be responsible for such inhibition involves the binding of anti-apoptotic Bcl-2 proteins to the Bcl-2 homology 3 (BH3) domain of Atg6, thus interfering with the initiation of autophagy. One group has suggested that this protein family affects autophagy only indirectly, through inhibition of the pro-apoptotic Bcl-2 proteins Bax and Bak (Lindqvist et al., 2014). The sequestration of Atg6 by Bcl-2 proteins can be reversed by phosphorylation

of the former's BH3 domain by death-associated protein kinase (Zalckvar et al., 2009). Atg6 contains the APG6 domain that binds class III PI3K to affect its role in vacuolar protein sorting and autophagy (Furuya et al., 2005). Autophagy is highly conserved, with single orthologs of many of the components involved in this mechanism present in *Drosophila melanogaster* (McPhee and Baehrecke, 2009; Zirin and Perrimon, 2010). For example, the Atg6-interaction complex seems to be well conserved and is central to autophagy (Juhász et al., 2008; Chang and Neufeld, 2010). Orthologs of Atg14L, UVRAG, Rubicon, and Bcl-2 are present in the fly and may play the same roles as their mammalian counterparts by forming various autophagy regulatory complexes.

PD counts among those diseases in which the dysregulation of autophagy has been implicated. Their vesicular nature and the dominant pathological features of surviving neurons in patients with PD has led to the suggestion that the autophagy-lysosome pathway contributes to the formation or dissolution of Lewy bodies (LBs) (Perrett et al., 2015; Xilouri and Stefanis, 2015). The implication of autophagy in human disease, especially in neurodegenerative disorders, makes *Drosophila* an attractive model to study the consequences of altering genes involved in this process, such as *Atg6* and the class III PI3K *Pi3K59F*. The first fly model of PD utilized a human *α-synuclein* transgene to induce PD-like symptoms (Feany and Bender, 2000). This model has become one of the most successful systems for the study of this disease, owing to accurate replication of the degeneration and loss of DA neurons, age-dependent loss of locomotor ability, presence of LB-like inclusions, and compromised survival (Staveley, 2014). We determined the effect of *Atg6* or *Pi3K59F* inhibition via directed expression of stable RNA interference (RNAi) transgenes in *Dopa decarboxylase* (*Ddc*)-*Gal4*-expressing *Drosophila* neurons. Furthermore, these transgenes were co-expressed with *α-synuclein* to investigate the role of *Atg6* and *Pi3K59F* in the original *Drosophila* model of PD. In addition, loss of function of these autophagy genes was evaluated in the context of overexpression of *Buffy*, the only pro-survival *Bcl-2* homolog in *Drosophila*.

MATERIAL AND METHODS

Bioinformatic analysis

Protein sequences were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein), and domains were identified using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd; Marchler-Bauer et al., 2015) and the Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org; Dinkel et al., 2016), which focuses on annotation and detection of ELMs, also known as short-linear motifs. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo; Goujon et al., 2010; Sievers et al., 2011) was used for multiple-sequence alignment and to identify the conservation of domains across selected organisms. The presence of nuclear export signals (NESs) was predicted using the NetNES 1.1 server (http://www.cbs.dtu.dk/services/NetNES; la Cour et al., 2004).

Drosophila stocks and media

The *UAS-Atg6-RNAi* (w^{1118}; *P{GD11647}v22122*) and *UAS-Pi3K59F-RNAi* (*P{KK107602}VIE-260B*) lines were obtained from the Vienna Drosophila Resource Center (Vienna, Austria). *UAS-α-synuclein* flies (Feany and Bender, 2000) were generously provided

by Dr. M. Feany of the Harvard Medical School, *UAS-Buffy* flies (Quinn et al., 2003) by Dr. L. Quinn of the University of Melbourne, and *Ddc-Gal4* flies (Li et al., 2000) by Dr. J. Hirsch of the University of Virginia. *GMR-Gal4* (Freeman, 1996) and *UAS-lacZ* flies were obtained from the Bloomington Drosophila Stock Center at Indiana University, IN, USA. Stocks and crosses were maintained on standard cornmeal/molasses/yeast/agar medium treated with propionic acid and methylparaben to inhibit fungal growth. Stocks were maintained at room temperature (22° ± 3°C), whereas crosses and experiments were carried out at 25° and 29°C.

Derivative *Drosophila* lines

UAS-α-synuclein/CyO; *Ddc-Gal4/TM3* and *UAS-α-synuclein/CyO*; *GMR-Gal4* flies, in which *α-synuclein* was overexpressed in neurons under the direction of *Ddc-Gal4* and the developing eye under the control of *GMR-Gal4*, respectively, were generated according to standard homologous recombination methods. The *UAS-Buffy/CyO*; *Ddc-Gal4* and *UAS-Buffy/CyO*; *GMR-Gal4* lines were used to overexpress *Buffy* in neurons and the developing eye, respectively. Polymerase chain reaction (PCR) was employed to detect the presence of the *α-synuclein* construct using primers designed with the NCBI primer design tool according to the *Homo sapiens* synuclein, alpha (non-A4 component of amyloid precursor), transcript variant 1 mRNA sequence (NCBI reference sequence NM_000345.3). The 5'- to 3'-sequence of the forward primer was GTGCCCAGTCATGACATTT, and that of the reverse primer was CCACAAAATCCACAGCACAC; both primers were synthesized by Invitrogen (Carlsbad, CA, USA). The *D. melanogaster Buffy* mRNA sequence (NM_078978.2) was used to design a set of primers targeting the endogenous gene and transgenic construct. The 5'- to 3'-sequences of the forward primers were CACAGCGTTTATCCTGCTGA and CGGGTGGTGAGTTCCATACT, while those of the reverse primers were TCGCAGTGTGAAGATTCAGG and TTAATCCACGG AACCAGCTC; these primers were ordered from Eurofins MWG Operon (Louisville, KY, USA). Gel electrophoresis was performed for confirmation of recombination events based on the presence of the corresponding PCR product.

Survival assay

Fly survival was assessed following a standard protocol, as previously described (Todd and Staveley, 2012; M'Angale and Staveley, 2016). Briefly, over 200 flies were monitored per genotype and scored every 2 days for the presence of deceased adults (Staveley et al., 1990). Longevity data were analyzed using GraphPad Prism version 5.04 (GraphPad Software, Inc., La Jolla, CA, USA), and survival curves were compared by the log-rank (Mantel-Cox) test. Significance was determined at a 95% confidence level ($P \leq 0.05$) with Bonferroni correction.

Climbing assay

The climbing assay was performed as previously described, and repeated three times (Todd and Staveley, 2004). Climbing indices were computed and subsequently analyzed using GraphPad Prism version 5.04. The 5-climbing index is a model employed for graded climbing analysis using non-linear regression, "5" being the highest level that flies can climb. Data were compared using 95% confidence intervals (CI) and a P value threshold of 0.05.

Scanning electron microscopy of *Drosophila* eyes

Drosophila eyes were prepared for scanning electron microscopy following a standard protocol, as previously described (M'Angale and Staveley, 2016). For each cross, at least 10 different images of eyes from adult flies 5 days post-eclosion per genotype were analyzed using the ImageJ software (National Institutes of Health; Schneider et al., 2012), and biometric analysis was performed using GraphPad Prism version 5.04. The relative size of the disrupted eye area was calculated as detailed in a previous publication (M'Angale and Staveley, 2012). Statistical comparisons comprised one-way analyses of variance and Dunnett multiple comparison tests. P values less than 0.05 were considered significant.

RESULTS

Atg6 and Pi3K59F are evolutionarily conserved

Bioinformatic analysis of Atg6 (also known as Beclin 1) and Pi3K59F protein sequences revealed remarkable conservation of domains and highlights the importance of these two autophagy proteins (Figure 1). The *Drosophila* Atg6 protein consists of 422 amino acids, and shares 50% identity and 67% similarity with human Beclin 1, comprising 450 amino acids, alignment with which revealed conserved APG6/Vps30 and NES domains (Figure 1A). A putative non-canonical BH3 domain appears to be present in the *Drosophila* sequence that may function in Atg6 inhibition through sequestration by the pro-survival Bcl-2 proteins, as occurs in mammals (Figure 1B). Alignment of the *Drosophila* Pi3K59F sequence with its human homolog showed evolutionary conservation of these class III PI3K proteins, which demonstrated 64% identity and 79% similarity (Figure 1C). Moreover, both sequences contain C2, PIK, and kinase catalytic domains. ELM prediction identified an NES, a nuclear localization signal, several Atg8-binding motifs, and a di-arginine endoplasmic reticulum retention motif in the *Drosophila* sequence (Dinkel et al., 2016).

Inhibition of *Atg6* or *Pi3K59F* in the developing *Drosophila* eye results in different eye phenotypes

The directed inhibition of *Atg6* or *Pi3K59F* in the developing eye caused significant disruption of the ommatidial array, and whereas inhibition of *Atg6* resulted in a higher mean number of ommatidia, that of *Pi3K59F* had the opposite effect (Figure 2A, I-III). The suppression of these two autophagy genes in the developing eye greatly disrupted the ommatidia (Figure 2B), implying a role for these proteins in normal eye development.

Co-expression of *Atg6-RNAi* or *Pi3K59F-RNAi* and *α-synuclein* in the eye intensified developmental eye defects (Figure 2A, IV-VI). Disruption of the ommatidial array was pronounced, and the mean number of ommatidia was reduced (Figure 2C). These results indicate that inhibition of *Atg6* or *Pi3K59F* in the eye, coupled with the toxic effects of α-synuclein, enhances developmental abnormalities in this organ.

In contrast, co-expression of *Atg6-RNAi* or *Pi3K59F-RNAi* and *Buffy* in developing eye resulted in a slight increase in the number of ommatidia and a marked decrease in disruption of the ommatidial array (Figure 2A and D, VII-IX).

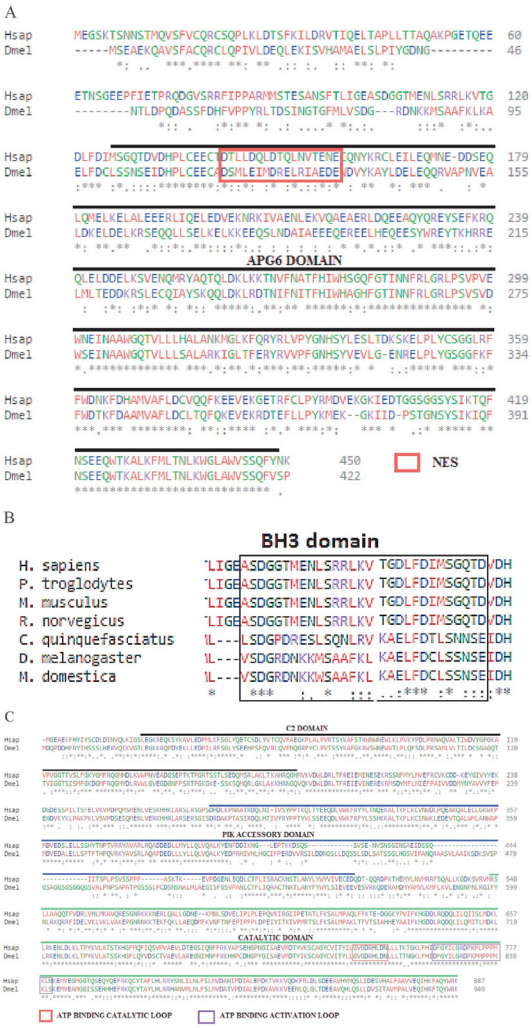

Figure 1. Autophagy proteins Atg6 and Pi3K59F are evolutionarily conserved. **A.** Multiple-sequence alignment of human Beclin 1 and its *Drosophila melanogaster* homologue Atg6 reveals conservation of the APG6/Vps30 domain important for initiation of autophagy, and presence of a nuclear export signal (NES). Hsap = *Homo sapiens* NP_003757.1, Dmel = *D. melanogaster* NP_651209.1. **B.** *Drosophila* sequence contains a putative BH3 domain, which is conserved with the exception of a small number of amino acid substitutions, as demonstrated by Clustal Omega (Goujon et al., 2010; Sievers et al., 2011) multiple-sequence alignment of the *Drosophila* Atg6 protein sequence with that of mammalian and insect homologues. H. sapiens = *H. sapiens* NP_003757.1, P. troglodytes = *Pan troglodytes* XP_511522.2, M. musculus = *Mus musculus* NP_062530.2, R. norvegicus = *Rattus norvegicus* NP_001029289.1, C. quinquefasciatus = *Culex quinquefasciatus* XP_001861442.1, D. melanogaster = *D. melanogaster* NP_651209.1, M. domestica = *Musca domestica* XP_005189450.1. **C.** Pi3K59F is highly conserved, with sequences from both organisms containing C2, PIK accessory, and kinase catalytic domains. Hsap = *H. sapiens* NP_002638.2, Dmel = *D. melanogaster* NP_477133.1. Domains were identified using the National Center for Biotechnology Information Conserved Domain Database (Marchler-Bauer et al., 2015). Asterisks indicate identical residues, colons indicate conserved substitutions, and periods indicate semi-conserved substitutions. Colors represent the chemical properties of each amino acid, as follows: red = small hydrophobic (including aromatic), blue = acidic, magenta = basic, green = basic with hydroxyl or amine groups.

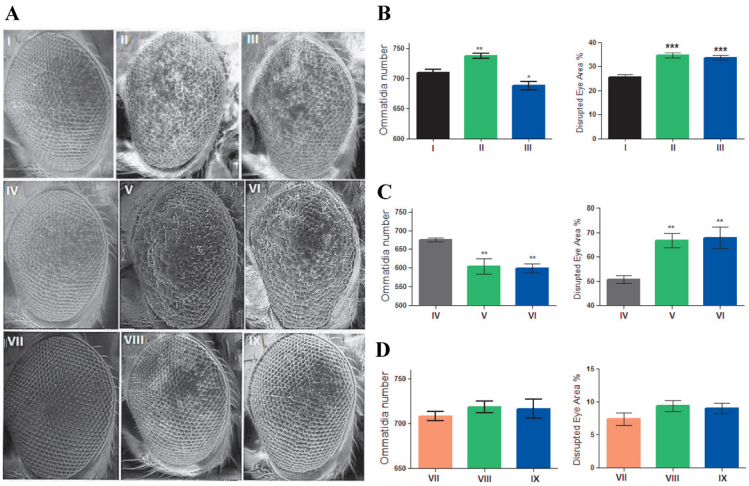

Figure 2. Conditional expression of *Atg6-RNAi* or *Pi3K59F-RNAi* in the developing *Drosophila* eye results in different phenotypes. **A.** Scanning electron micrographs of developing eyes in which *Atg6* or *Pi3K59F* expression has been suppressed in isolation, or in the context of *α-synuclein* or *Buffy* overexpression; (I) *GMR-Gal4/UAS-lacZ*, (II) *GMR-Gal4/UAS-Atg6-RNAi*, (III) *GMR-Gal4/UAS-Pi3K59F-RNAi*, (IV) *UAS-α-synuclein*; *GMR-Gal4/UAS-lacZ*, (V) *UAS-α-synuclein*; *GMR-Gal4/UAS-Atg6-RNAi*, (VI) *UAS-α-synuclein*; *GMR-Gal4/UAS-Pi3K59F-RNAi*, (VII) *UAS-Buffy*; *GMR-Gal4/UAS-lacZ*, (VIII) *UAS-Buffy*; *GMR-Gal4/UAS-Atg6-RNAi*, (IX) *UAS-Buffy*; *GMR-Gal4/UAS-Pi3K59F-RNAi*. **B.** Biometric analysis shows a significant difference in the number of ommatidia and disrupted area of the ommatidial array, with *Atg6-RNAi* and *Pi3K59F-RNAi* flies demonstrating higher and lower mean numbers of ommatidia, respectively, compared to control flies. **C.** Inhibition of *Atg6* or *Pi3K59F* combined with *α-synuclein* overexpression results in lower ommatidial count and larger disrupted eye areas than those observed in the control group. **D.** Co-expression of *Buffy* and *Atg6-RNAi* or *Pi3K59F-RNAi* in the eye did not significantly increase the mean number of ommatidia and the disrupted eye area compared to the control group that expressed both *Buffy* and the benign *lacZ* transgene. Comparisons were performed by one-way analysis of variance and the Dunnett multiple-comparison test ($P < 0.05$; $N = 10$). Error bars show standard error of the mean. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Inhibition of *Atg6* or *Pi3K59F* in neurons replicates PD phenotypes

Inhibition of *Atg6* or *Pi3K59F* in *Ddc-Gal4*-expressing neurons by RNAi under control of the *Ddc-Gal4* transgene decreased fly survival (Figure 3A). The median lifespans of *Atg6-RNAi* and *Pi3K59F-RNAi* flies were 58 and 64 days, compared to 73 days for the control group. The directed inhibition of *Atg6* or *Pi3K59F* in these neurons impaired climbing ability, as indicated by comparison of the climbing curves and associated 95%CIs (Figure 3B). Thus, neuronal inhibition of *Atg6* or *Pi3K59F* appears to decrease *Drosophila* longevity and climbing ability; normal levels of the corresponding proteins are therefore required for the correct functioning of neurons.

Inhibition of *Atg6* or *Pi3K59F* in the *α-synuclein*-induced PD model alters survival but not climbing ability

Expression of *α-synuclein* in DA neurons impaired the climbing ability of flies and shortened their lifespan. In comparison, inhibition of *Atg6* or *Pi3K59F* in the neurons of flies expressing *α-synuclein* lowered lifespan but did not affect locomotor ability (Figure 4A and

B). *Atg6-RNAi* flies had a median lifespan of 56 days, as did *Pi3K59F-RNAi* flies, compared to the control group that had a median of 62 days. Non-linear fitting of curves with 95%CIs revealed that climbing indices did not significantly differ between the *Atg6-RNAi*, *Pi3K59F-RNAi*, and control groups, although slightly lower values were observed in the former two than in the latter. This indicates that inhibition of these autophagy genes somewhat modifies the phenotype induced by expression of *α-synuclein* in *Ddc-Gal4*-expressing *Drosophila* neurons.

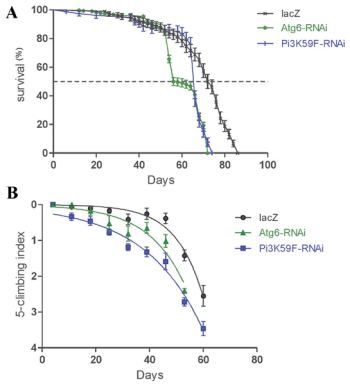

Figure 3. Inhibition of *Atg6* or *Pi3K59F* in neurons reproduces Parkinson's disease-like phenotypes. **A.** Directed inhibition of *Atg6* or *Pi3K59F* in *Ddc-Gal4*-expressing neurons decreases survival compared to control flies overexpressing the benign *UAS-lacZ* transgene. Fly genotypes were *Ddc-Gal4/UAS-lacZ*, *Ddc-Gal4/UAS-Atg6-RNAi*, and *Ddc-Gal4/UAS-Pi3K59F-RNAi.* Longevity is reported as the percentage of surviving flies [$P < 0.05$, determined by the log-rank (Mantel-Cox) test; $N \geq 200$]. **B.** Inhibition of *Atg6* or *Pi3K59F* in these neurons significantly decreases climbing ability, as determined by non-linear fitting of the climbing curves and comparisons of 95% confidence intervals. Fly genotypes were *Ddc-Gal4/UAS-lacZ*, *Ddc-Gal4/UAS-Atg6-RNAi*, and *Ddc-Gal4/UAS-Pi3K59F-RNAi*. Error bars indicate standard error of the mean (N = 50).

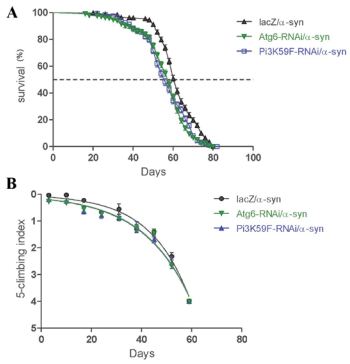

Figure 4. Inhibition of *Atg6* or *Pi3K59F* in an *α-synuclein*-induced model of Parkinson's disease (PD) slightly alters survival, but not climbing ability. **A.** Inhibition of *Atg6* or *Pi3K59F* together with *α-synuclein* (α-syn) overexpression in *Ddc-Gal4*-expressing neurons slightly decreases median lifespan compared to control flies. Fly genotypes were *UAS-α-synuclein*; *Ddc-Gal4/UAS-lacZ*, *UAS-α-synuclein*; *Ddc-Gal4/UAS-Atg6-RNAi*, and *UAS-α-synuclein*; *Ddc-Gal4/UAS-Pi3K59F-RNAi*. Longevity is reported as the percentage of surviving flies [$P < 0.05$, determined by log-rank (Mantel-Cox) test; $N \leq 200$]. **B.** Expression of *Atg6-RNAi* or *Pi3K59F-RNAi* in the *α-synuclein*-induced model of PD did not significantly affect age-dependent loss of climbing ability compared to the control group. Fly genotypes were *UAS-α-synuclein*; *Ddc-Gal4/UAS-lacZ*, *UAS-α-synuclein*; *Ddc-Gal4/UAS-Atg6-RNAi*, and *UAS-α-synuclein*; *Ddc-Gal4/UAS-Pi3K59F-RNAi*. Error bars indicate standard error of the mean (N = 50).

Buffy overexpression enhances *Atg6-RNAi*- and suppresses *Pi3K59F-RNAi*-induced phenotypes

Co-expression of *Atg6-RNAi* or *Pi3K59F-RNAi* and the pro-survival *Bcl-2 Drosophila* homolog *Buffy* enhanced the phenotype caused by reduction of *Atg6* expression, and rescued loss of *Pi3K59F* expression (Figure 5). *Buffy* overexpression combined with RNAi inhibition of *Atg6* reduced survival to a median lifespan of 46 days, compared to 66 days for control flies (Figure 5A). The impaired climbing ability exhibited by the *Atg6-RNAi* flies was amplified as determined by non-linear fitting of climbing curves and 95%CIs (Figure 5B). This may be due to interaction between Buffy and the BH3 domain of Atg6, restricting the latter's ability to initiate autophagy. Interestingly, overexpression of *Buffy* in *Pi3K59F-RNAi* flies suppressed the reduced survival and climbing ability resulting from *Pi3K59F* inhibition. The median lifespan of these flies (66 days) was the same as that of the control flies (Figure 5A). In addition, their climbing ability improved to normal levels (Figure 5B).

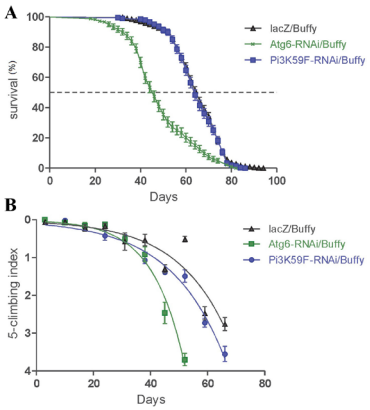

Figure 5. *Buffy* overexpression enhances and suppresses phenotypes induced by *Atg6-RNAi* and *Pi3K59F-RNAi*, respectively. **A.** Co-expression of *Buffy* and *Atg6-RNAi* exacerbates the decrease in survival caused by suppression of *Atg6*, whereas co-expression of *Buffy* and *Pi3K59F-RNAi* negates the lowered median survival resulting from reduced *Pi3K59F* expression. Fly genotypes were *UAS-Buffy*; *Ddc-Gal4/UAS-lacZ*, *UAS-Buffy*; *Ddc-Gal4/UAS-Atg6-RNAi*, and *UAS-Buffy*; *Ddc-Gal4/UAS-Pi3K59F-RNAi*. **B.** Co-expression of *Atg6-RNAi* and *Buffy* in *Ddc-Gal4*-expressing neurons worsens age-dependent loss of climbing ability. In contrast, co-expression of *Pi3K59F-RNAi* and *Buffy* suppresses age-dependent loss of climbing ability compared to flies expressing *Pi3K59F-RNAi* alone. Fly genotypes were *UAS-Buffy*; *Ddc-Gal4/UAS-lacZ*, *UAS-Buffy*; *Ddc-Gal4/UAS-Atg6-RNAi*, and *UAS-Buffy*; *Ddc-Gal4/UAS-Pi3K59F-RNAi*. Error bars indicate standard error of the mean (N = 50).

DISCUSSION

Biometric analysis following inhibition of *Atg6* or *Pi3K59F* showed significant differences in *Drosophila* eye phenotypes. Reduced *Atg6* expression significantly increased the number of ommatidia and the size of the disrupted eye area. However, when *Pi3K59F* was inhibited, fewer ommatidia were observed, along with a high degree of disruption to the ommatidial array. Although these two autophagy proteins are known to be members of the same complex, it is possible that when each is conditionally inhibited, they exert different effects on the developing eye. Altered *Atg6* or *Pi3K59F* expression in the *α-synuclein-*

expressing developmental eye defect model resulted in an enhanced phenotype. Both RNAi transgenes caused reduced numbers of ommatidia and ommatidial disarray. It is interesting that inhibition of *Atg6* or *Pi3K59F* in an α-synuclein-overexpression background enhanced the eye development phenotype, a trend that we did not observe with *Ddc-Gal4*-expressing neurons. The toxic effects of α-synuclein in the developing eye have been well documented (Feany and Bender, 2000), and demonstrate an additive effect with eye phenotype worsening under additional assaults (M'Angale and Staveley, 2012). It seems therefore that inhibition of the autophagy genes *Atg6* or *Pi3K59F* in tandem with *α-synuclein* overexpression produces severe eye disruption. Overexpression of *Buffy* in *Drosophila* is known to suppress stress phenotypes and improve the "healthspan" of flies (Quinn et al., 2003; M'Angale and Staveley, 2016). Notably, inhibition of *Pi3K59F* in the developing eye resulted in fewer ommatidia, whereas co-expression of *Pi3K59F-RNAi* and *Buffy* raised the number of ommatidia and improved the degeneration of the eye tremendously. Expression of *Atg6-RNAi* in the developing eye caused higher numbers of ommatidia coupled by high levels of ommatidial degeneration, and its co-expression with *Buffy* resulted in reduced ommatidial counts and lower disrupted eye areas.

Conditional expression of *Atg6-RNAi* or *Pi3K59F-RNAi* in *Drosophila* neurons under the direction of the *Ddc-Gal4* transgene reduced survival and increased the age-dependent loss of climbing ability, phenotypic attributes that are strongly associated with *Drosophila* models of PD. The reduction in Atg6 and Pi3K59F activity in these neurons may be detrimental to the health of these flies, manifesting as PD-like symptoms. Atg6 and Pi3K59F are involved in the nucleation phase of autophagy during autophagosome formation (Levine and Kroemer, 2008). Inhibition of these two important autophagy genes may lead to decreased autophagic activity that appears to disrupt neuronal function.

Flies in which *α-synuclein* is expressed in *Ddc-Gal4*-expressing neurons demonstrate impaired locomotor function. In previous studies, α-synuclein accumulation in LBs and Lewy neurites has been attributed to failure of the ubiquitin proteasome degradation system (Auluck et al., 2002). However, recent investigations have shown that α-synuclein may be degraded via the lysosomal pathway, in particular, by macroautophagy and chaperone-mediated autophagy (CMA; Xilouri and Stefanis, 2011; Jiang and Mizushima, 2014). Co-expression of *Atg6-RNAi* or *Pi3K59F-RNAi* and *α-synuclein* slightly reduced longevity and climbing ability compared to flies overexpressing *α-synuclein* alone. Inhibition of autophagy leads to the accumulation of α-synuclein, demonstrating an important role for this process in normal α-synuclein turnover (Webb et al., 2003). Post-translational modifications of α-synuclein interfere with its degradation and that of other proteins by CMA (Martinez-Vicente et al., 2008), and inhibit autophagosome formation at an early stage (Winslow et al., 2010). These findings indicate a compromised autophagy system; therefore, α-synuclein toxicity and *α-synuclein*-induced phenotypes are only slightly enhanced by a further reduction in autophagic activity. Such observations highlight the complexity of PD pathogenesis, and demonstrate the involvement of numerous failed cellular mechanisms.

Members of the Bcl-2 family bind Atg6, a BH3-only protein, in a multimeric complex involved in the vesicle nucleation stage of autophagosome formation. Discovery of the BH3 domain in Atg6, a binding site allowing interaction with the anti-apoptotic Bcl-2 proteins and required for Atg6 inhibition, revealed that Bcl-2 proteins not only regulate apoptosis, but also have an anti-autophagic function (Sinha and Levine, 2008). Bcl-2 proteins appear to play a crucial role in maintaining autophagic homeostasis, as weakening their interaction with Atg6 by its phosphorylation promotes autophagy (Zalckvar et al., 2009). Atg6 contains a Pi3K59F

binding site, and it is this interaction that leads to the production of the phosphatidylinositol-3-phosphate pivotal to the nucleation process (Furuya et al., 2005). Overexpression of the *Bcl-2* homolog *Buffy*, together with *Atg6-RNAi*, resulted in lower survival and climbing scores compared to flies expressing *Atg6-RNAi* alone. Notably, however, *Buffy* overexpression rescued the effects of inhibiting *Pi3K59F*. Taken together, these results indicate that *Buffy* magnifies *Atg6-RNAi*-induced phenotypes and suppresses those resulting from *Pi3K59F-RNAi*. Since Bcl-2 proteins have been shown to block Atg6-dependent autophagy by inhibiting the formation of the Atg6/Pi3K59F complex (Pattingre et al., 2005), we are inclined to speculate that Buffy plays a similar role in *D. melanogaster*. Excessive autophagy leads to cell death; therefore, Buffy may act to balance autophagic activity via the Atg6 pathway involved in the elimination of dysfunctional mitochondria, counteracting PD-like symptoms in *Drosophila*. The accumulation of α-synuclein promotes excessive autophagy levels, leading to removal of mitochondria, the depletion of which from most neurons may interrupt synaptic function.

CONCLUSIONS

Inhibition of either *Atg6* or *Pi3K59F* resulted in reduced longevity and impaired locomotor function, possibly representing a novel model of PD. It is important to establish other roles for these proteins, besides those in autophagy, so as to further understand their functions. Buffy, similar to most pro-survival Bcl-2 proteins, demonstrates anti-autophagic effects that enhance and suppress phenotypes associated with inhibition of *Atg6* or *Pi3K59F*, respectively. The mechanism by which Buffy affects this response remains unclear. However, it may involve interaction with the putative BH3 domain in Atg6, disrupting formation of the Atg6/Pi3K59F complex that is key to the initiation of autophagy. Further studies are required to elucidate the roles of Atg6, Pi3K59F, and Buffy in the autophagy-lysosome pathway.

Conflicts of interest

The authors declare no conflict of interest.

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