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Rbf and healthy ageing in Drosophila: interactions with *park*in, *Buffy* and *Debcl*

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ABSTRACT. Mitochondrial health depends upon proteins that counteract dysfunction generated by cellular stress due to impairment of essential cellular pathways. The expression of the *Bcl-2* family genes, the E3 ubiquitin ligase encoding, *parkin*, and the transcription regulator gene, *Rbf/Rb*, play significant roles in mitochondrial and cellular survival. Under conditions of extreme cellular stress, mitochondria can act to promote apoptosis. The overexpression of *Rbf* in the dopaminergic neurons of Drosophila directed by *Ddc-Gal4* can result in flies with a reduced median lifespan and impaired climbing ability over time: a well-established Drosophila model of Parkinson Disease (PD). The inhibition of *Rbf* can lead to a premature reduction in locomotor ability compared to control. The overexpression of *Rbf* can rescue the neurodegenerative phenotypes induced due to the loss of function of *parkin*. The expression of the pro-cell survival *Bcl-2* family member *Buffy* and inhibition of the anti-apoptotic *Debcl* can rescue the longevity and impaired locomotor ability over time observed in a model of PD induced by *Rbf-RNAi*. Overall, the alteration of expression of *Rbf* in selected neurons can produce a novel model of PD in Drosophila; the directed expression of *Buffy* acts to protect to counteract the *Rbf-RNAi*-induced deficits in lifespan and climbing ability.

Key Words: *Drosophila melanogaster*; Aging; Climbing Ability; Mitochondria; Drosophila model of Parkinson disease

INTRODUCTION

Mitochondria are essential for aerobic respiration and many signalling processes. Poorly functioning mitochondria can contribute to a wide range of pathologies, including Parkinson Disease (PD) and other age-related neurodegenerative diseases (Perier and Vila, 2012). In turn, the health of the mitochondrial population is dependent upon a number of crucial cellular processes. Cells utilize a variety of diverse mechanisms to upkeep a healthy and efficient mitochondrial network, including the mitochondrial unfolded protein response (UPRmt), Ubiquitin Proteasome System, mitophagy and other aspects of mitochondrial dynamics (Chan, 2012; Jovaisaite et al., 2014; Sugiura et al., 2014). The *parkin*-encoded protein is a key regulator of the ubiquitin-proteasome system and of mitophagy; importantly, mutant variants of *parkin* are responsible for some familial forms of PD. The Parkin protein is a part of an E3 ubiquitin ligase complex that functions to target protein and organelles for degradation (Yoshii et al., 2011; Bingol and Sheng, 2016). The loss of *parkin* gene activity, a common cause identified for the pathology of Parkinson disease, can lead to the accumulation of substrates, such as Cyclin E, and the upregulation of the Akt pathway. Cyclin E phosphorylates the retinoblastoma (Rb) tumour suppressor protein, which then causes the release of the transcription factor E2F-1 (Höglinger et al., 2007; Feng et al., 2015). The unencumbered E2F-1 can trigger apoptosis in post-mitotic neurons, which likely underlies the death of dopaminergic (DA) neurons in PD patients. The Rb protein is a crucial regulator of cellular proliferation and apoptosis.

The well-studied nuclear function of the *Rb*-encoded protein is that of transcriptional regulation via interactions with E2F1. The overexpression or inhibition of the *Rb* homologue in mice throughout development can have catastrophic effects that can include lethality (Vooijs and Berns, 1999; Lipinski and Jacks, 1999). Interestingly, the examination of Rb protein activity has established direct interactions with mitochondria as the Rb protein can 1) localize to near the mitochondrial surface; 2) induce the MOMP; 3) bind with the Bcl-2 family member Bax (*in vitro* and *in vivo*); 4) induce apoptosis when in a form designed to be deficient of nuclear function and is targeted to mitochondria; and 5) suppress tumorigenesis (Hilgendorf et al., 2013). In *Drosophila melanogaster*, *Rbf*, the fly orthologue of *Rb*, can decrease the transcription of anti-apoptotic *Bcl-2* family protein *Buffy* (Clavier et al., 2014) and promote the interaction of the Debcl protein, encoded by *Debcl* the sole Drosophila pro-apoptotic Bcl-2 family protein and the product of *Drp1*, to promote apoptosis via JNK pathway by induction of the production of ROS (Clavier et al., 2015). The Drp1 protein has a central role in mitochondrial quality control and mitochondrial apoptosis (Sebastián et al., 2017; Favaro et al., 2019). Altered forms of the Rb protein cause the cells to be sensitized with a predilection towards apoptosis (Ariss et al., 2018). The extent of the molecular mechanisms through which the Rb/Rbf proteins function have much to reveal.

The roles of the *Bcl-2* family encoded proteins in the protection of the mitochondria is very well established. However, little is known about the contribution of *Rbf* to mitochondrial health. Here, we propose that the phenotypes associated with the altered expression of *Rbf* are due to excessive apoptosis and can be rescued by the appropriate regulation of this process. *Drosophila melanogaster* was exploited as a model organism to study the phenotypic effects of the interactions of these genes. In these experiments, the *UAS-Gal4* system was used to direct the expression and inhibition of the *Rbf* gene in selected neuronal tissues by *Ddc-Gal4* and, in supportive experiments, in the developing eye tissue via the *GMR-Gal4* transgenes. In our experiment, the expression and inhibition of *Rbf* have led to apparent toxic effects, compromised lifespan and the diminishment of the ability to climb overtime. Interestingly, the overexpression of *Rbf* can act to rescue the consequences of *parkin* inhibition. Importantly, the phenotypic effects of *Rbf* inhibition can be rescued by the expression of the anti-apoptotic *Bcl-2* family protein gene, *Buffy*.

MATERIALS AND METHODS

Bioinformatic analysis

The *H. sapiens* and *D. melanogaster* protein sequences were obtained from the National Center of Biotechnology Information (NCBI) protein database *(https://www.ncbi.nlm.nih.gov/protein/).* The conserved domains were identified through the use of the Eukaryotic Linear Motif *(http://elm.eu.org/)* and NCBI Conserved Domain Database *(https://www.ncbi.nlm.nih.gov/cdd/).* Multiple sequence alignment was accomplished via the Clustal Omega on-line tool *(https://www.ebi.ac.uk/Tools/msa/clustalo/)* to reveal the conservation of domains. The nuclear localization signal was predicted with cNLS Mapper (*http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi*) (Kosugi et al. 2009). The phosphorylation sites were identified using: KinasePhos (*http://kinasephos.mbc.nctu.edu.tw/index.php*) it computationally predicts phosphorylation site using HMM (Huang et al., 2005); PhosphositePlus (*https://www.phosphosite.org/homeAction*) and Eukaryotic Linear Motif (ELM) *(*http://elm.eu.org/*)*, it is a comprehensive information tool to study post-translational modifications (Hornbeck et al., 2015). The phosphorylation site found conserved between *D. melanogaster,* and *H. sapiens* were highlighted.

Drosophila melanogaster stocks and media

The *GMR-Gal4*^{12}; *Ddc-Gal4*$^{4.3D}$*(w[1118]; P{w[+mC]=Ddc-GAL4.L}4.3D); UAS-lacZ*$^{4-1-2;}$ the *UAS-Rbf1 (w[*]; P{w[+mC]=UAS-Rbf.D}II*); the *UAS-Rbf RNAi1*^{HMS03004} *(y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS03004}attP2/TM3, Sb[1]);* the *UAS-Rbf RNAi2*^{GL01293} *(y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL01293}attP40)*; the *UAS-Buffy (w[*]; P{w[+mC]=UAS-Buffy.S}E1); UAS-Buffy-RNAi (w[*]; P{w[+mC]=UAS-Buffy.RNAi}3);* and *UAS-Debcl (y[1] w[67c23]; P{y[+mDint2] w[+mC]=EPgy2}Debcl[EY05743])*; stocks were obtained from Bloomington Drosophila Stock Center at Indiana University, Bloomington, Indiana, USA. The UAS-Debcl-RNAi^{v47515} (w1118; P{GD 1637}v47515) stock were obtained from Vienna Drosophila Resource Center. The *UAS-parkin-RNAi* line was obtained from Dr. B. Lu (Yang et al., 2006). The *Ddc-Gal4 UAS-parkin-RNAi* and *Ddc-Gal4 UAS-Rbf-RNAi*^{HMS03004} transgene lines were produced through standard methods (M'Angale and Staveley, 2016). All flies were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben for fungal resistance. Stocks were maintained at room temperature of 22° ± 3° C, whereas crosses and experiments were kept at 25°C.

Survival assay

Several crosses of virgin females and males were made, and a cohort of critical class males collected upon eclosion Male progeny of critical class was collected from mating until approximately 250 flies of each genotype were obtained. The flies were maintained in cohorts of 25 or less per vial on standard media, to avoid over-crowding. Flies were scored every second day for viability and were transferred to new food every two to five days. The tally continued until all flies were observed to be dead (Todd and Staveley, 2004, 2012). Longevity data were analyzed using GraphPad Prism version 8 statistical software (*graphpad.com*), and the Mantel-Cox test compared survival curves. Significance was determined at a 95% confidence level ($P \leq 0.05$) with Bonferroni correction.

Locomotor analysis

Approximately 70 male flies of the critical class were collected over 24 hours from the crosses similar to survival assay and maintained as cohorts of 10 flies in each vial. The media was changed twice a week. The climbing assay was performed as previously described according to a standard protocol (Todd and Staveley, 2004, 2012). Every week 50 males were assayed, in groups of 10, for their ability to climb a glass tube divided into five levels of 2 cm each. The climbing index was calculated for each week using GraphPad prism version 8 statistical software. The climbing curve was fitted using non-linear regression and determined at a 95% confidence interval ($P \leq 0.05$).

Biometric analysis of the *Drosophila melanogaster* eye

Female virgins of the *GMR-Gal4* genotype were collected every 8 to 12 hours for several days. The confirmed virgins were then crossed with the males of the following genotypes: *UAS-lacZ*, *UAS-Rbf1*, *UAS-Rbf2, UAS-Rbf-RNAi1* and *UAS-Rbf-RNAi2*. Critical class male progeny was collected from each genotype. The collected flies were kept as cohorts of 10 flies or less in each vial upon fresh media. Flies were allowed to age for three to four days and then frozen at -80°C. The flies were prepared for scanning electron microscopy following the standard protocol (M'Angale and Staveley, 2017). Ommatidia and interommatidial bristle counts were performed on ten or more flies of each genotype using the National Institute of Health (NIH) ImageJ software. The Biometric analysis was performed using GraphPad Prism version 8 statistical software. Significance was determined at a 95% confidence level ($P \leq 0.05$) using unpaired t-test.

RESULTS

Rbf is conserved between *Homo sapiens* and *Drosophila melanogaster*

The *D. melanogaster Rbf* (CAA65661.1) and the *H. sapiens,* retinoblastoma-like protein 1 (NP_899662.1) protein sequence were sourced from the NCBI protein database and the conserved sequences were identified using NCBI CDD. The multiple sequence alignment of the two proteins derived by Clustal Omega (Figure 1) shows a highly conserved domain of the unknown function (DUF3452), an RB-A and RB-B domain. The conserved phosphorylation sites identified using KinasePhos, PhosphoSitePlus and ELM and listed in Table 1. Towards the amino-terminus region, a nuclear localization signal has been identified using cNLS Mapper and re-confirmed by Eukaryotic Linear Motif (ELM), which localized NLS in the overlapping region. The cNLS score of the sequence was 6.5, proteins with scores as high as 8, 9 and 10 are exclusively localized in nucleus; proteins with scores 1 and 2 are solely localized in the cytoplasm, and in-between scores are supposedly co-localized in nucleus and cytoplasm depending on their scores. As the amino acid sequence of these proteins is highly conserved, the cellular functions are likely to be nearly identical (Figure 1).

The phosphorylation sites were identified using KinasePhos and PhosphoSitePlus and ELM resources. The KinasePhos computationally predict the phosphorylation site using HMM. The PhosphoSitePlus is a comprehensive information resource to study and document post-translational modifications. The ELM scans user-submitted protein sequence and matches corresponding sequences could be false positive. The kinases found or predicted to do the phosphorylation are listed with the respective phosphorylation site.

Table 1. List of the phosphorylation site found conserved between the Rbf/Rb protein sequence of *Homo sapiens* and *Drosophila melanogaster*.

Phosphorylation site *D. melanogaster* /*H. sapiens*	Amino acid	Kinase	Identified using
85/74	Threonine	CK1	ELM
674/879	Tyrosine	EGFR	KinasePhos
700/938	Threonine	INSR	KInasePhos
742/980	Threonine/Serine	PKC and PKA	KinasePhos
749/988	Serine	CDK2	PhosphoSitePlus (Zhou et al. 2013)
771/1009	Serine	cdc2 and CDK2	KinasePhos; PhosphoSitePlus (Leng et al. 2002)

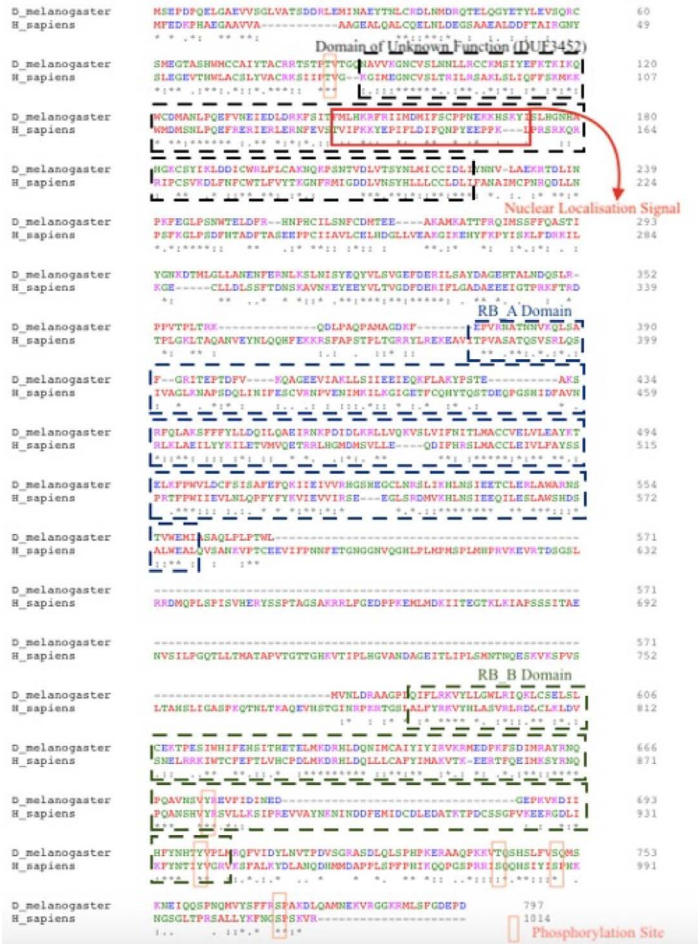

Figure 1. Rbf/Rb evolutionarily is conserved between *D. melanogaster* and *H. sapiens*. Clustal Omega multiple sequence alignment of *D. melanogaster* Rbf (CAA65661.1) protein with the *H. sapiens* (NP_899662.1) shows evolutionarily conserved domains identified using the NCBI Conserved Domain Database (CDD) and further confirmed by the Eukaryotic Linear Motif (ELM) resource. The conserved domains, Nuclear localization Signals and phosphorylation sites found conserved between the two proteins are highlighted in different colours. The asterisks indicate the identical residues; the colons indicate the conserved substitutions; the dots indicate the semi-conserved substitutions. Colour differences indicate the chemical nature of amino acids: red indicates small hydrophobic (includes aromatic) residues; blue indicates acidic; magenta indicates basic; green indicates basic with hydroxyl or amine groups.

Alteration of the expression of *Rbf* with *Ddc-Gal4*

The directed expression and inhibition of *Rbf1* by the *Ddc-Gal4* transgene result in decreased lifespan compared to the control (Figure 2A). The overexpression of *Rbf* resulted in median lifespans of 42 days in 311 flies, which is significantly less than control. The inhibition of *Rbf* by two distinct RNAi transgenes, via the *UAS-Rbf-RNAi1* and *UAS-Rbf-RNAi2,* results in a reduced median lifespan of approximately 56 days in 250 flies compared to 68 days observed in control (Figure 2A) as determined by log-rank (Mantel-Cox) test at a P-value at <0.0001. The climbing ability of flies with altered *Rbf* expression is severely compromised as determined in the non-linear fitting of the climbing curve by 95% confidence interval (Figure 2B).

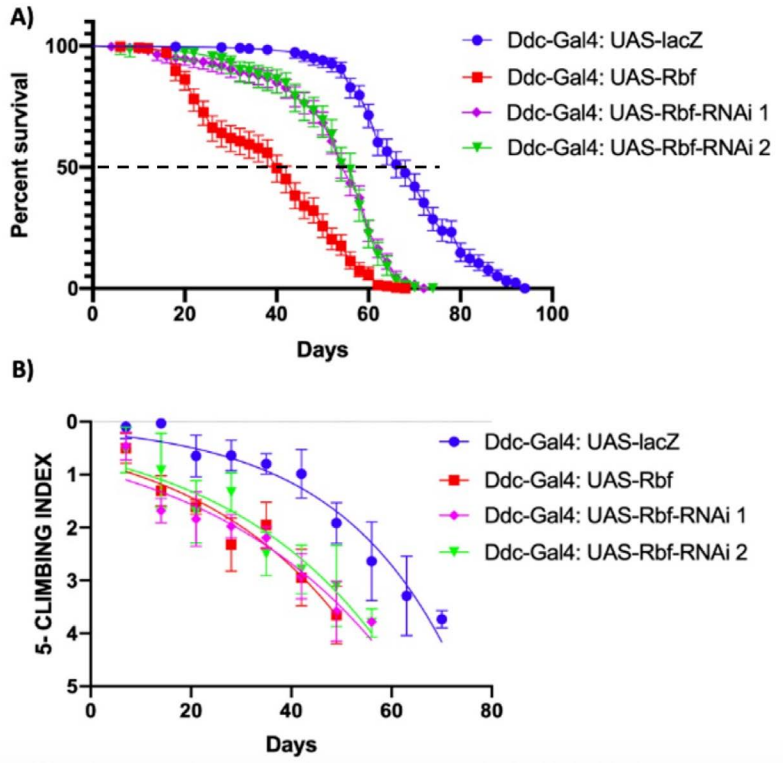

Figure 2. Altered *Rbf* expression under the control of *Ddc-Gal4*$^{4.3D}$ influences the survival and climbing ability of flies. **A).** The GraphPad prism8 generated graph of the longevity assay for the directed expression of *Rbf, Rbf RNAi's* under the control of *Ddc-Gal4*$^{4.3D}$ transgene. The overexpression results in decreased median lifespan of 40 days compared to 68 days of control calculated by the Log-rank Mantel-Cox test, with Bonferroni correction. The inhibition of *Rbf* under the control of the *Ddc-Gal4* transgene results in a decreased lifespan of 56 days with *UAS-Rbf-RNAi1*^{HMS03004} and *UAS-Rbf-RNAi2*^{GL01293} compares to 68 days of control done by Log-rank Mantel-Cox test, with Bonferroni correction. **B)**. The GraphPad prism8 generated graph of the climbing abilities of flies with overexpression of *Rbf, Rbf RNAi's* and control. The climbing ability of *Rbf* overexpression and *Rbf RNAi's* flies is significantly compromised compared to control as determined in the non-linear fitting of the climbing curve by a 95% confidence interval.

The directed expression of *Rbf* by the *Ddc-Gal4* transgene results in a decreased lifespan of 252 flies to 50 days compared to 68 days observed in control. The *Ddc-Gal4 UAS-Rbf-RNAi UAS-lacZ* critical class males have a lifespan of 60 days. The overexpression of *Rbf* in the *Ddc-Gal4 UAS-Rbf-RNAi* transgene has a median lifespan of 72 days in 272 flies, similar to 68 days of control done by Log-rank Mantel-Cox test, with Bonferroni correction (Figure 3A) as determined by log-rank (Mantel-Cox) test at a P value at <0.0001. The comparison of the climbing ability of flies shows an intermediate phenotype when *Rbf* is overexpressed along with *Rbf-RNAi*^{HMS03004} as determined in the non-linear fitting of the climbing curve by 95% confidence interval (Figure 3B).

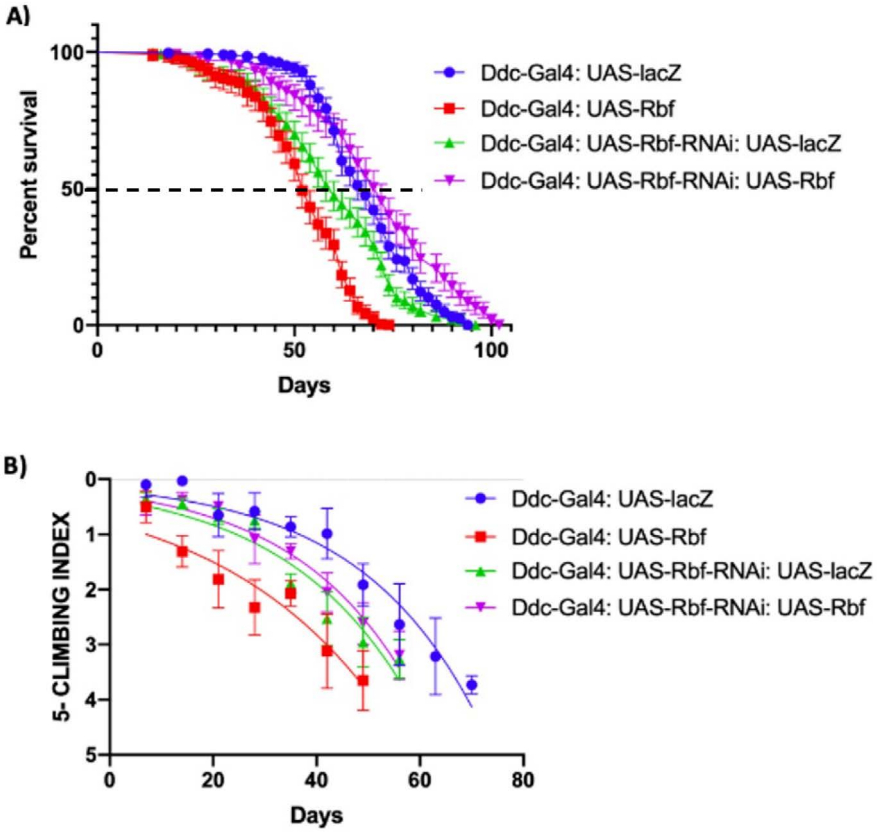

Figure 3. Directed co-expression of *Rbf* and *Rbf-RNAi* rescues diminished median lifespan A) The graph of longevity assay generated by GraphPad prism8 in *Ddc-Gal4/ UAS-lacZ; Ddc-Gal4/ UAS-Rbf; Ddc-Gal4 UAS-Rbf-RN*^{HMS03004}*/ UAS-lacZ;* and *Ddc-Gal4* median *UAS-Rbf-RNAi*^{HMS03004}*/ UAS-Rbf*. The overexpression of *Rbf* resulted in a decreased lifespan of 50, and inhibition of *Rbf* resulted in a decreased lifespan of 60 days compared to 68 days of control done by the Log-rank Mantel-Cox test, with Bonferroni correction. The overexpression of *Rbf* in neurons using *Ddc-Gal4 Rbf-RNAi*^{HMS03004} transgene results in a lifespan of 72 days, similar to 68 days of control done by Log-rank Mantel-Cox test, with Bonferroni correction. **B)** The GraphPad prism8 generated graph of the climbing abilities of flies with expression *Rbf, Rbf-RNAi*^{HMS03004}*/lacZ, Rbf-RNAi*^{HMS03004}*/ Rbf* and control. There is an intermediate phenotype when *Rbf* is overexpressed along with *Rbf-RNAi*^{HMS03004}, as determined in the non-linear fitting of the climbing curve by a 95% confidence interval.

The altered expression of *Rbf* influences the *Ddc-Gal4 Gal4$^{4.3D}$ UAS-parkin-RNAi* model of PD

The loss of function of the parkin has led to the establishment of several Drosophila models of PD. The *Ddc-Gal4 UAS-parkin-RNAi UAS-lacZ* critical males have a median lifespan of 60 days in 256 flies. Overexpression of *Rbf* in the *Ddc-Gal4 UAS-parkin-RNAi* expressing flies results in a much-increased median life span of 72 days (n=283) compared to the control, as determined by log-rank (Mantel-Cox) test at a P value at <0.0001. The *UAS-Rbf-RNAi1* transgene when expressed along with *UAS-parkin-RNAi* transgene, result in median lifespan of 50 (n=328) days much less compared to control flies (P-value=<0.0001) (Figure 4A). The *UAS-Rbf-RNAi2* transgene when expressed along with *Ddc-Gal4 parkin-RNAi*, results in a median life span of 58 days (n=308) (Figure 4A) similar to 60 days of control, as determined by log-rank (Mantel-Cox) test at a P-value at 0.8425. The overexpression of *Rbf* by *Ddc-Gal4* along with *parkin-RNAi* slightly ameliorates the decline in climbing ability over time. However, the locomotor activity of the critical classes with the directed expression of the *UAS-Rbf-RNAi* transgenes are decreased compared to control as determined in the non-linear fitting of the climbing curve by 95% confidence interval (Figure 4B).

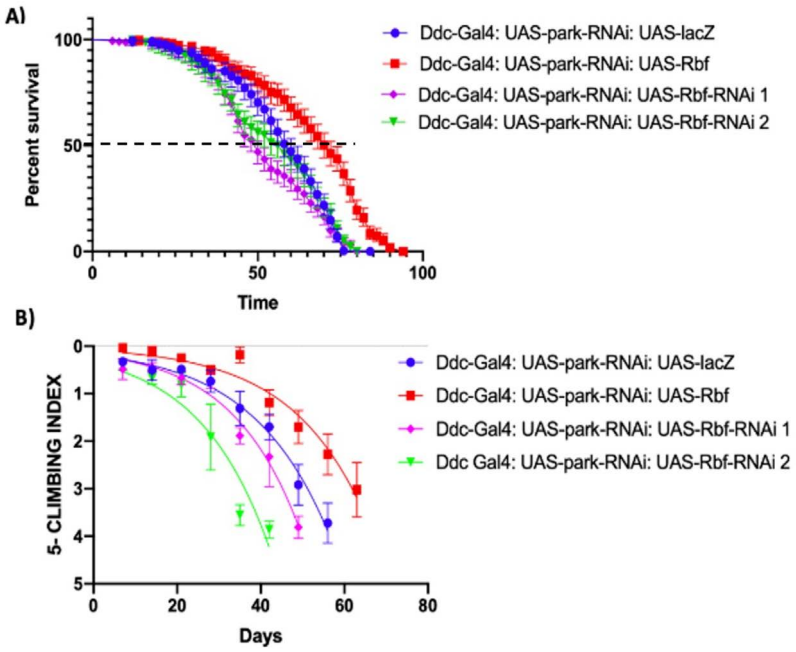

Figure 4. Altered expression of *Rbf* can enhance and suppress the *Ddc-Gal4 UAS-parkin-RNAi* model of PD. **A**. The graph of longevity assay generated by GraphPad prism8 with altered expression of *Rbf* in *Ddc-Gal4 parkin-RNAi* expressing flies. The overexpression of *Rbf* results in a median lifespan of 72 days compare to 60 days of control *(lacZ/parkin-RNAi*); the inhibition of *Rbf* by two distinct RNAi transgenes, via *UAS- Rbf-RNAi1*^{HMS03004} and *UAS-Rbf-RNAi2*^{GL01293} directed by the *Ddc-Gal4* transgene, result in the median lifespan of 50 and 58 days, respectively; similar to control, determined by Log-rank Mantel-Cox test, with Bonferroni correction. **B.** The GraphPad prism8 generated graph of the climbing abilities of *Ddc-Gal4 parkin-RNAi* flies with the expression of *Rbf, Rbf RNAi's* and control. The climbing ability of flies overexpressing *Rbf* have significantly increased compared to control as determined in the non-linear fitting of the climbing curve by a 95% confidence interval.

The altered co-expression of *Buffy* and *Debcl* with *Rbf-RNAi* via *Ddc-Gal4*$^{4.3D}$

The loss of function of *Rbf* leads to compromised lifespan and climbing ability over time. The control *Ddc-Gal4; UAS-Rbf-RNAi; UAS-lacZ* critical class males have a median lifespan of 58 days (n=351). Overexpression of *Buffy* in the *Ddc-Gal4; UAS-Rbf-RNAi; UAS-Buffy* flies have a median lifespan of 88 days in 392 flies (P-value=<0.0001), which is significantly greater when compared to the controls. The inhibition of *Buffy* in the *Ddc-Gal4; UAS-Rbf-RNAi; UAS-Buffy-RNAi* critical class male flies have a median lifespan of 58 days in 275 flies which is very similar to the control (Figure 5A) as determined by log-rank (Mantel-Cox) test at a P value at 0.0007. Overexpression of *Debcl* in the *Ddc-Gal4; UAS-Rbf-RNAi; UAS-Debcl* critical class flies have a median lifespan of 54 days in 317 flies (P-value=0.0093), which is not very different from the *lacZ*-expressing controls. The inhibition of *Debcl* in the *Ddc-Gal4 UAS-Rbf-RNAi*; *UAS-Debcl-RNAi* expressing flies have a median lifespan of 64 days in 288 sample size, which is increased compared to control flies (Figure 5A) as determined by log-rank (Mantel-Cox) test at a P value at <0.0001. The overexpression of *Buffy* in *Ddc-Gal4; UAS-Rbf-RNAi; UAS-Buffy flies* rescue the climbing ability defects over time. The inhibition of *Buffy* increases the loss of climbing ability throughout the life of critical class flies as determined in the non-linear fitting of the climbing curve by 95% confidence interval (Figure 5B). The overexpression of *Debcl* by *Ddc-Gal4 UAS-Rbf-RNAi; UAS-Debcl* results in an increase in impairment of the climbing ability defect over time. The inhibition of *Debcl* ameliorates the defects in climbing abilities as determined in the non-linear fitting of the climbing curve by 95% confidence interval at a P-value= 0.0220 (Figure 5B).

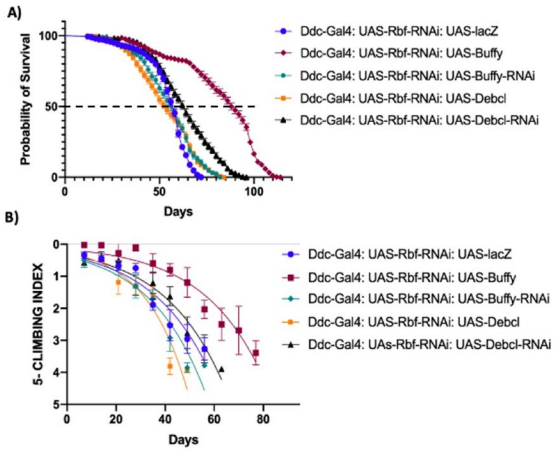

Figure 5. Altered expression of *Buffy* and *Debcl,* can suppress the *Ddc-Gal4*$^{4.3D}$ *UAS-Rbf-RNAi*^{HMS03004} potential model of PD. **A**. The graph of longevity assay generated by GraphPad prism8 with altered expression of *Buffy* and *Debcl* in *Ddc-Gal4 Rbf-RNAi*^{HMS03004} expressing flies. The overexpression of *Buffy* results in a median lifespan of 96 days compare to 58 days of control *(lacZ/Rbf-RNAi*^{HMS03004}); the inhibition of *Buffy* result in the median lifespan of 58 days similar to control, determined by Log-rank Mantel-Cox test, with Bonferroni correction. The overexpression of *Debcl*^{EY05743} in neurons using *Ddc-Gal4* transgene along with *Rbf-RNAi*^{HMS03004} results in a lifespan of 54 days similar to control and inhibition of *Debcl* ^{EY05743} resulted in the increased lifespan of 64 days compared to 58 days of control done by Log-rank Mantel-Cox test, with Bonferroni correction. **B.** The GraphPad prism8 generated graph of the climbing abilities of *Ddc-Gal4 Rbf-RNAi*^{HMS03004} flies with the expression of *Buffy, Buffy RNAi, Debcl* ^{EY05743}*, Debcl RNAi* ^{v47515} and control. The climbing abilities of *Buffy* flies have significantly increased compared to control as determined in the non-linear fitting of the climbing curve by a 95% confidence interval.

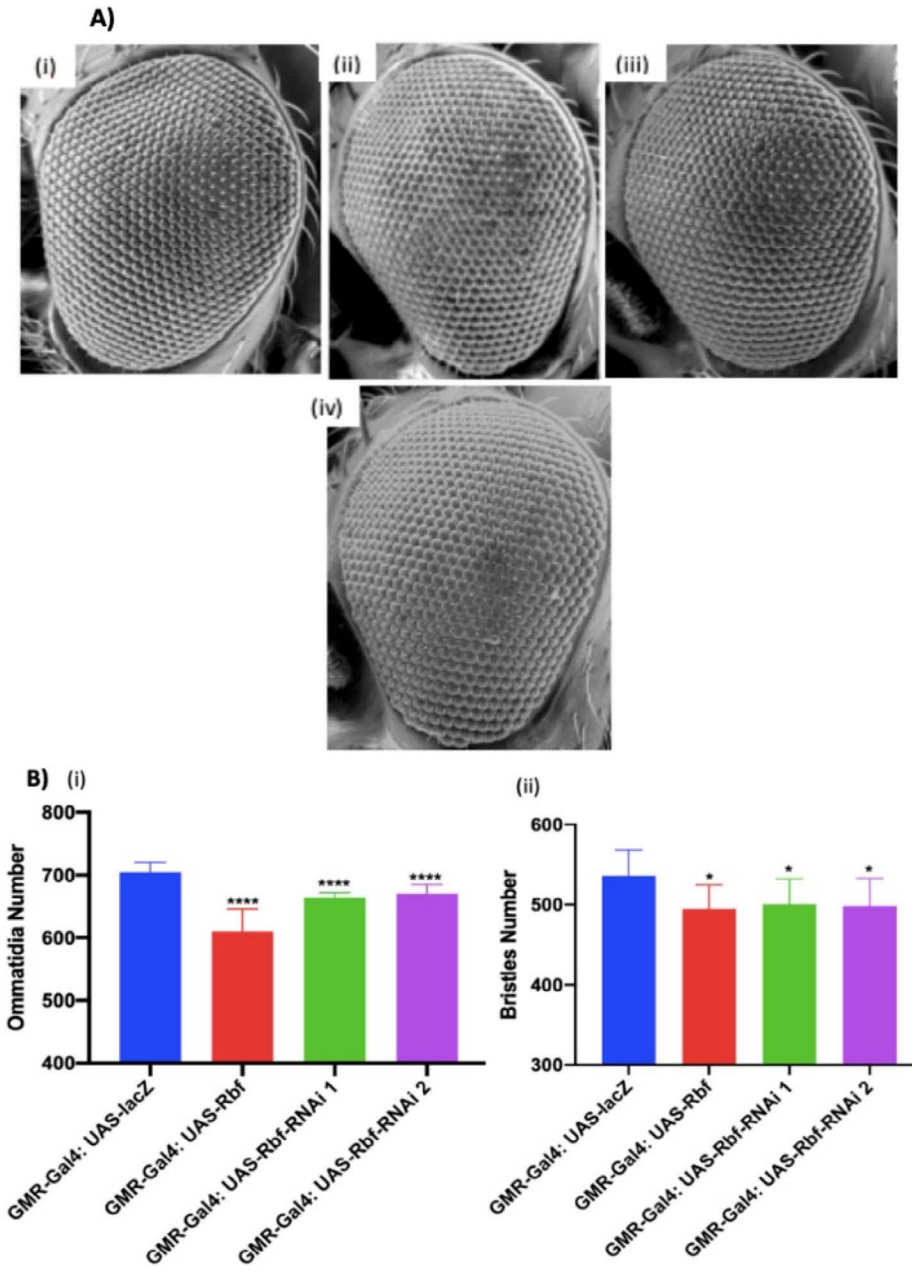

Figure 6. The phenotypic effects of altered *Rbf* expression in *D. melanogaster* eye. **A**. Scanning electron micrograph of the altered *Rbf* expression under the control of *GMR-Gal4* transgene. The genotypes are (a) *GMR-Gal4/UAS-lacZ* (Control); (b) *GMR-Gal4/UAS-Rbf*; (c) *GMR-Gal4/UAS-Rbf-RNAi1*^{HMS03004} (d) *GMR-Gal4/UAS-Rbf-RNAi2*^{GL01293}. **B.** The ommatidia number for control is 704.4±15.4; the inhibition and expression of *Rbf* results in a decrease in ommatidial count compared to control. **C.** The interommatidial bristle count for the control is 536±32.5; the overexpression and RNAi inhibition lines (*GMR-Gal4 UAS-Rbf-RNAi1*^{HMS03004} and *GMR-Gal4 UAS-Rbf-RNAi2*^{GL01293}) results in a decrease interommatidial bristle count compared to control.

The overexpression and inhibition of *Rbf* during eye development

In complementary experiments, the inhibition and overexpression of *Rbf,* directed by the *GMR-Gal4* transgene in the neuron-rich developing eye of flies' influences development. The numbers of ommatidia are decreased with either overexpression or inhibition lines of *Rbf* analyzed in 15 flies of each group. The ommatidia count of *Rbf*, *Rbf-RNAi1*^{HMS03004} and *Rbf-RNAi2*^{GL01293} is 610, 664.2 and 670.6, respectively, compared to 704.4 for the *lacZ* control flies as shown in Figure 6B as determined by unpaired t-test with P values of 0.0198, 0.0677 and 0.8151. The mean of interommatidial bristle produced through inhibition by the *UAS-Rbf-RNAi1* and *UAS-Rbf-RNAi2* transgene was lower at 500.6 (P-value= 0.0320) and 498.1 (P-value=0.0.0296) compare to 536 of control flies as determined by an unpaired t-test. The mean number of interommatidial bristles for *UAS-Rbf* flies was 494.9 less compared to 536 of control, as determined by an unpaired t-test (P-value= 0.0.0128). The decrease in the interommatidial bristle number is consistent with the reduction of ommatidial numbers produced through overexpression and inhibition by the *Rbf and Rbf-RNAi* bearing transgenes compare to control (Figure 6B ii).

DISCUSSION

The role of the *Rbf/Rb* homologues is crucial to a healthy life. In mice, the loss of function *Rb* mutant mouse displays reduced mitochondrial function, including defects of the TCA cycle and in oxidative phosphorylation (Nicolay et al., 2015). The Rb protein is required for the activation of a number of mitochondrial protein genes. Cells deficient in *Rb* are more sensitive to the damaging effects of ROS and require elevated levels of glutathione peroxidase to thrive. In these experiments, the flies with altered expression of *Rbf* display compromised longevity. Of interest is that the diminishment of the median lifespan of flies was more severe in response to the overexpression of *Rbf* than with the directed loss of function genotype. This may be due to the induction of apoptotic cell death as has been observed with post-mitotic proliferating cells (Milet et al., 2010) or the consequences of *Rbf* overexpression may act in a manner similar to those that promote tumour progression (Shi et al., 2000). The differentiation defects due to loss of *pRb* can be rescued by normalizing mitochondrial activity with the help of PGC-1 alpha expression (Váraljai et al., 2015). The development of eyes compromised during *Rbf* overexpression, the eyes develop in a manner that reduces numbers of ommatidia and bristles as compared to control. Most likely, the overexpression of *Rbf* acts to promote apoptosis through transcription activation and regulation of the pro-apoptotic sub-cellular machinery. Interestingly, our novel results show that *Rbf* overexpression can rescue the *parkin*-inhibition phenotype. The *Rbf* induced protection could be due to its role in blocking fly version of the E2F1 transcription factor. Afterall, patients with PD demonstrate E2F1 activation in DA neurons and mediate neuronal death (Höglinger et al., 2007). Altering *Rbf* expression with parkin changes the dynamics of its phenotypic effects.

Unexpectedly, the inhibition of *Rbf* leads to consistently reduced longevity and loss of climbing ability phenotypes that are milder than those generated by the gain of *Rbf* function. The *Rbf/Rb* mutants may promote transcription of apoptotic genes in an *E2F1*-dependent manner in eukaryotes (Moon et al., 2006; Milet et al., 2014). Afterall, the inactivation of the Rb protein allows E2F1 dependent transcription of pro-apoptotic genes, including *Apaf-1/PUMA* (Polager and Ginsberg, 2009). Interestingly, *Rb* can contribute to neuronal apoptosis, both dependently and independently of E2F1 transcription activity (Andrusiak et al., 2012). The inhibition and overexpression phenotypes associated with *Rbf/Rb* is likely due to excessive apoptosis promoted through distinct mechanisms.

While the role of *Rbf* in transcription may be well studied, the understanding of a largely non-nuclear role of *Rbf*/*Rb* is limited. In humans, the function seems to be dependent upon a pro-apoptotic Bcl-2 family protein, Bax, which regulates mitochondrial intrinsic apoptosis (Hilgendorf et al., 2013). In flies, when the overexpression of the pro-apoptotic Bcl-2 family member, *Debcl*, was directed along with the inhibition of *Rbf1*, both the median lifespan and climbing ability over the life of the flies were severely compromised. However, the defects generated by the inhibition of *Rbf* was rescued by overexpression of the anti-apoptotic Bcl-2 family protein, *Buffy*. It is unclear if the rescue of the phenotype is due to the inactivation of the E2F1 apoptotic activity or a distinct anti-apoptotic activity of Buffy. The endogenous role of *Rb/Rbf* seems to promote apoptosis in non-mitotic cells with the help of Bcl-2 family protein. The nuclear role of regulating transcriptional factor E2F1 is very complex. However, the overall effect is dependent on the result of different pathways regulated by endogenous and nuclear Rbf protein.

The function of *Rbf* is mainly nuclear, but a small fraction of the protein tends to localize near mitochondria in the cytoplasm. The overexpression of *Rbf* gene in neurons results in reduced survival and an age-dependent decline in locomotor ability. The knockdown of *Rbf* in the *Ddc-Gal4* transgenes of Drosophila results in an age-dependent loss in locomotor function, phenotypes that are strongly associated with neuronal degeneration and Parkinson disease. Thus, the compromised climbing abilities in flies with directed inhibition of *Rbf* have produced a novel model of Parkinson Disease and can be used to investigate further the mechanisms underlying PD and other neurodegenerative diseases. The overexpression of *Rbf* rescue the *parkin* inhibition phenotype; the Rbf and parkin protein products may activate similar downstream targets for cell survival. Similarly, the anti-apoptotic *Bcl-2* proteins rescued the PD phenotypes induced by *Rbf* inhibition. Further studies are required to understand better the interaction between *parkin, Bcl-2* family members, and *Rbf* in these neurons. Overall, these experiments allow us to contribute to the understanding of mitochondrial health and enhanced conditions of homeostasis.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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