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Article

Altered *Parkin* and *Bcl-2* Expression Suppresses *Drp1* Gain and Loss of Function Age-Related Phenotypes in *Drosophila*

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Abstract: The relationship of Amyotrophic Lateral Sclerosis, Parkinson Disease and other age-related neurodegenerative diseases associated with mitochondrial dysfunction has led to study of the mitochondrial fission gene *Drp1* in *Drosophila*. *Drp1* can interact with mitochondrial proteins including parkin and the *Drosophila* Bcl-2 proteins. Mutations in *Drp1* can lead to mitochondrial dysfunction and neuronal loss. Ddc-Gal4 was exploited to direct the expression of *Drp1* and the inhibition of *Drp1* in neurons. Here, the inhibition of *Drp1* seems to compromise locomotor function throughout life but does not alter longevity. The consequences of *Drp1* overexpression can be suppressed through overexpression of pro-survival *Bcl-2* gene *Buffy* or by inhibition of the pro-cell death *Bcl-2* homologue *Debcl*. The expression of *Buffy* suppresses the poor climbing induced by loss of *Drp1* function. Interestingly, the loss of parkin function is rescued by the directed inhibition of *Drp1*. Alteration of the expression of *Drp1* acts to phenocopy neurodegenerative disease phenotypes in *Drosophila*, while overexpression of *Buffy* can counteract these phenotypes to improve overall health. The pro-survival effect of *Buffy* can rescue the phenotypic effect due to the *Drp1*-induced apoptosis. The diminished health due to either overexpression or inhibition of *Drp1* has produced a novel model to investigate mechanisms underlying neurodegenerative disease.

Keywords: *Drp1*; *Buffy*; *Debcl*; *parkin*; *Drosophila melanogaster*; Amyotrophic Lateral Sclerosis; Parkinson Disease; mitochondrial fission; mitochondrial dysfunction

1. Introduction

The mitochondrial network is critical for many aspects of the subcellular survival mechanisms of organisms. Known to be the powerhouse of the cell, mitochondria are responsible for various aspects of energy homeostasis, oxidative stress, calcium handling, cell signalling, and, thus, cell survival [1]. The dynamic nature of the mitochondria population is critical to the integrity of the subcellular network structures that these organelles maintain and to the control of the quality of mitochondrial proteins and other components [2–4]. For example, the early dynamic events that occur during apoptosis include cristae remodeling, mitochondrial fragmentation, and membrane “blebbing” or zeiosis [5]. Inhibition of these processes, either through downregulation directed by RNA-interference or by expression of a dominant-negative mutant form of *Drp1*, can slow the rate of mitochondrial fragmentation and, in turn, the cascade of apoptotic events [6–8]. The activity of the *Drp1* protein promotes caspase-independent mitochondrial fission and cristae remodeling to amplify the process of apoptosis whether cell death is instigated by either the specific activity of the pro-apoptotic protein BID or by the general consequences of oxidative stress [9]. Overall, the mechanics of mitochondrial fission plays a crucial role in the amplification of aspects of the essential cellular process of apoptosis.

Modifications to the mitochondrial network seem to differentially influence a number of signalling pathways. In response to a series of molecular cues [10–12], the mitochondrial network participates in a delicate balance between continuous division and fusion processes [13,14]. Mitochondrial fusion helps compromised mitochondria, with likely bearing highly damaged DNA and proteins, to actively exchange components with other more healthy mitochondria to decrease the severity of heteroplasmy, and help with functional complementation [15,16]. Mitochondrial fission

allows for the segregation of irreversibly damaged portions of the mitochondrial network and subsequent degradation [17]. Mitochondrial fission necessarily requires *dynammin-related protein 1* (*Drp1*) and *FIS1* [18]. Nevertheless, an explicit understanding of the factors promoting fission and fusion remains limited.

The Bcl-2 family proteins interact with *Drp1*, and *Drp1* can promote apoptosis in Bcl-2 protein-dependent and independent manners [9,19]. The two Bcl-2 family homologues in *Drosophila melanogaster* are *Buffy* (anti-apoptotic) and *Debel* (pro-apoptotic). The *Debel* protein can interact with *Drp1* in *Drosophila* to activate apoptosis via the JNK pathway [20]. *Drp1* is required for a standard rate of Cyt-c release and caspase activation during programmed cell death [21]. *Drp1* increases the mitochondrial fragmentation under nitrosative stress in primary cerebrocortical neuron culture [22]. The *Drp1* protein interacts with other proteins involved in a number of mitochondrial processes, such as protein product of *Bax* [10,12,22], the products of *Pink1* (*PTEN-induced putative kinase 1*) and *park* in Cos-7 cells [23]. Mutation of the *Pink1* and *park* genes are among the most prominent causes of early onset of PD [24]. The roles of *Pink1* and *park* are vital to ubiquitin-dependent mitophagy [25]. The *Pink* and *parkin* loss of function mutation increase the mitochondrial localization of *Drp1* in flies [26]. The *parkin* protein ubiquitinates the *Drp1* for proteasomal degradation, incriminate the *Drp1* for dysregulated mitochondrial dynamics in *parkin* loss of function induced mitochondrial morphology in HeLa and SH-SY5Y cells [27]. Number of stresses can increase the mitochondrial translocation of *Drp1* in neurons and initiate apoptosis or mitophagy [28]. In a number of models of ALS, dephosphorylation of *Drp1* through the activity of protein phosphatase 1, has been identified as causative of the disease-associated phenotypes [29]. As well, *Drp1*-mediated mitochondrial fragmentation caused by the administration of rotenone has been identified in a rat model of PD-like changes to the olfactory bulb [30]. As adjustment of *Drp1* activity may be key to the inhibition of the pathology of ALS and PD, investigation of such alterations is essential to knowledge required for the development of therapies. Excessive activity of *Drp1* increase mitochondrial fission and consequently promote cell death.

Here we propose that alteration of *Drp1* expression results in a degenerative phenotype. We utilize *Drosophila* to model neurodegenerative disease because it is an excellent model system to study the genes and proteins affected in ALS and PD [31]. The anticipated role of mitochondria in PD pathogenesis has made the study of the interactions of *Drp1* gene important to modelling this disease in *Drosophila*. In these experiments, we exploited the UAS-Gal4 system to direct the overexpression and inhibition of the genes of interest in selected neuronal tissues using the *Ddc-Gal4* transgene [32,33]. The proposal is that the *Drp1* overexpression phenotype is due to excessive activities related to apoptosis and can be rescued by the appropriate regulation by anti-apoptotic Bcl-2 gene, *Buffy*. In an established *park-RNAi* model of PD [34], we directed and inhibited the expression of the *Drp1* gene. The ND-like phenotypes of *Ddc-Gal4 park-RNAi* were rescued by the expression of *Drp1-RNAi* transgenes. The phenotype produced in response to the expression of *Drp1-RNAi* is due to the diminishment of mitochondrial integrity and maybe rescued through modification of the responsible signalling pathway. The careful regulation of mitochondrial dynamics is important to control mitochondrial induced defects. The strategy is to identify the basic mechanisms in the fly model to encourage further validation in mammalian model organisms.

2. Materials and Methods

Bioinformatic Analysis

Protein sequences were obtained from the National Center of Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/protein/>). The conserved domains were identified using NCBI Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/cdd/>), and Eukaryotic Linear Motif (<http://elm.eu.org/>). Multiple sequence alignment was done using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) to reveal the conservation of domains. The *Homo sapiens* Dynammin-1 like protein (DLP-1/*Drp1*) structure (PDB ID 4BEJ) was obtained from NCBI structure database (<https://www.ncbi.nlm.nih.gov/structure/>) and *Drosophila melanogaster Drp1* protein structure was developed using Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) modeling tool. The final models were

edited with the PyMOL software (<https://pymol.org/2/>) to highlight the N-terminus, C-terminus and LIR regions.

Drosophila Stocks and Media

All stocks were maintained on a standard media prepared from cornmeal/molasses/yeast/agar medium treated with propionic acid and methylparaben to resist fungal growth. Aliquots of media were poured into plastic vials, allowed to solidify, and refrigerated at 4°C until used. Stocks are kept at room temperature while crosses and experiments were carried out at 25°C. The *UAS-Drp1* (*y*[1] *w*[*]; *P*{*w*[+*mC*]=FLAG-FLAG-HA-*Drp1*}3, *Ki*[1]); the *UAS-Drp1-RNAi*¹^{IF02762} (*y*[1] *v*[1]; *P*{*y*[+*t*7.7] *v*[+*t*1.8]=*TRiP.JF02762*}attP2)), *UAS-Drp1-RNAi*²^{HMC03230} (*y*[1] *v*[1]; *P*{*y*[+*t*7.7] *v*[+*t*1.8]=*TRiP.HMC03230*}attP40), the *UAS-Buffy* (*w*[*]; *P*{*w*[+*mC*]=*UAS-Buffy.S*}E1); *UAS-Buffy-RNAi* (*w*[*]; *P*{*w*[+*mC*]=*UAS-Buffy.RNAi*}3), *UAS-Debc1*^{EY05743} (*y*[1] *w*[67c23]; *P*{*y*[+*mDint2*] *w*[+*mC*]=*EPgy2*}*Debc1*[*EY05743*]), *Ddc-Gal4*^{4.3D}(*w*[1118]; *P*{*w*[+*mC*]=*Ddc-GAL4.L*}4.3D) and *UAS lacZ*⁴⁻¹⁻²; stocks were obtained from Bloomington Drosophila Stock Center at Indiana University, Bloomington, Indiana, USA. The *UAS-Debc1-RNAi*^{v47515} (*w*[1118]; *P*{*GD* 1637}v47515) was obtained from Vienna Drosophila Resource Center. The *UAS-park-RNAi* line was obtained from Dr. B. Lu [51,52]. The *Ddc-Gal4 park-RNAi* line was produced through standard methods [53]. The *Ddc-Gal4/CyO*; *UAS-Drp1*/TM3, *Ddc-Gal4/CyO*; *UAS-Drp1-RNAi* /TM3, derivative lines were generated using standard recombination methods and used to overexpress or inhibit *Drp1* in the selected DA neurons using the *Ddc-Gal4*^{4.3D} transgene.

Ageing Assay

Crosses of select virgin females and males were made, and a cohort of critical class males collected upon eclosion. At least 250 flies were aged per genotype in the cohorts of 25 or less per vial on fresh media, replenished every two to five days to avoid crowding. Flies were observed and scored every two days for the presence of deceased adults. As a rule, flies were considered dead when they did not display movement upon agitation [54]. Longevity data were analyzed using GraphPad Prism version 8 statistical software (graphpad.com), and survival curves were compared by the Mantel-Cox test. Significance were determined at a 95% confidence level ($P \leq 0.05$) with Bonferroni correction.

Climbing Assay

A cohort of 70 critical class male flies were collected within 24 hours and maintained as a maximum of ten flies per vial at low density. The food was changed twice every week. Every week 50 males of each genotype were assayed, in groups of 10 or less, for their ability to climb a glass tube divided into five levels of 2 cm each according to standard protocol [54]. 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 ($P \leq 0.05$).

3. Results

2.1. Drp1 Is Highly Conserved between Homo Sapiens and Drosophila Melanogaster

The *D. melanogaster* Drp1 protein sequence was sourced from NCBI protein, and the conserved sequences were identified using NCBI CDD. NCBI protein Blast of Drp1 protein of *D. melanogaster* (NP_608694.2) with the *H. sapiens*, identified dynamin-1-like protein (isoform 4) (NP_001265392.1), it is 65% identical with a bit score of 957. The multiple sequence alignment of the two proteins derived by Clustal Omega (Figure 1A) shows a highly conserved dynamin-like protein family domain, a dynamin central domain, and a dynamin GTPase effector domain. Two well-documented phosphorylation sites are identified; S606 and S627 in *dynamin-1-like protein* isoform 4 of *H. sapiens*; and S616 and T637 in *Drp1* of *D. melanogaster*. A template-based modeling of *D. melanogaster* Drp1 protein by use of a combination of empirically derived energy functions and physics-based simulated folding was produced using Phyre2. The modeled *D. melanogaster* Drp1 protein (i) and the *H. sapiens* Dynamin-1 like protein (ii) from the NCBI database share a near identical structure (Figure 1B). The amino-terminus region of the Drp1 protein is highly conserved and has a consensus LC3-interacting

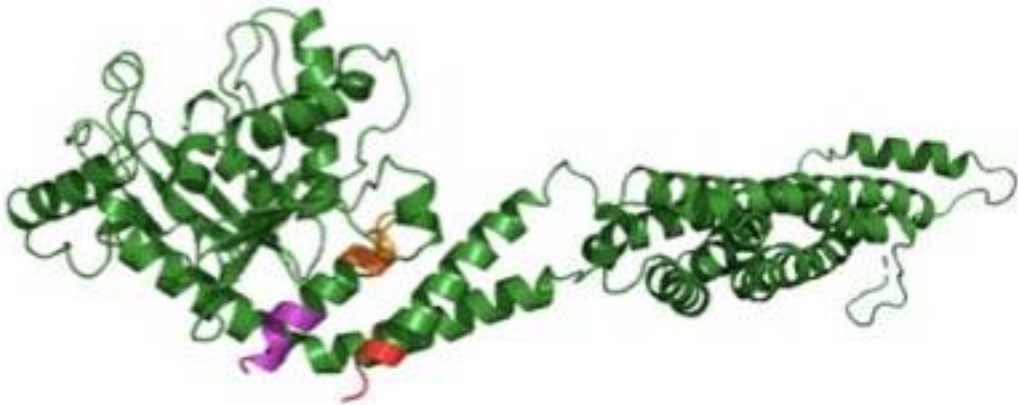
region (LIR) sequence for binding to the ATG8/LC3 protein as determined by the Eukaryotic Linear Motif (ELM) resource. As this protein structure is so highly conserved, it seems very likely that the functions are highly conserved.

	ATG8 binding region	Dynamain like protein family domain	
D.melanogaster	MEALIPVINKLQDVFNVTGSDSIQLPQIVVLGSQSSGKSSVIESVVGSRFLPRGTGIVTR		60
H.sapiens	MEALIPVINKLQDVFNVTGADIQLPQIVVVGTSQSSGKSSVLESVGRDLLPRGTGIVTR		60
	*****[LIR]*****	*****:*****:*****:*****:*****	
D.melanogaster	RPLVLQLIYSPDLLDRENRSANGTSNAEEWGRFLHTK-KCFTDFDEIRKEIENETERAAG		119
H.sapiens	RPLILQLVHVSQEDKRKTTGEENGVEAEWGRFLHTKNKLYTDFDEIRQEIENETERISG		120
	::***:***:***:***:***:***:***:***:***:***:***:***:***:***		
D.melanogaster	SNKGICPEPINLKIFSTHVNLTLVDLPGITKVPVGDQPEDIAEQIKELVLKYIENPNNSI		179
H.sapiens	NNKGVSPPEIHLKIFSPNVNLTLVDLPGMTKVPVGDQPKDIELQIRELILRFISNPNSI		180
	::***:***:***:***:***:***:***:***:***:***:***:***:***:***		
D.melanogaster	ILAVTAANTDMATSEALKLAKDVDPGRRTLAVTKLMDLMDAGTDAIDILCGRVIPVKLG		239
H.sapiens	ILAVTAANTDMATSEALKISREVDPDGRRTLAVTKLMDLMDAGTDAMDVLMGRVIPVKLG		240
	*****:*****:*****:*****:*****:*****:*****:*****:*****:*****		
		Dynamain central region domain	
D.melanogaster	IIIVMNRSQKDIMDQKHIDDQMKDEAAFLQRKYPTLATRNGTPYLAKTLNRLMHHIRDC		299
H.sapiens	IIIGVNRSQLDINNKKSVTDSIRDEYAFLLQKKYPSLANRNGTKYLARTLNRLMHHIRDC		300
	::***:***:***:***:***:***:***:***:***:***:***:***:***:***		
D.melanogaster	LLDLKTRVNIMATQFQSLLSYGEDVSDKSQTLQIITKFSSAYCCTIEGTARNIETTEL		359
H.sapiens	LLDLKTRINVLAQYQSLLSYGEVPDDKSATLLQLITKFATEYCNTIEGTAKYIETSEL		360
	::***:***:***:***:***:***:***:***:***:***:***:***:***:***		
D.melanogaster	CGGARMGYIFHETFGRTLDSIHPLAGLSKMDILTAINRATGPRPALFVPEVSFELLVKRQ		419
H.sapiens	CGGARICYIFHETFGRTLESVDPLGGLNTDILTAINRATGPRPALFVPEVSFELLVKRQ		420
	*****:*****:*****:*****:*****:*****:*****:*****:*****:*****		
D.melanogaster	IRREEPSLRCVELIHEEMQRIVQHCNEVQQEMLRFPKLHEKIVDVVTQLRRRLPHTN		479
H.sapiens	IKRLEEPSLRCVELVHEEMQRIIQHCSNYSTQELLRFPKLHDAIVEVVTCLLRKLPVTN		480
	*****:*****:*****:*****:*****:*****:*****:*****:*****:*****		
D.melanogaster	VMVENIVAIELAYINTKHPDFHKDAALVPSLLKTDSDPYSQINLGQRRANTPRNHMSPQI		539
H.sapiens	EMVHNLVAIELAYINTKHPDFADACGLMNNNIEEQRRNRLARELP-----SAVSRDK		532
	::*****:*****:*****:*****:*****:*****:*****:*****		
D.melanogaster	SSHSAGSQPQQQPPQPQNSSQQYSQV-HEQNHVAENSTPSMASTWLSNLPAPTRPD		598
H.sapiens	SSKVPSPALAPASQE-PSPAASAEADGKVASGGGGVGDGVQEPTTGNWRGMLKTSKAE---		588
	**:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*.:.:*		
	Conserved Phosphorylation sites	GTPase effector domain	
D.melanogaster	SIENSTNTPVHNIVSPV--KPVNLLPDVPANHNPRRITPKEQKDCDVIEHLIKSYFYI		656
H.sapiens	ELLAEKSKPIPIPPASPKGHAVNL-LDVP-VPVARKISAREQFDCEVIERLIKSYFLI		646
	:.:..:..:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***:***		
D.melanogaster	VRKSIQDSVPKAIMHFLVNYVKDNLQSELVTHLYKSDKAETLLNESDHIAVRRKEAADML		716
H.sapiens	VRKNIQDSVPKAVMHFLVNHVKDTLQSELVGQLYKSSLLDDLLTESDMAQRKKEAADML		706
	:**:*****:*****:*****:*****:*****:*****:*****:*****		
D.melanogaster	KALTRANHIISEIRETHMW 735		
H.sapiens	KALQGASQIIAEIRETHLW 725		
	.:.::***:***:***:***:***:***:***:***:***:***:***:***:***:***:***		

(a)



(bi)



(bii)

Figure 1. Drp1 is evolutionarily conserved between *Drosophila* and humans. (a) Clustal Omega multiple sequence alignment of *D. melanogaster* Drp1 (NP_608694.2) protein with the *H. sapiens* (NP_001265392.1) shows evolutionarily conserved domains identified using the NCBI Conserved Domain Database (CDD) and further confirmed by the Eukaryotic Linear Motif (ELM) resource. The two well documented phosphorylation sites are identified, S606 and S627 in dynamin-1-like protein (DLP-1) isoform 4 of *H. sapiens*; and S616 and T637 in Drp1 of *D. melanogaster*. The asterisks indicate the residues that are identical; the colons indicate the conserved substitutions; and the dots indicates 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; and green indicates basic with hydroxyl or amine groups. (bi) The original Dynamin-1 like protein (DLP-1) structure of *H. sapiens* (NP_001265392.1) from the NCBI structure database. (bii) The Phyre2 web portal for protein modelling, prediction and analysis mediated the development of a model of the Drp1 protein of *D. melanogaster* (NP_608694.2) from a 76% identical protein with a confidence of 100%. The N terminus is coloured in Magenta; C terminus is coloured in Red and a consensus ATG8 binding region at N terminus is coloured in orange.

2.2. The Overexpression and Inhibition of Drp1 with Ddc-Gal4^{4.3D}

In these experiments, the control *Ddc-Gal4^{4.3D}; UAS-lacZ* critical class males were determined to have a median lifespan of 68 days (n=340). The overexpression of *Drp1* by the *Ddc-Gal4* transgene results in a decreased lifespan of 56 days in 314 flies, much lower compared to the control as determined by log-rank (Mantel-Cox) test with a P-value at <0.0001 (Figure 2A). Inhibition of *Drp1* by two distinct RNAi transgenes, via the *UAS-Drp1-RNAi1* and *UAS-Drp1-RNAi2* directed by the *Ddc-Gal4* transgene, results in median lifespans of 70 (n=377) and 72 days (n=323), respectively; very similar to the control (Figure 2A) as determined by log-rank (Mantel-Cox) test with P-value 0.0566 and 0.0213. The non-linear fitting of the climbing curve shows that altering the *Drp1* expression have compromised the climbing ability phenotype compared to control at 95% CI (P- value <0.0001) (Figure 2B) (n=50).

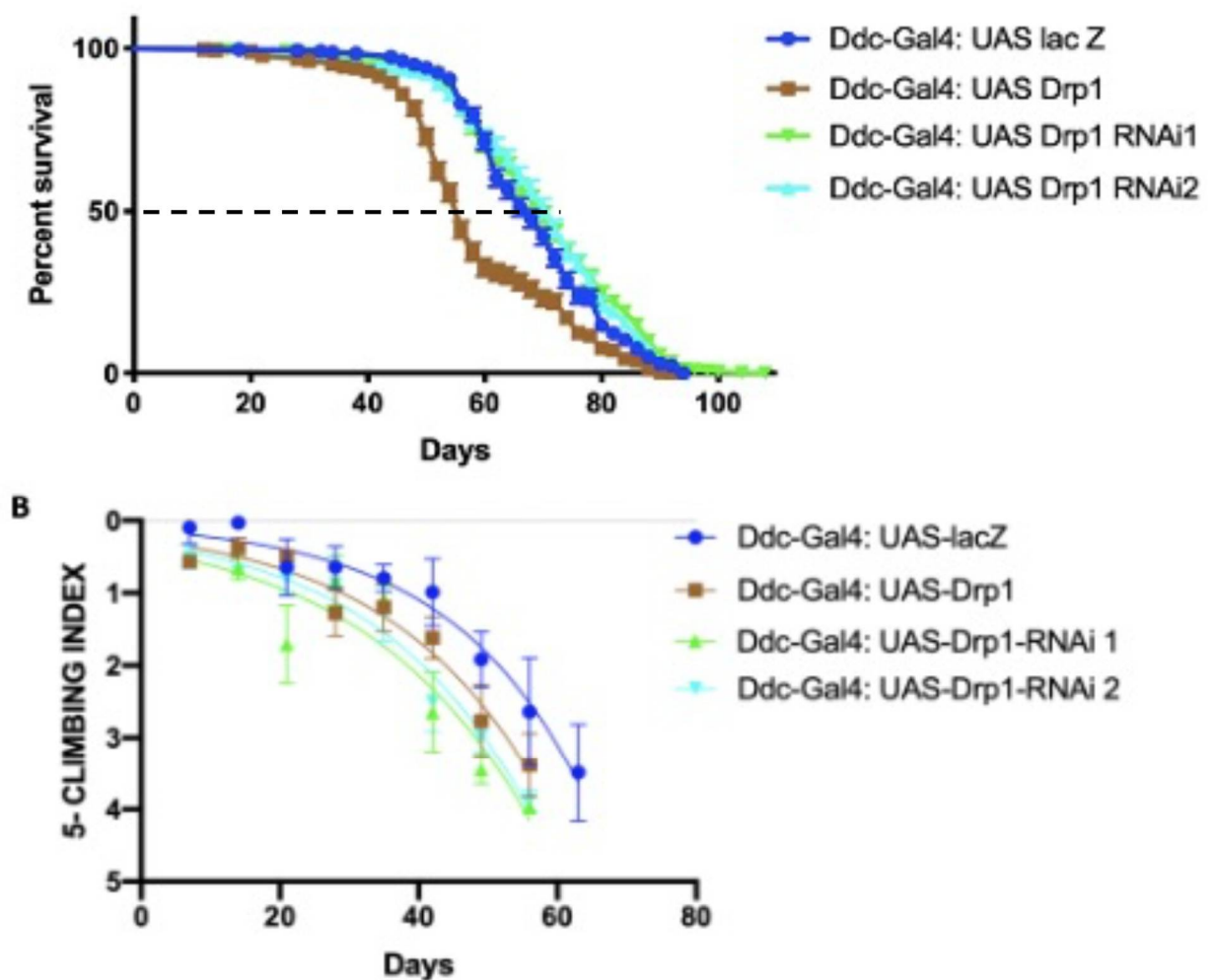
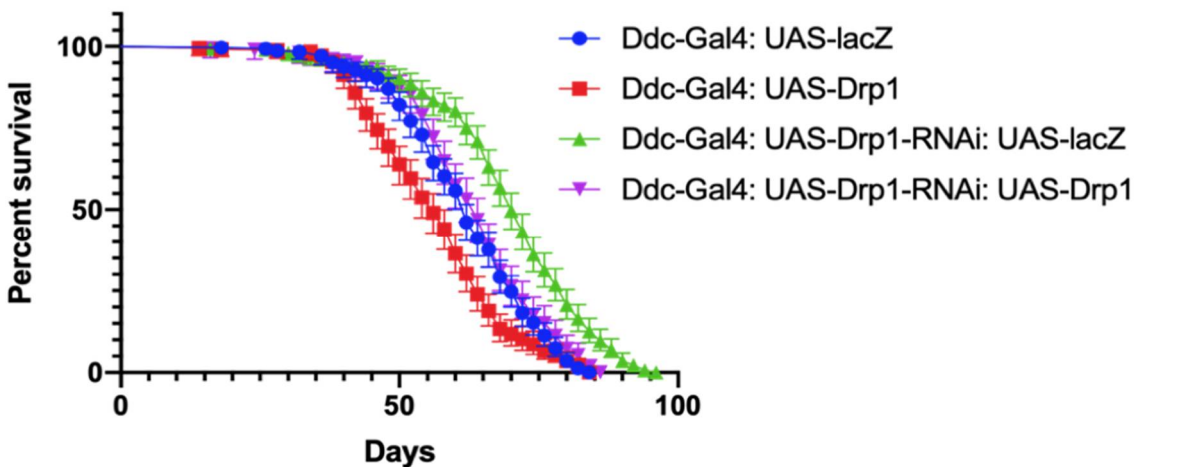


Figure 2. Altered *Drp1* expression under the control of *Ddc-Gal4^{4.3D}* influences the survival and climbing ability of flies. (a) The GraphPad prism8 generated graph of the longevity assay for the expression of *Drp1*, *Drp1* RNAi's under the control of *Ddc-Gal4* transgene. The directed expression results in decreased median lifespan of 56 days compare to 68 days of control calculated by Log-rank Mantel Cox test, with Bonferroni correction. The inhibition of *Drp1* under the control of *Ddc-Gal4* transgene results in lifespan of 70 days with *UAS-Drp1-RNAi1* and 72 days with *UAS-Drp1-RNAi2* compared 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 *Drp1*, *Drp1* RNAi's and control. The climbing ability of *Drp1* overexpression and *Drp1* RNAi's flies have decreased compared to control as determined in nonlinear fitting of the climbing curve by 95% confidence interval (p-value <0.0001).

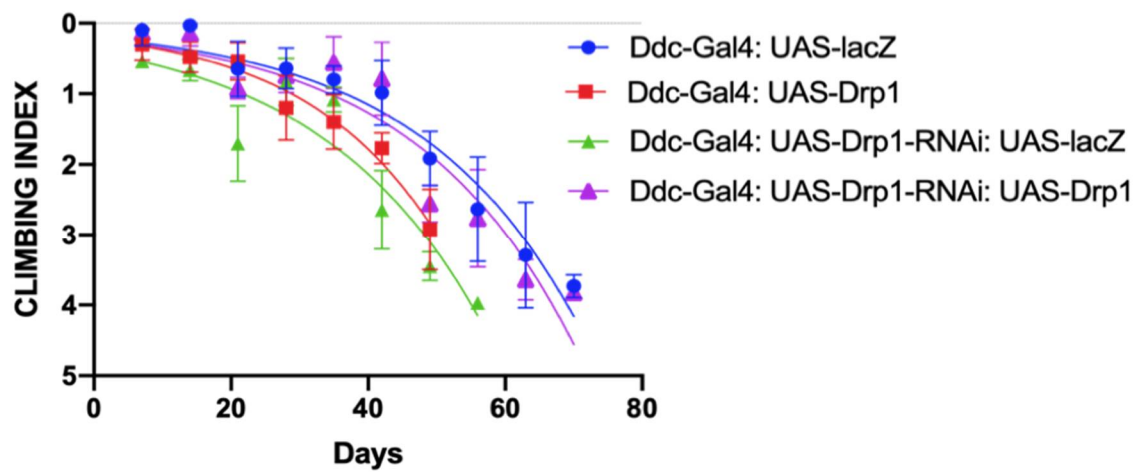
2.3. Alteration of the Expression of Drp1 and Drp1-RNAi Directed by Ddc-Gal4^{4.3D}

In these experiments, the control *Ddc-Gal4*^{4.3D}; *UAS-lacZ* critical class males were determined to have a median lifespan of 62 days (n = 308). The directed inhibition of *Drp1* by the *Ddc-Gal4* transgene results in a greater median lifespan of 70 days in 321 flies compared to the control as determined by log-rank (Mantel-Cox) test with P-value <0.0001 (Figure 3). In contrast, the directed expression of *Drp1* by the *Ddc-Gal4* transgene results in a reduced median lifespan of 56 days in 255 flies compared to the control as determined by log-rank (Mantel-Cox) test with P-value <0.0001 (Figure 3A). Furthermore, the *Ddc-Gal4* *UAS-Drp1-RNAi* *UAS-lacZ* critical class males have a median lifespan of 70 days in 310 flies. The overexpression of *Drp1* along with *UAS-Drp1-RNAi* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4*; *UAS-Drp1-RNAi*; *UAS-Drp1*) has a median lifespan of 64 days, similar to control (*Ddc-Gal4*; *UAS-lacZ*) with a P value of 0.0633 as determined by the Log-rank Mantel-Cox test with a Bonferroni correction (Figure 3A). The *Ddc-Gal4* *UAS-Drp1* *UAS-lacZ* critical class males have a median lifespan of 58 days in 294 flies. The inhibition of *Drp1* along with *UAS-Drp1* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4*; *UAS-Drp1*; *UAS-Drp1-RNAi*) has a median lifespan of 64 days (n=327), similar to control (*Ddc-Gal4*; *UAS-lacZ*) with a P value of 0.0582 as determined by the Log-rank Mantel-Cox test with a Bonferroni correction (Figure 3C). The non-linear fitting of the climbing ability curve shows the *Drp1* expression and inhibition both have compromised the climbing ability phenotype compared to control at 95% CI (p <0.0001) (Figure 3B & D). The climbing ability curve of *Ddc-Gal4* *UAS-Drp1-RNAi* *UAS-Drp1* and *Ddc-Gal4* *UAS-Drp1* *UAS-Drp1-RNAi* is very close to the control (*Ddc-Gal4*; *UAS-lacZ*) as determined by the non-linear fitting of the climbing curve at a 95% CI at P-value 0.2752 and 0.0589, respectively. The co-expression of *Drp1-RNAi* along with *Drp1* overexpression in flies has resulted in the phenotypes that are similar to the control and suggests that these phenotypes are primarily due the changes in expression of *Drp1*.

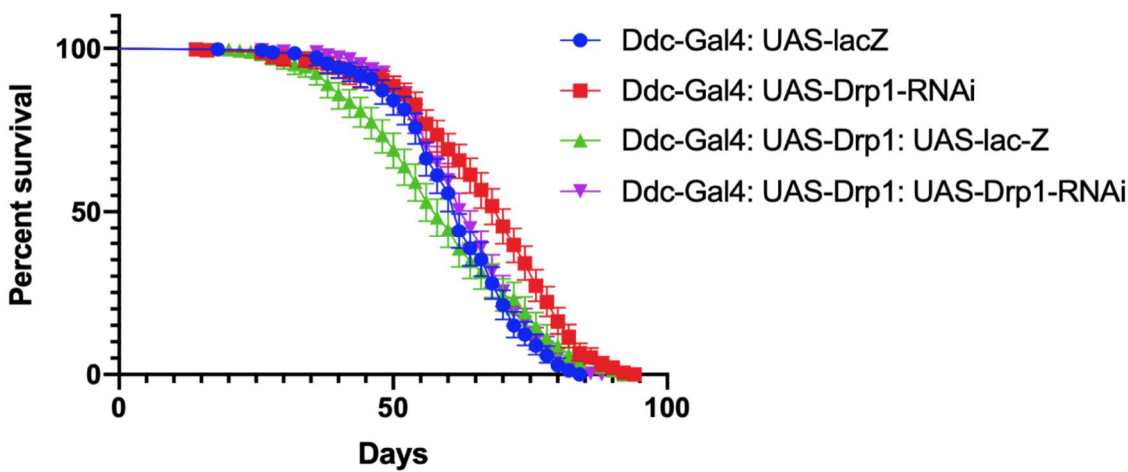
A)



B)



C)



D)

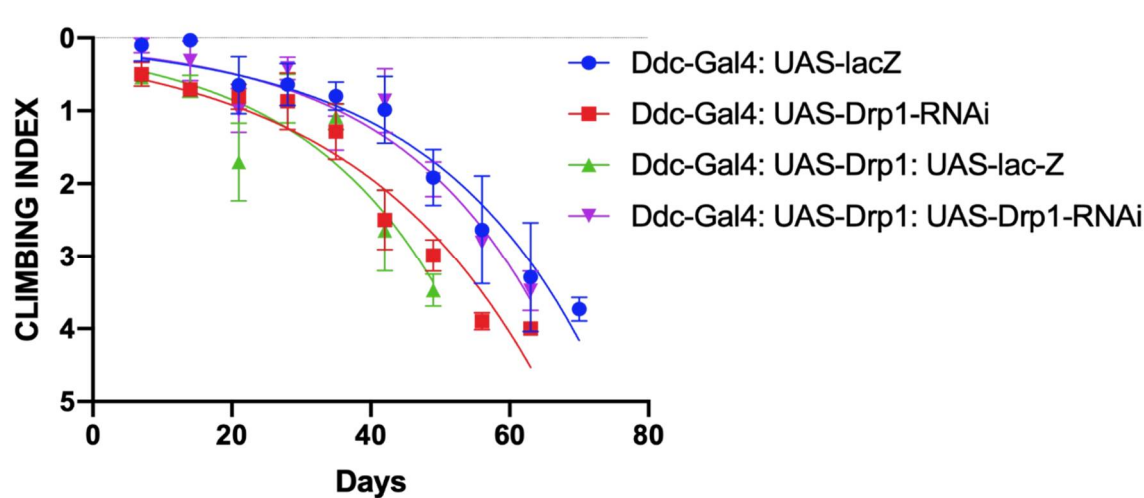


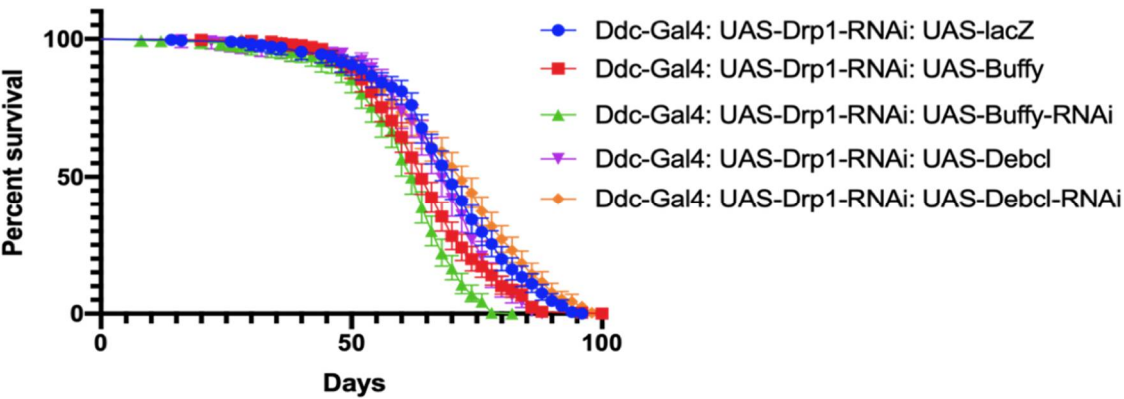
Figure 3. The expression of *Drp1-RNAi*, directed by *Ddc-Gal4^{4.3D}*, can increase median lifespan and decrease climbing. (a) In control, *Ddc-Gal4^{4.3D}* *UAS-lacZ* critical class males resulted in a median life span of 62 days (n=308). Expression of *Drp1* in *Ddc-Gal4^{4.3D}* resulted in a median life span of 56 days (n=310), much lower than the *lacZ*-expressing control; expression of *Drp1* in *Ddc-Gal4^{4.3D}* *UAS-Drp1*-

RNAi resulted in a median life span of 64 days (n=250) very similar to control (Ddc/lacZ) as determined by the Log-rank Mantel-Cox test (p value=0.0633) with Bonferroni correction. The graph of the longevity assay was generated by GraphPad prism8. (b) The *Ddc-Gal4^{4.3D}* flies express *UAS-lacZ* in control flies. The climbing abilities of *Ddc-Gal4^{4.3D}* *UAS-Drp1* expressing flies have decreased compared to control as determined in the non-linear fitting of the climbing curve by a 95% confidence interval (p<0.0001). The flies' climbing ability expressing *Drp1* in *Ddc-Gal4^{4.3D}* *UAS-Drp1-RNAi* transgene is similar to control as determined in the non-linear fitting of the climbing curve by a 95% confidence interval at P value=1.309. The graph of longevity assay was generated by GraphPad prism8 non-linear regression curve. (c) In control, *Ddc-Gal4^{4.3D}* *UAS-lacZ* critical class males resulted in a median life span of 62 days (n=308). Expression of *Drp1-RNAi* in *Ddc-Gal4^{4.3D}* resulted in a median life span of 70 days (n=321), much higher compared to the control; expression of *Drp1-RNAi* in *Ddc-Gal4^{4.3D}* *UAS-Drp1* resulted in a median life span of 64 days (n=327), very similar to control (Ddc/lacZ) as determined by the Log-rank Mantel-Cox test (p value=0.0582) with Bonferroni correction. The graph of the longevity assay was generated by GraphPad prism8. (d) The *Ddc-Gal4^{4.3D}* flies express *UAS-lacZ* in control flies. The climbing abilities of *Ddc-Gal4^{4.3D}* *UAS-Drp1-RNAi* expressing flies have decreased compared to control as determined in the non-linear fitting of the climbing curve by a 95% confidence interval (p<0.0001). The flies' climbing ability expressing *Drp1-RNAi* in *Ddc-Gal4^{4.3D}* *UAS-Drp1* transgene is similar to control (Ddc/lacZ) as determined in the non-linear fitting of the climbing curve by a 95% confidence interval at p value=0.0027. The graph of longevity assay was generated by GraphPad prism8 non-linear regression curve.

2.4. Alteration of the Expression of *Buffy* and *Debcl* in Combination with *Drp1* Directed by the *Ddc-Gal4^{4.3D}* Transgene

The control *Ddc-Gal4^{4.3D}*; *UAS-Drp1*; *UAS-lacZ* critical class males were determined to have a median lifespan of 58 days (n=282). The overexpression of *Buffy* along with *UAS-Drp1* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4*; *UAS-Drp1*; *UAS-Buffy*) has a median lifespan of 68 days (n=255), much higher compared to control with a P value of <0.0001 as determined by log-rank (Mantel-Cox) test with a Bonferroni correction. The inhibition of *Buffy* along with *UAS-Drp1* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4*; *UAS-Drp1*; *UAS-Buffy-RNAi*) has a median lifespan of 52 days (n=274), much less compared to the control (Figure 4A) with a P value 0.0125 as determined by log-rank Mantel-Cox test with a Bonferroni correction. The overexpression of *Buffy* in neurons rescued the early onset of impairment in the climbing ability of *Ddc-Gal4*; *UAS-Drp1* flies. The non-linear fitting of the climbing curve shows *Buffy* overexpression has rescued the climbing ability defect compared to control at 95% CI (p <0.0001) (Figure 4B). The inhibition of *Buffy* by *Ddc-Gal4* *UAS-Drp1*; *UAS-Buffy-RNAi* further contributes to loss of the climbing ability throughout the life of critical class flies compared to control at 95% CI at a P-value 0.0125 (n=50) (Figure 4B).

A)



B)

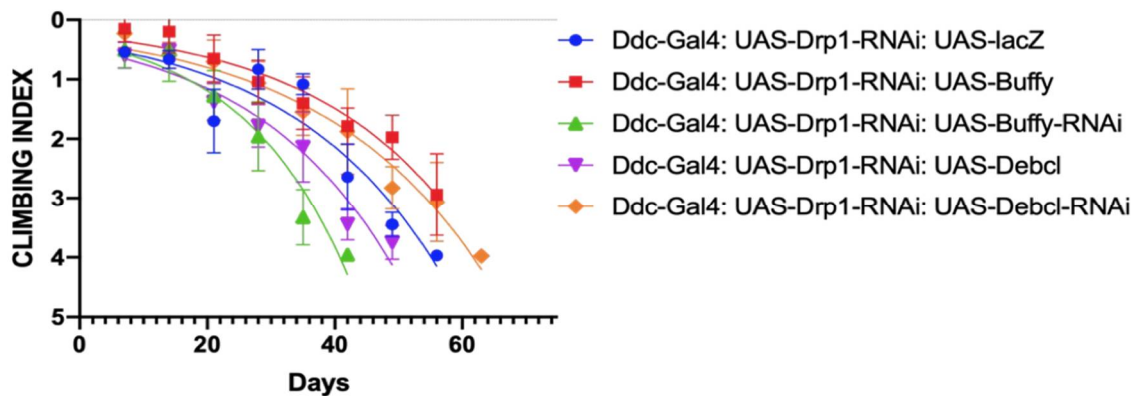


Figure 4. Altered expression of *Buffy* and *Debcl* can enhance and suppress climbing ability in *Drp1* over-expression flies. (a) In control, *Ddc-Gal4^{4.3D}; UAS-Drp1 UAS-lacZ* critical class males resulted in a median life span of 58 days (n=282). The overexpression of *Buffy* results in a median lifespan of 68 days (n=375) compares to 58 days of control (P value=0.0002); the inhibition of *Buffy* directed by the *Ddc-Gal4^{4.3D} UAS-Drp1* transgene results in the median lifespan of 52 (n=274), much less compared to control, determined by Log-rank Mantel-Cox test at P-value <0.0001, with Bonferroni correction. The overexpression of *Debcl^{EY05743}* results in a median lifespan of 60 days (n=331) similar to 58 days of control determined by Log-rank Mantel-Cox test at P-value 0.3293; the inhibition of *Debcl* directed by the *Ddc-Gal4 UAS-Drp1* transgene result in the median lifespan of 66 (n=303); much higher than control, determined by Log-rank Mantel-Cox test at P value 0.0057, with Bonferroni correction. (b) The GraphPad prism8 generated graph of the climbing abilities of *Ddc-Gal4 -Drp1* flies with the expression of *Buffy*, *Buffy-RNAi*, *Debcl^{EY05743}*, *Debcl-RNAi^{v47515}* and control. The climbing abilities of flies overexpressing *Buffy* have rescued compared to control as determined in the climbing curve's non-linear fitting by a 95% confidence interval (p<0.0001). The climbing ability of the flies was further weakened by the expression of *UAS-Buffy-RNAi* as determined in the non-linear fitting of the climbing curve by a 95% confidence interval at a p-value 0.0125 and 0.03293 respectively (n=50). The climbing abilities of flies expressing *Debcl-RNAi^{v47515}* has rescued compared to control as determined by the non-linear fitting of the climbing curve by a 95% confidence interval (p value=0.0057). The graph of longevity assay was generated by GraphPad prism8 non-linear regression curve.

The overexpression of *Debcl* along with *UAS-Drp1* under the direction of *Ddc-Gal4* transgene (*Ddc-Gal4; UAS-Drp1; UAS-Debcl^{EY05743}*) has a median lifespan of 60 days (n=331 flies), similar to the control (Figure 4A) with a P value at 0.0114 as determined by log-rank Mantel-Cox test with Bonferroni correction. The inhibition of *Debcl*, along with *UAS-Drp1* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4; UAS-Drp1; UAS-Debcl-RNAi^{v47515}*) has a median lifespan of 66 days (n=303), much higher compared to the control (Figure 4A) with a P value at 0.0004 as determined by log-rank Mantel-Cox test with Bonferroni correction. The non-linear fitting of the climbing curve shows *Debcl* overexpression has no change in the climbing ability defect compared to control at 95% CI (p=0.3293) (Figure 1.4B). The inhibition of *Debcl* by *Ddc-Gal4 UAS-Drp1; UAS-Debcl-RNAi^{v47515}* has rescued the climbing ability throughout the life of critical class flies compared to control at 95% CI at a P-value 0.0057 (n=50) (Figure 1.4B).

2.5. Altering the Expression of *Buffy* and *Debcl* along with *Drp1-RNAi* by *Ddc-Gal4^{4.3D}* Transgene

The control *Ddc-Gal4^{4.3D}; UAS-Drp1-RNAi; UAS-lacZ* critical class males were determined to have a median lifespan of 70 days (n=323). The overexpression of *Buffy* along with *UAS-Drp1-RNAi* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4; UAS-Drp1-RNAi; UAS-Buffy*) has a median lifespan of 64 days (n=308), much lower compared to control with a P value of <0.0001 as determined by log-rank (Mantel-Cox) test with a Bonferroni correction. The co-inhibition of *Buffy* and *Drp1* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4; UAS-Drp1-RNAi; UAS-Buffy-RNAi*) has a median lifespan of 62 days (n=273), much less compared to the control (Figure 5A) with a P value at <0.0001 as determined by log-rank Mantel-Cox test with a Bonferroni correction. The non-linear fitting of the

climbing curve shows *Buffy* overexpression has rescued the climbing ability defect compared to control at 95% CI ($p < 0.0001$) (Figure 5B). The inhibition of *Buffy* by *Ddc-Gal4 UAS-Drp1-RNAi; UAS-Buffy-RNAi* further contributes to loss of the climbing ability throughout the life of critical class flies compared to control at 95% CI at a P-value < 0.0001 ($n=50$) (Figure 5B).

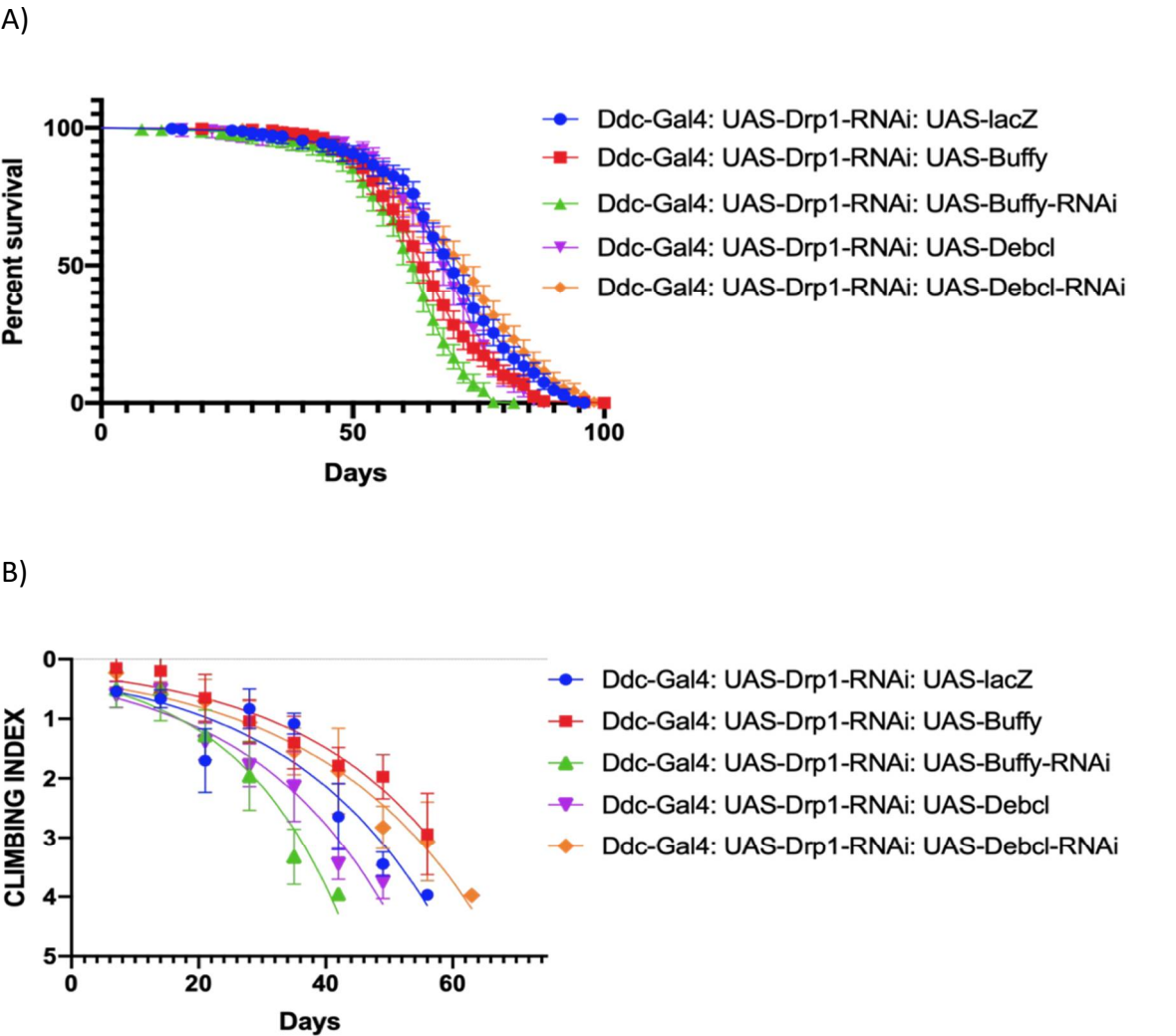


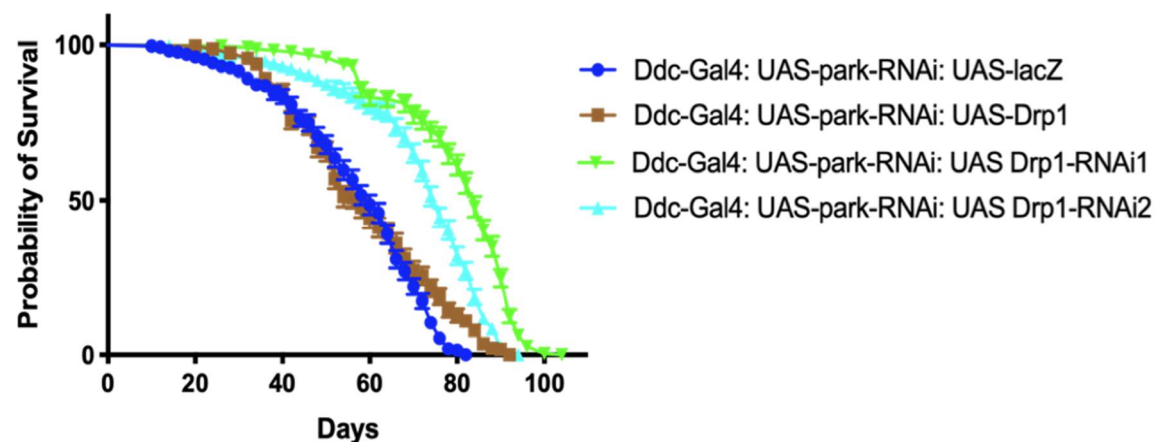
Figure 5. Altered expression of *Buffy* and *Debcl* can enhance and suppress climbing ability in *Drp1* loss of function flies. (a) In control, *Ddc-Gal4^{4.3D}; UAS-Drp1* transgene results in the median lifespan of 62 ($n=273$), determined by Log-rank Mantel-Cox test at P-value < 0.0001 , with Bonferroni correction. The overexpression of *Debcl^{EY05743}* results in a median lifespan of 68 days ($n=331$) much higher compared to control as determined by Log-rank Mantel-Cox test at P-value 0.0003; the inhibition of *Debcl* directed by the *Ddc-Gal4; UAS-Drp1* transgene result in the median lifespan of 72 ($n=303$); similar to control, determined by Log-rank Mantel-Cox test at p-value 0.021, with Bonferroni correction. (b) The GraphPad prism8 generated graph of the climbing abilities of *Ddc-Gal4 UAS-Drp1* flies with the expression of *Buffy*, *Buffy-RNAi*, *Debcl^{EY05743}*, *Debcl-RNAi^{v47515}* and control. The climbing abilities of flies overexpressing *Buffy* have rescued compared to control as determined in the climbing curve's non-linear fitting by a 95% confidence interval ($p < 0.0001$). The climbing ability of the flies has further diminished through the expression of *UAS-Buffy-RNAi* and *UAS-Debcl^{EY05743}* as determined in the non-linear fitting of the climbing curve by a 95% confidence interval at a p-value 0.0004 and 0.0002 respectively ($n=50$). The climbing abilities of flies expressing *Debcl-RNAi^{v47515}* has rescued compared to control as determined by the non-linear fitting of the climbing curve by a 95% confidence interval (p value < 0.0001). The graph of longevity assay was generated by GraphPad prism8 non-linear regression curve.

The overexpression of *Debcl* along with *UAS-Drp1-RNAi* under the direction of *Ddc-Gal4* transgene (*Ddc-Gal4; UAS-Drp1-RNAi; UAS-Debcl*^{EY05743}) has a median lifespan of 68 days (n=156 flies), similar to the control (Figure 1.5A) with a P value at 0.0003 as determined by log-rank Mantel-Cox test with Bonferroni correction. The inhibition of *Debcl*, along with *UAS-Drp1-RNAi* under the direction of the *Ddc-Gal4* transgene (*Ddc-Gal4; UAS-Drp1-RNAi; UAS-Debcl-RNAi*^{v47515}) results in a median lifespan of 72 days (n=321), higher compared to the control (Figure 5A) with a P value at 0.0211 as determined by log-rank Mantel-Cox test with Bonferroni correction. The non-linear fitting of the climbing curve shows *Debcl* overexpression has further increased the climbing ability defect compared to control at 95% CI (p=0.0004) (Figure 5B). The inhibition of *Debcl* by *Ddc-Gal4 UAS-Drp1-RNAi; UAS-Debcl-RNAi*^{v47515} has rescued the climbing ability throughout the life of critical class flies compared to control at 95% CI at a P-value 0.0211 (n=50) (Figure 5B).

2.6. The Altered Co-Expression of *Drp1* along with *Park-RNAi* with *Ddc-Gal4*^{4.3D}

The loss of function of *park* has led to the establishment of a number of *Drosophila* models of PD. The *Ddc-Gal4*^{4.3D} *park-RNAi* *UAS-lacZ* critical males were determined to have a median lifespan of 60 days (n=259). The overexpression of *UAS-Drp1* along with *UAS-park-RNAi* under the direction of the *Ddc-Gal4* transgene has a median life span of 58 days (n=258), similar to the control with a P-value of 0.0026. The inhibition of *Drp1* by the two *RNAi* transgenes, *UAS-Drp1-RNAi1*^{IF02762} and *UAS-Drp1-RNAi2*^{HMC03230}, results in much longer median lifespans of 84 (n=283) and 76 days (n=277) respectively, compared to the control (Figure 6A) as determined by log-rank (Mantel-Cox) test with a P-value at <0.0001. The overexpression of *Drp1* by *Ddc-Gal4* along with *park-RNAi* slightly increases the climbing ability over time. However, the locomotor activity of the critical classes with the directed expression of the *Drp1-RNAi* transgenes are rescued compared to control (Figure 6B).

A)



B)

3 A

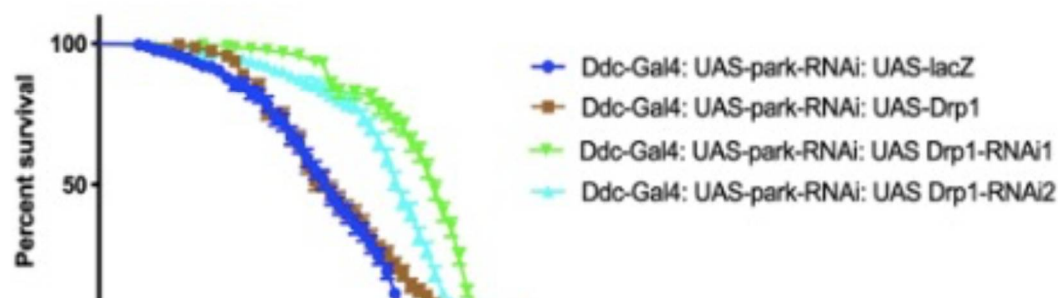


Figure 6. Inhibition of *Drp1* expression enhances lifespan and climbing ability of *parkin* loss of function in *Ddc-Gal4^{4.3D} park-RNAi* flies. (a) The graph of longevity assay generated by GraphPad prism8 with altered *Drp1* expression in *Ddc-Gal4 park-RNAi* expressing flies. The overexpression results in median lifespan of 58 days similar to 60 days of control (*lacZ/park-RNAi*) determined by Log-rank Mantel-Cox test, with Bonferroni correction. The inhibition of *Drp1* in neurons using *Ddc-Gal4* transgene along with *park-RNAi* results in increased lifespan of 84 days with *UAS-Drp1-RNAi1* and lifespan of 76 days with *UAS-Drp1-RNAi2* compare to 60 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 *Drp1*, *Drp1 RNAi* and control. The climbing abilities of and *Drp1 RNAi* flies has rescued compared to control as determined in non-linear fitting of the climbing curve by 95% confidence interval.

4. Discussion

The protein product of the *Drp1* gene, along with the participation of other mitochondrial protection proteins, is involved in the processes of mitochondrial fission, apoptosis, and mitophagy. Excessive mitochondrial fragmentation can be associated with dysfunctional metabolic diseases whereas a “hyper-fused” mitochondrial network can serve to protect cells from metabolic insult and autophagy [35]. In the skeletal muscle of mice, *Drp1* overexpression can cause a severe impairment of post-natal muscle growth as the production of protein may become attenuated and growth hormone pathways may be down regulated [36]. Conditions of high fat and/or high glucose levels can cause excessive oxidative stress along with mitochondrial fragmentation as mediated by the *Drp1* protein [37,38]. These phenotypes are similar to the increased activity of *Drp1* as observed with Cos and PC12 cells [39]. In humans, protein kinase A (or PKA) can phosphorylate and inactivate the pro-apoptotic Bcl-2 family member protein Bad [40] and the *Drp1* protein [41] in a complex effort to promote cell survival. The effect of *Drp1* overexpression and consequently excessive mitochondrial fragmentation can be toxic to many physiological processes.

The Bcl-2 family proteins assist the pro-fission activity of the *Drp1* protein during apoptosis in nematodes and mammals [42]. However, in non-apoptotic cells of mammals, the Bcl-2 family proteins have both pro-fission and pro-fusion activities. The overexpression of *Drp1* in selected neurons along with the overexpression of *Buffy* or the inhibition of *Debcl* has resulted in an increase in median lifespan and of the ability to climb over the increased lifespan. In complementary experiments, *Buffy* inhibition and *Debcl* overexpression have resulted in reduced lifespans accompanied with impaired climbing abilities, consistent with the conclusion that *Buffy* can function as the antithesis of *Debcl* [43]. The rescue of the *Drp1* expression phenotype is in accordance with the role of the *Buffy* protein as “the guardian of mitochondria”. As proteins, *Buffy* can interact with *Debcl* to inhibit *Debcl*-induced cell death. As a mechanism, this process could be due to decreased activity of the *Debcl* protein to influence cooperation with *Drp1* in the promotion of cell death [20]. The pro-apoptotic *Debcl* protein acts to induces apoptosis through a caspase-independent mechanism that triggers the release of Cytochrome C [44] in an activity that resembles the loss of *Drp1* [9]. The overexpression of *Debcl* and *Drp1* together in selected neurons does not alter the phenotype generated by overexpression of *Drp1* without *Debcl*. This may not be surprising as *Drp1* and *Debcl* functions seem to cooperate to promote apoptosis [20]. Indeed, this and earlier studies have demonstrated that *Drp1* can play various roles

in mitochondrial fragmentation and apoptosis, to act in concert with anti- and pro-survival proteins, dependent upon the stimuli.

The directed inhibition in *Drosophila melanogaster* of *Drp1* in a subset of neurons results in an age-dependent loss in climbing ability, a phenotype strongly associated with the modelling of ND in flies. The overexpression of *Buffy* in neurons that co-express *Drp1-RNAi* led to a decrease in the median lifespan accompanied with a rescue of the impaired locomotor ability. The recovery in age-dependent climbing ability over time may be evidence of a complicated regulatory relationship. A study shows *Drp1* inhibition reduces the total accumulation of pro-apoptotic Bcl-2 protein, Bax, on mitochondria outer membrane in HeLa cell lines [12]. This intermediate phenotype was not expected but may be important in the determination of the pathology of neurological diseases [45,46]. The inhibition of anti-apoptotic *Buffy* or overexpression of pro-apoptotic *Debcl* enhanced the loss of *Drp1*-induced phenotype. The interaction of Bax with Drp1 in mammals seems to evolve from the *Debcl* and Drp1 protein interactions during the course of evolution. Drp1 protein interacts directly and indirectly with Bcl-2 family protein to facilitate MOMP in apoptotic cells [42]. Overall, we believe that we have established that *Buffy* confers survival advantage to flies overexpressing *Drp1* and provides a partially rescued intermediate phenotype in flies with a loss of *Drp1* function.

The *park* gene is crucial to the function of *Pink1*-dependent mitochondrial mitophagy. The loss of the parkin protein is a cause of great cellular stress as a major mechanism that controls mitophagy is compromised. Under normal circumstances, the *park*-encoded ubiquitin ligase recruits the Drp1 protein to mediate mitochondrial fragmentation during mitophagy [23,47]. In mouse embryonic fibroblasts, loss of *park* does not produce visible effects upon the net number of mitochondria [48]. Furthermore, the loss of *park* along with the loss of *Drp1* increases the number of mitochondria by threefold, which can be interpreted that *park* controls mitochondria fragmentation in a *Drp1* knockout background. Therefore, *park* may regulate, negatively, the *Drp1*-independent mitochondrial division. Alternatively, the parkin protein may direct the ubiquitination of the mitochondrial localised Drp1 protein to lead its proteasomal mediated degradation [26] and, hence, the activity of Drp1 protein on mitochondria is higher in the *Drp1-parkin* co-inhibition state as compared to the inhibition of Drp1 alone. In these experiments, the critical class flies that have the directed co-expression of *Drp1-RNAi* and *park-RNAi* inhibitory transgenes live longer than those that express *park-RNAi* and *Drp1-RNAi* under the *Ddc-Gal4* transgene individually. This support the hypothesis that the basic mechanism of PD-like phenotypes may be conserved among mammals [49,50] and the Diptera. This could be due to the establishment of an altered mitochondrial network to enhance homeostasis and benefit cellular health. The inhibition of *Drp1* has been shown to suppress *parkin* mutant phenotypes which suggests a role for this protein in 1) in a pathway that regulates mitochondrial health and integrity and 2) in interactions with mitochondrial proteins. Regardless of the underlying mechanism, this work provides strong evidence of *Drp1* as an important therapeutic target for maintaining mitochondrial health.

5. Conclusions

Closely associated with cell death pathways in neurons, recently *Drp1* has been associated with ALS, Parkinson disease and age-related disease. Our studies demonstrate that the overexpression and inhibition of *Drp1* activity in selected neurons can phenocopy some neurodegenerative-like symptoms in *Drosophila* and, therefore, may represent a novel model of disease. Importantly, the decrease in lifespan and age-dependent loss in climbing ability observed with overexpression of *Drp1* in flies is “rescued” to near controls either by overexpression of *Buffy* or by *Debcl* inhibition. The age-dependent loss of climbing ability in flies expressing *Drp1-RNAi* can be rescued by *Buffy* overexpression or *Debcl-RNAi*-directed inhibition. The co-inhibition of the *parkin* with *Drp1* results in the rescue of the phenotypes observed, thus it is possible that *Drp1* and parkin can participate in cellular pathways that promote cell death. Future studies of these interactions will be required to chart out a pathway for *Drp1*, Bcl-2 and *parkin* interaction in *Drosophila* and, importantly, the molecular changes associated with the loss-of-function of these genes in the development, function and aging of neurons and of the organism.

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