Mitochondrial dynamics and aging: conditions and molecular mechanisms by which genetic alterations of mitochondrial fission and fusion genes promotes longevity

Annika Traa

Department of Neurology and Neurosurgery

McGill University, Montreal, Canada

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Abstract

Aging is the greatest risk factor for multiple chronic human pathologies, including diabetes, neurodegeneration, cancer and heart disease. Using *C. elegans* as a model organism, we investigate the cell and molecular mechanisms involved in longevity in order advance our understanding of the aging process and the mechanisms by which healthy aging can be promoted.

While the biology of aging is incompletely understood, accumulating evidence indicates that mitochondria play a significant role in the aging process. Multiple mitochondria exist in the form of an interconnected network within a single cell, where they undergo fission, to break away from the network, or fusion, to join the network. The dynamicity of the mitochondrial network is crucial for meeting the ever-changing metabolic and energy needs of the cell. Mitochondrial fission promotes the degradation and distribution of mitochondria, while mitochondrial fusion maintains mitochondrial function through the complementation of mitochondrial components. Age-associated mitochondrial network fragmentation is seen in both humans and *C. elegans* and is also a hallmark of multiple age-associated diseases, including neurodegeneration. In contrast, healthy long-lived humans and *C. elegans* are both reported to have more highly connected mitochondrial networks.

In this work, we find that disruption of the mitochondrial fission gene *drp-1* extends *C. elegans* lifespan. We hypothesize that inhibition of *drp-1* increases lifespan by decreasing mitochondrial fragmentation. To test this, we examine whether decreasing mitochondrial fragmentation through increasing mitochondrial fusion, rather than inhibiting mitochondrial fission, is sufficient to increase lifespan. We find that overexpression of mitochondrial fusion genes increases lifespan and resistance to stress, but without decreasing mitochondrial fragmentation. We also determine that overexpression of mitochondrial fusion genes increases activation of multiple stress response pathways, facilitating the expression of pro-longevity genes.

Additionally, we investigate the conditions and mechanisms by which disruption of *drp-1* increases lifespan. In examining how disruption of *drp-1* affects the longevity of already long-

lived mutants, we find that disruption of *drp-1* can drastically extend the lifespan of already long-lived *daf-2* insulin-like signaling mutants. We determine that disruption of *drp-1* during development is required for lifespan extension in *daf-2* mutants and that tissue-specific inhibition of *drp-1* in the neurons, muscle or intestine is not sufficient to extend *daf-2* lifespan. By contrast, we find that inhibition of *drp-1* during adulthood and in the neurons is most beneficial in wild-type animals. Although we find that disruption of *drp-1* increases mitochondrial network fusion in both *daf-2* and wild-type animals, we find that disruption of *drp-1* does not have the same effects on indicators of mitochondrial health in both *daf-2* and wild-type animals. In *daf*-2 animals, we report increased mitochondrial function and quality control while in wild-type animals we find increased levels of reactive oxygen species. Together, these findings suggest that disruption of *drp-1* increases wild-type and *daf-2* lifespan through distinct mechanisms.

Overall, this work demonstrates that altering the expression of mitochondrial fission and fusion genes in *C. elegans* can promote longevity. Furthermore, this work defines the conditions required for altered mitochondrial dynamics to increase lifespan and determines the effects on mitochondrial form and function.

Résumé

Le vieillissement est le plus grand facteur de risque pour de nombreuses pathologies humaines chroniques, notamment le diabète, la neurodégénération, le cancer et les maladies cardiaques. En utilisant *C. elegans* comme organisme modèle, nous étudions les mécanismes cellulaires et moléculaires impliqués dans la longévité afin de faire progresser notre compréhension du processus de vieillissement et des mécanismes par lesquels un vieillissement bonne santé peut être favorisé.

Tandis que la biologie du vieillissement est incomplètement comprise, des preuves de plus en plus nombreuses indiquent que les mitochondries jouent un rôle important dans le processus de vieillissement. De multiples mitochondries existent sous la forme d'un réseau interconnecté à l'intérieur d'une seule cellule, où elles subissent une fission, pour se détacher du réseau, ou une fusion, pour rejoindre le réseau. Le dynamisme du réseau mitochondrial est essentiel pour répondre aux besoins métaboliques et énergétiques en constante évolution de la cellule. La fission mitochondriale favorise la dégradation et la distribution des mitochondries, tandis que la fusion mitochondriale maintient la fonction mitochondriale par la complémentation des composants mitochondriaux. La fragmentation du réseau mitochondrial associée à l'âge est observée chez l'homme et chez *C. elegans* et est également une caractéristique de plusieurs maladies associées à l'âge, comme la neurodégénération. En plus, les humains et les *C. elegans* en bonne santé et ayant une longue durée de vie ont tous deux des mitochondries plus étroitement connectées.

Dans ce travail, nous constatons que la perturbation du gène de fission mitochondriale *drp-1* prolonge la durée de vie de *C. elegans*. Nous émettons l'hypothèse que la diminution de la fragmentation mitochondriale, plutôt que l'inhibition de *drp-1*, augmente la durée de vie. Pour tester cette hypothèse, nous examinons si l'augmentation de la fusion mitochondriale, plutôt que l'inhibition de la fission mitochondriale, est suffisante pour augmenter la durée de vie. Nous constatons que la surexpression des gènes de fusion mitochondriale augmente la durée de vie et la résistance au stress, mais sans diminuer la fragmentation mitochondriale.

De plus, nous étudions les conditions et les mécanismes par lesquels la perturbation de *drp-1* augmente la durée de vie. En examinant comment la perturbation de *drp-1* affecte la longévité de mutants ayant déjà une longue durée de vie, nous constatons que la perturbation de *drp-1* peut prolonger considérablement la durée de vie des mutants *daf-2* de la signalisation de type insuline ayant déjà une longue durée de vie. Nous déterminons que la perturbation de *drp-1* au cours du développement est nécessaire pour prolonger la durée de vie des mutants *daf-2* et que l'inhibition de *drp-1* spécifiquement dans les neurones, les muscles ou l'intestin n'est pas suffisante pour prolonger la durée de vie des mutants *daf-2*. Cependant, nous constatons que l'inhibition de *drp-1* à l'âge adulte et dans les neurones est plus bénéfique chez les animaux de type sauvage. Alors que la perturbation de *drp-1* augmente la fusion du réseau mitochondrial chez les animaux *daf-2* et de type sauvage, nous constatons que la perturbation de *drp-1* n'a pas les mêmes effets sur les indicateurs de santé mitochondriale chez les animaux *daf-2* et de type sauvage. L'ensemble de ces résultats indique que la perturbation de *drp-1* augmente la durée de vie des animaux de type sauvage et *daf-2* par des mécanismes distincts.

Dans l'ensemble, ce travail démontre que la modification de l'expression des gènes de fission et de fusion mitochondriales chez *C. elegans* peut favoriser la longévité. En plus, ce travail définit les conditions requises pour que l'altération de la dynamique mitochondriale augmente la durée de vie et détermine les effets sur la forme et la fonction mitochondriales.

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Contributions to original scientific knowledge

This doctoral thesis work has generated novel contributions to scientific knowledge in the research topics of aging and mitochondria. Key findings from each manuscript-based chapter are described below. All together, these findings advance our understanding of how altering mitochondrial dynamics affects longevity. During my doctoral studies, I have also published 12 additional papers that are not included in this thesis, but which also contribute to advancing our understanding of the biology of aging.

Chapter 2: Overexpression of mitochondrial fusion genes enhances resilience and extends longevity

I. Overexpression of both mitochondrial fission and fusion genes increases *C. elegans* lifespan
II. Overexpression of mitochondrial fusion genes can increase lifespan without improving mitochondrial network connectivity
III. Overexpression of fusion genes enhances biological resilience and activates multiple stress response pathways
IV. Combined overexpression of fission and fusion genes extends lifespan to a lesser extent than overexpression of a single fission or fusion gene

Chapter 3: Developmental disruption of the mitochondrial fission gene *drp-1* extends the longevity of *daf-2* insulin/IGF-1 receptor mutant

I. Inhibition of *drp-1* in the neurons extends wild-type lifespan
II. Inhibition of *drp-1* during development extends *daf-2* lifespan
III. Inhibition of *drp-1* increases both mitochondrial and peroxisomal network connectivity in *daf-2* mutants
IV. Inhibition of *drp-1* increases mitochondrial function and quality control in *daf-2* mutants

Author contributions

This doctoral thesis was prepared by Annika Traa in accordance with the guidelines described on the McGill University website as well as the "Manuscript-based thesis" section of McGill University's initial thesis submission checklist. All work presented in this thesis was supervised by Dr. Jeremy Van Raamsdonk. Annika Traa is the first author of each manuscript presented in chapters 2 and 3. The detailed contributions of each author are listed below. Authors are designated by their initials.

Chapter 2: Overexpression of mitochondrial fusion genes enhances resilience and extends longevity. Annika Traa, Allison Keil, Zenith Rudich, Shusen Zhu, Jeremy M. Van Raamsdonk.

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AT performed all experiments pertaining to figures 1-5, S2 and S3. JMVR conducted lifespan assays for figure 6. SZ performed RT-qPCR for figure 7. AK performed experiments for figures S1, S3 and S4. ZR designed and obtained the overexpression worm strains. Figures were assembled by AT, AK and JMVR. The manuscript was written by AT and JMVR. AT, JMVR, AK and ZR edited the manuscript.

Chapter 3: Developmental disruption of the mitochondrial fission gene *drp-1* extends the longevity of *daf-2* insulin/IGF-1 receptor mutant. Annika Traa, Jeremy M. Van Raamsdonk.

JMVR performed lifespan assays for figures 1A, 5 and S3. AT performed all experiments for the rest of the figures. Figures were assembled by AT and JMVR. The manuscript was written and edited by AT and JMVR.

List of figures and tables

Chapter 2

Chapter 3

List of abbreviations

AD	Alzheimer's Disease
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
EMS	ethyl methane sulfonate
ER	endoplasmic reticulum
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide
FOXO	forkhead box transcription factors – class O
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTPase	guanosine triphosphate hydrolase enzyme
HD	Huntingtin's disease
IIS	insulin/IGF-1 signaling
IGF	insulin growth factor
IMM	inner mitochondrial membrane
LC3	microtubule-associated protein 1A/1B-light chain 3
MAPK	mitogen-activated protein kinase
MCU	mitochondrial calcium uniporter
mdivi-1	mitochondrial division inhibitor 1
MDV	mitochondrial-derived vesicle
Mff	mitochondrial fission factor
Mfn	mitofusin protein
MTS	mitochondrial targeting sequence
NADH	nicotinamide adenine dinucleotide
NGM	nematode growth media
OMM	outer mitochondrial membrane
PD	Parkinson's disease
PTPC	mitochondrial permeability transition pore complex

RNAi	RNA interference
ROS	reactive oxygen species
TF	transferrin protein
TFEB	transcription factor EB
TIM	inner membrane translocase protein
TOM	outer membrane translocase protein
TOR	target of rapamycin
SUMO	small ubiquitin-like modifier
UPR	unfolded protein response
VDAC	voltage-dependent anion channel

Chapter 1

General Introduction

1.1 Aging

Aging is characterized by a progressive decline in physiologic function resulting in impaired maintenance of biological homeostasis and increased susceptibility to disease and death. Although it is not known when aging starts during an organism's life, it is known that aging is a major risk factor for many chronic diseases that manifest later in life, including neurodegenerative disease, cardiovascular disease, diabetes and many cancers [1].

Currently the aging process is poorly understood. Thus, it is imperative that we advance our understanding of the aging process, as the average age of the world's population is increasing [2]. This urgency has given rise to a new focus on research that aims to improve our understanding of the cell and molecular mechanisms contributing to aging in order to determine how healthy aging can be promoted in humans [3].

Many theories on the mechanism of aging have been devised but a consensus has not been reached amongst the scientific community [4]. Harman's Free Radical Theory of Aging, for example, proposes that aging is caused by an accumulation of oxidative damage incurred by reactive oxygen species which are generated during the metabolic reactions required for cellular energy production [5]. However, Weismann's Wear and Tear Theory of Aging proposes that over time, key cellular components wear out from repeated use until they eventually become dysfunctional [6].

In order to be able to characterize aging despite not being able to define its cause, researchers in aging have identified cellular and molecular hallmarks of aging. Some of these hallmarks include mitochondrial dysfunction, loss of proteostasis, epigenetic alterations, inflammation and genomic instability [3, 7]. Many hallmarks of aging are seen in age-associated disease, and interventions aiming to ameliorate some of these processes can be beneficial in preventing or treating disease [8, 9]. Furthermore, interventions that extend lifespan in model organisms can often lengthen the healthy portion of their lifespan, otherwise known as healthspan, such that the onset of pathology is delayed [8, 10, 11]. Altogether, accumulating data indicates that

targeting biological processes in aging may provide novel therapeutic mechanisms by which chronic age-associated diseases can be prevented, delayed or minimized [3].

1.1.1 Genetics of aging

Gene and molecular pathways that regulate aging have been identified and studied in model organisms such as Drosophila [12], *C. elegans* [13]and *M. musculus* [14]. This indicates that genetic alterations can increase or decrease lifespan. For example, in *C. elegans* mutating the *daf-2* gene of the insulin/IGF-1 signaling (IIS) pathway, activates the DAF-16/FOXO transcription factor, which promotes the expression of pro-survival genes and doubles lifespan [15-17].

The influence of genetics on lifespan is further supported by genomic studies in twins which determined that variations in lifespan can correspond to genetic variants [18]. Furthermore, studies on the genomes of families with centenarians determined that living long can be hereditary [19-21]. Thus, the merits of using genetics to study mechanisms of lifespan extension have been illustrated by the identification of multiple genetic factors that affect aging.

1.2 *C. elegans*

Caenorhabditis elegans is a microscopic free-living nematode found in ecosystems around the world. In the 1960s, South African biologist Sydney Brenner developed *C. elegans* as a model organism for research in molecular biology [22]. Since then, *C. elegans* have been extensively used for research on programmed cell death, neurodevelopment, RNAi interference, aging and many other areas of research [23].

1.2.1 *C. elegans* physiology

The post-embryonic developmental time of wild-type *C. elegans* is approximately 2 days. L1 larvae hatch from eggs and grow through the L2, L3 and L4 larval stages. Pre-fertile young adults are small adult worms that have completed development but have not yet begun producing eggs [24]. *C. elegans* exist as self-fertilizing hermaphrodites in which gonadal structures hold both oocytes and spermatheca and fertilized eggs are released through the vulva [25]. However, males housing only one gonadal structure for sperm development arise rarely due to spontaneous non-disjunction during germline development [26]. Males can also be generated for crosses by exposing parental worms to temperatures between 25 ℃ and 30 ℃ to increase the rate of non-disjunction.

C. elegans are maintained at 20℃ on Nematode Growth Media (NGM) and are most often fed the OP50 strain of *Escherichia coli* [27]. Wild-type worms produce progeny of approximately 300 over the course of 4 days and thus worms used for experiments must be transferred to NGM plates with new OP50 to avoid starvation. Under poor growth conditions such as low nutrient availability or heat stress, developing worms can enter the dauer larval stage [28, 29]. Worms can be stored and survive for many months in this stage as their development has been arrested, they do not eat, and they have produced an enhanced cuticle for protection. Additionally, young worms can be frozen and stored at -80℃, then recovered years later [30].

The cuticle is the outer most covering of the worm and is secreted by the epidermis which encloses a pseudocoelomic cavity in which the main organ systems are found. Just inside the epidermis are bands of body wall muscle cells which control the movement of the worm in conjunction with the dorsal and ventral nerve cords. The intestine runs along the length of the body, connecting to the mouth and pharynx where food is ground down. The nervous system in *C. elegans* consists mainly of chemosensory and mechanosensory neurons arranged at the head of the worm, as well as the nerve ring, which surrounds the pharynx [31].

1.2.2 *C. elegans* genetics

The *C. elegans* genome is diploid and consists of five autosomal chromosomes and one sex chromosome. Females have both copies of the X chromosome, while males have only one. The entire *C. elegans* genome was fully sequenced as early as 1998 and contains 20 444 protein-coding genes [32]. *C. elegans* genes follow the traditional eukaryotic gene structure, where ORFs have introns and exons that are subject to alternative splicing and are delimited by 5' and 3' untranslated areas [33].

Since *C. elegans* self-fertilize, mutant alleles can be generated in a hermaphrodite and then easily maintained by self-propagation in subsequent generations of progeny. Ethyl methane sulfonate (EMS) and CRISPR are widely used to generate mutations. Microinjection of DNA constructs is used to induce extrachromosomal transgene expression and UV irradiation can then be applied to promote the integration of a transgene into the *C. elegans* genome [34]. Genes and mutants for specific genes are both referred to using an italicized three letter and one number designation (the *drp-1* gene and mutant for example). The resulting protein encoded by the gene is denoted with capitalization (the *drp-1* gene encodes the DRP-1 protein) [35].

1.2.3 Advantages of *C. elegans* in aging research

With a short lifespan of approximately 21 days, *C. elegans* are an excellent model organism for assays that measure longevity. Additionally, their small size, short generation time and large brood size allows for a high number of assay replications.

C. elegans undergo recognizable physiologic changes as they age, some of which include reduced motility, reduced feeding, deterioration of the cuticle and intestinal distention. Like humans, worms also experience a decline in nervous system function, muscle function, memory, reproduction and immunity as they age [36, 37]. Additionally, *C. elegans* exhibit many of the cell and molecular hallmarks of aging, including mitochondrial dysfunction and loss of

proteostasis [38]. Approximately 60-80% of the *C. elegans* genome is homologous to the human genome and an estimated two-thirds of genes associated with human disease can be found within this homology [39-41]. Similarly, many molecular pathways seen in the worm are conserved in humans [42]. Thus, the molecular mechanisms of human pathologies can be investigated in *C. elegans*.

The many tools that facilitate the manipulation of *C. elegans* genetics have given rise to entire globally shared databases of strains with specific gene mutations or transgene expression. Additionally, a genome-wide library exists for RNAi by feeding which allows for the knockdown of gene expression within a population of worms, without requiring any genome alterations [43]. Altogether, these methods benefit a genetic approach to studying aging.

1.2.4 Limitations of *C. elegans* in aging research

While the short life cycle and easy generation of new *C. elegans* populations is advantageous for aging research, it can also lead to the accumulation of background mutations that can impact phenotypes of interest. This problem must be addressed by periodically thawing fresh vials of worms [44].

Additionally, modeling human disease in *C. elegans* can be challenging. Many organs and physiological systems that play key roles in human health and pathology are absent from *C. elegans.* In particular, *C. elegans* lack a brain, a heart or circulatory system as well as an adaptive immune response or lymphatic system. Furthermore, *C. elegans* lack homologues of some mammalian genes that play key roles in human disease [42].

1.3 Mechanisms of lifespan extension

1.3.1 Reduced insulin/IGF-1 signaling

Mutations in the *C. elegans age-1* and *daf-2* genes extend lifespan such that worms live twice as long, on average [15, 45]. The *age-1* gene encodes a phosphatidylinositol 3-kinase (PI3K), and *daf-2* encodes the insulin /IGF-1 receptor, both of which are key components within the insulin/IGF-1 signaling (IIS) pathway [46]. Reduced IIS promotes lifespan extension due to increased nuclear translocation of DAF-16, a homolog of the mammalian FOXO transcription factor. Once DAF-16 can access the genome, it promotes expression of pro-survival genes, which encode components such as chaperone proteins and ROS scavengers [47]. Reduced IIS also promotes increased activation of SKN-1, a homolog of the mammalian Nrf2 (nuclear factor erythroid 2-related factor 2) transcription factor, mediating increased resistance to oxidative stress [48]. In addition to having improved proteostasis and redox homeostasis, mutants with reduced IIS also have improved mitochondrial function [49] and increased rates of autophagy [50]. Increased mitochondrial function leads to increased generation of ROS in *daf-2* mutants and this increase in ROS is at least partially required for the long lifespan of *daf-2* mutants [51].

The IIS pathway is highly conserved and its role in the regulation of longevity is also seen in Drosophila [52] and mice [53]. In humans, specific genetic variations in the insulin/insulin-like growth factor I receptor (*IGF1R*) gene are enriched in long-lived individuals. Furthermore, genetic variants of FOXO3A and FOXO1 are associated with longevity within specific geographic populations [54, 55].

1.3.2 Mild impairment of mitochondrial function

Although severe defects in mitochondrial function are known to cause disease, mild impairment of mitochondrial function allows for lifespan extension. Mutations in components of the mitochondrial electron transport chain (ETC) extend *C. elegans* lifespan. Lifespan is increased due to mild impairment of mitochondrial function in the *C. elegans clk-1, isp-1* and *nuo-6* mutants, which have defects in ubiquinone synthesis, complex III and complex I, respectively

[56-58]. Mild impairment of mitochondrial function is proposed to increase lifespan by activation of multiple stress response pathways, including the DAF-16-mediated stress response pathway as well as the mitochondrial unfolded protein response (mitoUPR) [59-61]. Activation of these pathways is thought to be signaled by increased levels of mitochondrial ROS due to defects in ETC function. This is supported by evidence that increased ROS levels are required for the long lifespan and the increased DAF-16 target gene expression in *isp-1* and *nuo-6* mutants [51, 62].

Mild impairment of mitochondrial function is a conserved mechanism of lifespan extension in other organisms. In Drosophila, the use of RNAi to target multiple genes encoding components of the ETC complexes resulted in increased lifespan [63]. In mice, loss of one copy of *Mclk1*, the murine homolog of the *C. elegans clk-1* gene, increases lifespan [64].

1.3.3 Mild elevation of reactive oxygen species

ROS can act as a mitochondria-to-nucleus signal that indicates the need for increased expression of stress response genes to deal with the less favorable consequences of meeting high energy demands [65]. Increased expression of mitochondrial heat shock proteins and antioxidants allows the cell to prevent a disruption in mitochondrial proteostasis due to increased oxidants [66].

In *C. elegans*, increased ROS levels can increase activation of multiple pathways, especially the DAF-16 pathway, the SKN-1-mediated oxidative stress response pathway and the mitoUPR [65, 67]. This is seen in mutants with mild impairment of mitochondrial function as well as IIS mutants. Additionally, disruption of the mitochondrial superoxide dismutase gene *sod-2* yields a lifespan extension that is dependent on increased levels of ROS [68]. Using low levels of the mitochondrial oxidant paraquat, it has also been determined that in *C. elegans*, there is a threshold, after which increased levels of ROS become detrimental to lifespan [69]. It has also been determined that while increases in mitochondrial ROS can increase lifespan, increasing cytoplasmic ROS is detrimental [70].

Whether or not a mildly elevated ROS level is conserved as a mechanism of lifespan extension in other organisms remains unclear. In flies and yeast, disruption of the mitochondrial superoxide dismutase decreases lifespan [71, 72]. In mice, loss of *Sod2* is embryonic lethal, but *Sod2* heterozygosity does not affect lifespan [73, 74]. However, multiple examples exist where genetic alterations or compounds that generate increased ROS levels lead to an increased lifespan that can be reversed by treatment with antioxidants in yeast, worms, flies and mice [75].

1.3.4 Dietary restriction

In *C. elegans*, dietary restriction is thought to increase lifespan by altering key nutrient sensing pathways to promote longevity. Lifespan extension is achieved by restricting the worm's food intake but can also be seen in the *eat-2* mutant which consumes less food due to defects in pharyngeal pumping [76]. Increased mitochondrial function and increased ROS generation are both seen in response to dietary restriction [77]. Additionally, increased activation of SKN-1, DAF-16 and AMP-activated protein kinase (AMPK) are seen, and target of rapamycin (TOR) signaling is inhibited, all of which contribute to increased longevity [9, 78].

Lifespan extension by dietary restriction is conserved in multiple organisms, including yeast [79], worms [80], flies [81], mice [82] and primates [83]. In humans, there is evidence that dietary restriction lowers blood pressure as well as cholesterol and decreases the risk of cardiovascular disease and diabetes [84-86].

1.3.5 Germline ablation

Lifespan extension mediated by germline ablation in *C. elegans* is dependent on repressed IIS and increased DAF-16 activity [87]. Long-lived germline-less worms can be achieved by laser ablation of germ line precursor cells but are also seen in *mes-1* mutants, which lack germ cells, and *glp-1* mutants, which are defective in the receptor for molecular signals promoting germline proliferation [88]. Germline ablation increases resistance to stress but also improves innate

immunity, independently of DAF-16 [89]. Additionally, increased lipid metabolism contributes to lifespan extension by germline ablation in *C. elegans* [90].

Lifespan extension by germline ablation is also seen in Drosophila [91]. Early life castration increases the lifespan of male mice [92]. In humans, there is evidence that castrated men have extended lifespans [93].

1.4 Aging and biological resilience

In *C. elegans*, it is frequently observed that animals that are resistant to stress live longer or that genetic alterations which increase longevity also increase stress resistance by activation of stress response pathways [94]. Many of the long-lived mutants with well-studied mechanisms of lifespan extension, such as *daf-2, isp-1, eat-2* and *glp-1* also have increased resistance to multiple exogenous stressors such as heat stress, oxidative stress, bacterial pathogen stress, osmotic stress and hypoxic stress [95]. In a screen using EMS for random mutagenesis, it was discovered that most mutants that were more resistant to heat stress also exhibited significantly extended lifespans [96]. Additionally, the loss of transcription factors such as DAF-16 and SKN-1, which mediate key longevity regulating pathways, decreases both lifespan and resistance to stress [47, 97]. Gene expression induced by activation of the mitochondrial unfolded protein response, mediated by ATFS-1 (activating transcription factors associated with stress-1) in *C. elegans* [98], as well as of the p38-mediated innate immune signaling pathway [99] have also both been found to significantly overlap with gene expression associated with increased longevity. The extended lifespans of long-lived *C. elegans* mutants frequently depend on these pathways.

1.4.1 Hormesis and longevity

The association of activated stress response pathways and increased longevity indicates that hormesis can contribute to lifespan extension [100]. A hormetic response is one where a mild

level of stress or toxic stimuli gives rise to a beneficial rather than a detrimental effect on organismal survival [101]. In *C. elegans*, it has been observed that mild or short-term exposure to stressors such as heat, bacterial pathogen stress, osmotic stress or oxidative stress earlier in life can subsequently increase resistance to the same stress later in life and promote increased longevity [102-104]. Exposure to such stressors allows for sustained activation of multiple stress response pathways, including the DAF-16-mediated stress response pathway, for several days after exposure to the stress. Importantly, the ability to activate stress response pathways, to mount a hormetic response to mild stress exposure and overall stress resistance declines with age [103].

Further support for the theory that hormesis contributes to mechanisms of lifespan extension comes from long-lived *C. elegans* mutants. Rather than being detrimental, mild stress due to elevated levels of reactive oxygen species in *clk-1, isp-1, nuo-6* and *sod-2* mutants as well as due to reduced nutrient signaling in *eat-2* and *daf-2* mutants leads to an increase in lifespan by activation of various stress response pathways [59, 70, 76, 98]. It is therefore important to consider whether hormesis may be a contributor when investigating novel mechanisms of lifespan extension.

1.4.2 DAF-16-mediated stress response

DAF-16 is a *C. elegans* transcription factor of the Fox (Forkhead box) family of transcription factors. Fox transcription factors are evolutionarily conserved and are known to regulate longevity, cell growth and stress response due to their involvement in the transcription of genes involved in key cellular processes such as DNA repair, autophagy and metabolism [105]. In *C. elegans,* several identified DAF-16 target genes were determined to regulate longevity. For example, the *sod-3* gene, which encodes a mitochondrial superoxide dismutase is normally expressed at low levels but is upregulated in response to DAF-16 activation and contributes to the long lifespan of *daf-2* mutants [17]. Increased expression of *sod-3* is frequently used as a marker for activation of the DAF-16 pathway.

DAF-16 activity is regulated by multiple pathways. In the IIS pathway, binding of insulin-like proteins to the DAF-2 receptor triggers autophosphorylation to begin a downstream signaling cascade [106]. AGE-1 is recruited to the DAF-2 receptor where it catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the plasma membrane [107]. Increased levels of PIP3 activates PDK-1 (3-phosphoinositide-dependent kinase-1) which activates AKT-1 and AKT-2 by phosphorylation. AKT-1 and AKT-2 then phosphorylate DAF-16, keeping it inactive and out of the nucleus [108, 109].

The TOR signaling pathway can also regulate DAF-16 activity independently of IIS. The TORC1 complex contains the DAF-15 coactivator and inhibits DAF-16 activity. Activated DAF-16 can in turn inhibit *daf-15* expression [110]. Inhibition of the TOR pathway increases lifespan in *C. elegans,* Drosophila and mice [111].

The AMPK nutrient-signaling pathway is also closely linked to DAF-16. *In vitro* studies show that AMPK can directly phosphorylate DAF-16 at a residue that leads to activation and that is different from the residue phosphorylated by the AKT kinases [112]. Additionally, constitutively active AMPK leads to upregulated DAF-16 target gene expression as well as a DAF-16-dependent increase in longevity [112]. Activation of the AMPK pathway increases lifespan in *C. elegans* and Drosophila [113].

The JNK (Jun N-terminal kinase) signaling pathway is activated in response to various stressors, including oxidative stress. Activated JNK is proposed to directly activate DAF-16 via phosphorylation [114]. Additionally, JNK signaling is thought to promote DAF-16 activation by antagonizing IIS [115]. Activation of the JNK pathway increases lifespan in *C. elegans* and Drosophila [114, 116].

1.4.3 p38-mediated innate immune response

A conserved p38 MAPK (mitogen-activated protein kinase) pathway was first identified in *C. elegans* using a genetic screen to identify mutants that were more susceptible to the bacterial pathogen *Pseudomonas aeruginosa* (PA14) [117]. Mitogens associated with *Pseudomonas* infection bind to the toll-interleukin receptor domain adaptor protein TIR-1, activating a signaling cascade that includes the MAPKKK NSY-1, the MAPKK SEK-1 and finally the MAPK PMK-1 [118]. PMK-1 can then activate two transcription factors SKN-1 and ATF-7 (activating transcription factor 7) [118, 119]. While SKN-1 target gene expression yields a protective response against oxidative stress, ATF-7 target gene expression mounts an innate immune response to protect the host against pathogen infection [120].

Gene expression induced by ATF-7 activation includes c-type lectins which function in pathogen recognition as well as antimicrobial peptides. Other targets of ATF-7 include *xbp-1*, a regulator of the unfolded protein response, as well as *lgg-1* a regulator of autophagy [119]. ATF-7 also upregulates *hlh-30* expression, which encodes the TFEB homolog HLH-30 (helix-loop-helix protein 30) that acts to limit nutrient signaling and cell growth in favor of upregulating autophagy in response to stress. Gene expression mediated by HLH-30 significantly overlaps with gene expression associated with longevity [121]. The ATF-7 transcription factor also upregulates expression of the *atf-7* genes, and thus increased expression of *atf-7* as well as the anti-bacterial *sysm-1* gene is used as a marker for activation of the p38 MAPK pathway in *C. elegans*.

1.4.4 SKN-1-mediated oxidative stress response

SKN-1 is the *C. elegans* homolog of the mammalian Nrf2 transcription factor, which plays a significant role in promoting the expression of genes encoding detoxifiers in response to stressors such as oxidative and xenobiotic stress [97]. Like DAF-16, SKN-1 is located predominantly in the cytoplasm under normal conditions but translocates to the nucleus under stressful conditions [122]. Of the well-known SKN-1 target genes, examples include glutathione

synthesizing enzymes and glutathione-S-transferase (GST), which each contribute to the maintenance of the glutathione cycle, an important antioxidant defense mechanism [97]. Increased expression of the *gst-4* gene is often used as a marker for activation of the SKN-1 pathway in *C. elegans.*

The p38 MAPK pathway is required for the activation of SKN-1 in response to most stressors, including oxidative stress. PMK-1, a kinase in the p38 signaling pathway, activates SKN-1 by phosphorylating it at two residues. Impairment of the p38 pathway or ablation of those residues inhibits SKN-1 activation [118].

While PMK-1-mediated phosphorylation may be the primary mechanism to activate SKN-1, there are several mechanisms to inhibit SKN-1. IIS signaling leads to inhibitory phosphorylation of SKN-1 by the AKT kinases [48]. Phosphorylation by GSK-3 (glycogen synthase kinase 3) also inhibits SKN-1 [123]. As with DAF-16, TOR signaling also inhibits SKN-1 activity [110].

1.4.5 ATFS-1-mediated mitochondrial unfolded protein response

The mitochondrial unfolded protein response (mitoUPR) is mediated by the ATFS-1 transcription factor, a homolog of the mammalian ATF-5 (activating transcription factor 5). ATFS-1 acts as a line of communication between mitochondria and the nucleus under conditions of stress [124, 125]. Under normal physiological conditions, ATFS-1 is imported into the mitochondria where it is degraded by the mitochondrial protease LONP1 (Lon peptidase 1). Under stressful conditions where mitochondria may become susceptible to dysfunction, ATFS-1 translocates into the nucleus, where it forms a complex with the transcription factor DVE-1 and the small ubiquitin-like protein UBL-5 to promote expression of genes that can mitigate mitochondrial stress [126].

Activation of the mitochondrial unfolded protein response can be triggered by mitochondrial perturbations such as defects in oxidative phosphorylation, disruptions in mitochondrial import or elevated levels of mitochondrial ROS [127]. Activation of the mitoUPR upregulates expression of genes which encode mitochondrial-targeted chaperones, proteases or detoxifiers [128]. The

activated mitoUPR pathway can also coordinate with the regulation of mitochondrial dynamics and mitochondrial quality control to support a return to mitochondrial homeostasis [129]. Activation of the mitoUPR is commonly measured by monitoring the expression of the *hsp-6* gene which encodes a mitochondrial chaperone protein [61]. Constitutive activation of the mitoUPR provides increased resistance to stress but not lifespan extension in *C. elegans* [130].

1.5 Mitochondrial biology

1.5.1 Mitochondrial structure

Mitochondria are double membraned organelles within the cell that are best known for their role in cellular energy metabolism. They are thought to have originated after an endosymbiotic event whereby an ancestral eukaryotic cell engulfed an aerobic bacterial cell [131]. Mitochondria contain multiple copies of their own genome consisting of a few protein-coding genes, most of which are subunits of the electron transport chain complexes that carry out oxidative phosphorylation [132]. Additional mitochondrial genes are encoded in the nuclear genome and their protein products include a mitochondrial targeting sequence (MTS) allowing them to be recognized for transportation to the mitochondria [133].

The outer mitochondrial membrane (OMM) contains porins which allow materials to pass from the cytoplasm into the intermembrane space. Additionally, outer membrane translocase proteins (TOMs) import nuclear-encoded proteins through the OMM, into the intermembrane space [134]. To increase surface area available for oxidative phosphorylation, the inner mitochondrial membrane (IMM) forms invaginations called cristae [135]. Inner membrane translocase proteins (TIMs) transport proteins from the intermembrane space into the mitochondrial matrix [134]. Multiple processes take place within the mitochondrial matrix, including the citric acid cycle, fatty acid metabolism, protein folding and iron storage [136].

Importantly, the cell contains multiple mitochondria, which form an interconnected network that responds to the cell's needs in coordination with other cellular components such as the endoplasmic reticulum (ER), the peroxisomes and the lysosomes. For example, mitochondrial-

derived vesicles (MDVs) are thought to allow for the transportation of mitochondrial components to lysosomes and peroxisomes for degradation [137]. Furthermore, the mitochondrial network must reshape itself to respond to cellular needs through the process of mitochondrial fission or fusion. Mitochondrial fission or fusion events can therefore occur quickly, sometimes with one quickly following the other [138, 139].

1.5.1.1 Mitochondrial fission

Mitochondrial fission is the process by which one or more mitochondrion separates from the network of mitochondria within the cell. In *C. elegans* this process is governed by the evolutionarily conserved dynamin-related GTPase DRP-1 (dynamin-related protein 1), which is recruited to the constriction site where it dimerizes and performs the scission of both mitochondrial membranes [140-142]. The constriction site is marked by contact with the ER which forms a tubule around the mitochondria to aid with constriction [143, 144]. Importantly, although additional fission proteins may contribute to membrane scission in other organisms, such as dynamin-2 in mammals [145], no other protein has been found to contribute to mitochondrial membrane scission in nematodes.

Mammalian studies indicate that the transcription of *Drp1* is regulated by PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), a transcription factor which also regulates mitochondrial biogenesis, and which can be activated by stress pathways such as FoxO/DAF-16 and Nrf2/SKN-1 [146, 147]. Additionally, Drp1 activity can be modulated post-translationally by phosphorylation, nitrosylation or SUMOylation at various residues and is ubiquitinated by mitochondrial E3 ubiquitin ligases Parkin and MARCH5 (membrane-associated RING-CH protein 5) for proteasomal degradation [148, 149].

Mitochondrial fission is needed for several physiologic purposes. Firstly, mitochondrial fission allows for the generation of new mitochondria, the proliferation of mitochondria within newly divided cells and the dispersion of mitochondria throughout a cell [150]. Additionally, mitochondrial fission is an early event in apoptosis leading to the fragmentation of the

mitochondrial network and contributing to the activation of the apoptotic signaling cascade [151-153]. Mitochondrial fission is also crucial for breaking off damaged mitochondria from the network, such that they can be cleared from the cell [154].

1.5.1.2 Mitochondrial fusion

Mitochondrial fusion is performed in two synchronized steps. In *C. elegans,* fusion of the outer mitochondrial membrane is carried out by the cytosolic FZO-1 protein, homolog of the mammalian Mfn1 and Mfn2 GTPases [152]. Fusion of the inner mitochondrial membrane is carried out by the mitochondrial EAT-3 protein, homolog of the mammalian Opa1 (optic atrophy 1 protein) GTPase [155]. Thus, FZO-1 proteins on opposite membranes must first dimerize, tethering the two membranes together to catalyze their fusion. Then, EAT-3 proteins in the inner mitochondrial membranes of opposing mitochondria can dimerize and catalyze the fusion of the inner mitochondrial membranes [156].

As with mitochondrial fission machinery, mitochondrial fusion machinery can be transcriptionally regulated, namely by PGC-1α [146], but is largely regulated by protein turnover systems [149]. Mammalian studies indicate that since mitofusins are on the outer mitochondrial membrane, they are subject to cytosolic ubiquitin-proteasome degradation [149]. During mitophagy, mitofusins are degraded via Parkin-mediated ubiquitination which occurs when membrane potential is lost [157, 158]. Mitofusin degradation is also mediated via ubiquitination by Huwe1 (HECT domain-containing E3 ubiquitin ligase 1) under conditions of stress, where mitochondrial fission is favored [159]. By contrast, proteolytic regulation of Opa1 in the inner mitochondrial membrane is governed by proteases Yme1L and Oma1 [160]. Under normal physiologic conditions, the full-length form of Opa1 (L-Opa1) is cleaved into its shorter form (S-Opa1) by Yme1L, yielding an approximate 50/50 ratio of L-Opa1 to S-Opa1. However, under conditions of stress, the Oma-1 protease promotes increased cleavage of L-Opa1 to S-Opa1 in order to inhibit mitochondrial fusion [161-163]. Interestingly, it is proposed that increased levels of S-Opa1 can help promote mitochondrial fission [160]. In *C. elegans,* there is no evidence to

suggest that the proteolytically cleaved form of EAT-3 can promote mitochondrial fission. EAT-3 is cleaved by proteases YMEL-1 (homolog of Yme1L in mammals) and SPG-7 (spastic paraplegia 7 protease) [164, 165]. Mechanisms by which fusion proteins are increasingly cleaved under conditions of stress, as seen with Huwe1 for mitofusins and Oma1 for Opa1 in mammals, are not as well characterized in *C. elegans.*

Mitochondrial fusion is thought to benefit high energy demands due to complementation of mitochondrial components such as electron transport chain subunits, metabolites and mitochondrial transcripts [166, 167]. This is supported by the finding that disruption of mitochondrial fusion decreases respiratory capacity in yeast and mammals [168]. Under certain conditions such as low nutrient availability and other types of stress, mitochondria can become hyperfused, which may further increase the efficiency of oxidative phosphorylation and ATP production [169].

1.5.2 Mitochondrial functions

Though mitochondria are best known as the powerhouse of the cell, they also contribute to multiple other key cellular functions including apoptosis, calcium buffering, iron homeostasis and lipid metabolism.

1.5.2.1 Energy production

Mitochondria are known as the powerhouse of the cell due to their production of the majority of the cell's energy by oxidative phosphorylation. Under aerobic conditions, glycolysis products are imported into mitochondria and are metabolized by the citric acid cycle which produces ATP, as well as NADH and $FADH_2$ [170]. The electrons carried by NADH and $FADH_2$ are then transferred to the electron transport chain where the transport of electrons through complex I to IV releases protons into the intermembrane space. Maintenance of a proton gradient

provides a charge gradient across the inner mitochondrial membrane that is crucial as it provides the energy required for ATP synthase (complex V) to convert ADP to ATP [171].

1.5.2.2 Apoptosis

Programmed cell death is triggered first by the permeabilization of the mitochondrial membrane and release of mitochondrial proteins, including cytochrome c or mitochondrial DNA, which act as caspase activators [153]. Mitochondrial permeability is regulated by the mitochondrial permeability transition pore complex (PTPC) at the inner mitochondrial membrane [172]. The PTPC is in turn regulated by pro- and anti- apoptotic proteins of the Bcl-2 (B-cell lymphoma 2) protein family located at the inner mitochondrial membrane [173]. Thus, the activation of programmed cell death is regulated by the mitochondrial response to apoptotic stimuli.

1.5.2.3 Calcium buffering

In coordination with the endoplasmic reticulum, mitochondria regulate the second messenger, Ca^{2+} in cellular signaling by buffering the level of Ca^{2+} available throughout the cytoplasm. The re-uptake of calcium by mitochondria after physiologic stimuli trigger the release of calcium from the ER is particularly important to maintain efficient cell signaling [174]. The proximity between the ER and mitochondria gives rise to cytoplasmic domains with high calcium concentrations, allowing mitochondria to rapidly import calcium using the voltage-dependent anion channels (VDACs) located on the outer mitochondrial membrane [175, 176]. Calcium can then be stored in the mitochondrial matrix after import by the mitochondrial calcium uniporter (MCU) at the inner mitochondrial membrane [177, 178]. Thus, calcium signaling within the cell is regulated by the fine-tuning of calcium levels by mitochondria.

1.5.2.4 Iron homeostasis

Ferrous iron (Fe^{2+}) is imported into the mitochondrial matrix by metal transporters with the help of metallochaperones or by “kiss and run” contact sites with endosomes containing the iron carrier protein transferrin (TF) [179-181]. Within the mitochondria, iron is used for heme and iron-sulfur (Fe-S) cluster biosynthesis, both of which are essential cofactors required for multiple functions including ribosome assembly, DNA repair and mitochondrial respiration [182]. Additionally, iron can be stored in the mitochondria by mitochondrial ferritin [183].

1.5.2.5 Lipid metabolism

Long-chain fatty acids are converted to acylcarnitine in the cytoplasm before being imported into the mitochondria [184]. Beta oxidation then yields NADH and $FADH_2$ molecules which can be used to generate ATP by oxidative phosphorylation, as well as acetyl-CoA (acetyl coenzyme A) which can enter the citric acid cycle to generate additional energy [185]. Thus, a key process by which the cell harnesses energy from lipids is localized to mitochondria.

1.5.3 Mitochondrial quality control

When mitochondrial damage exceeds a certain threshold after which activation of the mitochondrial unfolded protein response or complementation of mitochondrial components by mitochondrial fusion can no longer restore homeostasis, a selective autophagy process called mitophagy clears dysfunctional mitochondria from the cell [186]. Although increased mitophagy is induced under conditions of stress, clearance of dysfunctional mitochondria is required to maintain mitochondrial homeostasis under basal conditions as well [187]. In *C. elegans*, mitophagy is required to maintain resistance to multiple stressors, including oxidative stress and heat stress. Additionally, mitophagy is required for lifespan extension in multiple long-lived mutants [50, 188].

In *C. elegans*, mitophagy is mediated by the PINK-1/PDR-1 signaling pathway [189]. PDR-1, which is a homolog of the mammalian Parkin, is a component of an E3-ubiquitin ligase that tags proteins in the cytoplasm for degradation as part of the ubiquitin-proteasome system [190]. Under normal conditions, PDR-1 works in the cytoplasm, but when mitochondria become stressed, PDR-1 is recruited by PINK-1 to the outer mitochondrial membrane in order to ubiquitinate outer mitochondrial membrane proteins [191]. PINK-1, which is a homolog of the mammalian PINK1 (PTEN-induced kinase 1), is a kinase that is localized to the mitochondria. In mammalian studies, it has been determined that under normal conditions PINK1 is imported into the intermembrane space and degraded by the PARL protease [192]. However, when mitochondrial homeostasis is perturbed and mitochondrial membranes become depolarized, PINK1 remains at the outer mitochondrial membrane to recruit the necessary machinery required for mitophagy [193]. In *C. elegans,* PINK-1 signaling recruits the mitophagy receptor DCT-1, which is a homolog of the mammalian BNIP3 (Bcl-2 interacting protein 3), to the outer mitochondrial membrane [188]. Importantly, the expression of DCT-1, and other mitophagy machinery is upregulated upon activation of the DAF-16 and SKN-1 stress response pathways. DCT-1 interacts with the autophagosomal protein LGG-1, which is homologous to the mammalian LC3, thus helping to recruit autophagosome formation around the mitochondria [188].

Mitochondrial fission is thought to prime mitochondria for mitophagy by separating dysfunctional mitochondria from the network. In Drp1 knockout cells, it has been seen that mitophagy still occurs, but with larger segments of mitochondria being targeted. In mammalian models, it is therefore thought that Drp1-mediated fission is required for selective autophagy to spare healthy mitochondria from being autophagocytosed, but that mitophagy can proceed without Drp1-mediated mitochondrial fission [194]. Importantly, this indicates that Drp1-independent mitochondrial fission can occur during autophagy, however the mechanism by which this occurs remains incompletely understood [195].

1.5.4 Mitochondria and peroxisomes

The peroxisome is another sub-cellular organelle within the cell where many metabolic processes take place. Like mitochondria, peroxisomes have the ability to alter their shape and interconnectivity in response to the cell's needs [196]. Mitochondria and peroxisomes coordinate with each other to carry out multiple key cellular metabolic functions. Both organelles share the responsibility of being primary sites for the regulation of redox homeostasis, given that both produce several kinds of ROS and also host multiple ROS scavengers [197]. Peroxisomes are home to the catalase enzyme, which catalyzes the metabolism of hydrogen peroxide, one of the products generated by superoxide dismutase [198]. Fatty acid oxidation also occurs in both mitochondria and peroxisomes [199].

Lipids, ROS, and other metabolites generated from these reactions can move between mitochondria and peroxisomes, sometimes via mitochondrial-derived vesicles (MDVs), acting as messengers in a process known as crosstalk [200]. Additionally, mitochondria and peroxisomes are often seen in close proximity and can even form contact sites with one and other [201]. The biogenesis of both organelles is closely linked under the regulation of PGC-1α [202]. In mammalian studies, it was determined that in addition to being a key component of mitochondrial fission, Drp1 can promote peroxisomal division, a process which is important for both peroxisomal biogenesis and clearance of peroxisomes by pexophagy [203]. Thus, in addition to their functions, the dynamics of mitochondria and peroxisomes are likewise coordinated.

1.6 Mitochondrial dynamics and longevity

1.6.1 *In vivo* visualization of mitochondria in *C. elegans*

C. elegans are transparent such that dissection and fixation are not required for microscopy. Therefore, visualization of subcellular organelles such as mitochondria is possible *in vivo* by temporarily immobilizing the worms [204]. Visualization of mitochondrial networks in *C. elegans* is easiest in the body wall muscle cells which form a sheath of muscle along the body wall, just

under the epidermis and cuticle [205]. To visualize these mitochondria, we express a GFP with a mitochondrial targeting sequence, or fused to the outer mitochondrial membrane transporter TOMM-20, using a muscle-specific promoter. Because mitochondrial morphology varies from head to tail, we always image mitochondria in the tail region just below the vulva, for consistency.

Mitochondrial morphology is quantified in this work by ImageJ. Parameters measured include the number of mitochondria per region of interest, the average mitochondrial area, the circularity of mitochondria, and the length of mitochondria. When mitochondrial networks go from being fused to fragment, the number of mitochondria increases, the average mitochondrial area decreases, mitochondrial circularity increases and mitochondrial length decreases.

1.6.2 Mitochondrial dynamics during aging

Age-associated increases in mitochondrial fragmentation have been observed in multiple organisms, including Drosophila [206], *C. elegans* [207, 208] and mice [209]. However, paradoxical evidence shows that mitochondrial fission machinery activity and expression is reduced with age in mice and human endothelial cells, giving rise to mitochondrial dysfunction and defects in mitophagy [210-212].

Increased mitochondrial fusion is often associated with lifespan extension. In *C. elegans*, increased mitochondrial fusion is seen in a number of long-lived mutants, including *daf-2* insulin/IGF-1 signaling mutants, and is required for their long lifespan. Additionally, increased fusion is required for lifespan extension by germline ablation, dietary restriction, AMPK activation, TORC-1 inhibition and mild impairment of mitochondrial function [165, 213, 214]. In humans, it was determined that the mitochondria of dermal fibroblast from long-lived individuals (about 100 years old) were more fused compared to young individuals (about 27 years old) and old individuals (about 75 years old). Mitochondrial hyper-fusion in long-lived individuals was paired with decreased mitophagy but increased basal autophagy, a mild

increase in hydrogen peroxide levels, increased mitochondrial mass and a maintenance of bioenergetic efficiency [215].

1.6.3 Mitochondrial dynamics during stress

In response to stress, mitochondrial networks become hyperfused or fragmented in order to adopt the bioenergetic state required to maintain cellular homeostasis. A fused mitochondrial network allows for the maintenance of high bioenergetic efficiency, prioritizing ATP production, protecting mitochondria from autophagy and preventing programmed cell death [169, 216]. This state is often observed in response to mild or short-term stressors, including nutrient starvation or activation of the ER unfolded protein response (ER-UPR) [217]. In mouse embryonic fibroblasts, mitochondrial fission is blocked in response to starvation conditions, by post-translational inactivation of Drp1 [218]. In mouse and human cells, it was determined that stress-induced mitochondrial hyperfusion increases ATP production and bioenergetic efficiency to help combat protein misfolding stress upon activation of the ER-UPR and is accompanied by activation of the NF-κB stress response pathway [219, 220].

A fragmented mitochondrial network is associated with low bioenergetic efficiency, resulting in membrane depolarization, low ATP production and increased degradation of mitochondria [216]. This state is often seen in response to severe or prolonged stressors, such as oxidative stress, nutrient overload or mitochondrial proteotoxicity. PINK1 is known to accumulate on the mitochondrial surface in response to stresses that cause mitochondrial depolarization, thereby signaling mitophagic degradation of those mitochondria [191]. Mitochondrial fragmentation also mobilizes ROS intracellular signaling molecules, which can activate various cellular stress response pathways [221, 222].

Dysregulation of mitochondrial dynamics and abnormal mitochondrial morphology coincides with loss of mitochondrial protein homeostasis during aging in *C. elegans* [223]. Given that mitochondrial dynamics become dysregulated with age, and that the mitochondrial network's ability to dynamically respond to stress is crucial for cellular stress response, age-associated loss

of mitochondrial dynamics may contribute to the decreased resistance to stress that is seen in older organisms.

1.6.4 Mitochondrial dynamics in age-associated disease

Abnormal mitochondrial morphology, dysregulation of mitochondrial dynamics, mitochondrial dysfunction and defects in mitochondrial quality control are seen in various age-associated diseases.

Genetic forms of Parkinson's disease (PD) arise as a result of mutations in mitophagy machinery which give rise to mitochondrial dysfunction [224]. Additionally, excessive Drp1-mediated fragmentation is seen in PD patients [225-227]. In Huntington's disease (HD) patients, a decline in mitochondrial function and an increase in levels of mitochondrial fission proteins is seen [228]. Additionally, in models of HD across multiple organisms, mitochondrial fragmentation is seen [229-231]. Reduced expression of mitofusins and Opa1 is associated with cardiac disease and excessive Drp-1-mediated mitochondrial fission drives cerebral ischemia-reperfusion injury [232-235]. Insulin resistance in type II diabetes is associated with mitochondrial dysfunction, high levels of ROS and reduced mitofusin levels [236, 237]. Additionally, aberrant mitochondrial dynamics and elevated levels of ROS are associated with defective cell signaling in cancer cells [238]. Thus, perturbations in mitochondrial fission and fusion are central to many mechanisms of pathology that manifest later in life, though the extent of their contribution to pathology remains largely unknown.

1.6.5 Targeting mitochondrial dynamics to promote healthy aging

1.6.5.1 Modulation of longevity

Disruption of the mitochondrial fission gene Drp1 extends lifespan in yeast [239, 240] and fungal [241] models but has not been reported to extend lifespan in Drosophila or wild-type *C. elegans*. In yeast, inhibition of peroxisomal fission without blocking mitochondrial fission can

extend chronological lifespan [242]. In mice, loss of Drp1 is embryonic lethal and Drp1 has been determined to be required for proper brain development [243, 244].

Notably, in both Drosophila and *C. elegans*, there is evidence that timing is important when it comes to altering mitochondrial fission, as maintaining mitochondrial fission may be beneficial during certain times in the life cycle. In Drosophila, midlife but not early adulthood induction of mitochondrial fission by overexpression of *Drp1* extends lifespan while midlife upregulation of mitofusins shortens lifespan [206]. In *C. elegans*, disruption of mitochondrial fission during development or in old adults (day 7 and 11), but not during early adulthood (day 3) impairs neuronal function. By contrast, disruption of mitochondrial fusion at any of these timepoints consistently impairs neuronal function [245].

The maintenance of balanced mitochondrial fission and fusion is also proposed to play an important role in lifespan extension. In *C. elegans,* disruption of both *drp-1;fzo-1* double mutants maintain a youthful-looking mitochondrial network and have an increase lifespan compared to wild-type animals [213]. However, in both yeast and *C. elegans*, disruption of both fission and fusion appears to force mitochondrial networks into a non-dynamic state in which they are unable to respond to stress or changes in nutrient availability, resulting in reduced biological resilience [213, 246].

Increased levels of the mitochondrial fusion protein EAT-3 are thought to contribute to increased mitochondrial fusion required for lifespan extension in the *C. elegans* IIS mutant *daf-2.* The proposed mechanism by which EAT-3 levels are elevated in *daf-2* mutants is by DAF-16 related inhibition of mitochondrial protease expression, including SPG-7 which cleaves EAT-3 [165]. However, overexpression of the *C. elegans* mitofusin homolog FZO-1 in wild-type animals does not promote lifespan extension [213].

In *C. elegans* IIS mutants *daf-2* and *age-1,* disruption of the mitochondrial fission gene *drp-1* further extends their already long lifespans. This finding indicates that mitochondrial dynamics may coordinate with insulin/IGF-1 signaling. Furthermore, given that *daf-2* mutants already have elongated, tubular and interconnected mitochondrial networks, this finding also indicates

that disruption of mitochondrial fission can still be beneficial when mitochondria are in a fused state [247]. However, the mechanism by which disruption of *drp-1* extends the lifespan of long-lived IIS mutants remains unknown.

Importantly, the contribution of changes in peroxisomal biology is not considered in many of these studies despite Drp1 and Fis1 (mitochondrial fission 1 protein) being modulators of peroxisomal dynamics. Thus, the contribution of peroxisomal dynamics to the modulation of lifespan is not well understood.

1.6.5.2 Modulation of stress resistance

Altering mitochondrial dynamics affects the organism's ability to respond to stress. In *C. elegans* disruption of the mitochondrial fusion genes *fzo-1* and *eat-3* increases resistance to heat stress, and oxidative stress while disruption of *drp-1* increases resistance to oxidative stress only. Disruption of either fission or fusion significantly reduces resistance to osmotic and anoxic stress [208]. Notably, it was shown that disruption of the fusion genes induces activation of multiple stress response pathways, including the mitochondrial unfolded protein response, the SKN-1-mediated oxidative stress response, the cytosolic unfolded protein response, the HIF-1-mediated (hypoxia-inducible factor-1) response, and the DAF-16-mediated response. By contrast, disruption of *drp-1* only activated the SKN-1-mediated oxidative stress response [208]. In another study, inhibition of either fission or fusion activated the mitochondrial unfolded protein response [248].

Little is known about how disruption of mitochondrial dynamics affects stress response pathways in other organisms. In mice, neuronal *Drp1* deletion impairs endoplasmic reticulum morphology and activates ER stress pathways [249]. In yeast, disruption of the *Opa1* homolog *mgm-1* made cells less resistant to stress-induced apoptotic cell death while disruption of the *Drp1* homolog *dnm1* yielded increased resistance to this stress. In response to heat stress, UV stress and oxidative stress by paraquat treatment, both *dnm1* and *mgm1* strains were less resistant than wild type [246]. Together, these data indicate that mitochondrial dynamics play a

crucial role in resistance to stress, and that disruption of mitochondrial fission or fusion can yield beneficial or detrimental responses depending on the type of stress.

1.6.5.3 Outcomes in age-onset disease models

Inhibition of Drp1 ameliorates phenotypes in multiple disease models. In a *C. elegans* models of Huntington's disease (HD), decreasing mitochondrial fragmentation without disrupting *drp-1* improved mitochondrial morphology, and improved motility [205]. Additionally, in *C. elegans*, disruption of *drp-1* increased lifespan, improved movement and decreased mitochondrial fragmentation in a neuronal model of polyglutamine toxicity [250]. In two mouse model of HD, inhibition of Drp1-mediated mitochondrial fission decreases mitochondrial fragmentation, diminishes neurodegeneration and reduces behavioral deficits [251, 252]. In mouse models of Parkinson's disease (PD), inhibition of Drp1 attenuates loss of dopaminergic neurons and upregulates PINK1 and Parkin expression thus improving mitochondrial network morphology [253, 254]. Inhibition of Drp1 using the pharmacological inhibitor mdivi-1, ameliorates synaptic degeneration and reduces both amyloid-beta deposits and cognitive impairment in mouse models of Alzheimer's disease (AD) [255]. Similar results were obtained by genetically reducing Drp1 expression and by decreasing Drp1 activity by blocking post-translational phosphorylation of one of its residues [256-258]. Inhibitions of Drp1 may also be neuroprotective in cerebral ischemia [259].

Inhibition of Drp1 by mdivi-1 has also been shown to improve insulin sensitivity as well as mitochondrial fragmentation, oxidative stress, inflammation, retinopathy and atherosclerosis in diabetic mice [260-263]. Similarly, the use of mdivi-1 in models of various types of cancer interrupts cell metabolism and cell cycle progression, sensitizing cancer cells to induction of apoptotic cell death [264-267]. Furthermore, inhibition of Drp1 protects against myocardial injury in multiple models of cardiac disease by enhancing mitochondrial function, decreasing mitochondrial fragmentation as well as regulating ROS and calcium homeostasis [268-272].

Thus, inhibition of Drp1 is beneficial in multiple models of age-associated disease where mitochondrial fragmentation contributes to disease mechanisms.

1.7 Study Rationale

1.7.1 Gaps in knowledge

The mechanism by which altering mitochondrial dynamics modulates lifespan is not well understood. Disruption of Drp1-mediated mitochondrial fission can extend yeast chronological lifespan and is beneficial in multiple models of age-onset diseases experienced by humans. However, inhibition of Drp1 can also be detrimental in some models, including mouse embryogenesis. In *C. elegans* disruption of *drp-1* has been found to have no effect on wild-type lifespan but significantly extends the already long lifespan of IIS mutants. The mechanism by which lifespan extension occurs in this model remains unknown.

Additionally, little is known about the conditions required for disruption of Drp1 to modulate longevity. For example, it is not known whether disrupting Drp1 after an organism's developmental stages are complete may be sufficient to extend lifespan. Similarly, it is not clear whether decreasing mitochondrial fragmentation or increasing mitochondrial fusion without inhibiting Drp1 can modulate longevity.

1.7.2 Objective of research

The objective of this research is to improve our understanding of how altering mitochondrial dynamics affects longevity and response to stress. In chapter 2, we hypothesize that decreasing mitochondrial fragmentation without disrupting *drp-1* is sufficient to promote longevity in *C. elegans*. We aim to determine whether increasing mitochondrial fusion by increasing expression of the mitochondrial fusion genes *eat-3* and *fzo-1* can promote longevity in *C. elegans*.

In chapter 3, to examine the conditions required for disruption of mitochondrial fission to extend lifespan, we explore how inhibition of *drp-1* in specific tissues and during specific times affects *C. elegans* lifespan. We hypothesize that inhibition of *drp-1* during either development or adulthood and in either neurons, muscles or intestine is sufficient to extend *daf-2* lifespan. We also aim to determine whether *daf-2* lifespan can be increased by decreasing mitochondrial fragmentation without disrupting *drp-1*. Additionally, we explore what mitochondrial mechanisms may be involved in modulation of longevity by disruption of *drp-1* in *C. elegans*.

Preface to Chapter 2

Though increased mitochondrial network connectivity is seen in healthy long-lived individuals and in multiple models of lifespan extension, little is known about how promoting mitochondrial fusion can affect lifespan in a wild-type background. We hypothesize that decreasing age-associated mitochondrial network fragmentation is sufficient to extend lifespan. Thus, in this work, we examine whether increased expression of mitochondrial fusion machinery, rather than decreased expression of mitochondrial fission machinery, extends lifespan. Surprisingly, this work demonstrates that increased expression of mitochondrial fusion machinery extends lifespan through mechanisms that do not depend on decreased mitochondrial network fragmentation. This work can be viewed as a preprint on BioRxiv, was initially submitted to *Aging Cell* in November 2023 and is invited for resubmission after revisions in 2024.

Chapter 2

Overexpression of mitochondrial fusion genes enhances resilience and extends longevity

Overexpression of mitochondrial fusion genes enhances resilience and extends longevity

Annika Traa[1,2,3], Allison Keil[1,2,3], Zenith Rudich[1,2,3], Shusen Zhu[1,2,3], Jeremy M. Van Raamsdonk[1,2,3,4]

[1]Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada
[2]Metabolic Disorders and Complications Program, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada
[3]Brain Repair and Integrative Neuroscience Program, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada
[4]Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, Quebec, Canada

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Corresponding Author:

Jeremy Van Raamsdonk
Research Institute of the McGill University Health Centre
1001 Decarie Blvd., Montreal, Quebec, Canada
1-514-934-1934 ext. 76157, jeremy.vanraamsdonk@mcgill.ca

2.1. Abstract

The dynamicity of the mitochondrial network is crucial for meeting the ever-changing metabolic and energy needs of the cell. Mitochondrial fission promotes the degradation and distribution of mitochondria, while mitochondrial fusion maintains mitochondrial function through the complementation of mitochondrial components. Previously, we have reported that mitochondrial networks are tubular, interconnected and well-organized in young, healthy *C. elegans*, but become fragmented and disorganized with advancing age and in models of age-associated neurodegenerative disease. In this work, we examine the effects of increasing mitochondrial fission or mitochondrial fusion capacity by ubiquitously overexpressing the mitochondrial fission gene *drp-1* or the mitochondrial fusion genes *fzo-1* and *eat-3*, individually or in combination. We then measured mitochondrial function, mitochondrial network morphology, physiologic rates, stress resistance and lifespan. Surprisingly, we found that overexpression of either mitochondrial fission or fusion machinery both resulted in an increase in mitochondrial fragmentation. Similarly, both mitochondrial fission and mitochondrial fusion overexpression strains have extended lifespans and increased stress resistance, which appears to be at least partially due to the upregulation of multiple stress response pathways in these strains. Overall, our work demonstrates that increasing the expression of mitochondrial fission or fusion genes extends lifespan and improves biological resilience without promoting the maintenance of a youthful mitochondrial network morphology. This work highlights the importance of the mitochondria for both resilience and longevity.

Keywords: Mitochondrial fusion, mitochondrial fission, lifespan, biological resilience, *C. elegans*, mitochondria, genetics, aging

2.2. Introduction

While mitochondria have well-established roles in cellular metabolism and energy production, mitochondria also contribute to other crucial processes in the cell including calcium homeostasis, redox signalling, autophagy, innate immunity and programmed cell death [1]. The importance of mitochondria is further highlighted by how defects in multiple mitochondrial processes such as mitochondrial gene expression, redox homeostasis, respiratory chain assembly or membrane structure can cause inherited metabolic disorders, and contribute to age-related diseases such as neurodegeneration, diabetes and cancer [2].

The dynamicity of the mitochondrial network, where single mitochondrion connect and separate from an interconnected web of mitochondria, is governed by the fusion and fission of the inner and outer mitochondrial membranes [3]. During mitochondrial fusion in *C. elegans*, the inner mitochondrial membrane is fused by the Opa-1 homolog, EAT-3, while the outer mitochondrial membrane is fused by the mitofusin homolog, FZO-1. Mitochondrial fission occurs at ER contact sites, where ER tubules begin to constrict the mitochondria and a host of proteins, including FIS-1, FIS-2, MFF-1 and MFF-2 in *C. elegans*, recruit DRP-1 to the mitochondrial constriction site [4-8]. DRP-1 oligomerizes into a ring structure around the mitochondrial constriction site and completes the scission of both the inner and outer mitochondrial membrane using the energy produced by its hydrolysis of GTP [9-12].

Tight regulation of mitochondrial fission and fusion allows the mitochondrial network to dynamically respond to changing cellular needs. For example, conditions demanding a high energy output can be adapted to by promoting mitochondrial fusion, which allows for complementation of mitochondrial components and improved mitochondrial function [13, 14]. Alternatively, mitochondria may become dysfunctional under conditions of stress, in which case mitochondrial fission is important to facilitate the clearing of defective components by mitophagy [15, 16].

The role of mitochondrial fission and fusion during the aging process remains incompletely understood. As in other model organisms, the mitochondrial networks of *C. elegans* become

fragmented and disorganized with age or in models of neurodegenerative diseases [17-26]. While the fragmentation of the mitochondrial network is presumed to be due to mitochondrial fission, large aggregates of swollen mitochondria frequently occur and are thought to be a product of overactive fusion and dysfunctional mitophagy [27]. However, despite age-associated increases in mitochondrial fragmentation, fission proteins DRP-1 and FIS1 are downregulated in aged mice and aged human endothelial cells [28-31]. In Drosophila, increased expression of Drp1 in midlife extends lifespan and health span, and aged flies display improved mitophagy and mitochondrial function [32]. Therefore, mitochondrial fission may be beneficial for healthy aging. In *C. elegans*, inhibition of the mitochondrial fusion gene *fzo-1* is reported to have no effect on lifespan, while inhibition of *eat-3* may increase lifespan [33, 34]. Furthermore, we previously reported that disruption of either *fzo-1* or *eat-3* activates multiple stress response pathways that are known to be tightly linked with mechanisms of longevity extension, including the DAF-16-mediated stress response, the SKN-1-mediated oxidative stress response and the ATFS-1-mediated mitochondrial unfolded protein response [17, 35, 36].

Paradoxically, disruption of mitochondrial fission can also promote lifespan extension. Inhibition of Drp1 is neuroprotective in mouse models of neurodegeneration [37-39]. In yeast, loss of the mitochondrial fission protein Dnm1p decreases mitochondrial fission, causes a tubular elongated mitochondrial network, and delays aging phenotypes [40]. In *C. elegans,* disrupting *drp-1* in wild-type animals has little or no effect on lifespan but disrupting *drp-1* in a neuronal model of polyglutamine toxicity improves both lifespan and health span, and decreases mitochondrial fragmentation [19]. The benefits of *drp-1* disruption may be tissue-specific as disruption of *drp-1* in a body wall muscle model of polyglutamine toxicity worsened lifespan, health span and mitochondrial network morphology [18]. Notably, disrupting *drp-1* in already long-lived *C. elegans* mutants, such as the insulin signaling mutants *daf-2* and *age-1*, drastically extends lifespan [41].

Other data suggests that promoting a balance between mitochondrial fission and fusion by disrupting both *drp-1* and *fzo-1* can extend lifespan and generates a mitochondrial network that does not become fragmented with age but instead remains elongated [33]. Furthermore,

increased mitochondrial fusion is seen in multiple long-lived mutants and is required for their extended lifespan, including *daf-2* insulin signaling mutants, feeding defective *eat-2* mutants, germlineless *glp-1* mutants, and mildly impaired mitochondrial function mutant *clk-1* [42]. Together, these data suggest that decreasing mitochondrial fragmentation, or promoting mitochondrial network elongation can increase lifespan but that a balance between mitochondrial fission and fusion components may need to remain.

In this work, we evaluated how ubiquitous overexpression of mitochondrial fission and fusion machinery affects mitochondrial network morphology, animal physiology, lifespan and resistance to stress. We hypothesized that while animals with increased expression of mitochondrial fusion genes would have elongated mitochondrial networks and increased lifespans, overexpression of both fission and fusion machinery would give animals an increased capacity to perform both fission and fusion, maintain a dynamic mitochondrial network and thus best respond to stress and cellular needs. Surprisingly, we found that overexpression of either mitochondrial fission or fusion genes individually extended lifespan and increased stress resistance despite generating a fragmented mitochondrial network. Additionally, while animals with overexpression of both fission and fusion machinery did have enhanced longevity and stress resistance compared to wild-type animals, these animals exhibit decreased longevity and stress resistance compared to animals overexpressing either *drp-1, fzo-1* or *eat-3* individually. Thus, our findings suggest that overexpression of either a single mitochondrial fission or fusion gene can promote increased longevity and stress resistance, likely through the activation of key cellular stress response pathways, but that overexpression of multiple fission or fusion genes does not enhance this effect.

2.3. Results

Overexpression of mitochondrial fission or fusion genes causes mitochondrial fragmentation

To determine how overexpression of mitochondrial fission or fusion genes would affect mitochondrial network morphology, we expressed the mitochondrial fusion genes *eat-3* and *fzo-1* and the mitochondrial fission gene *drp-1* using ubiquitous promoters (*pro-1, rpl-28* and *eft-3* respectively). Additionally, we expressed a fluorescent reporter from tissue-specific promoters such that each overexpression (OE) strain had its own marker to facilitate crossing. Thus, *drp-1* OE was identified by fluorescence in the muscle, *fzo-1* OE by fluorescence in the pharynx, and *eat-3* OE by fluorescence in the intestine (**Figure 1A**).

To validate whether the created strains had a significant increase in expression of their respective overexpressed genes, quantitative RT-PCR was used to measure the transcript levels of *drp-1, fzo-1* and *eat-3*. We found that each strain had increased mRNA levels for their corresponding gene and that overexpression of the mitochondrial fusion genes was nearly 4 times higher than overexpression of the mitochondrial fission gene *drp-1* (**Figure 1B**).

The mitochondrial network morphology of the overexpression strains was evaluated by crossing each strain with animals expressing mitochondrially-targeted GFP in body wall muscle cells. At day 1 of adulthood, overexpression of *drp-1, fzo-1,* or *eat-3* significantly increased mitochondrial fragmentation as evaluated by mitochondrial number, area, circularity and length (**Figure 2A,B**).

The impact of overexpression of mitochondrial fission or fusion genes on mitochondrial network morphology during aging was also examined, using day 8 adult worms. As we previously reported [17], wild-type worms exhibit increased mitochondrial fragmentation at this aged time point (**Figure 2C,D**). While worms overexpressing *drp-1, fzo-1* or *eat-3* also exhibit increased mitochondrial fragmentation with age, at the aged time point their mitochondrial morphology was no longer different from wild-type (**Figure 2C,D**).

Overexpression of mitochondrial fission or fusion genes decreases physiologic rates

To examine the overall health impact of overexpressing mitochondrial fission and fusion genes, general phenotypic traits such as movement, fertility, and development time were evaluated. As motility is frequently used as an indicator of worm health span [43, 44], thrashing rates were quantified for animals at day 1, day 4, and day 8 of adulthood. Overexpression of either *drp-1* or *fzo-1* significantly increased the thrashing rate at all three timepoints, while overexpression of *eat-3* significantly decreased the thrashing rate at all three timepoints (**Figure 3A-C**). Fertility was evaluated by the number of viable progeny per worm. All three overexpression strains had significantly reduced brood sizes (**Figure 3D**). Development times were measured as the time from hatching to adulthood. While overexpression of *drp-1* did not affect development time, overexpression of the mitochondrial fusion genes *fzo-1* and *eat-3* slowed development (**Figure 3E**).

To ensure that the observed phenotypes are specific to overexpression of the mitochondrial fission or fusion genes rather than being an artifact of overexpression in general, we evaluated whether overexpression of an unrelated, control protein could produce similar effects on worm physiology. To do so, we expressed the first exon of the huntingtin protein with a non-disease length polyglutamine tract from the *rpl-28* promoter, which is the same promoter used for the overexpression of *fzo-1*. We found that *rpl-28p::htt19Q* control worms exhibit an increased thrashing rate but a brood size and post-embryonic development time similar to that of wild-type animals (**Figure S1A-C**). This indicates that the deficits in movement, fertility and development that we observed are specific to the overexpression of mitochondrial dynamics genes.

Overexpression of mitochondrial fission or fusion genes affects mitochondrial function

It has previously been reported that mitochondrial network conformation affects mitochondrial function. We and others have reported findings which suggest that a fragmented mitochondrial network results in a decrease in mitochondrial oxygen consumption and ATP production, both

indicators of mitochondrial function [17]. Therefore, we tested whether overexpression of mitochondrial fission or fusion genes would affect mitochondrial function in day 1 and day 8 adults. We found that overexpression of mitochondrial fusion gene *eat-3,* but not *fzo-1,* increased oxygen consumption linked to mitochondrial respiration (**Figure 3F**) and increased ATP content (**Figure 3G**) in day 1 adults. Additionally, we found that overexpression of the mitochondrial fission gene *drp-1* decreased oxygen consumption linked to mitochondrial respiration (**Figure 3F**) as well as ATP content (**Figure 3G**) in day 1 adults, though not to a statistically significant extent. By contrast, at day 8 of adulthood, overexpression of *eat-3* significantly decreased both oxygen consumption linked to mitochondrial respiration as well as ATP content, while overexpression of *drp-1* and *fzo-1* both had increased ATP levels but without significant differences in in oxygen consumption (**Figure 3H, 3I**).

Overexpression of mitochondrial fusion genes increases resistance to exogenous stressors

Mitochondrial network morphology changes in response to environment, including conditions of stress, in order to adapt to the needs of the cell. Mitochondrial fragmentation can facilitate clearing of damaged mitochondria by mitophagy, while mitochondrial fusion can promote complementation of mitochondrial components and increased mitochondrial function. We therefore examined whether overexpression of mitochondrial fission or fusion genes impacted organismal resistance to heat stress (37°C), osmotic stress (600 mM NaCl), acute oxidative stress (300 µM juglone), chronic oxidative stress (4 mM paraquat), anoxic stress (72 hours, 24 hours recovery) and bacterial pathogen stress (*P. aeruginosa* strain PA14).

Overexpression of the mitochondrial fusion genes *eat-3* and *fzo-1* significantly increased resistance to all six exogenous stressors that we tested (**Figure 4A-F**). Overexpression of the mitochondrial fission gene *drp-1* also provided increased resistance to all the exogenous stressors except for heat stress, where a trends towards increased resistance was observed but failed to reach significance (**Figure 4A-F**). For all stress assays, *eat-3* OE worms exhibited the greatest resistance to stress, followed by *fzo-1* OE worms and then *drp-1* OE worms.

The *rpl-28p::htt19Q* control survived similarly to wild-type animals in response to heat stress, osmotic stress, chronic oxidative stress and anoxic stress (**Figure S1**). In response to acute oxidative stress, *rpl-28p::htt19Q* worms have increased survival compared to wild-type animals and performed similarly to *drp-1* OE and *fzo-1* OE worms (**Figure S1F**). In response to bacterial pathogen stress, *rpl-28p::htt19Q* worms again had increased survival compared to wild-type worms and performed similarly to *drp-1* OE animals (**Figure S1I**).

Overexpression of mitochondrial fission or fusion genes extends lifespan

To evaluate whether overexpression of mitochondrial fission or fusion genes could benefit *C. elegans* longevity, we measured the lifespan of animals overexpressing mitochondrial fusion genes *eat-3* and *fzo-1* as well as animals overexpressing the mitochondrial fission gene *drp-1*. Somewhat unexpectedly, we found that overexpression of *drp-1* significantly increases lifespan (**Figure 5A**). Additionally, overexpression of *eat-3* and *fzo-1* nearly doubled wild-type lifespan (**Figure 5B,C**). In contrast, the *rpl-28p::htt19Q* control strain only had a slight increase in lifespan compared to wild-type animals, and lived significantly shorter than animals overexpressing *drp-1, fzo-1* or *eat-3* (**Figure S1J,K**). This indicates that the lifespan extension we observed is specific to the overexpression of mitochondrial fission or fusion genes and not due to overexpression of any gene.

Disruption of mitochondrial fission or fusion genes can extend lifespan

Having shown that overexpression of mitochondrial fission and fusion genes extends longevity, we next examined how disruption of mitochondrial fission and fusion genes affects lifespan. While disruption of *fzo-1* did not affect lifespan, disruption of either *drp-1* or *eat-3* significantly extended lifespan, though to a much lesser extent than overexpression of these genes (**Figure 6A-C**). Additionally, we observed that *fis-1;fis-2* and *mff-1;mff-2* double mutants had reduced

lifespans while their single mutant counterparts had no change in lifespan (**Figure 6D-I**), suggesting the possibility of functional redundancy.

Overexpression of mitochondrial fusion genes activates multiple pathways of cellular resilience

Activation of key pathways of cellular resilience such as the DAF-16-mediated stress response, the p38-mediated innate immune signaling pathway, the mitochondrial unfolded protein response, the cytosolic unfolded protein response, the SKN-1-mediated oxidative stress response and the HIF-1-mediated hypoxia response can enhance resistance to stress and contribute to lifespan extension [35, 36, 45-48]. To determine if activation of cellular resilience pathways contributes to the lifespan extension and stress resistance in the mitochondrial fission and fusion overexpression strains, we measured mRNA levels of target genes for each pathway using quantitative RT-PCR.

We examined target genes from the mitochondrial unfolded protein response (*cdr-2, F15B9.10, hsp-6;* **Figure 7A-C**), the cytoplasmic unfolded protein response (*hsp-16.2;* **Figure 7D**), the ER-unfolded protein response (*hsp-4*; **Figure 7E**), the hypoxia response (*nhr-57, F22B5.4*; **Figure 7F,G**), the DAF-16-mediated stress response (*mtl-1, sod-3, dod-3, sodh-1*; **Figure 7H-K**), the p38-mediated innate immune signaling pathway (*sysm-1, Y9C9A.8*; **Figure 7L,M**) and the SKN-1-mediated oxidative stress response (*gst-4*; **Figure 7N**). Overexpression of the mitochondrial fusion gene *eat-3* resulted in upregulated expression of multiple DAF-16 target genes, including *mtl-1, sod-3* and *dod-3* (**Figure 7H-J**), as well as target genes from the innate immune signaling pathway (**Figure 7L**) and the SKN-1-mediated oxidative stress response (**Figure 7N**). Overexpression of the mitochondrial fusion gene *fzo-1* also exhibited a trend towards increased expression of these target genes but it only reached significance for the SKN-1 pathway. Overexpression of *drp-1* did not significantly increase the expression of any of the target genes. With the exception of *hsp-4* and *F22B5.4*, the *rpl-28p::htt19Q* control strain did not increase

expression of any target genes, indicating that overexpression of the mitochondrial fission or fusion proteins specifically activated these stress response pathways.

Overexpression of *drp-1* does not ameliorate mitochondrial morphology or enhance increased stress resistance and lifespan caused by overexpression of mitochondrial fusion genes

As *drp-1* and *fzo-1/eat-3* perform opposite functions within the cell, we hypothesized that overexpression of *drp-1* could restore mitochondrial network morphology in *fzo-1* OE and *eat-3* OE worms, and revert the phenotypic differences observed in these worms to those of wild-type worms. Accordingly, we crossed *drp-1* OE worms to both *fzo-1* OE and *eat-3* OE worms to generate *fzo-1* OE;*drp-1* OE and *eat-3* OE;*drp-1* OE double transgenic worms.

At day 1 of adulthood, *drp-1* OE failed to revert mitochondrial morphology towards wild-type morphology in either *fzo-1* OE or *eat-3* OE animals (**Figure S2A,B**). In fact, *drp-1* OE further decreased mitochondrial area and length and further increased circularity in *eat-3* OE worms. Similarly, at day 8 of adulthood, overexpression of *drp-1* decreased mitochondrial area and length and increased circularity in both *fzo-1* OE and *eat-3* OE worms (**Figure S2A,C**).

In examining the effect of *drp-1* OE on physiologic rates, we found that it was able to restore the thrashing rate of *eat-3* OE worms towards wild-type levels (**Figure S2D**) but did not improve either fertility (**Figure S2E**) or development time (**Figure S2F**). Despite increasing resistance to stress in wild-type worms, overexpression of *drp-1* in *eat-3* OE worms decreased resistance to heat stress (**Figure S3A**), acute oxidative stress (**Figure S3C**), chronic oxidative stress (**Figure S3D**) and bacterial pathogen stress but did not significantly reduce resistance to osmotic stress (**Figure S3B**) or anoxia (**Figure S3E**). *drp-1* OE also significantly decreased the lifespan of both *fzo-1* OE and *eat-3* OE worms (**Figure S3G,H**), despite increasing wild-type lifespan.

Overexpression of *fzo-1* and *drp-1* in *eat-3* OE worms decreases stress resistance and lifespan

As FZO-1 fuses the outer mitochondrial membrane and EAT-3 fuses the inner mitochondrial membrane, it may be necessary to increase the expression of both genes to increase mitochondrial fusion. To determine if some of the phenotypes we observed might result from an imbalance in fusion of the inner and outer mitochondrial membrane, we characterized *eat-3* OE;*fzo-1* OE double mutants. We also examined *eat-3* OE;*fzo-1* OE;*drp-1* OE triple mutants, which have increased expression of all three of the major mitochondrial fission and fusion genes, and might be predicted to have increased mitochondrial fission and fusion capacity. Similar to overexpression of *eat-3* or *fzo-1* individually, *eat-3* OE;*fzo-1* OE double mutants and *eat-3* OE;*fzo-1* OE;*drp-1* OE triple mutants exhibited decreased thrashing (**Figure S4A**), decreased fertility (**Figure S4B**), and slow post-embryonic development time (**Figure S4C**). Resistance to heat stress (**Figure S4D**), acute oxidative stress (**Figure S4E**), chronic oxidative stress (**Figure S4F**), bacterial pathogens (**Figure S4G**), osmotic stress (**Figure S4H**) and anoxia (**Figure S4I**) in *eat-3* OE;*fzo-1* OE and *eat-3* OE;*fzo-1* OE;*drp-1* OE worms was all significantly diminished compared to *eat-3* OE worms. Similarly, while *eat-3* OE;*fzo-1* OE double mutants and *eat-3* OE;*fzo-1* OE;*drp-1* OE triple mutants live longer than wild-type worms, their lifespan is significantly decreased compared to *eat-3* OE worms (**Figure S4J**). Overall, the overexpression of *fzo-1* or of both *fzo-1* and *drp-1* decreases resistance to stress and lifespan in *eat-3* OE worms.

2.4. Discussion

Overexpression of mitochondrial fusion genes can cause mitochondrial fragmentation

Contrary to our prediction that overexpression of mitochondrial fusion genes would result in a more fused mitochondrial network, we found that overexpression of either mitochondrial fission or fusion genes both caused mitochondrial fragmentation. However, other groups have also observed mitochondrial fragmentation in response to overexpression of mitochondrial fusion genes. In mammalian cells, the overexpression of the outer mitochondrial fusion proteins MFN1 and MFN2 causes either clustering of spherical mitochondria or elongated mitochondria [49, 50]. Overexpression of the mammalian inner mitochondrial fusion protein OPA1 increases mitochondrial network fragmentation, likely due to an imbalance in the generation of long and short OPA-1 isoforms, the latter of which has been suggested to promote fission [16, 51-55]. Additionally, modest overexpression of OPA1 induces mitochondrial fusion and mitochondrial network elongation while high levels of OPA1 overexpression induces mitochondrial fragmentation, indicating that outcomes from fusion machinery overexpression may be dependent upon the degree of overexpression [56].

In *C. elegans*, the overexpression of either of the mitochondrial fusion genes *fzo-1* or *eat-3* using a heat-shock inducible promoter produces mitochondrial fragmentation in embryos and it was suggested that, as with OPA1, EAT-3 may contribute to fission activity in certain conditions [57]. In the same study, it was reported that overexpression of the outer mitochondrial fusion gene *fzo-1* specifically in the muscle results in mitochondrial fragmentation in the muscle, indicating that overexpression of either of the mitochondrial fusion genes can induce mitochondrial fragmentation in *C. elegans*.

Overexpression of mitochondrial fusion genes activates multiple stress response pathways.

We found that overexpression of the mitochondrial fusion genes *fzo-1* and *eat-3* increases resistance to all tested exogenous stressors and activated multiple stress response pathways.

Overexpression of *drp-1* also increases resistance to all stressors other than heat stress, though to a lesser degree compared to *eat-3* OE and *fzo-1* OE. However, we did not observe an increase in the activation of stress response pathways in *drp-1* OE animals, suggesting that altering the expression of *drp-1* does not induce resistance to stress in the same manner as overexpression of the mitochondrial fusion genes.

Overexpression of mitochondrial fission or mitochondrial fusion genes increases lifespan

Others previously reported that ubiquitous overexpression of the mitochondrial fusion gene *fzo-1* is not sufficient to extend lifespan in *C. elegans* [33]. However, tissue-specific overexpression of *fzo-1* in either the neurons, the muscle or the intestine of animals with a *fzo-1* null mutant background resulted in a slight but significant lifespan extension, suggesting that specific conditions may be required in order for overexpression of *fzo-1* to increase longevity [33].

We predicted that increasing the capacity for mitochondrial fusion events to occur by increasing mitochondrial fusion gene expression could benefit lifespan by decreasing age-associated mitochondrial fragmentation and improving mitochondrial function. We found that, despite increasing mitochondrial fragmentation, ubiquitous overexpression of the mitochondrial fission gene *drp-1* or either of the mitochondrial fusion genes *eat-3* and *fzo-1* significantly extends *C. elegans* lifespan. Thus, our findings suggest that lifespan extension by overexpression of mitochondrial fusion genes may not be dependent on mitochondrial network morphology or function, but rather on activation of stress response pathways triggered by excess levels of mitochondrial fission and fusion proteins. Furthermore, our findings suggest that increasing mitochondrial fragmentation by increasing expression of *drp-1* can extend lifespan and somewhat improve mitochondrial function during aging, contrary to our prediction that decreasing mitochondrial fragmentation benefits longevity.

Increasing or decreasing expression of mitochondrial fission or fusion genes extends lifespan and increases resistance to stress

Previous reports as to how the deletion of mitochondrial fission and fusion genes affects *C. elegans* lifespan differ [33, 34, 42, 58]. We find that in the case of *eat-3* and *drp-1,* either deletion or overexpression increases lifespan, though deletion of these genes extends lifespan to a much lesser extent compared to overexpression (**Table S1**). Previously, we have also shown that disruption of mitochondrial fusion genes *fzo-1* and *eat-3* increases resistance to stress and activates multiple stress response pathways, similarly to overexpression of mitochondrial fusion genes. We have also observed that while disruption of *drp-1* increases resistance to stress, similarly to overexpression of *drp-1,* it does not activate multiple stress response pathways[17].

We previously reported that disruption of *drp-1* does not affect mitochondrial morphology and that disruption of *eat-3* causes mitochondrial fragmentation. Here, we find that overexpression of either *drp-1* or *eat-3* induces segmentation of the mitochondrial network. Combined, these findings suggest that lifespan extension by increasing or decreasing mitochondrial fission and fusion gene expression is not dependent on either mitochondrial fragmentation or mitochondrial elongation.

Furthermore, while disruption of *drp-1* somewhat decreases mitochondrial function and disruption of *eat-3* significantly decreases mitochondrial function, overexpression of *drp-1* provides an age-associated improvement in mitochondrial function and overexpression of *eat-3* results in an age-associated decline in mitochondrial function [17]. Together, this suggests that lifespan extension by manipulation of mitochondrial fission and fusion gene expression is not specifically linked to the increased or decreased mitochondrial function that may be expected with morphological changes.

Altogether, these findings suggest that an imbalanced expression level of mitochondrial fission or fusion genes may lead to a hormetic response that acts independent of mitochondrial structure or function, leading to increased lifespan possibly through increased expression of pro-survival genes. However, our findings suggest that while increasing or decreasing

mitochondrial fusion gene expression benefits lifespan by activating stress response pathways, increasing or decreasing fission gene expression may act to extend lifespan through an alternative mechanism. Given that disruption of *drp-1* significantly extends the lifespan of already long-lived mutants such as *daf-2,* and we have shown that overexpression of *drp-1* significantly extends the lifespan of wild-type animals, future investigations should determine how overexpression of *drp-1* may affect the lifespan of *daf-2* worms and other long-lived mutants [41].

Simultaneous overexpression of mitochondrial fission and fusion genes is not more beneficial than overexpression of either a fission or a fusion gene.

We predicted that the simultaneous overexpression of both *drp-1* and either *fzo-1* or *eat-3* would maintain the dynamicity of mitochondria that is lost with age, improving an organism's ability to respond to stress, and thus allowing for an increase in lifespan. However, we found that while *fzo-1;drp-1* OE animals still survived longer than wild-type animals, their lifespans were shorter than *fzo-1* OE animals and their mitochondrial networks were more fragmented than wild-type animals. Likewise, the lifespans of *eat-3;drp-1* OE animals were shorter than *eat-3* OE animals and like *eat-3* OE worms, their mitochondrial networks were highly fragmented. Similarly, while *fzo-1;drp-1* OE and *eat-3;drp-1* OE animals still had increased resistance to multiple stressors, they did not survive as well as animals that overexpressed only one gene, suggesting that a more unbalanced expression of mitochondrial fission and fusion genes may promote a stronger stress response despite producing similar mitochondrial morphologies.

We also evaluated whether the benefits of overexpressing both mitochondrial fusion genes *fzo-1* and *eat-3* could further extend lifespan and stress resistance. We again found that the overexpression of both genes resulted in reduced resistance to stress and reduced lifespan extension compared to overexpression of only one fusion gene. The overexpression of all three mitochondrial dynamics genes performed similarly to the overexpression of only the fusion

genes, further indicating that a stronger imbalance in the expression of mitochondrial fission or fusion genes may yield a stronger activation of stress response genes and improved survival.

2.5. Conclusion

Overall, our findings demonstrate that overexpression of mitochondrial fission genes or mitochondrial fusion genes both result in mitochondrial fragmentation, increased resilience and extended longevity. We find that as with the deletion of mitochondrial fusion genes, the overexpression of mitochondrial fusion genes activates multiple stress response pathways, which likely contributes to their enhanced resistance to multiple stressors. Combined with our previous work, this indicates that increasing or decreasing the expression of genes involved in mitochondrial fission or mitochondrial fusion can lead to increased stress resistance and lifespan.

2.6. Experimental procedures

Strains

WT/N2	
MQ1753	*drp-1(tm1108)IV*
FX1133	*fzo-1(tm1133)II*
JVR063	*eat-3(tm1107)II*
FX2227	*fis-1(tm2227)II*
JVR078	*fis-2(gk414)X*
JVR108	*fis-1(tm2227) II;fis-2(gk414)X*
JVR330	*mff-1(tm2955)X*
JVR331	*mff-2(tm3041)I*
JVR375	*mff-1(tm2955)X;mff-2(tm3041)I*
JVR587	*drp-1 o/e sybIs3765[Peft-3::drp-1::unc-54 3'UTR]*
JVR588	*eat-3 o/e sybIs3770[Ppro-1-eat-3-unc-54 3'UTR]*
JVR589	*fzo-1 o/e sybIs3776[Prpl-28-fzo-1-unc-54 3'UTR]*
JVR617	*drp-1 o/e sybIs3765[Peft-3::drp-1+Pmyo-3::mCherry]; fzo-1 o/e sybIs3776[Prpl-28::fzo-1+Pmyo-2::mCherry]*
JVR618	*drp-1 o/e sybIs3765 [Peft-3::drp-1+Pmyo-3::mCherry]; eat-3 o/e sybIs3770[Ppro-1::eat-3+Pvha-6::mCherry]*
JVR122	*bcIs78(pMyo-3::mitoGFP(matrixGFP) + pRF4); rol-6(su1006)II*
JVR622	*drp-1 o/e sybIs3765[Peft-3::drp-1+Pmyo-3::mCherry]; bcIs78(pmyo-3::mitoGFP (matrix GFP) + pRF4); rol-6(su1006)II*
JVR623	*fzo-1 o/e sybIs3776[Prpl-28::fzo-1+Pmyo-2::mCherry]; bcIs78(pmyo-3::mitoGFP (matrix GFP) + pRF4); rol-6(su1006)II*
JVR624	*eat-3 o/e sybIs3770[Ppro-1::eat-3+Pvha-6::mCherry]; bcIs78(pmyo-3::mitoGFP (matrix GFP) + pRF4); rol-6(su1006)II*

JVR625 *drp-1 o/e sybIs3765 [Peft-3::drp-1+Pmyo-3::mCherry]; fzo-1 o/e sybIs3776[Prpl-28::fzo-1+Pmyo-2::mCherry]; bcIs78(pmyo-3::mitoGFP (matrix GFP) + pRF4); rol-6(su1006)II*

JVR626 *drp-1 o/e sybIs3765 [Peft-3::drp-1+Pmyo-3::mCherry]; eat-3 o/e sybIs3770 [Ppro-1::eat-3+Pvha-6::mCherry]; bcIs78(pmyo-3::mitoGFP (matrix GFP) + pRF4); rol-6(su1006)II*

JVR642 *fzo-1 o/e sybIs3776[Prpl-28::fzo-1+Pmyo-2::mCherry]; eat-3 o/e sybIs3770[Ppro-1::eat-3+Pvha-6::mCherry]; drp-1 o/e sybIs3765[Peft-3::drp-1+Pmyo-3::mCherry]*

JVR643 *fzo-1 o/e sybIs3776[Prpl-28::fzo-1+Pmyo-2::mCherry]; eat-3 o/e sybIs3770[Ppro-1::eat-3+Pvha-6::mCherry]*

sybIs6440 Prpl-28::HTT(Q19)::wrmScarlet::unc-54 3'UTR

Quantitative real-time RT-PCR

To perform quantitative RT-PCR, we first collected worms in M9 buffer and extracted RNA using Trizol as previously described [59]. Using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814), the collected mRNA was then converted to cDNA. Quantitative PCR was performed using a PowerUp SYBR Green Master Mix (Applied Biosystems A25742) in a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems N8010560) and a Viia 7 Applied Biosystems qPCR machine. mRNA levels were calculated as the copy number of the gene of interest relative to the copy number of the endogenous control, *act-3*, then expressed as a percentage of wild-type. Primer sequences for each target gene are as follows: *drp-1* (L-GAGATGTCGCTATTATCGAACG, R- CTTTCGGCACACTATCCTG) *fzo-1* (L- GCTTTCTGCAGGTTGAAGGT, R- CGACACCAGGGCTATCAAGT) *eat-3* (L- GCGAAGTTTTGGACTTGCTC, R-CGATCGAATCCGAACTGTTT).

Confocal imaging and quantification

Mitochondrial morphology was imaged using worms that express mitochondrially-targeted GFP in the body wall muscle cells as well as a *rol-6* mutant background. The *rol-6* mutation results in animals moving in a twisting motion, allowing one side of the sheaths of muscle cells to always be facing the objective lens and thus facilitating imaging of mitochondrial networks within cells.

Without the *rol-6* mutation, only the longitudinal edges of the muscle will often be visible, thus making it difficult to observe mitochondrial organization. Worms at day 1 or day 8 of adulthood were mounted on 2% agar pads and immobilized using 10 µM levamisole. Worms were imaged under a 40× objective lens on a Zeiss LSM 780 confocal microscope. Single plane images were collected for a total of 24 worms over 3 biological replicates for each strain. Imaging conditions were kept the same for all replicates and images. Quantification of mitochondrial morphology was performed using ImageJ. Segmentation analysis was carried out using the SQUASSH (segmentation and quantification of subcellular shapes) plugin. Particle analysis was then used to measure number of mitochondria, mitochondrial area, mitochondrial circularity, and maximum Feret's diameter (an indicator of particle length).

Thrashing rate

Thrashing rates were determined manually by transferring 20 worms onto an unseeded agar plate. One milliliter of M9 buffer was added and the number of body bends per 30 seconds was counted for 3 biological replicates of 6-8 worms per strain.

Brood size

Brood size was determined by placing individual prefertile young adult animals onto NGM plates. Worms were transferred to fresh NGM plates daily until progeny production ceased. The resulting progeny was allowed to develop to the L4 stage before quantification. Three biological replicates of 5 animals each were completed.

Post-embryonic development

Postembryonic development (PED) was assessed by transferring ~ 50-100 eggs to agar plates. After 3 h, newly hatched L1 worms were transferred to a new plate. Starting at 28 hours after hatching, worms were scored approximately every 2 hours and worms that reached young adulthood were removed from the plate. The hours from hatching to young adulthood were measured as the PED time. Three biological replicates of 20 animals each were completed.

Oxygen consumption rate

Oxygen consumption measurements were taken using a Seahorse XFe96 analyzer. The night before the assay, probes were hydrated in 200 µL Seahorse calibrant at 37 degrees while the analyzer's heater was turned off to allow the machine to cool. Day 1 and day 8 worms were collected in M9 buffer and washed three times before being pipetted into a Seahorse 96 well plate (Agilent Technologies Seahorse Flux Pack 103793-100). We pipetted approximately 5-25 worms into each well, a number which others have previously determined to be optimal for this assay [60]. Calibration was performed after 22 µL of FCCP and 24 µL sodium azide was loaded into the drug ports of the sensor cartridge. Measurements began within 30 minutes of worms being added to the wells. Basal oxygen consumption was measured 5 times before the first drug injection. FCCP-induced oxygen consumption was measured 9 times, then sodium-azide-induced oxygen consumption was measured 4 times. Measurements were taken over the course of 2 minutes and before each measurement the contents of each well were mixed for an additional 2 minutes. Non-mitochondrial respiration (determined by sodium-azide-induced oxygen consumption rate) was subtracted from basal respiration to calculate mitochondrial respiration.

ATP determination

Day 1 and day 8 adult worms were collected, washed 3 times and frozen in 50 µL of M9 buffer using liquid nitrogen. Samples were then immersed in boiling water for 15 minutes followed by ice for 5 minutes and finally spun down at 14,800g for 10 minutes at 4 ℃. Supernatants were diluted 10-fold before ATP measurements using a Molecular Probes ATP determination kit (A22066) and TECAN plate reader. Luminescence was normalized to protein content measured using a Pierce BCA protein determination kit.

Heat stress assay

To measure resistance to heat stress, approximately 25 pre-fertile young adult worms were transferred to new NGM plates freshly seeded with OP50 bacteria and were incubated at 37℃.

Starting at 2 hours, survival was measured every hour for a total of 10 hours of incubation. Three biological replicates were completed.

Osmotic stress assay

To measure resistance to osmotic stress, approximately 25 pre-fertile young adult worms were transferred to NGM plates containing 500 mM NaCl and seeded with OP50 bacteria. Worms were kept at 20℃ for 24 hours before survival was scored. Five biological replicates were completed.

Oxidative stress assays

Resistance to acute oxidative stress was measured by transferring approximately 25 pre-fertile young adult worms to 300 µM juglone plates seeded with OP50 bacteria. Worms were kept at 20℃ and survival was monitored every 2 hours for a total of 8 hours. Resistance to chronic oxidative stress was performed by transferring 30 pre-fertile young adult worms to freshly prepared plates containing 4 mM paraquat, 25 µM FUdR and seeded with concentrated OP50. Survival was monitored daily. Three biological replicates were completed for both assays.

Anoxic stress assay

To measure resistance to anoxic stress, approximately 50 pre-fertile young adult worms were transferred to new NGM plates seeded with OP50 bacteria. To create a low-oxygen environment for the worms, we utilized Becton-Dickinson Bio-Bag Type A Environmental Chambers. Plates with young adult worms of each strain were placed in the Bio-Bags for 48 hours at 20℃, then removed from the bags and allowed to recover for 24 hours at 20℃ before survival was measured. Five biological replicates were completed.

Bacterial pathogen stress assay

We tested for nematode resistance to death by bacterial colonization of the intestine. The slow kill assay was performed as previously described [61, 62]. OP50 bacteria was seeded to the center of NGM plates containing 100 mg/L FUdR and plates were left at room temperature for

two days. PA14 cultures were grown with aeration at 37℃ for 16 hours, then seeded to the center of NGM agar plates containing 20 mg/L FUdR. The plates seeded with PA14 bacteria were allowed to dry, then incubated at 37℃ for 24 hours and then at room temperature for 24 hours. Approximately 40 L4 worms were transferred to plates containing 100 mg/L FUdR that were seeded with OP50 bacteria, and the worms were grown at 20℃ until they reached day 3 of adulthood. Day 3 adult worms were then transferred from these plates onto plates containing 20 mg/L FUdR that were seeded with PA14 bacteria. The assay was conducted at 20℃ and survival was monitored daily until all worms died. Three biological replicates were completed.

Lifespan assay

Lifespan assays were completed at 20℃ and on NGM agar plates that contained FUdR to inhibit the development of progeny and limit internal hatching. We used a low concentration of 25 μM FUdR, which we have previously shown does not affect the longevity of wild-type worms [63]. For each lifespan assay, 40 pre-fertile young adult worms were transferred to 25 μM FUdR plates seeded with OP50 bacteria and were kept at 20℃. Four biological replicates were started on four subsequent days and all replicates were scored every other day to monitor survival until all worms died. Worms were excluded from the assay if they crawled off the agar and died on the side of the plate, had internal hatching of progeny or expulsion of internal organs. Raw lifespan data are provided in **Table S2**.

Statistical analysis

A minimum of three biological replicates were completed for all assays. Where possible, the experimenter was blinded to the genotype during the course of the experiment, to ensure unbiased results. Statistical significance of differences between groups was determined by computing a t-test, a one-way ANOVA, a two-way ANOVA or a log-rank test using Graphpad Prism, as indicated in the Figure legends. All error bars indicate the standard error of the mean.

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2.10. Figures

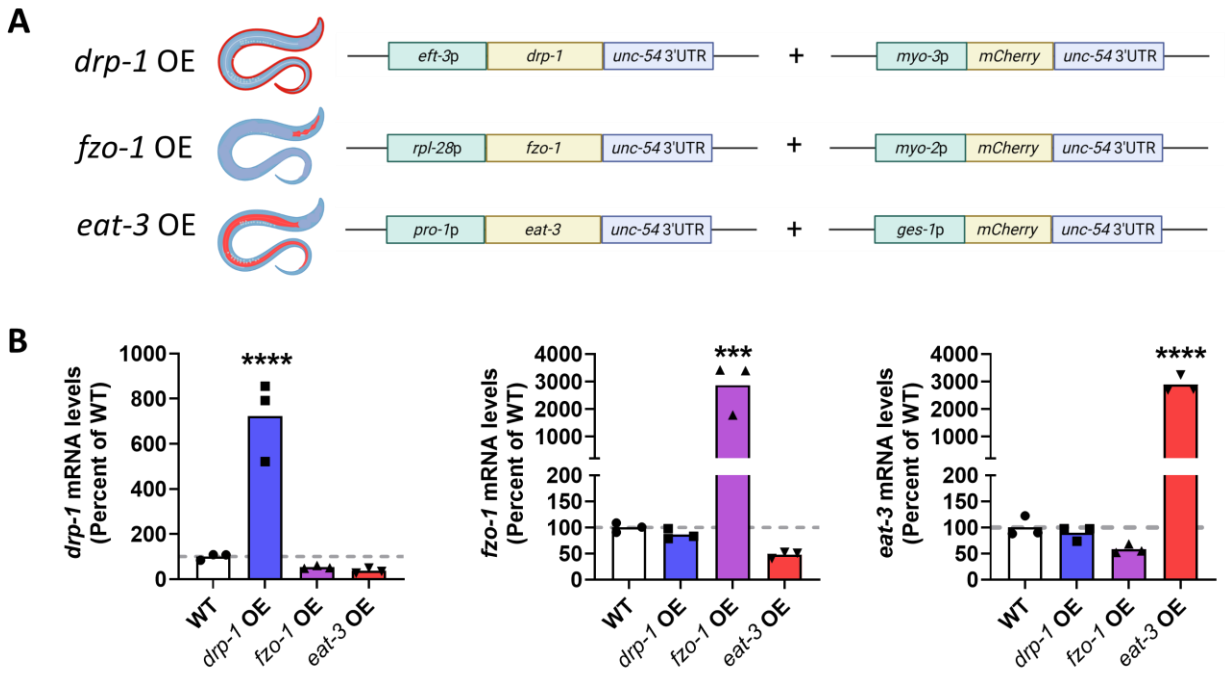

Figure 1. Overexpression of mitochondrial fission or fusion genes in *C. elegans*. *C. elegans* strains that overexpress mitochondrial fission or fusion genes were generated by microinjection and integration of the extrachromosomal array. (**A**) Diagram of constructs used to generate overexpression strains. The mitochondrial fission and fusion genes *drp-1, fzo-1* and *eat-3* were overexpressed under the ubiquitous promoters *eft-3, rpl-28* and *pro-1,* respectively (indicated by blue colouring in worm diagram). Each strain also expressed a red fluorescent co-injection marker in intestine (*drp-1* OE), pharynx (*fzo-1* OE) or body wall muscle (*eat-3* OE). (**B**) Quantitative RT-PCR confirmed that each overexpression strain exhibits increased expression of the intended mitochondrial fission or fusion gene. Three biological replicates were performed. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparisons test. OE = overexpression. ***p<0.001, ****p<0.0001.

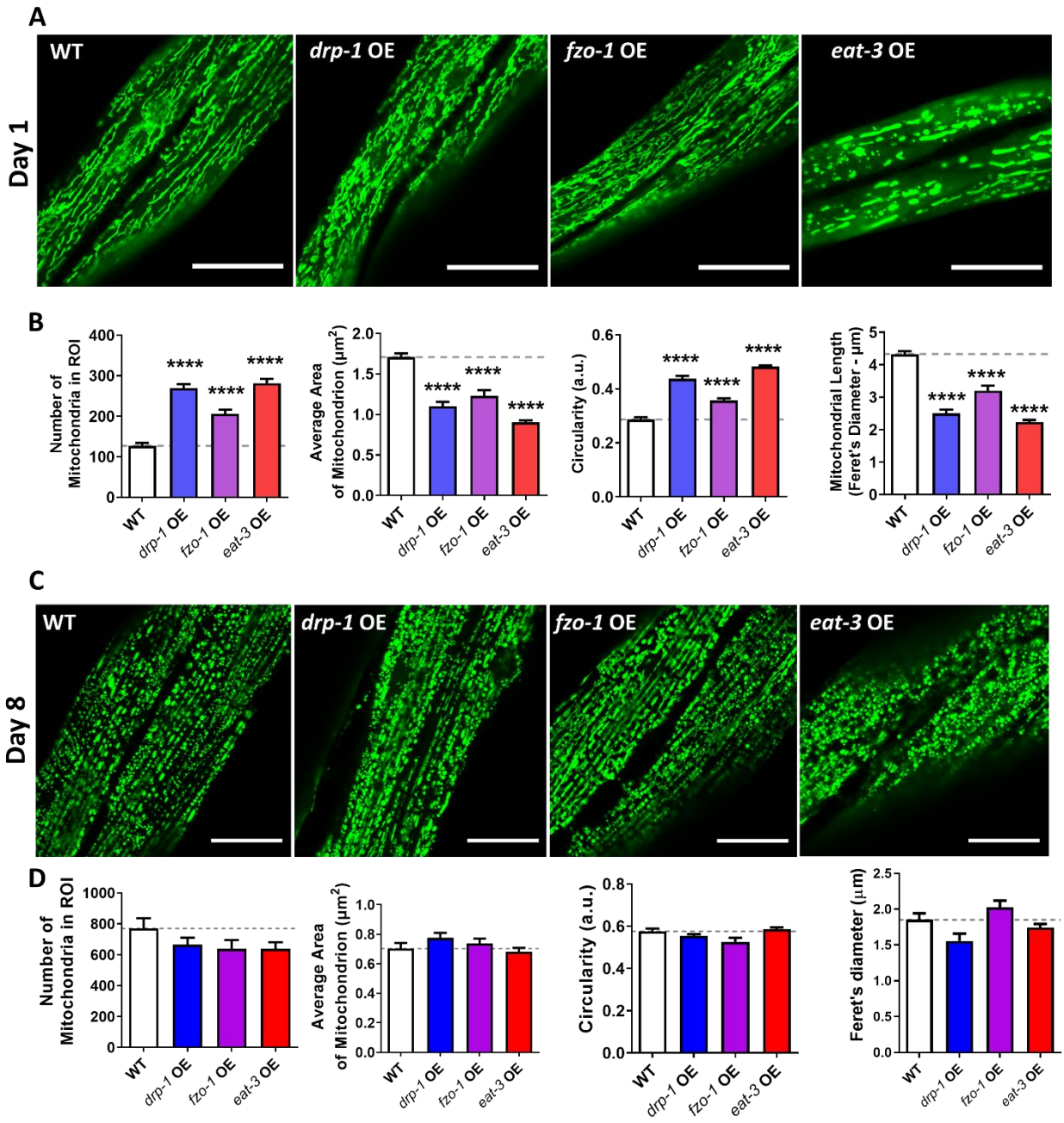

Figure 2. Overexpression of mitochondrial fission or fusion genes causes mitochondrial fragmentation. The mitochondrial morphology resulting from overexpression of mitochondrial fission and fusion genes was assessed at day 1 and day 8 of adulthood. (**A**) At day 1 of adulthood, overexpression of *drp-1, fzo-1* or *eat-3* resulted in mitochondrial fragmentation. Scale bar indicates 25 μm. (**B**) Quantification of mitochondrial morphology showed that these worms have an increased number of mitochondria, decreased mitochondrial area, increased

circularity and decreased length, all consistent with increased fragmentation. (**C**) At day 8 of adulthood, mitochondrial morphology in the strains overexpression *drp-1, fzo-1* or *eat-3* was similar to that in wild-type worms. (**D**) Quantification of mitochondrial morphology at day 8 of adulthood revealed no significant differences between the overexpression strains and wild-type worms. Error bars indicate SEM. Three biological replicates were performed. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparisons test. OE = overexpression. ****p<0.0001.

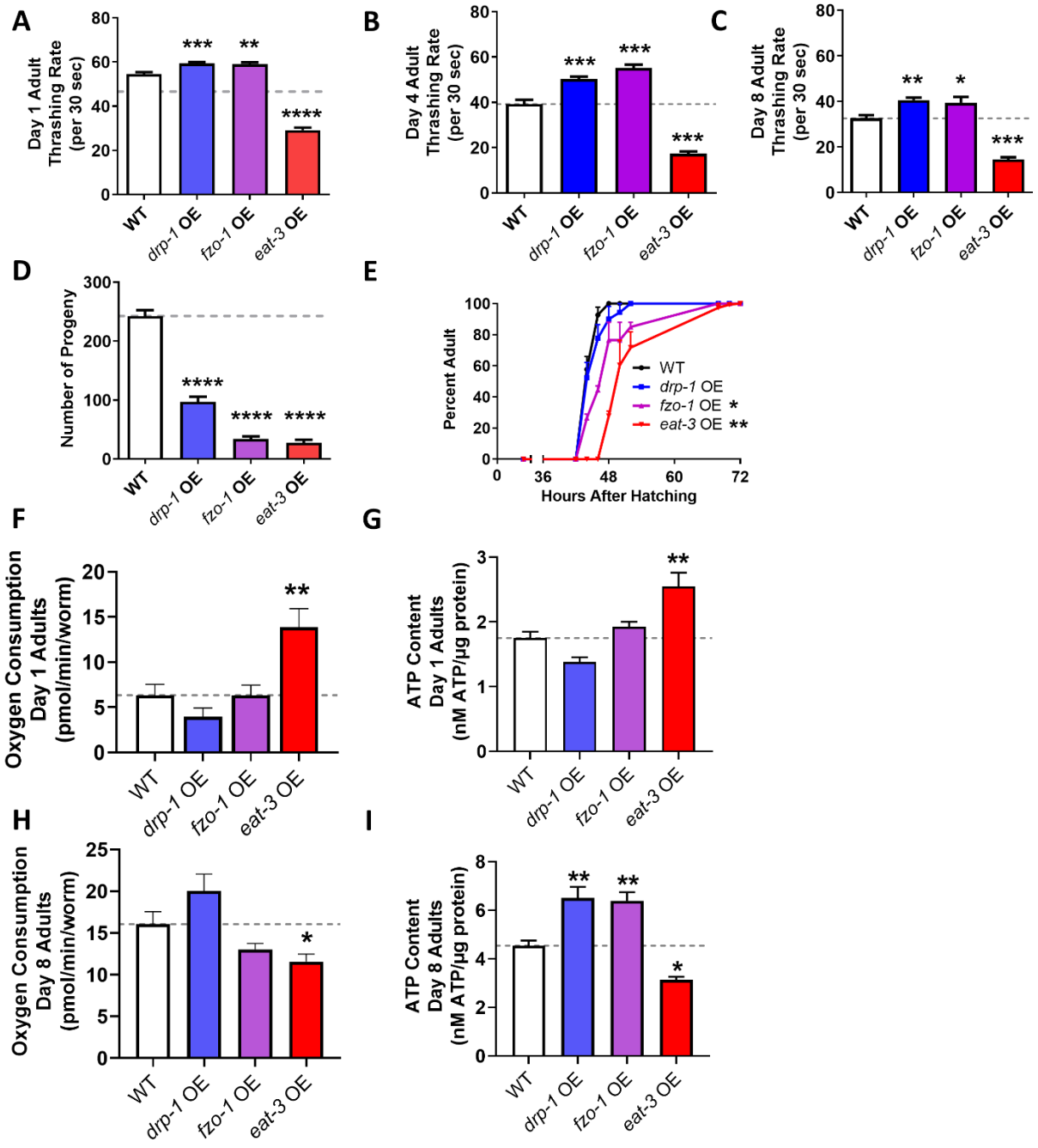

Figure 3. Overexpression of mitochondrial fission or fusion genes results in slowed physiologic rates. The effect of overexpressing mitochondrial fission and fusion genes on the general health of worms was assessed by measuring physiologic rates. (**A-C**) Overexpression of *drp-1* or *fzo-1* resulted in a significant increase in movement as measured by thrashing rate in liquid. In contrast, *eat-3* OE worms exhibited a significant decrease in thrashing rate. (**D**) All three overexpression strains have markedly decreased fertility as indicated by a decreased brood size.

(**E**) Overexpression of either of the mitochondrial fusion genes, *fzo-1* or *eat-3,* results in slow post-embryonic development. At day 1 of adulthood, *eat-3* OE worms have increased oxygen consumption (**F**) and increased levels of ATP (**G**). In contrast, at day 8 of adulthood, *eat-3* OE worms have decreased oxygen consumption (**H**) and decreased ATP levels (**I**). Error bars indicate SEM. A minimum of three biological replicates were performed. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparisons test. OE = overexpression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

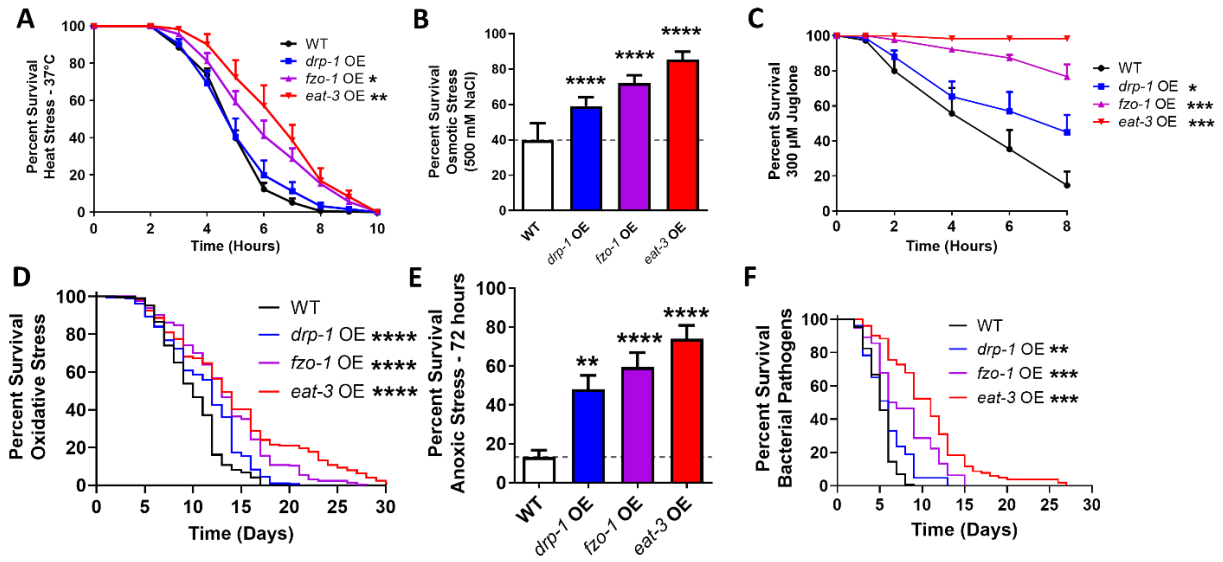

Figure 4. Overexpression of mitochondrial fusion genes increases resistance to exogenous stressors. (**A**) Overexpression of the mitochondrial fusion genes, *fzo-1* or *eat-3,* results in increased resistance to heat stress at 37°C. *drp-1* OE, *fzo-1* OE and *eat-3* OE worms all have increased resistance to osmotic stress (500 mM NaCl, **B**), acute oxidative stress (300 µM juglone, **C**), chronic oxidative stress (4 mM paraquat, **D**), anoxia (72 hours, **E**), and bacterial pathogen stress (*P. aeruginosa* strain PA14, **F**). Error bars indicates SEM. A minimum of six biological replicates were performed. Statistical significance was assessed using a repeated measures ANOVA with Tukey's multiple comparison test in panels A and C, a log-rank test in panels B, D and F, and a one-way ANOVA with Dunnett's multiple comparison test in panel E. OE = overexpression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

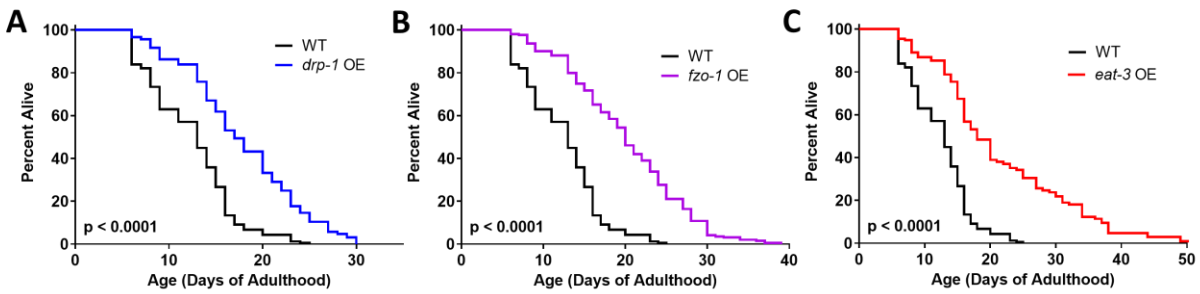

Figure 5. Overexpression of mitochondrial fission or fusion genes extends lifespan.

Overexpression of the mitochondrial fission gene *drp-1* or either of the mitochondrial fusion genes *fzo-1* or *eat-3* significantly increases lifespan. Six biological replicates were performed. Statistical significance was assessed using a log-rank test.

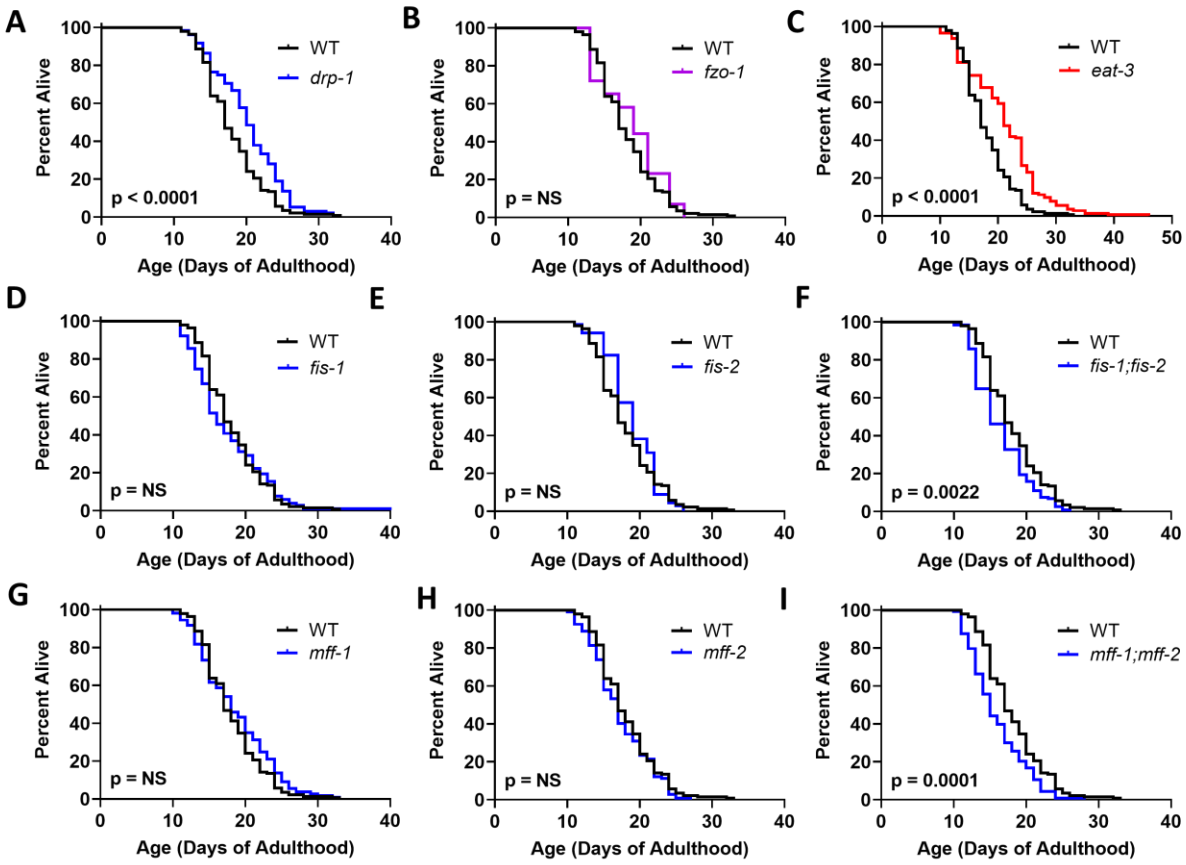

Figure 6. Disruption of mitochondrial fission or fusion genes can extend lifespan. (**A**) Disruption of the mitochondrial fission gene *drp-1* resulted in a small increase in lifespan. While deletion of the mitochondrial fusion gene *fzo-1* did not affect lifespan (**B**), disruption of *eat-3* extended longevity (**C**). Disruption of either *fis-1* (**D**), or *fis-2* (**E**) individually did not affect lifespan, while *fis-1;fis-2* double mutants exhibited a slight decrease in longevity (**F**). Similarly, *mff-1* (**G**) and *mff-2* (**H**) single mutants have a normal lifespan, while *mff-1;mff-2* double mutants exhibit decreased longevity (**I**). At least three biological replicates were performed. Statistical significance was assessed using a log-rank test.

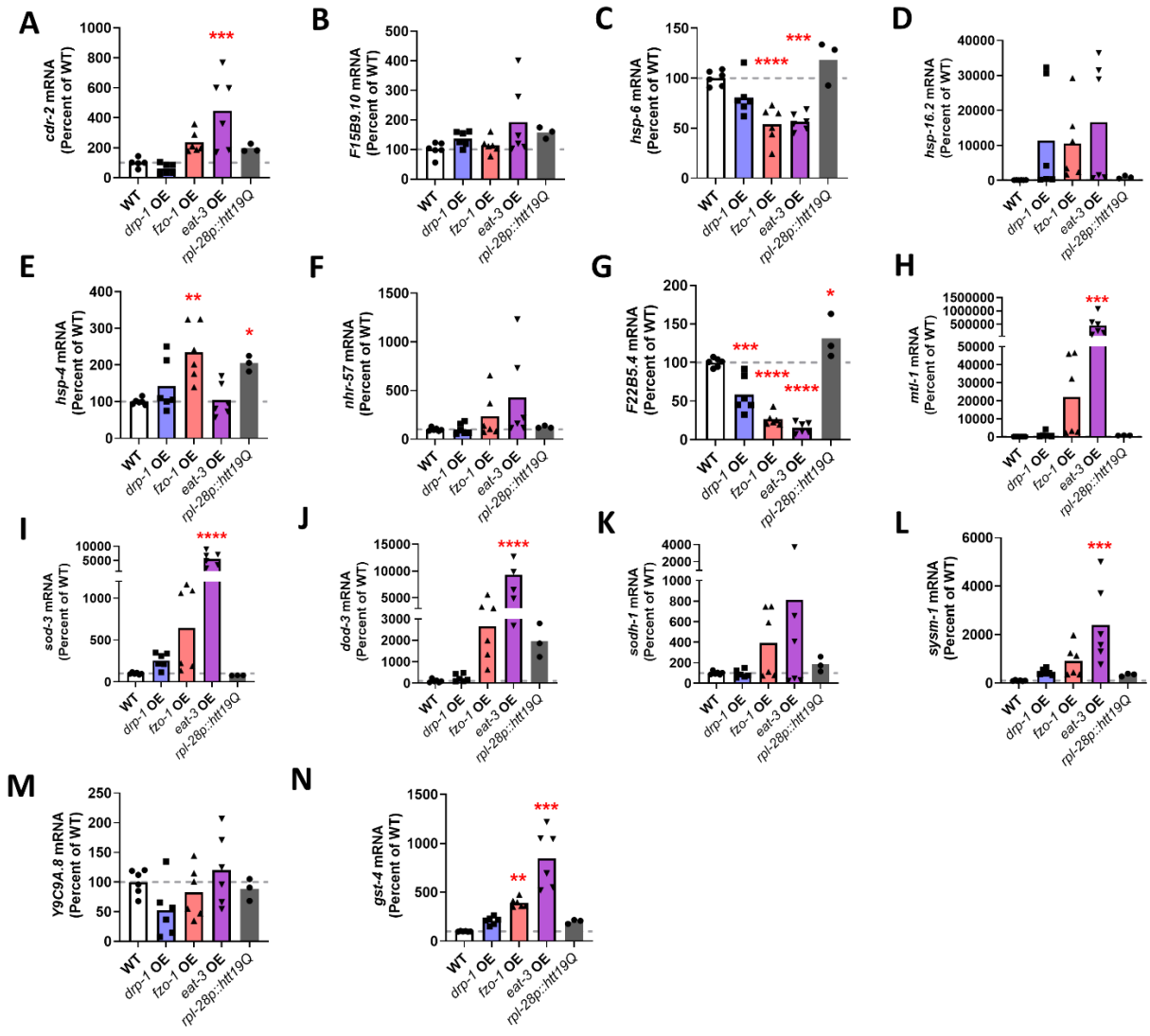

Figure 7. Overexpression of mitochondrial fusion genes activates multiple pathways of cellular resilience. Quantitative real-time PCR was used to assess the expression of target genes from different pathways of cellular resilience in the mitochondrial fission and fusion overexpression strains (*drp-1* OE, *fzo-1* OE and *eat-3* OE). As a control, expression was also examined in worms overexpressing a control protein under the *rpl-28* promoter, which was used in the *fzo-1* OE strain. We examined target genes from the mitochondrial unfolded protein response (*cdr-2, F15B9.10, hsp-6;* **A-C**), the cytoplasmic unfolded protein response (*hsp-16.2;* **D**), the ER-unfolded protein response (*hsp-4*; **E**), the hypoxia response (*nhr-57, F22B4.5*; **F,G**), the DAF-16-mediated stress response (*mtl-1, sod-3, dod-3, sodh-1*; **H-K**), the p38-mediated innate immune signaling pathway (*sysm-1, Y9C9A.8*; **L,M**) and the SKN-1-mediated oxidative stress response

(*gst-4*; **N**). At least on of the mitochondrial fusion mutants exhibited a significant increase in expression of targets of the DAF-16-mediated stress response pathway (*mtl-1, sod-3, dod-3*), the innate immune signaling pathway *(sysm-1*) and the SKN-1-mediated oxidative stress response *(gst-4*), which was not observed in the control strain. This indicates that multiple pathways of cellular resilience are activated in mitochondrial fusion overexpression strains. Six biological replicates were performed. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.11. Supplementary figures

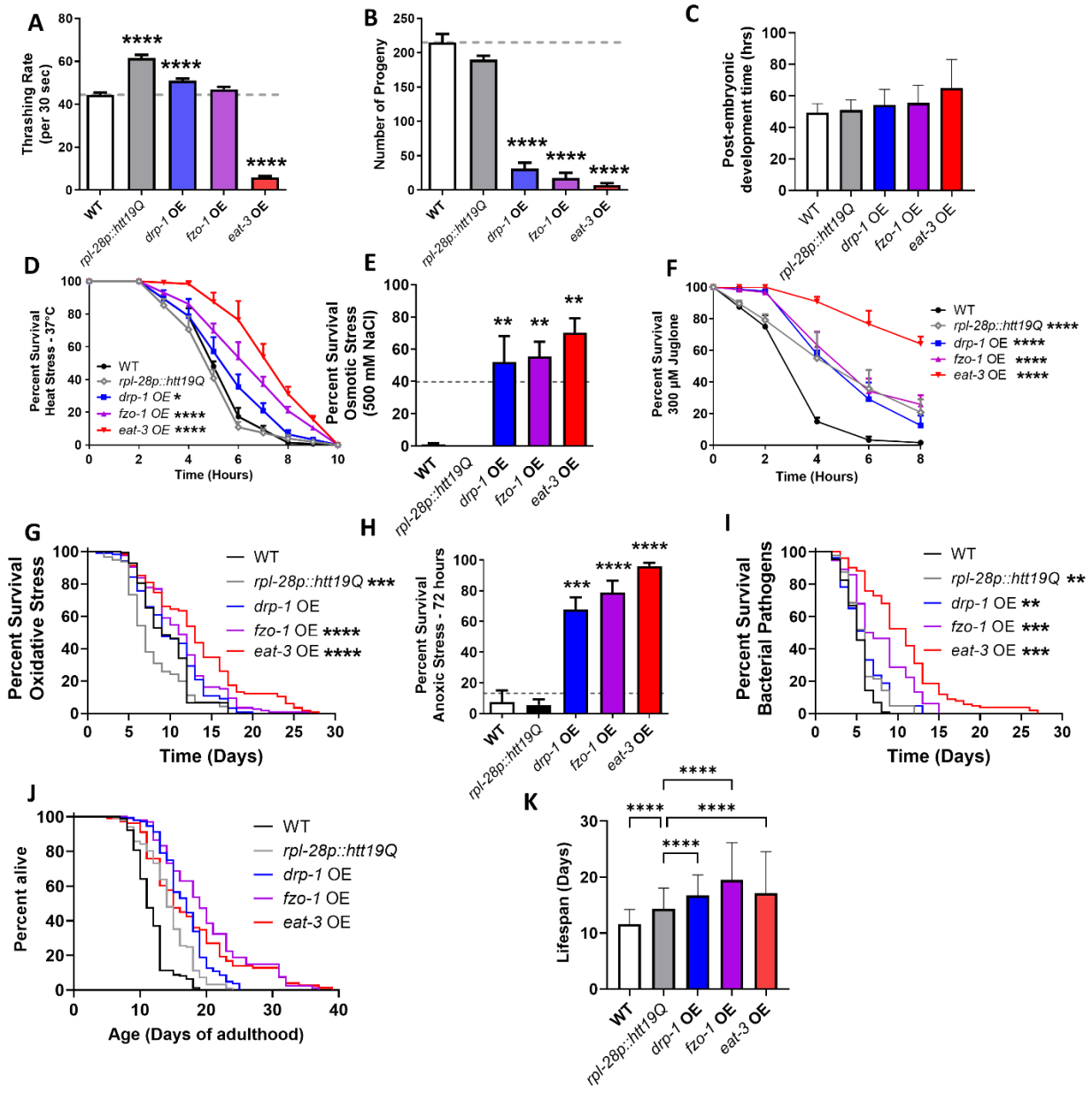

Figure S1. Overexpression of a control protein from *rpl-28* promoter does not recapitulate phenotypes in strains overexpressing mitochondrial fission or fusion genes. To control for overexpression, we compared the phenotype of *drp-1* OE, *fzo-1* OE and *eat-3* OE worms to worms overexpression a control protein (exon 1 of wild-type huntingtin) under the *rpl-28* promoter that was used to generate *fzo-1* OE worms. *rpl-28p::htt19Q* worms did not have decreased movement (**A**) or decreased fertility (**B**). These worms also exhibited wild-type post-

embryonic development (**C**) as well as resistance to heat stress (**D**) and osmotic stress (**E**). *rpl-28p::htt19Q* did show a significantly increased resistance to acute oxidative stress (**F**) suggesting that this phenotype might not be specific to overexpression of mitochondrial fission and fusion genes. Expression of *htt19Q* from the *rpl-28* promoter did not increase resistance to oxidative stress (G) or anoxia (H). While *rpl-28::htt19Q* worms have a small increase in resistance to bacterial pathogens (**I**) and lifespan (**J,K**), this increase is less than observed in the mitochondrial fusion overexpression strains. Error bars indicate SEM. A minimum of six biological replicates were performed. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparison test in A,B, C, E, H and K; a repeat measures ANOVA with Tukey's multiple comparison test in D and F; and a log-rank test in G, I and J. OE = overexpression. Data on overexpression strains from Figures 3,4 and 5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

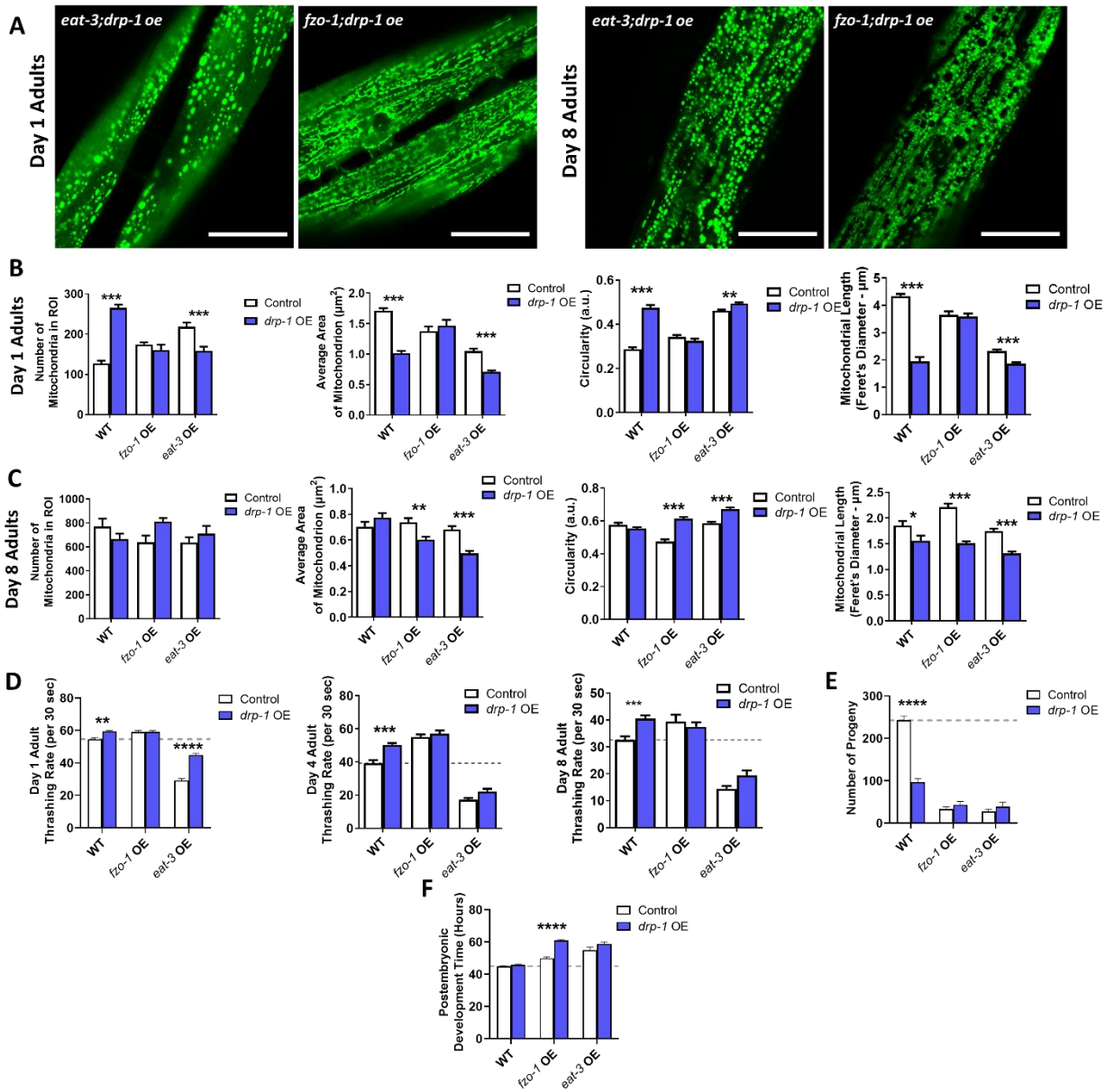

Figure S2. Overexpression *drp-1* causes further mitochondrial fragmentation in *eat-3* OE worms. To determine if overexpression of the mitochondrial fission *drp-1* would diminish phenotypes caused by overexpression of mitochondrial fusion genes, *drp-1* OE worms were crossed with *fzo-1* OE and *eat-3* OE worms. (**A**) Images of mitochondrial morphology in *eat-3;drp-1* and *fzo-1;drp-1* worms at day 1 and day 8 of adulthood. Scale bar indicates 25 μm. (**B**) At day 1 of adulthood, overexpression of *drp-1* decreased mitochondrial number, decreased mitochondrial area, increased mitochondrial circularity and decreased mitochondrial length in *eat-3* OE worms. *drp-1* OE did not affect the mitochondrial morphology of *fzo-1* OE worms. (**C**)

At day 8 of adulthood, overexpression of *drp-1* decreased mitochondrial area, increase mitochondrial circularity and decreased mitochondrial length in both *fzo-1* OE and *eat-3* OE worms.(**D**) *drp-1* OE ameliorated the decreased movement of *eat-3* OE worms on day 1 of adulthood but not at day 4 or day 8. (**E**) Overexpression of *drp-1* did not affect fertility in *fzo-1* OE or *eat-3* OE worms and resulted in a slowing of post-embryonic development time in *fzo-1* OE worms (**F**). Errors bars indicate SEM. A minimum of three biological replicates were performed. Statistical significance was assessed using a two-way ANOVA with Šidák's multiple comparisons test. OE = overexpression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

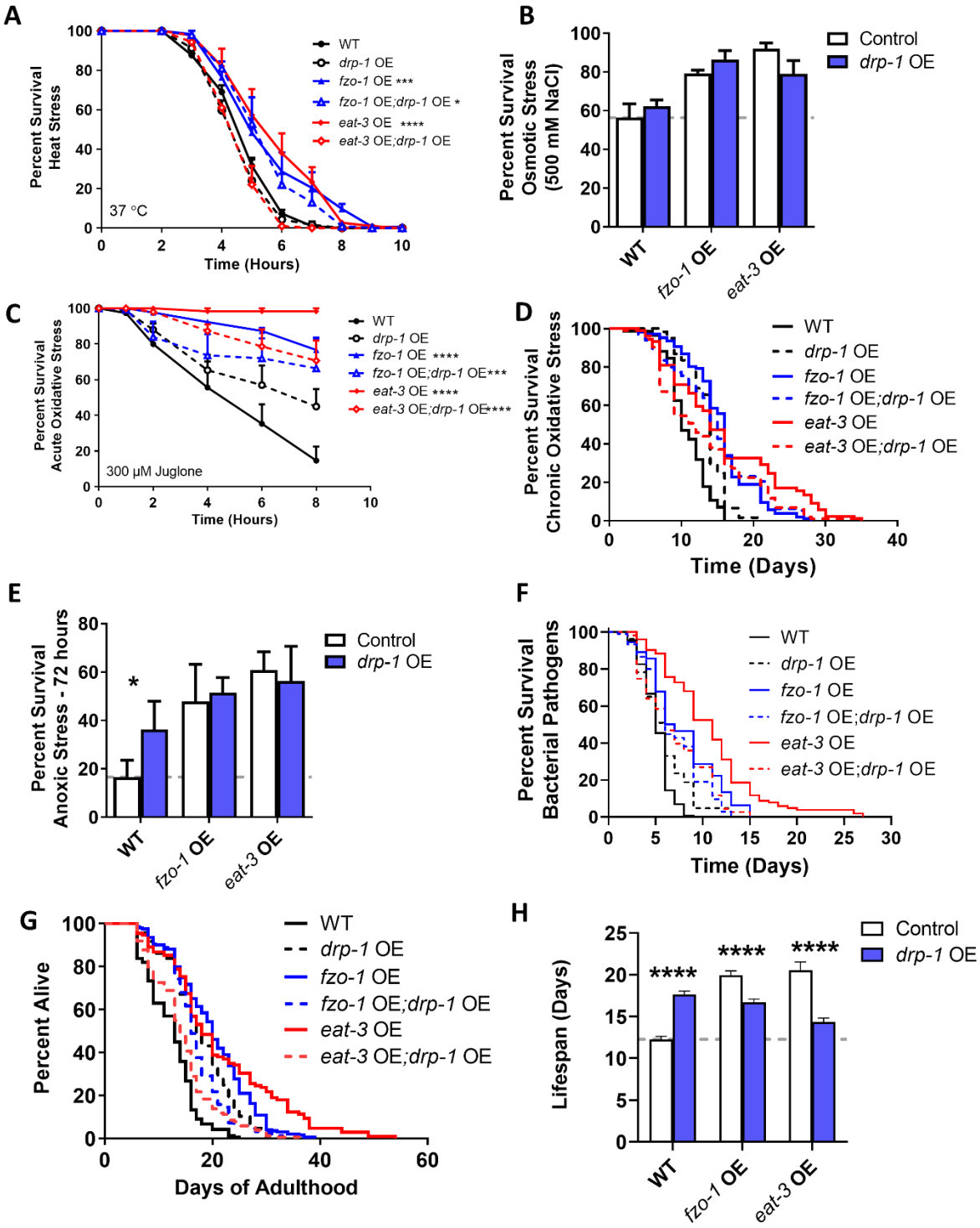

Figure S3. Overexpression *drp-1* decreases stress resistance and lifespan in worms overexpressing *eat-3.* (**A**) Overexpression of *drp-1* reverts heat stress resistance to wild-type in *eat-3* OE worms. (**B**) *eat-3* OE;*drp-1* OE worms exhibit a trend toward decreased osmotic stress

resistance compared to *eat-3* OE worms. (**C**) *drp-1* OE decreases acute oxidative stress resistance in *fzo-1* OE and *eat-3* OE worms. (**D**) *drp-1* OE also reduces resistance to chronic oxidative stress in *eat-3* OE worms. (**E**) Overexpression of *drp-1* does not affect anoxia resistance in *fzo-1* OE or *eat-3* OE worms. While *drp-1* OE increases bacterial pathogen resistance (**F**) and lifespan (**G,H**) in wild-type worms, it decreases resistance to bacterial pathogens and lifespan in worms overexpressing mitochondrial fusion genes. Error bars indicate SEM. A minimum of three biological replicates were performed. Statistical significance was assessed using a repeated measures ANOVA with Tukey's multiple comparison test in A and C; a two-way ANOVA with Šidák's multiple comparisons test in B, E, and H; and a log-rank test in D, F and G. OE = overexpression. Data on single overexpression strains from Figures 4 and 5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

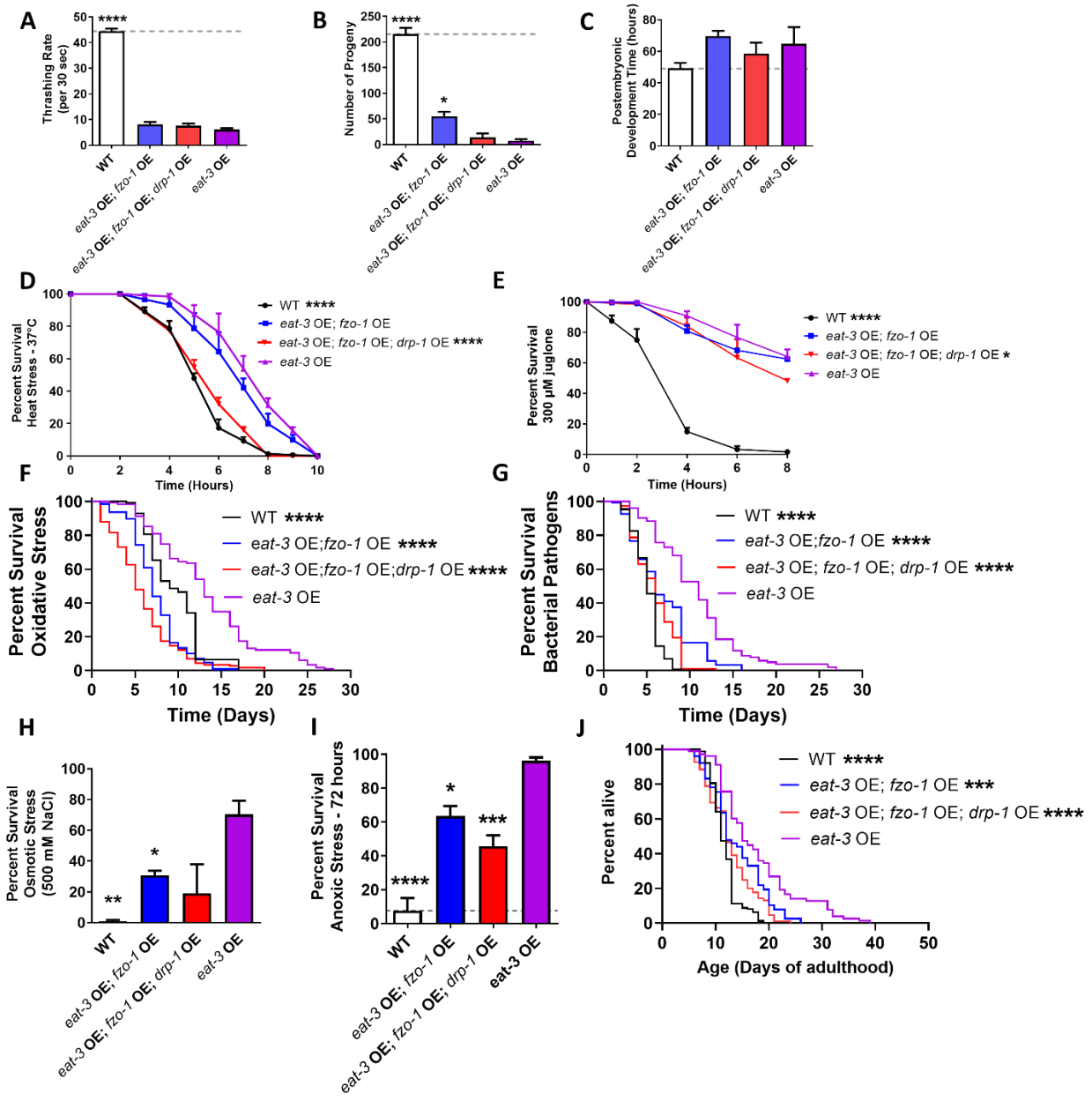

Figure S4. Effects of overexpressing *drp-1, fzo-1* and *eat-3* are not additive. To examine the effects of increasing the expression of *drp-1, fzo-1* and *eat-3* simultaneously, we generated *eat-3* OE; *fzo-1* OE; *drp-1* OE worms. Similar to *eat-3* OE worms, *eat-3* OE; *fzo-1* OE; *drp-1* OE worms exhibit decreased movement (**A**), reduced fertility (**B**), and slow post-embryonic development (**C**). These worms have only a small increase in resistance to heat stress (D). While *eat-3* OE; *fzo-1* OE; *drp-1* OE worms have increased resistance to acute oxidative stress (E), they have increased sensitivity to chronic oxidative stress (F). *eat-3* OE; *fzo-1* OE; *drp-1* OE worms have a

small increase in resistance to bacterial pathogens, which is less than observed in *eat-3* OE worms (G). These worms also exhibit a small increase in resistance to osmotic stress (H) and anoxia (I). Although *eat-3* OE; *fzo-1* OE; *drp-1* OE worms have increased lifespan compared to wild-type worms, the magnitude of increase is much less than in *eat-3* worms. Error bars indicate SEM. A minimum of three biological replicates were performed. Statistically significant differences from *eat-3* OE worms are indicated. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparison test in A, B, C, H and I; a repeated measures ANOVA with Tukey's multiple comparison test in D and E; and a log-rank test in F, G and J. OE = overexpression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.12. Supplementary tables

	drp-1 OE	*fzo-1* OE	*eat-3* OE	*drp-1* DEL	*fzo-1* DEL	*eat-3* DEL
Mito number	↑	↑	↑	=	↑	↑
Mito. size	↓	↓	↓	=	↓	↓
Circularity	↑	↑	↑	=	↑	↑
Feret's diameter	↓	↓	↓	=	↓	↓
ATP levels (day 1)	=	=	↑	↓	↓	↓
Oxygen consumption (day 1)	=	=	↑	=	↓	↓
Movement (day 1)	↑	↑	↓	↓	=	↓
Fertility	↓	↓	↓	↓	↓	↓
Development time	=	↑	↑	↑	↑	↑
Heat stress	=	↑	↑	↓	↑	↑
Osmotic stress	=	=	↑	↓	↓	↓
Acute oxidative stress	↑	↑	↑	↑	↑	↑
Chronic oxidative stress	↑	↑	↑	↑	↑	↑
Anoxia	↑	=	=	↓	↓	↓
Bacterial pathogen stress	↑	↑	↑	↑	↑	↑
Lifespan	↑	↑	↑	↑	=	↑

Table S1. Summary of results. ↑ = increased, ↓ = decreased, "=" = unchanged, OE = overexpression.

Preface to Chapter 3

As outlined in chapter 1, disruption of Drp1-mediated mitochondrial fission can be both beneficial and detrimental, depending on the model. In this work we aim to define the conditions required for inhibition of the mitochondrial fission gene *drp-1* to extend wild-type and *daf-2* lifespan. We anticipate that determining where and when disruption of *drp-1* is required for lifespan extension will help in identifying the mechanism of lifespan extension itself. Additionally, given that disruption of *drp-1* can be detrimental, limiting inhibition of *drp-1* to certain time points or tissues may be more beneficial to the organism. We also evaluated how disruption of *drp-1* affects mitochondria and peroxisomes in order to identify possible pathways leading to lifespan extension. Overall, the findings from this study advance our understanding of how modulation of mitochondrial fission promotes longevity. This work was submitted to *Geroscience* in December 2023 and is currently under review.

Chapter 3

Developmental disruption of the mitochondrial fission gene *drp-1* extends the longevity of *daf-2* insulin/IGF-1 receptor mutant

Developmental disruption of the mitochondrial fission gene *drp-1* extends the longevity of *daf-2* insulin/IGF-1 receptor mutant

Annika Traa[1,2,3], Jeremy M. Van Raamsdonk[1,2,3,4]

[1]Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada
[2]Metabolic Disorders and Complications Program, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada
[3]Brain Repair and Integrative Neuroscience Program, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada
[4]Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, Quebec, Canada

Corresponding Author:

Jeremy M. Van Raamsdonk
Research Institute of the McGill University Health Centre
1001 Decarie Blvd., Montreal, Quebec, Canada
ORCID: 0000-0001-8376-9605
1-514-934-1934 ext. 76157, jeremy.vanraamsdonk@mcgill.ca

3.1. Abstract

The dynamic nature of the mitochondrial network is regulated by mitochondrial fission and fusion, allowing for re-organization of mitochondria to adapt to the cell's ever-changing needs. As organisms age, mitochondrial fission and fusion become dysregulated and mitochondrial networks become increasingly fragmented. Modulation of mitochondrial dynamics has been shown to affect longevity in fungi, yeast, *Drosophila* and *C. elegans*. While disruption of the mitochondrial fission gene *drp-1* only mildly increases wild-type lifespan, it drastically increases the already long lifespan of *daf-2* insulin/IGF-1 signaling (IIS) mutants. In this work, we determined the conditions required for *drp-1* disruption to extend *daf-2* longevity and explored the molecular mechanisms involved. We found that knockdown of *drp-1* during development is sufficient to extend *daf-2* lifespan, while tissue-specific knockdown of *drp-1* in neurons, intestine or muscle failed to increase *daf-2* longevity. Disruption of other genes involved in mitochondrial fission also increased *daf-2* lifespan as did treatment with a number of different RNAi clones that decrease mitochondrial fragmentation. In exploring potential mechanisms involved, we found that deletion of *drp-1* increases resistance to chronic stresses and slows physiologic rates in *daf-2* worms. In addition, we found that disruption of *drp-1* increased mitochondrial and peroxisomal connectedness in *daf-2* worms, increased oxidative phosphorylation and ATP levels, and increased mitophagy in *daf-2* worms, but did not affect their ROS levels or mitochondrial membrane potential. Overall, this work defined the conditions under which *drp-1* disruption increases *daf-2* lifespan and has identified multiple changes in *daf-2;drp-1* mutants that may contribute to their lifespan extension.

Keywords: Aging, mitochondrial fission, *C. elegans*, insulin/IGF-1 signaling, DRP1, biological resilience

3.2. Introduction

Mitochondria are important contributors to organismal health. In addition to being the primary producer of cellular energy, mitochondria also play key roles in apoptosis, calcium regulation, redox homeostasis and inter-organelle communication [1]. In aged organisms, mitochondrial function as well as mitochondrial morphology become dysregulated [2]. Though the mechanisms by which mitochondria influence longevity are not completely understood, defects in mitochondrial function contribute to multiple age-related metabolic [3-9], cardiovascular [10, 11] and neurodegenerative diseases [12-17].

Mitochondria form an interconnected network within the cell where fusion allows individual mitochondrion to join mitochondrial networks while fission allows mitochondrion to separate from each other. Regulation of mitochondrial fission and fusion allows mitochondria to dynamically respond to environmental conditions [18]. Mitochondrial fission facilitates the clearance of dysfunctional mitochondria as well as the generation of new mitochondria [19-21]. Mitochondrial fusion facilitates the complementation of mitochondrial components for optimal mitochondrial function [19, 22-24].

As organisms age, their mitochondrial networks become increasingly fragmented and lose the ability to switch between fused and fragmented networks [25-27]. Neurons from individuals with neurodegeneration have highly fragmented mitochondrial networks [28-31]. Furthermore, dysregulated expression of fission and fusion proteins occurs in both aging and age-related disease [2, 16, 32]. Notably, increased mitochondrial fusion has been associated with increased longevity. Healthy human centenarians have highly connected mitochondrial networks compared to 27- and 75-year-old individuals [33]. Additionally, in *C. elegans*, increased mitochondrial fusion is required for the longevity of multiple long-lived mutants [34-36] and overexpression of mitochondrial fusion genes is sufficient to extend longevity and enhance resistance to exogenous stressors (Traa et al., in revision for *Aging Cell*).

In *C. elegans,* the mammalian Opa1 homolog, EAT-3, fuses the inner mitochondrial membrane and the mammalian mitofusin homolog, FZO-1, fuses the outer mitochondrial membrane [37-

39]. The protein responsible for the scission of both mitochondrial membranes in *C. elegans*, is DRP-1, homolog to the mammalian Drp1 [32, 40]. DRP-1 is recruited by FIS-1, FIS-2, MFF-1 and MFF-2 to the mitochondrial constriction site [41-45], where ER tubules wrap around mitochondria to begin mitochondrial membrane constriction [46, 47]. Oligomerization of DRP-1 into a ring structure occurs at the constriction site where DRP-1 hydrolyzes GTP for the energy needed to complete the scission of the inner and outer mitochondrial membrane [48, 49]. In addition to mitochondrial fission, DRP-1 also mediates peroxisomal fission and thus contributes to the regulation of peroxisomal network morphology [50].

Altering mitochondrial dynamics can affects an organism's health and longevity. Disruption of mitochondrial fission increases lifespan in yeast and fungal models [51, 52]. In *C. elegans*, deletion of *drp-1* has little or no effect on lifespan in wild-type worms [35, 53] despite increasing resistance to specific exogenous stressors [25]. However, disruption of *drp-1* has been shown to affect longevity in other backgrounds. We found that disruption of *drp-1* increases lifespan and restores motility in a neuronal model of polyglutamine toxicity [54], but decreases lifespan in a model in which the toxic polyglutamine tract is expressed in muscle [55], suggesting that the beneficial effects of *drp-1* inhibition may be tissue-specific. Most notably, disruption of *drp-1* has been shown to extend the already long lifespan of *daf-2* insulin/IGF-1 receptor mutants and *age-1* phosphoinositide 3-kinase (PI3K) mutants [56].

The insulin/IGF-1 signaling (IIS) pathway is highly conserved among animals and links nutrient availability to organismal growth, metabolism and longevity [57]. The long lifespan of IIS pathway mutants, including *daf-2* and *age-1*, is dependent on the activation of the FOXO transcription factor DAF-16 [58]. DAF-16 upregulates the expression of pro-survival genes, such as chaperones and antioxidants, in response to stress, low nutrient availability, or when IIS is disrupted [59].

The mechanism by which *drp-1* extends *daf-2* lifespan is not known. Previous studies have indicated that *drp-1* deletion does not extend *daf-2* longevity by enhancing DAF-16 activity, as disruption of *drp-1* does not increase DAF-16 activity in wild-type animals [25] or in *daf-2* mutants [56]. It has also been shown that the interaction between *drp-1* and the IIS pathway is

not *daf-2*-specific as disruption of *drp-1* also extends the lifespan of *age-1* mutants [56]. Mutants of the IIS pathway have increased mitochondrial function, increased mitochondrial ROS production and increased induction of mitophagy, all of which may be affected by disrupting mitochondrial dynamics [60-62].

In this work, we define the conditions under which disruption of *drp-1* extends *daf-2* longevity and identify multiple factors that are altered by the loss of *drp-1* in *daf-2* mutants that may contribute to lifespan extension. We find that inhibition of *drp-1* during development is sufficient to increase *daf-2* lifespan but tissue specific inhibition of *drp-1* in the neurons, intestine or muscle fails to extend *daf-2* longevity. Additionally, decreasing mitochondrial fragmentation without disrupting *drp-1* is also sufficient to increase *daf-2* lifespan. We find that disruption of *drp-1* increases *daf-2* resistance to chronic stress, slows physiologic rates, increases mitochondrial function, increases mitophagy and enhances peroxisomal connectivity, all of which may contribute to the effect of *drp-1* on *daf-2* longevity.

3.3. Results

Disruption of *drp-1* extends lifespan and enhances resistance to chronic stress in *daf-2* mutants

To investigate the role of mitochondrial dynamics in the longevity of *daf-2* mutants, we disrupted the *drp-1* gene in *daf-2* mutants and measured their lifespan. While disruption of *drp-1* provides a slight but significant extension of lifespan in wild-type worms, disruption of *drp-1* drastically extends the lifespan of already long-lived *daf-2* mutants (**Fig. 1A**). In addition to having a significantly longer lifespan, *daf-2* is known to have an increased healthspan [63, 64] and increased resistance to stress [65-67] but reduced fecundity [68-70]. Thus, to evaluate how disruption of *drp-1* affects the physiology and biological resilience of *daf-2* worms, we measured motility (thrashing rate), fertility (brood size), and resistance to chronic oxidative stress (4 mM paraquat), acute oxidative stress (420 µM juglone), bacterial pathogen stress (*P. aeruginosa*, PA14), heat stress (37 ℃), osmotic stress (700 mM NaCl), and anoxic stress (72 hours, 24 hours recovery).

Disruption of *drp-1* slightly but significantly decreased the thrashing rate of *daf-2* animals at day 1, 4 and 8 of adulthood but had no effect at day 12 and 18 (**Fig. 1B**). Disruption of *drp-1* further decreased the brood size of *daf-2* worms such that a *daf-2;drp-1* double mutant produces few viable eggs (**Fig. 1C**). The *drp-1* deletion also slowed the development rate of *daf-2* worms.

We and others have shown that *daf-2* mutants have increased resistance to exogenous stressors [71, 72]. Disruption of *drp-1* in *daf-2* mutants further increased resistance to chronic stressors such as chronic oxidative stress (**Fig. 1D**) and bacterial pathogen stress (**Fig. 1E**). In contrast, disruption of *drp-1* decreased *daf-2* resistance to heat stress (**Fig. 1F**) and acute oxidative stress (**Fig. 1G**), but had no effect on resistance to osmotic stress (**Fig. 1H**) or anoxic stress (**Fig. 1I**). Combined these results show that the *drp-1* deletion slows physiologic rates and enhances resistance to specific stressors in *daf-2* worms, both of which have been associated with increased lifespan.

Disruption of *drp-1* increases the connectivity of the mitochondrial and peroxisomal network in *daf-2* mutants

Using a strain expressing mitochondrially-targeted GFP in muscle cells, we examined how disruption of *drp-1* affects mitochondrial network morphology at day 1 and day 8 of adulthood in *daf-2* and wild-type animals. Similarly, to determine if disruption of *drp-1* affects peroxisomal network morphology in in *daf-2* worms, we visualized peroxisomes using a strain expressing peroxisome-targeted GFP in the intestine.

At day 1 of adulthood, disruption of *drp-1* produced a hyperfused mitochondrial network morphology in *daf-2* mutants, where rather than being organized into parallel elongated tubules, larger tubules and aggregated mitochondria were interconnected by thin filaments (**Fig. 2A**). Quantification of mitochondrial morphology revealed that deletion of *drp-1* in *daf-2* worms resulted in a decrease in the number of mitochondria, an increase in the average area of a mitochondrion, an increase in mitochondrial circularity and a decrease in the length of mitochondria (**Fig. 2A**). At day 8 of adulthood, the mitochondrial network of *daf-2;drp-1* mutants continues to appear more connected compared to the tubular morphology of *daf-2* mitochondrial networks, but both show an increase in mitochondrial fragmentation compared to day 1 adult worms as indicated by an increase in the number of mitochondria (**Fig. 2B**).

Disruption of *drp-1* in wild-type animals also results in increased mitochondrial connectivity. At both day 1 (**Fig. S1A**) and day 8 (**Fig. S1B**) of adulthood, *drp-1* mutants exhibited a more fused mitochondrial network than wild-type worms as indicated by a decrease in the number of mitochondria, an increase in the average mitochondrial area, a decrease in the mitochondrial circularity and an increase in the average mitochondrial length.

Visualization of *daf-2* intestinal peroxisomes at day 1 of adulthood revealed that RNAi inhibition of *drp-1* increased peroxisomal network connectivity (**Fig. 2C**). In *daf-2* animals that were fed *drp-1* RNAi, peroxisomal tubules were visible resulting in a decrease in the number of peroxisomes, a decrease in peroxisomal circularity and an increase in peroxisomal length compared to *daf-2* animals that were fed empty vector bacteria. Thus, disruption of *drp-1*

increases the connectivity of both the mitochondrial and peroxisomal networks in *daf-2* mutants.

Inhibition of *drp-1* during development is sufficient to extend *daf-2* lifespan

In order to define the conditions under which *drp-1* disruption extends *daf-2* lifespan, we determined when during the animal's life disruption of *drp-1* is required to extend *daf-2* lifespan. To do this, we fed *daf-2* and wild-type animals *drp-1* RNAi either during development only, during adulthood only, or during both development and adulthood and we measured their lifespans. Knockdown during adulthood was achieved by growing worms on empty vector bacteria and transferring worms to *drp-1* RNAi plates at day 1 of adulthood (**Fig. 3A**). Development only knockdown of *drp-1* was achieved by growing worms on *drp-1* RNAi until adulthood and then transferring these worms to RNAi targeting dicer (*dcr-1*), which is required for RNAi activity, in order to inhibit the knockdown of *drp-1* [73-75].

Prior to beginning the lifespan experiments, we measured mRNA levels of *drp-1* and *dcr-1* to ensure that *drp-1* expression was being knocked down as expected. Adult only knockdown of *drp-1* significantly decreased the levels of *drp-1* mRNA within 1 day of transferring to *drp-1* RNAi (**Fig. 3B**). The level of knockdown achieved was equivalent to the level in worms with life long *drp-1* knockdown. For the development only paradigm, we confirmed that transferring to *dcr-1* RNAi decreased levels of *dcr-1* transcripts (**Fig. 3C**) and resulted in a recovery in the level of *drp-1* transcripts (**Fig. 3D**) one day after being transferred.

Wild-type animals with adulthood *drp-1* knockdown had no change in lifespan compared to those which were fed empty vector bacteria throughout their lives (**Fig. 3E**). However, wild-type animals with developmental or lifelong *drp-1* knockdown had shorter lifespans compared to those that were fed empty vector bacteria (**Fig. 3F**). In *daf-2* mutants, animals with adulthood *drp-1* knockdown did not live longer than animals with lifelong *drp-1* knockdown (**Fig. 3G**). However, *daf-2* mutants with developmental *drp-1* knockdown lived significantly longer than animals fed empty vector bacteria, such that their survival curve was not statistically different

from that of animals with lifelong knockdown of *drp-1* (**Fig. 3H**). Thus, disruption of *drp-1* during development is sufficient to increase *daf-2* lifespan.

Tissue specific inhibition of *drp-1* in the muscle, neurons or intestine is not sufficient to extend *daf-2* lifespan

To determine the extent to which inhibition of *drp-1* in specific tissues is sufficient to extend *daf-2* lifespan, we used RNAi to knockdown *drp-1* expression in the neurons, muscle and intestine of *daf-2* and wild-type animals. To achieve a tissue-specific knockdown of *drp-1*, we used *sid-1* mutants, which lack the dsRNA importer SID-1 in all of their tissues, and re-expressed *sid-1* from tissue specific promoters, resulting in tissue-specific RNAi sensitivity. The tissue-specific promoters that we used were *vha-6p* for the intestine, *myo-3p* for the muscle and *rgef-1p* for the neurons.

In order to confirm the tissue-specific RNAi sensitivity of each strain, we knocked down genes that act in each specific tissue to produce a clear phenotype. We knocked down the intestinal *elt-2* gene which causes L1 arrest (**Fig. 4A**), the hypodermal *bli-3* gene which causes cuticle blistering (**Fig. 4B**), the muscular *pat-4* gene which causes paralysis (**Fig. 4C**), and the neuronal *unc-70* gene which causes uncoordinated movement (**Fig. 4D**). We found that animals re-expressing *sid-1* from intestine-specific *vha-6p* were only sensitive to *elt-2* RNAi and animals re-expressing *sid-1* from the muscle-specific *myo-3p* were only sensitive to *pat-4* RNAi. Animals re-expressing *sid-1* from neuronal-specific *rgef-1p* were sensitive to *unc-70* RNAi and slightly sensitive to RNAi in the intestine.

In considering whether inhibition of *drp-1* in a specific tissue is sufficient to extend lifespan, we compared the survival curve of animals with tissue-specific *drp-1* knockdown to two controls: (1) animals with tissue-specific RNAi sensitivity that were grown on empty vector bacteria; and (2) *sid-1* mutants which have whole-body resistance to RNAi. We considered a tissue-specific knockdown of *drp-1* to affect lifespan if its curve was statistically different from that of its empty vector control as well as the *sid-1* mutant control.

In wild-type animals, inhibition of *drp-1* in the intestine increased lifespan compared to the empty vector control (**Fig. S2A**) but not compared to *sid-1* mutants (**Fig. 4E**). Inhibition of *drp-1* in the muscle of wild-type animals did not affect lifespan compared to the empty vector control (**Fig. S2B**) but increased lifespan compared to *sid-1* mutants (**Fig. 4F**). Pan-neuronal inhibition of *drp-1* in wild-type animals increased lifespan compared to both the empty vector control (**Fig. S2C**) and *sid-1* mutants (**Fig. 4G**). Combined, this indicates that disruption of *drp-1* in neurons promotes longevity.

In *daf-2* animals, inhibition of *drp-1* in the intestine, muscle or neurons had no effect on lifespan compared to both the empty vector (**Fig. S2D-F**) and *sid-1* control (**Fig. 4H-J**). Overall, these results demonstrate that *drp-1* knockdown in intestine, muscle or neurons is not sufficient to extend *daf-2* lifespan.

Disruption of multiple mitochondrial fission genes increases *daf-2* lifespan

To determine the extent to which *drp-1*'s role in mitochondrial fission contributes to lifespan extension in *daf-2;drp-1* double mutants, we evaluated whether disruption of other genes involved in mitochondrial fission could also increase longevity in *daf-2* worms. We found that disruption of *fis-1* or *fis-1* and *fis-2* together significantly increases *daf-2* lifespan, while a deletion of *fis-2* alone did not extend longevity (**Fig. 5A-C**). Similarly, disruption of *mff-1* and *mff-2* together extends *daf-2* lifespan, while single deletions in either gene have no effect (**Fig. 5D-F**). In contrast, disruption of *fis-1, fis-2, mff-1, mff-2* or combinations of these genes did not extend the lifespan of wild-type worms (**Fig. S3A-F**). Combined, these results demonstrate that disrupting other genes involved in mitochondrial fission can also increase the lifespan of *daf-2* worms.

Since disruption of mitochondrial fission increases *daf-2* lifespan, we next evaluated whether disruption of mitochondrial fusion genes in *daf-2* worms would have the opposite effect. We found that disruption of *fzo-1* did not affect lifespan in *daf-2* mutants (**Fig. 5G**) or wild-type worms (**Fig. S3G**). While disruption of *eat-3* caused *daf-2* mutants to become sterile, the sterile

daf-2;eat-3 double mutants lived significantly longer than *daf-2* worms (**Fig. 5H**). Disruption of *eat-3* also extends lifespan in wild-type worms (**Fig. S3H**). Given that disruption of *eat-3* has multiple effects in *daf-2* worms, it is unclear whether the mechanism underlying lifespan extension is due to *eat-3*'s role in mitochondrial fusion, the effect of the *eat-3*-induced sterility on *daf-2* lifespan or the effect of dietary restriction on *daf-2* longevity (*eat* mutants have decreased feeding).

Decreasing mitochondrial fragmentation without disrupting mitochondrial fission machinery can extend *daf-2* lifespan

As mitochondrial fission is important for the function of the cell, we tested whether decreasing mitochondrial fragmentation, without directly disrupting the mitochondrial fission machinery, could still extend *daf-2* lifespan. To do this, we treated *daf-2* worms with twenty-six RNAi clones that were previously shown to decrease mitochondrial fragmentation [76]. Of the twenty-six RNAi clones tested, ten RNAi clones significantly extended the lifespan of *daf-2* mutants (**Fig. 6A**), one decreased the lifespan of *daf-2* mutants and fifteen did not affect lifespan. RNAi clones that extended *daf*-2 lifespan include *sdha-2* (**Fig. 6B**), C34B2.8 (**Fig. 6C**), K02F3.2 (**Fig. 6D**), T10F2.2 (**Fig. 6E**), Y69F12A.b (**Fig. 6F**), Y69F12A.c (**Fig. 6G**), *timm-17B.1* (**Fig. 6H**), C33A12.1 (**Fig. 6I**), *cyp-35A1* (**Fig. 6H**) and *pgp-3* (**Fig. 6K**). These genes are involved in various pathways of metabolism, protein transport or oxidative phosphorylation and are not characterized as directly contributing to mitochondrial fission or fusion processes [76]. Combined, these results show that decreasing mitochondrial fragmentation, either through disruption of other mitochondrial fission genes (*fis-1, fis-2, mff-2, mff-2*) or genes that affect mitochondrial fragmentation but are not directly involved in mitochondrial fission, can be sufficient to increase *daf-2* lifespan without disrupting *drp-1.*

Disruption of *drp-1* does not affect levels of ROS in *daf-2* mutants

daf-2 worms and a number of other long-lived mutants have been shown to have increased levels of ROS, which contributes to their lifespan extension [62, 77-79]. As mitochondria are the primary site of ROS production in the cell, we asked whether disruption of *drp-1* might be altering ROS levels in *daf-2* worms and contributing to the extended lifespan of *daf-2;drp-1* double mutants. To determine whether ROS levels are altered by disruption of *drp-1* in either wild-type or *daf-2* mutants, we measured the fluorescence intensity of worms treated with the ROS-sensitive fluorescent dye dihydroethidium (DHE) at day 1 and day 8 of adulthood. While disruption of *drp-1* significantly increased the fluorescence intensity of DHE in wild-type animals, it did not further increase ROS levels in *daf-2* mutants (**Fig. 7A,B**). This suggests that altering ROS levels does not contribute to the effect of *drp-1* deletion on *daf-2* longevity.

Disruption of *drp-1* does not affect mitochondrial membrane potential in *daf-2* mutants

Alterations in mitochondrial membrane potential have been associated with longevity. *daf-2* mutants and several other long-lived mutants were shown to have lower mitochondrial membrane potential than wild-type worms, though others have reported the opposite [60, 61, 80]. Mitochondrial membrane potential decreases with age [81] and preventing this decline is sufficient to increase lifespan [82]. Moreover, dietary restriction appears to increase lifespan at least partially through the preservation of the mitochondrial membrane potential [83]. Accordingly, we examined the effect of *drp-1* disruption on mitochondrial membrane potential in wild-type and *daf-2* worms to assess its potential contribution to lifespan extension.

To quantify mitochondrial membrane potential, we imaged day 1 and day 8 worms treated with the TMRE (tetramethylrhodamine ethyl ester), a fluorescent indicator whose uptake is dependant on mitochondrial membrane potential. At both time points, we found that *daf-2* worms have decreased mitochondrial membrane potential compared to wild-type animals (**Fig. 7C,D**). While disruption of *drp-1* decreased TMRE fluorescence in wild-type animals, it had no

effect on TMRE fluorescence in *daf-2* mutants (**Fig. 7C,D**). This suggests that *drp-1* deletion does not increase *daf-2* lifespan through altering mitochondrial membrane potential.

Disruption of *drp-1* increases mitochondrial function during young adulthood in *daf-2* mutants

Previous studies have reported alterations in mitochondrial function are associated with increased lifespan [61, 84-91]. To investigate whether disruption of *drp-1* affects mitochondrial function in *daf-2* worms, we measured oxygen consumption and ATP content at day 1 and day 8 of adulthood.

In day 1 adults, disruption of *drp-1* increases the oxygen consumption rate of *daf-2* mutants, but not of wild-type animals (**Fig. 8A**). Furthermore, in day 1 adults, disruption of *drp-1* increases ATP levels in *daf-2* mutants, but not in wild-type animals (**Fig. 8B**). In day 8 adults, disruption of *drp-1* does not affect the oxygen consumption rate (**Fig. 8C**) or ATP levels (**Fig. 8D**) of either wild-type or *daf-2* mutants. Combined these results show that disruption of *drp-1* further improves the mitochondrial function of *daf-2* mutants early in adulthood.

Disruption of *drp-1* increases levels of mitophagy in *daf-2* mutants

Increased induction of mitophagy is observed in *daf-2* mutants [60] and has been shown to extend lifespan [92, 93]. As mitochondrial fission can facilitate mitophagy, we assessed whether disruption of *drp-1* affects levels of mitophagy in *daf-2* mutants. To do this, we generated *daf-2* animals expressing the mitochondrial-targeted Rosella (mtRosella) biosensor. mtRosella uses a pH-insensitive red fluorescent protein (dsRed) fused to a pH-sensitive green fluorescent protein (GFP) to monitor mitophagy levels in the body wall muscle cells of *C. elegans* [94]. After imaging the worms and quantifying the fluorescence intensity emitted by each protein, the dsRed to GFP ratio indicates levels of mitophagy. As expected, mitophagy levels were increased in *daf-2* mutants, compared to wild-type animals. Disruption of *drp-1* induces increased levels of

mitophagy in *daf-2* mutants but not in wild-type animals at day 1 of adulthood (**Fig. 8E; Fig. S4**). At day 8 of adulthood, disruption of *drp-1* did not affect mitophagy levels in either wild-type or *daf-2* mutants (**Fig. 8F; Fig. S4**). Therefore, disruption of *drp-1* further increases mitophagy in *daf-2* mutants early in adulthood but has no effect on mitophagy in wild-type animals.

3.4. Discussion

Disruption of *drp-1* increases mitochondrial and peroxisomal network connectivity

In this work, we show that disruption of *drp-1* increases mitochondrial network connectivity in both *daf-2* and wild-type young adults. Later in adulthood, disruption of *drp-1* continues to generate increased mitochondrial network connectivity, but to a lesser extent. Increased mitochondrial network connectivity as a result of disrupting *drp-1* in wild-type animals has previously been documented [35, 39], although we and others have also previously observed no significant difference from wild-type mitochondrial morphology [25, 53, 95].

Using TMRE staining, the mitochondrial network morphology of *daf-2* mutants has previously been observed to be altered by disruption of *drp-1* at day 3 of adulthood [56]. The ratio of circularity to branching of the mitochondria was quantified and deemed to be higher when *drp-1* was disrupted in wild-type, *daf-2* and *age-1* animals. Here, we similarly observe an increase in mitochondrial circularity, however, we also consistently observe a decrease in the total number of mitochondria, and an increase in the average area of a mitochondrion, indicating that mitochondria are fusing together into larger aggregates. Increased mitochondrial fusion in *C. elegans* has previously been observed in several long-lived mutants, including *daf-2* [34]. Elongated mitochondrial tubules as well as lifespans were reported to be dependent on increased expression of the mitochondrial fusion protein EAT-3.

Given the ability of DRP-1 to regulate peroxisomal fission, we also considered the peroxisomal morphology of *daf-2* mutants and found that peroxisomes in the intestine are more interconnected when *drp-1* is disrupted. We observed an increase in filamentous structures, as visualized by GFP targeted to peroxisomes and these filaments appeared to connect circular structures together. Filamentous peroxisomal structures have previously been observed in *drp-1;fzo-1* worms, which require peroxisomal function for their increased longevity [35]. Thus, morphological changes in both the mitochondrial and peroxisomal network could both contribute to the longevity of *daf-2;drp-1* mutants.

Disruption of *drp-1* further increases lifespan and stress resistance in *daf-2* mutants

In this work, we show that disruption of *drp-1* significantly enhances the already increased lifespan and stress resistance of *daf-2* mutants. The ability of *drp-1* disruption to increases *daf-2* and *age-1* lifespan was reported previously [56]. We also find that disruption of *drp-1* mildly increases wild-type lifespan. This is consistent with findings that disruption of *drp-1* can protect against age-associated pathologies in multiple models [54, 96-100], but contrasts with previous findings that *drp-1* mutants have a decreased lifespan [53] or have no change in lifespan in *C. elegans* [35].

To evaluate how disruption of *drp-1* affects the healthspan of *daf-2* mutants, we compared the thrashing rate of *daf-2* and *daf-2;drp-1* double mutants at multiple time points. We found that disruption of *drp-1* slightly but significantly decreases *daf-2* thrashing rate until day 8 of adulthood but does not affect thrashing rate later in adulthood. This data indicates that while disruption of *drp-1* may result in a slight decrease of *daf-2* motility early in life, it does not markedly affect *daf-2* healthspan. Disruption of *drp-1* does however significantly affect *daf-2* fertility, resulting in a drastic drop in the number of viable progeny produced by each worm. We and others have previously reported that *drp-1* mutants also have a significantly lower brood size compared to wild-type [25, 39]. This decrease in fertility could contribute to the ability of *drp-1* deletion to extend *daf-2* lifespan as many long-lived mutants have decreased fertility and complete ablation of the germline extends longevity [101, 102].

Though it was previously reported that disruption of *drp-1* decreases *age-1* resistance to chronic oxidative stress by paraquat exposure starting at day 3 of adulthood [56], we find that disruption of *drp-1* increases *daf-2* resistance to paraquat exposure starting at day 1 of adulthood. Additionally, we find that disruption of *drp-1* increases *daf-2* resistance to another chronic stress, bacterial pathogen stress by feeding worms *Pseudomonas aeruginosa*. We have not previously tested the resistance of *drp-1* mutants to bacterial pathogen stress, however, we have reported that *drp-1* mutants have increased resistance to paraquat exposure and increased activation of the SKN-1-mediated oxidative stress response pathway. We also showed

that disruption of other mitochondrial fission genes such as *fis-1*, *fis-2, mff-1* and *mff-2* increases resistance to paraquat exposure [25].

Mitochondrial hyper-fusion has been observed in response to oxidative stress in a murine cell model, indicating that increased mitochondrial fusion may be beneficial in response to chronic oxidative stress, perhaps due to mitochondrial complementation mitigating the detrimental effects caused by small amounts of damage. By contrast, we find that disruption of *drp-1* decreases *daf-2* resistance to oxidative stress by juglone exposure and heat stress, both of which are acute stressors. Disruption of *drp-1* also decreases *age-1* resistance to heat stress [56] . In comparison, we have previously found that *drp-1* mutants have increased resistance to juglone exposure and decreased resistance to heat stress [25]. Thus, the ability of *drp-1* disruption to further increase *daf-2* resistance to chronic forms of stress may contribute to the ability of *drp-1* disruption to extend *daf-2* lifespan.

Developmental disruption of *drp-1* increases *daf-2* lifespan

We find that developmental inhibition of *drp-1* is sufficient to increase *daf-2* lifespan. This finding is in agreement with our observation that disruption of *drp-1* affects *daf-2* mitochondrial morphology more significantly in early adulthood compared to later in life and indicates that the mechanism by which inhibition of *drp-1* extends *daf-2* lifespan takes place during development. Similarly, in long-lived mutants where altered mitochondrial function contributes principally to lifespan extension, genetic inhibition is required during development [75]. By contrast, *daf-2* inhibition is required during adulthood, and not during development, for lifespan extension to occur [74]. Thus, while mitochondrial dynamics and the IIS pathway converge to extend lifespan in *daf-2;drp-1* mutants, the mechanism by which disruption of *drp-1* extends *daf-2* lifespan occurs early in life, before disruption of *daf-2* contributes to lifespan extension.

Tissue-specific disruption of *drp-1* does not increase *daf-2* lifespan

Our findings indicate that tissue-specific inhibition of *drp-1* in muscles, neurons or intestine fails to increase *daf-2* lifespan. This suggests that extension of *daf-2* lifespan either requires knockdown of *drp-1* in multiple tissues or that tissues not tested here, such as the germline, could be essential for lifespan extension mediated by disruption of *drp-1*. Inhibition of *daf-2* in the intestine or re-expression of *daf-16* in *daf-2;daf-16* mutants is sufficient to extend longevity [70, 103, 104], while re-expression of *daf-2* in the neurons or to a lesser extent the intestines decreases *daf-2* lifespan [105]. Combined with our findings here, this suggests that disruption of *drp-1* is not acting specifically in the same tissues as the *daf-2* mutation to increase lifespan.

In wild-type animals, we found that RNAi inhibition of *drp-1* in the neurons extends lifespan. This observation provides a plausible mechanism for why whole-body RNAi inhibition of *drp-1* decreases lifespan while deletion of *drp-1* increases lifespan. In the deletion mutant, *drp-1* is disrupted in all tissues including the neurons and the beneficial effect of *drp-1* disruption in neurons mediates the overall increase in lifespan. In wild-type worms treated with RNAi against *drp-1*, *drp-1* will be inefficiently knocked down in the neurons as RNAi is less effective in neuronal tissue [106-108]. Without the beneficial effect of disrupting *drp-1* in neurons, *drp-1* knockdown in the rest of the body has an overall detrimental effect on lifespan. In future studies, it will be interesting to more closely examine the mechanisms by which neuronal knockdown of *drp-1* extends lifespan.

Decreasing mitochondrial fragmentation without disrupting *drp-1* can extend *daf-2* lifespan

We find that inhibiting genes known to be involved in mitochondrial fission as well as genes known to affect mitochondrial fragmentation is sufficient to extend *daf-2* lifespan without *drp-1* disruption. However, none of the targets tested were able to extend *daf-2* lifespan to the same magnitude as *drp-1*. This could be due to *drp-1* disruption having the strongest effect on mitochondrial fragmentation or that another activity of *drp-1* also contributes to its effect on *daf-2* lifespan.

Of the RNAi clones that decrease mitochondrial fragmentation and increased *daf*-2 lifespan, we previously reported that inhibition of *pgp-3* or *alh-12* is able to decrease mitochondrial network disorganization and improve movement in models of polyglutamine toxicity [55]. Furthermore, chemical inhibition of mitochondrial fission has previously been found to increase yeast lifespan during chronological aging [109], another example whereby decreasing mitochondrial fragmentation without inhibiting *drp-1* promotes lifespan extension. Limiting *drp-1* inhibition by identification of *drp-1*-alternative mechanisms to promote lifespan extension via decreased mitochondrial fragmentation may be useful given that *drp-1* disruption can be detrimental in mammals [110-112].

Disruption of *drp-1* improves mitochondrial function in young *daf-2* adults

Mitochondrial dysfunction and an abnormally high or low mitochondrial membrane potential have both been associated with mitochondrial fragmentation and increased generation of mitochondrial ROS. Under normal physiological conditions, it is thought that increased mitochondrial network fusion promotes a mitochondrial membrane potential that is sufficiently elevated to enhance mitochondrial function without crossing the threshold into ROS overproduction [113]. Decreased membrane potential, or increased ROS levels may act as signals to induce mitochondrial fragmentation and mitophagy [114-116].

Though differing outcomes have been reported for the membrane potential of *daf-2* mutants [60, 61, 80], increased ATP production and increased ROS generation are consistently observed [61, 62, 78]. We find that disruption of *drp-1* in *daf-2* mutants has no effect on mitochondrial membrane potential or ROS levels but does increase mitochondrial function during early adulthood. Thus, redox homeostasis, which is maintained by DAF-16 and SKN-1 activity in *daf-2* mutants [117, 118], remains unaffected by the enhanced mitochondrial function mediated by *drp-1* disruption. Increased mitochondrial function in young *daf-2;drp-1* mutants, but not later in life, corresponds with our finding that disruption of *drp-1* during development is sufficient to extend *daf-2* lifespan.

We have previously observed that disruption of *drp-1* has no effect on mitochondrial function at day 1 or day 7 of adulthood [25]. Similarly, others have previously reported that *drp-1* mutants do not have altered ATP-coupled respiration, although increased basal respiration and an increased proton leak is reported [95]. While *drp-1* mutants would be expected to have increased mitochondrial function, membrane potential and mildly elevated ROS levels due to their increased mitochondrial network fusion, in this work, we again show that disruption of *drp-1* in wild-type animals does not affect mitochondrial function. Additionally, while we do see a significant increase in ROS levels in *drp-1* mutants, we also see a significant decrease in mitochondrial membrane potential. This elevated level of ROS and lowered mitochondrial membrane likely contributes to activation of the SKN-1-mediated oxidative stress response and may explain why disruption of *drp-1* is beneficial only under certain conditions. Additionally, the absence of enhanced mitochondrial function may help to explain why disruption of *drp-1* in wild-type animals does not extend lifespan as significantly as in *daf-2* mutants, where mitochondrial function is enhanced.

Disruption of *drp-1* increases induction of mitophagy in young *daf-2* adults.

Increased DAF-16 and SKN-1 activity leads to increased mitochondrial quality control by selective autophagy in *daf-2* mutants [119]. Inhibition of mitophagy partially decreases the lifespan of *daf-2* mutants, indicating that increased mitophagy is required to maintain *daf-2* longevity [60]. Although DRP-1 is required for selective clearance of dysfunctional mitochondria by recruitment of mitophagy machinery to specific membrane domains, DRP-1-independent mitophagy is possible [20, 120]. However, inhibition of fission machinery has been found to decrease mitophagy, resulting in the accumulation of oxidized mitochondrial proteins and reduced mitochondrial respiration, indicating that mitochondrial fission machinery plays an important role in mitochondrial quality control [21, 121].

Given that inhibiting DRP-1 has previously been found to affect mitophagy activity, we evaluated whether disruption of *drp-1* affects mitophagy in *daf-2* mutants. Unexpectedly, we

find that disruption of *drp-1* further increases *daf-2* mitophagy but does not affect wild-type mitophagy levels. Furthermore, as with mitochondrial function, disruption of *drp-1* only enhances *daf-2* mitophagy in young adults and does not affect mitophagy later in life, corresponding with our finding that disruption of *drp-1* during development but not adulthood extends *daf-2* lifespan. While increased mitophagy in the absence of DRP-1, would be expected to be excessive and non-selective [20], the enhanced mitochondrial function and maintained ROS and membrane potential seen in *daf-2;drp-1* mutants suggest that the increased mitophagy remains beneficial.

3.5. Conclusion

Overall, this work defines the conditions under which disruption of *drp-1* extends *daf-2* lifespan and identifies multiple putative mechanisms that contribute to lifespan extension. *drp-1* inhibition acts during development to extend *daf-2* lifespan in multiple tissues or tissues other than intestine, neurons and muscle. Disruption of *drp-1* increases mitochondrial and peroxisomal connectedness, decreases reproduction, enhances resistance to chronic stress, increases mitochondrial function and increases mitophagy in *daf-2* mutants, all of which may contribute to lifespan extension. Similar to *drp-1*, decreasing mitochondrial fragmentation by targeting other mitochondrial fission genes or other genes whose disruption promotes mitochondrial connectivity is sufficient to increase lifespan.

3.6. Experimental procedures

Strains

N2	WT
MQ1753	*drp-1(tm1108) IV*
CB1370	*daf-2(e1370) III*
MQ1770	*daf-2(e1370) III;drp-1(tm1108) IV*
JVR122	*bcIs78(myo-3p::mitoGFP (matrix GFP) + pRF4) I*
JVR218	*drp-1(tm1108); bcIs78(myo-3p::mitoGFP (matrix GFP) + pRF4) I*
JVR644	*daf-2(e1370) III;bcIs78(myo-3p::mitoGFP (matrix GFP) + pRF4) I*
JVR645	*daf-2(e1370) III;drp-1(tm1108) IV;bcIs78(myo-3p::mitoGFP (matrix GFP) + pRF4) I*
IR1631	*Ex003[myo-3p::TOMM-20::Rosella]*
JVR607	*drp-1(tm1108) IV;Ex003[myo-3p::TOMM-20::Rosella]*
JVR646	*daf-2(e1370) III;Ex003[myo-3p::TOMM-20::Rosella]*
JVR647	*daf-2(e1370) III;drp-1(tm1108) IV;Ex003[myo-3p::TOMM-20::Rosella]*
JVR648	*daf-2(e1370) III;hjIs8[ges-1p::GFP-PTS1]*
HC196	*sid-1(qt9) V*
AGD855	*sid-1(qt9) V;uthIs237[myo-3p::tomato + myo3-p::sid-1]*
JVR635	*sid-1(qt9) V;aIxIs6[vha-6p::sid-1::sl2::gfp]*
JVR636	*sid-1(qt9) V;sqIs69[rgef-1p::GFP + rgef-1p::sid-1]*
AGD801	*daf-2(e1370) III;sid-1(qt9)*
MAH410	*daf-2(e1370) III;sid-1(qt9) V;aIxIs6[vha-6p::sid-1::sl2::gfp]*
MAH411	*daf-2(e1370) III;sid-1(qt9) V;uthIs237[myo-3p::tomato + myo-3p::sid-1]*
MAH727	*daf-2(e1370) III;sid-1(qt9);sqIs69[rgef-1p::GFP + rgef-1p::sid 1]*
JVR109	*daf-2(e1370) III;fis-1(tm2227) II*
JVR110	*daf-2(e1370) III;fis-2(gk414) X*
JVR111	*daf-2(e1370) III;fzo-1(tm1133) II*
JVR119	*fis-1(tm2227) II;daf-2(e1370) III;fis-2(gk414) X*
JVR376	*daf-2(e1370);mff-1(tm2955)*

JVR377 *daf-2(e1370);mff-2(tm3041)*

JVR378 *daf-2(e1370);mff-1(tm2955);mff-2(tm3041)*

JVR097 *fis-1(tm2227) II*

JVR079 *fis-2(gk414) X*

JVR076 *fzo-1(tm1133) II*

JVR108 *fis-1(tm2227) II; fis-2(gk414) X*

JVR353 *mff-1(tm2955)*

JVR354 *mff-2(tm3041)*

JVR375 *mff-1(tm2955);mff-2(tm3041)*

JVR079 *eat-3(tm1107)*

daf-2(e1370);eat-3(tm1107) (no strain name because sterile)

Quantitative real-time RT-PCR

To perform quantitative RT-PCR, we first collected worms in M9 buffer and extracted RNA using Trizol as previously described [122, 123]. Using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814), the collected mRNA was then converted to cDNA. Quantitative PCR was performed using a PowerUp SYBR Green Master Mix (Applied Biosystems A25742) in a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems N8010560) and a Viia 7 Applied Biosystems qPCR machine. mRNA levels were calculated as the copy number of the gene of interest relative to the copy number of the endogenous control, *act-3*, then expressed as a percentage of wild-type. Primer sequences for each target gene are as follows: *drp-1* (L-GAGATGTCGCTATTATCGAACG, R- CTTTCGGCACACTATCCTG) *dcr-1* (L-ATTTTCGCGTCGTTAGCAGT, R-CGCATCATGTGGAAAATCAC).

Confocal imaging and quantification

Mitochondrial morphology was imaged using worms that express mitochondrially-targeted GFP in the body wall muscle cells. In addition, we utilized a *rol-6* mutant background to facilitate imaging of the muscle cells [55]. The *rol-6* mutation results in animals moving in a twisting motion, allowing the sheaths of muscle cells to be facing the objective lens and thus more

completely facilitates the imaging of mitochondrial networks within cells. Without the *rol-6* mutation, only the longitudinal edges of the muscle will often be visible, thus making it difficult to observe mitochondrial organization. Peroxisomal morphology was imaged using worms that express peroxisome targeted GFP in the intestine. Worms at day 1 or day 8 of adulthood were mounted on 2% agar pads and immobilized using 10 µM levamisole. Worms were imaged under a 40× objective lens on a Zeiss LSM 780 confocal microscope. Single plane images were collected for a total of twenty-four worms over three biological replicates for each strain. Imaging conditions were kept the same for all replicates and images. Quantification of mitochondrial or peroxisomal morphology was performed using ImageJ. Segmentation analysis was carried out using the SQUASSH (segmentation and quantification of subcellular shapes) plugin. Particle analysis was then used to measure the number, area, circularity, and maximum Feret's diameter (an indicator of particle length) of the organelles.

The mtRosella mitophagy reporter was imaged as previously described in worms expressing the reporter in the body wall muscle [94]. The whole body of the worm was imaged under a 20x objective lens on a Zeiss LSM 780 confocal microscope. Quantification of dsRed and GFP fluorescence intensity was performed used ImageJ and representative images show both channels merged.

Thrashing rate

Thrashing rates were determined manually by transferring 20 age-synchronized worms onto an unseeded agar plate. One milliliter of M9 buffer was added and the number of body bends per 30 seconds was counted for 3 biological replicates of approximately 10 worms per strain.

Brood size

Brood size was determined by placing individual pre-fertile young adult animals onto NGM plates. Worms were transferred to fresh NGM plates daily until progeny production ceased. The resulting progeny was allowed to develop to the L4 stage before quantification. Three biological replicates of 5 animals each were completed.

Oxygen consumption rate

Oxygen consumption measurements were taken using a Seahorse XFe96 analyzer [25, 124]. The night before the assay, probes were hydrated in 200 µL Seahorse calibrant at 37 degrees while the analyzer's heater was turned off to allow the machine to cool. Day 1 and day 8 worms were collected in M9 buffer and washer three times before being pipetted into a Seahorse 96 well plate (Agilent Technologies Seahorse Flux Pack 103793-100). Others have previously determined that using between 5-25 worms per well is optimal [125]. Calibration was performed after 22 µL of FCCP and 24 µL sodium azide was loaded into the drug ports of the sensor cartridge. Measurements began within 30 minutes of worms being added to the wells. Basal oxygen consumption was measured 5 times before the first drug injection. FCCP-induced oxygen consumption was measured 9 times, then sodium-azide induced oxygen consumption was measured 4 times. Measurements were taken over the course of 2 minutes and before each measurement the contents of each well were mixed for an additional 2 minutes. Non-mitochondrial respiration (determined by sodium azide-induced oxygen consumption rate) was subtracted from basal respiration to calculate mitochondrial respiration.

ATP determination

Day 1 and day 8 adult worms were collected, washed 3 times and frozen in 50 µL of M9 buffer using liquid nitrogen. Samples were then immersed in boiling water for 15 minutes followed by ice for 5 minutes and finally spun down at 14,800g for 10 minutes at 4 ℃. Supernatants were diluted 10-fold before ATP measurements using a Molecular Probes ATP determination kit (A22066) and TECAN plate reader. Luminescence was normalized to protein content measured using a Pierce BCA protein determination kit.

Heat stress assay

To measure resistance to heat stress, approximately 25 pre-fertile young adult worms were transferred to new NGM plates freshly seeded with OP50 bacteria and were incubated at 37℃. Starting at 12 hours, survival was measured every hour for a total of 18 hours of incubation. Three biological replicates were completed.

Osmotic stress assay

To measure resistance to osmotic stress, approximately 25 pre-fertile young adult worms were transferred to NGM plates containing 700 mM NaCl and seeded with OP50 bacteria. Worms were kept at 20℃ for 24 hours before survival was scored. Five biological replicates were completed.

Oxidative stress assays

Resistance to acute oxidative stress was measured by transferring approximately 25 pre-fertile young adult worms to 420 µM juglone plates seeded with OP50 bacteria. Worms were kept at 20℃ and survival was monitored every 2 hours for a total of 8 hours. Resistance to chronic oxidative stress was performed by transferring 30 pre-fertile young adult worms to freshly prepared plates containing 4 mM paraquat, 100 µM FUdR and seeded with concentrated OP50. Survival was monitored daily. Three biological replicates were completed for both assays.

Anoxic stress assay

To measure resistance to anoxic stress, approximately 50 pre-fertile young adult worms were transferred to new NGM plates seeded with OP50 bacteria. To create a low-oxygen environment for the worms, we utilized Becton-Dickinson Bio-Bag Type A Environmental Chambers. Plates with young adult worms of each strain were placed in the Bio-Bags for 120 hours at 20℃, then removed from the bags and allowed to recover for 24 hours at 20℃ before survival was measured. Five biological replicates were completed.

Bacterial pathogen stress assay

We tested for nematode resistance to death by bacterial colonization of the intestine. The slow kill assay was performed as previously described [126, 127]. PA14 cultures were grown over night for a total of 16 hours and then seeded to the center of NGM agar plates containing 25 µM FUdR. Plates were left on the bench to dry overnight and were then incubated at 37℃ for 24 hours. Next the plates were left to adjust to the temperature at which the assay is

conducted at 25℃ overnight. To begin the assay, approximately 50 age synchronized L4 worms were transferred to each plate. To monitor survival, deaths were scored twice a day until all worms had died. Three biological replicates were completed.

Quantification of ROS levels

ROS levels were measured using dihydroethidium (DHE; ThermoFisher Scientific, D1168), as previously described [128, 129]. A 30 mM solution of DHE in DMSO was aliquoted and stored at −80 °C. When needed, 5 µl of DHE stock was diluted in 5 mL of PBS to create a 30 µM DHE solution. Age matched day 1 adult worms (approximately 50) were collected in PBS, transferred to a 1.5 mL centrifuge tube and washed 3 times in 1 mL PBS. To immerse worms in a final concentration of 15 µM DHE, 100 µL of PBS was left in the centrifuge tube after the final wash and 100 µL of 30 µM DHE was added to the tube. Centrifuge tubes were wrapped in tinfoil to protect from light and worms were incubated at room temperature, on a shaker for 1 h, then washed 3 times in PBS. For imaging, worms were mounted on a 2% agarose pad and immobilized with 10 mM levamisole. Worms were imaged with the 20 × objective using a Zeiss LSM 780 confocal microscope. A total of 60 worms were imaged over 3 biological replicates, per genotype. Image J was used to quantify the fluorescence intensity of ethidium-labelled ROS in the whole body of the worm. For each image (one worm per image), mean fluorescence intensity of the whole image and background intensity were measured. For each strain, autofluorescence was sampled from approximately 5-10 untreated worms and mean autofluorescence for the strain was determined. Fluorescence intensity of each worm was calculated by subtracting the image's background fluorescence and the strain's mean autofluorescence from the mean fluorescence of the image.

Tetramethylrhodamine (TMRE) staining

We used the potentiometric fluorescent indicator, TMRE, to measure mitochondrial membrane potential. TMRE was dissolved in DMSO to make a 50 mM stock solution. The TMRE stock solution was then diluted in M9 to make a 5 µM working solution. The working TMRE solution was then pipetted onto NGM plates seeded with OP50 and were left on a nutator for 30

minutes to allow the TMRE to spread evenly over the agar. Approximately 20 age matched L4 or day 7 worms were transferred to the TMRE plates which were then covered to protect from light and stored at 20 ℃ for 20 hours. To de-stain, worms were transferred to regular NGM plates seeded with OP50 and left at 20 ℃ for 4 hours. Worms were imaged with the 20 × objective using a Zeiss LSM 780 confocal microscope and ImageJ was used to quantify the fluorescence intensity of TMRE in the whole body of the worm.

Lifespan assay

Lifespan assays were completed at 20℃ and on NGM agar plates that contained FUdR to inhibit the development of progeny and limit internal hatching. We used a low concentration of 25 μM FUdR, which we have previously shown does not affect the longevity of wild-type worms [130]. For each lifespan assay, 40 pre-fertile young adult worms were transferred to 25 μM FUdR plates seeded with OP50 bacteria and were kept at 20℃. Four biological replicates were started on four subsequent days and all replicates were scored every other day to monitor survival until all worms died. Worms were excluded from the assay if they crawled off the agar and died on the side of the plate, had internal hatching of progeny or expulsion of internal organs. Raw lifespan data can be found in **Table S1**.

RNAi

We used RNAi to inhibit the expression of specific genes. RNAi clones from the Ahringer library were streaked onto LB plates containing 50 μg/mL carbenicillin and 10 ug/ml Tetracycline. Individual colonies were then inoculated in 2YT media with 50 μg/mL carbenicillin and grown with aeration at 37 ℃ for 18 hours. For experiments testing the tissue and timing requirements for *drp-1* knockdown to extend lifespan, RNAi clones were sequence verified and were concentrated before being seeded onto NGM plates containing 3 mM IPTG and 50 μg/mL carbenicillin. Seeded RNAi plates were left on the bench for two days to induce dsRNA expression in the bacteria. When conducting lifespan assays, we used the L4 parental paradigm where RNAi knockdown is begun in the parental generation. Age matched L4 worms were transferred to RNAi plates for 24 hours after which they were transferred to another RNAi plate

for 24 hours before being removed. The progeny from these worms were then transferred to the experimental RNAi plates containing 25 µM FUdR for lifespan assays. RNAi lifespan assays were conducted at 20 ℃ where deaths were scored every two days and a minimum of three biological replicates were completed.

Statistical analysis

A minimum of three biological replicates were completed for all assays. Where possible, the experimenter was blinded to the genotype during the course of the experiment, to ensure unbiased results. Statistical significance of differences between groups was determined by computing a t-test, a one-way ANOVA, a two-way ANOVA or a log-rank test using Graphpad Prism, as indicated in the figure legends. All error bars indicate the standard error of the mean.

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3.10. Figures

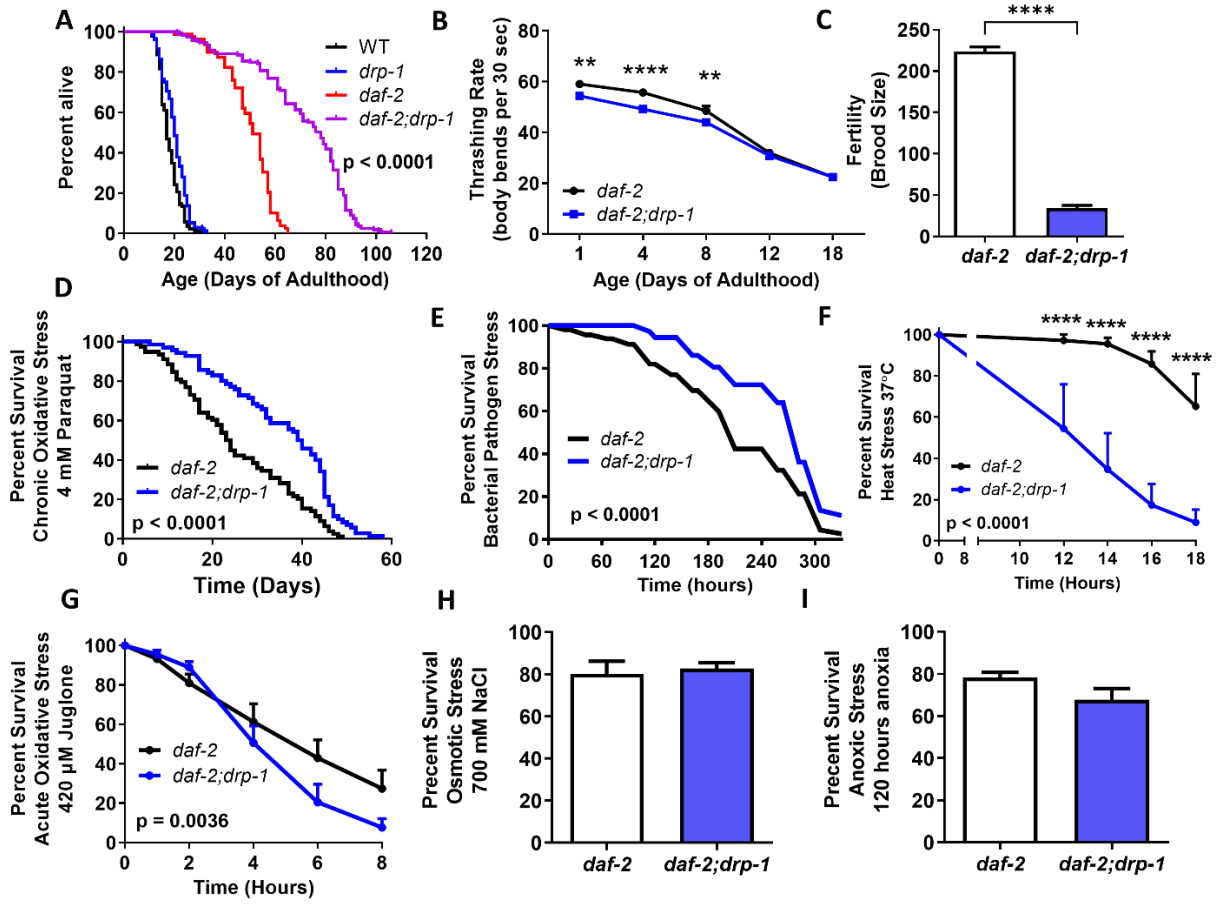

Figure 1. Disruption of *drp-1* extends the already long lifespan of *daf-2* mutants and increases resistance to bacterial pathogens and chronic oxidative stress. Deletion of *drp-1* markedly increases the lifespan of *daf-2* mutants **(A)**. Loss of *drp-1* reduces the rate of movement **(B)** and brood size **(C)** of *daf-2* worms. Deletion of *drp-1* increases *daf-2* resistance to chronic oxidative stress (**D**; 4 mm paraquat) and bacterial pathogen stress (**E**; *P. aeruginosa* strain PA14). In contrast, *daf-2;drp-1* mutants have increased sensitivity to heat stress (**F**) and acute oxidative stress (**G**). Deletion of *drp-1* does not affect resistance to osmotic stress (**H**) or anoxia (**I**) in *daf-2* worms. A minimum of three biological replicates were performed. Statistical significance was assessed using a log-rank test in panels A, D, E and G; a two-way ANOVA with Šidák's multiple comparisons test for panel B; and a student's t-test for panels C, H, and I. Error bars indicate SEM. **p<0.01, ****p<0.0001.

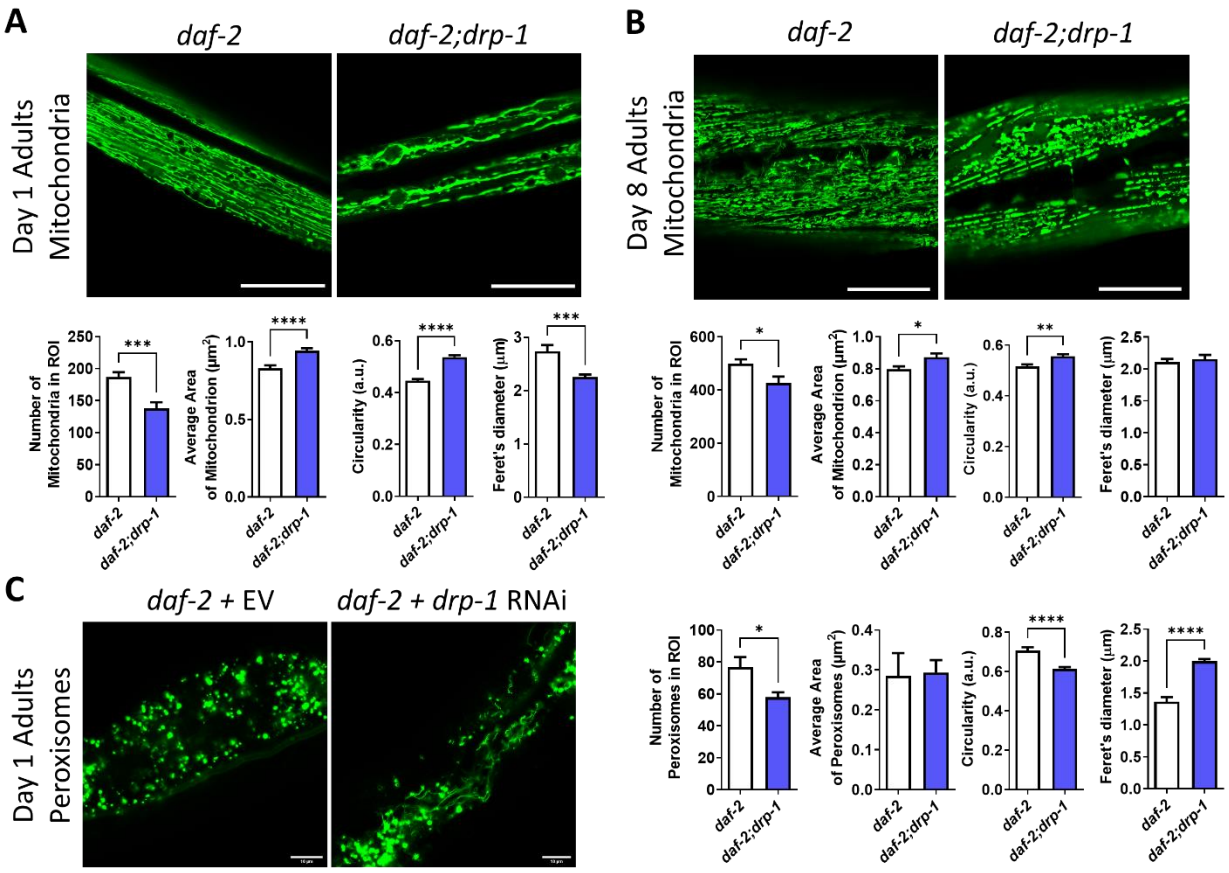

Figure 2. Disruption of *drp-1* increases mitochondrial and peroxisomal connectivity in *daf-2* mutants. (A) At day 1 of adulthood, deletion of *drp-1* decreases mitochondrial area, increases mitochondrial circularity and decreases mitochondrial length in *daf-2* worms. **(B)** At day 8 of adulthood, deletion of *drp-1* decreases mitochondrial number while increasing circularity in *daf-2* worms. **(C)** Decreasing *drp-1* levels with RNAi also affects peroxisome morphology in *daf-2* worms leading to decreased peroxisome numbers, decreased peroxisome circularity and increased peroxisome length. Three biological replicates were imaged. Statistical significance was assessed using a student's t-test. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar indicates 25 µm in panels A and B, and 10 µm in panel C.

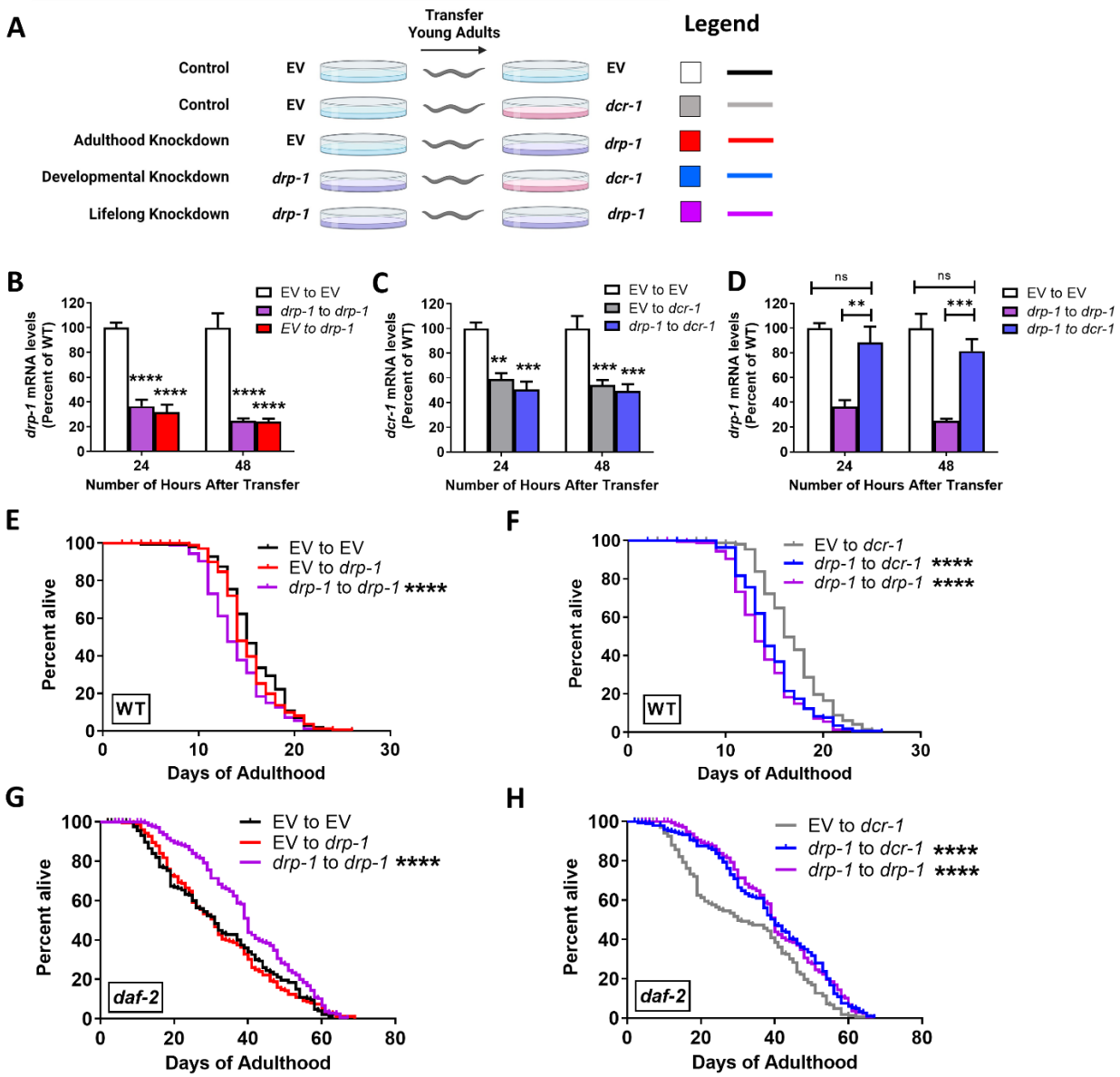

Figure 3. Inhibition of *drp-1* during development is sufficient to extend *daf-2* lifespan. (A) To determine when *drp-1* depletion acts to increase *daf-2* lifespan, *drp-1* levels were reduced during development only *(drp-1* to *dcr-1;* blue), during adulthood only (EV to *drp-1;* red) or during development and adulthood *(drp-1* to *drp-1;* purple) and compared to empty vector (EV) from development to adulthood (EV to EV; white bar or black line). **(B)** Measurement of *drp-1* levels by quantitative RT-PCR confirm that *drp-1* levels were decreased during adulthood by *drp-1* RNAi. **(C)** Worms transferred to *dcr-1* RNAi exhibit decreased levels of *dcr-1* mRNA. **(D)** Worms treated with *drp-1* RNAi during development and *dcr-1* RNAi during adulthood show a recovery

of *drp-1* mRNA levels during adulthood. In wild-type worms, adulthood only knockdown of *drp-1* did not affect lifespan **(E)**, while development only *drp-1* RNAi decreased longevity **(F)**. In *daf-2* mutants, adult only *drp-1* RNAi did not affect longevity **(G)**, while *drp-1* knockdown during development significantly increased lifespan **(H)**. Three biological replicates were performed for panels B, C, and D; and four biological replicates were performed for panels E-H. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparisons test in panels B, C, and D; and a log-rank test in panels E-H. Error bars indicate SEM. **p<0.01, ***p<0.001, ****p<0.0001.

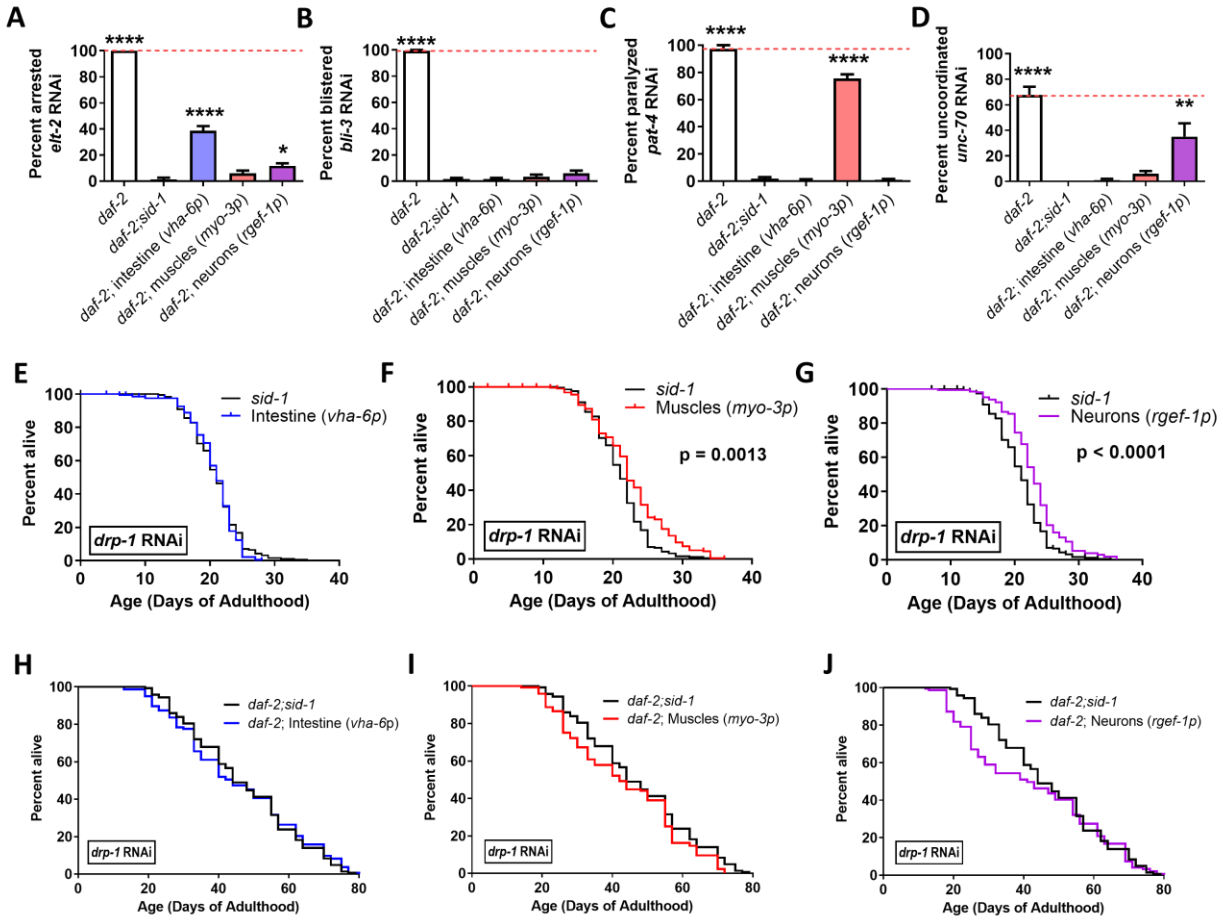

Figure 4. Inhibition of *drp-1* in individual tissues fails to extend *daf-2* lifespan. To identify the tissues in which decreasing the levels of *drp-1* acts to extend *daf-2* lifespan, tissue-specific RNAi was used to lower *drp-1* levels in different tissues of *daf-2* mutants and wild-type controls. To confirm that tissue-specific RNAi was working, all of the tissue-specific RNAi strains were treated with RNAi that should only produce a phenotype when knocked down in intestine (**A**; *elt-2* RNAi leading to arrestment and **B**; *bli-3* RNAi leading to blistering), body wall muscle (**C**; *pat-4* RNAi leading to paralysis) or neurons (**D**; *unc-70* RNAi leading to uncoordinated movement). In each case, treatment with RNAi caused a phenotype in *daf-2* worms and the corresponding tissue-specific RNAi strain but not *daf-2;sid-1* worms or any of the other untargeted tissue-specific RNAi strains, thereby indicating that the strains exhibit tissue specificity. In a wild-type background, knocking down *drp-1* in the intestine had no effect on lifespan (**E**), while knocking down *drp-1* in muscle (**F**) or neurons (**G**) resulted in a small increase in lifespan. In *daf-2* worms, knocking down *drp-1* in the intestine (**H**) body wall muscle (**I**) or

neurons (**J**) had no effect on longevity. Three biological replicates were performed in panels A-D and four biological replicates were performed in panels E-L. Statistical significance was assessed using a one-way ANOVA with Dunnett's multiple comparisons test in panels A-D and a log-rank test in panels E-L. In panels A-D, statistically significant differences from *daf-2;sid-1* are shown. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. A comparison of *drp-1* RNAi to empty vector can be found in Figure S2.

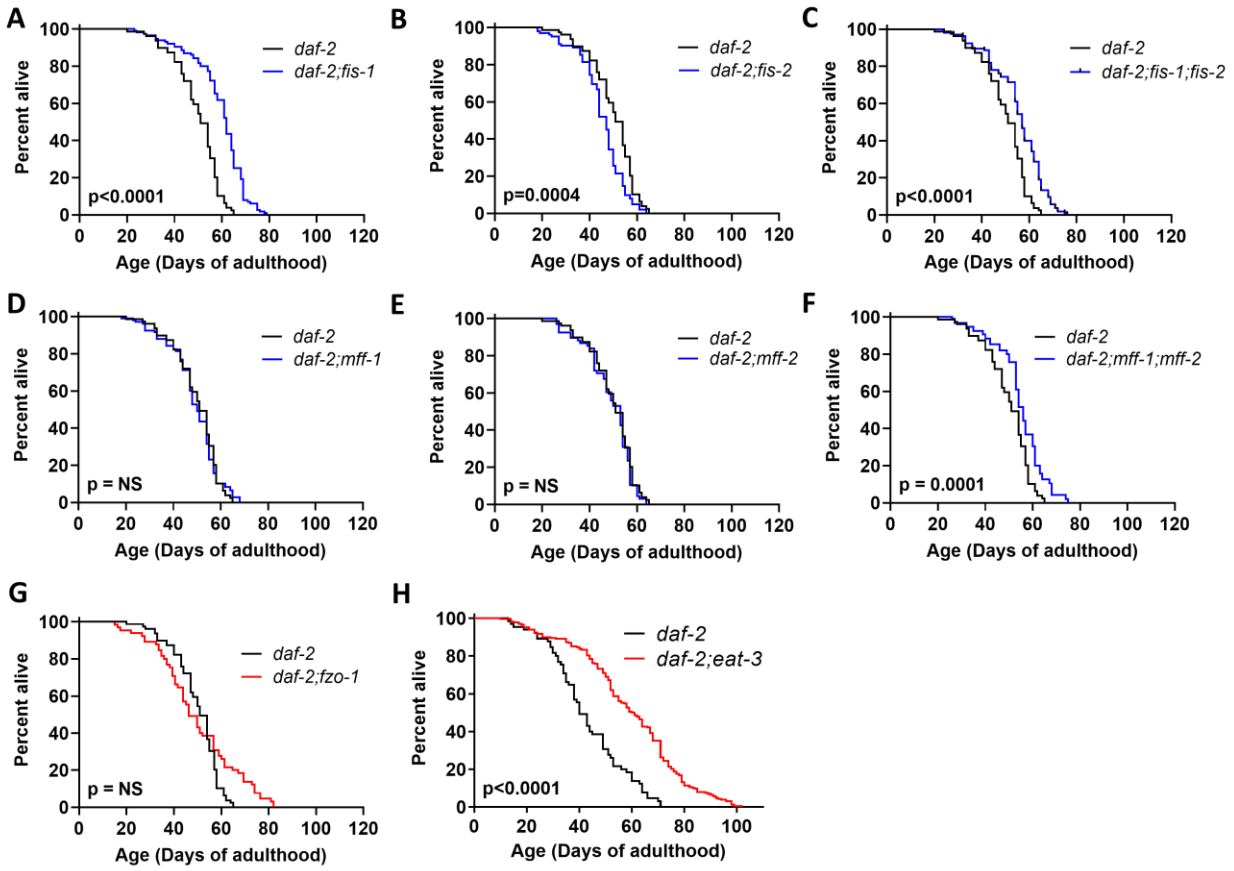

Figure 5. Disruption of mitochondrial fission genes increases *daf-2* lifespan. Deletion of the mitochondrial fission gene *fis-1* (**A**) or *fis-1* and *fis-2* together (**C**) increases *daf-2* lifespan, while disruption of *fis-2* results in a small decrease in *daf-2* lifespan (**B**). Disruption of the mitochondrial fission factor genes *mff-1* (**D**) or *mff-2* (**E**) do not affect *daf-2* lifespan individually but together result in a significant increase in *daf-2* longevity (**F**). In contrast to the ability of *drp-1* deletion to increase *daf-2* lifespan, disruption of *fzo-1* does not significantly affect *daf-2* lifespan (**G**). Disruption of *eat-3* markedly extends *daf-2* lifespan but also results in sterility (**H**). Three biological replicates were performed. Statistical significance was assessed using the log-rank test.

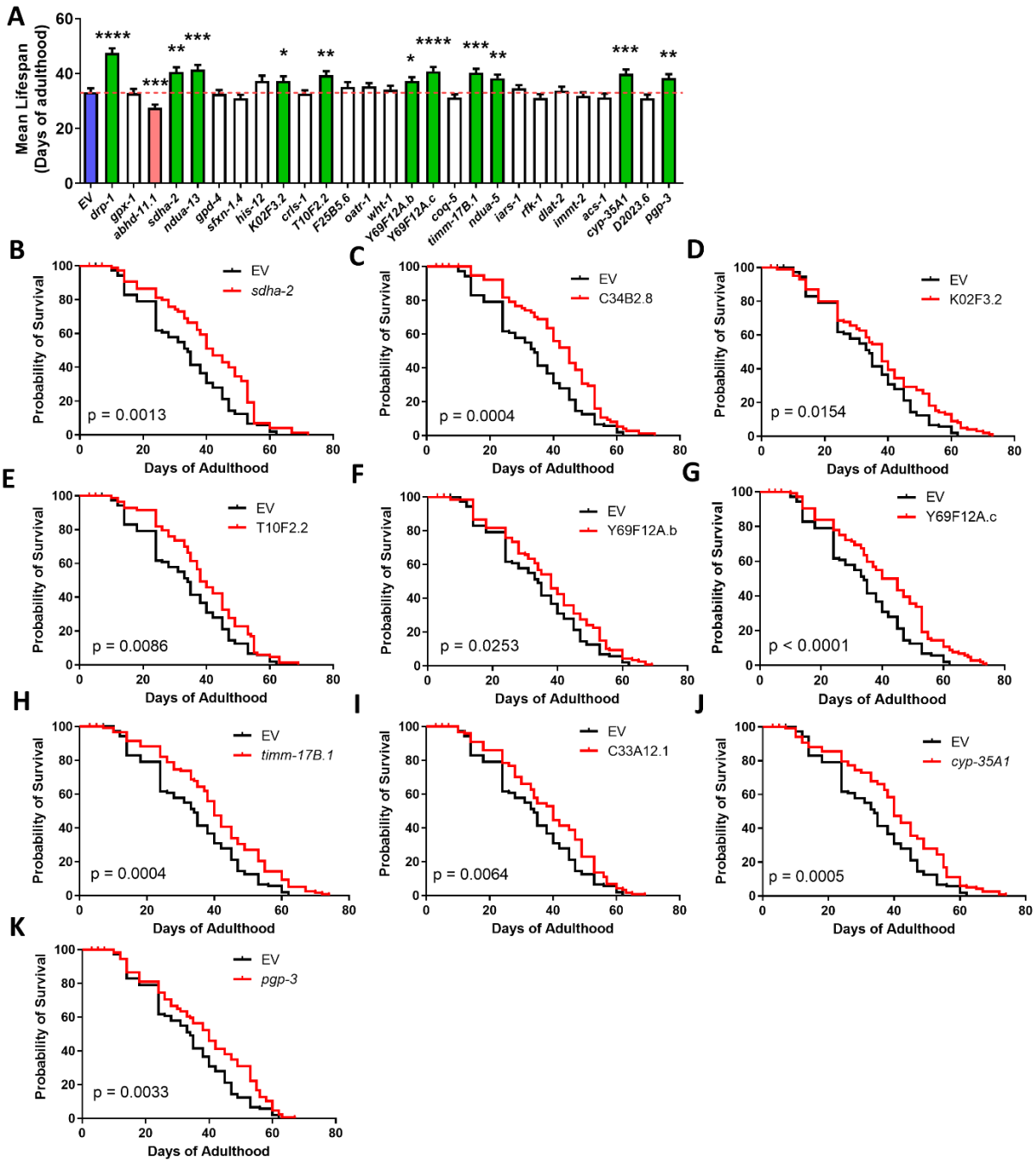

Figure 6. Decreasing mitochondrial fragmentation without disrupting *drp-1* can extend *daf-2* lifespan. To determine if decreasing mitochondrial fragmentation independently of *drp-1* could extend *daf-2* lifespan, *daf-2* worms were individually treated with 26 RNAi clones that were previously shown to decrease mitochondrial fragmentation in wild-type worms. Of these 26 RNAi clones, ten RNAi clones significantly increased the lifespan of *daf-2* mutants. This suggests

that decreasing mitochondrial fragmentation may be sufficient to extend longevity in *daf-2* worms. RNAi clones that increased lifespan compared to control (blue) are shown in green. Three biological replicates were performed. Statistical significance was assessed using the log-rank test. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

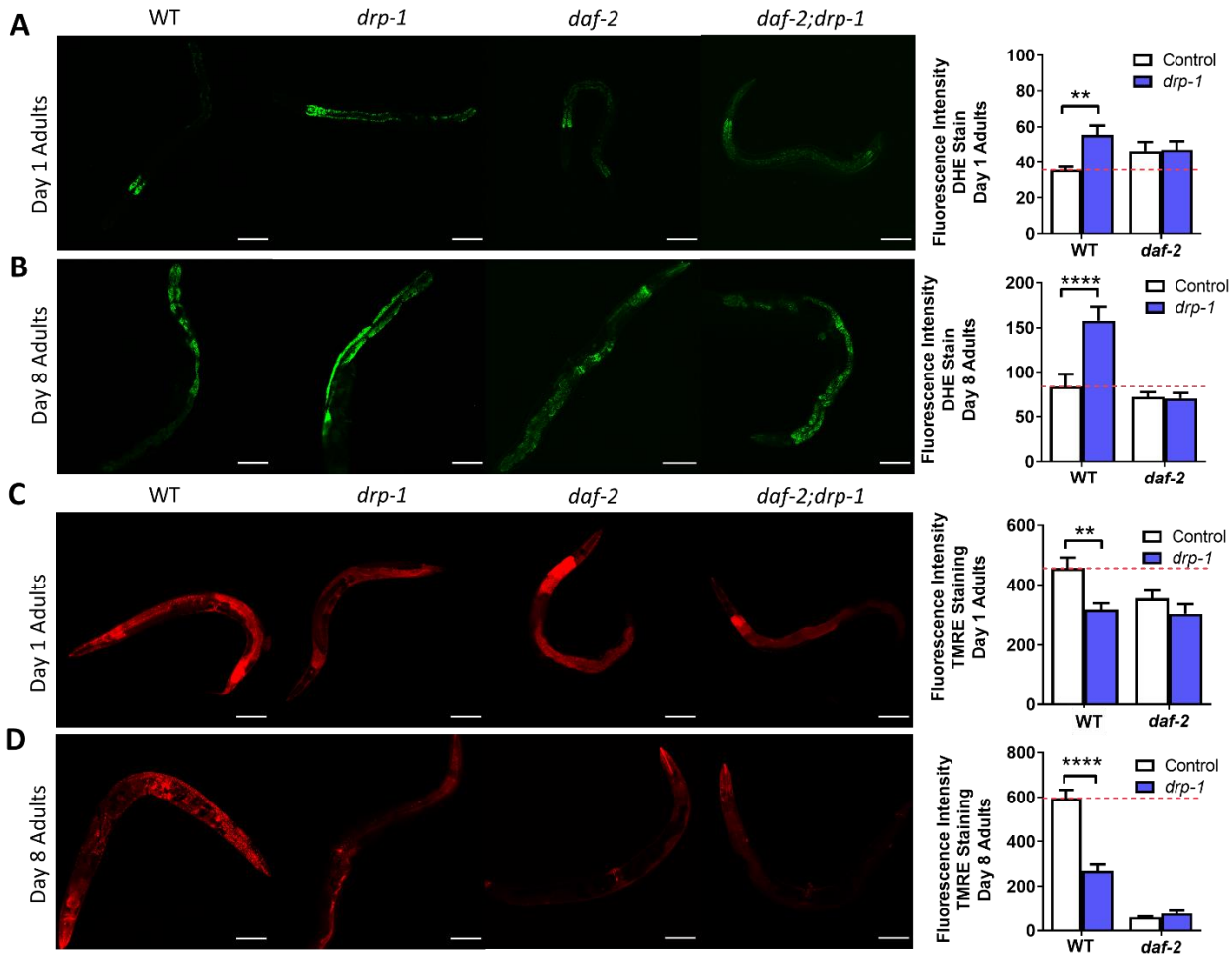

Figure 7. Disruption of *drp-1* increases ROS levels and decreases mitochondrial membrane potential in wild-type worms but not *daf-2* mutants. ROS levels indicated by whole-worm dihydroethidium (DHE) staining are higher in *drp-1* worms compared to wild-type worms at day 1 of adulthood, while *daf-2;drp-1* worms do not have increased ROS levels compared to *daf-2* worms (**A**). Similarly, at day 8 of adulthood, ROS levels remain higher in *drp-1* worms compared to wild-type, while *daf-2;drp-1* worms continue to show ROS levels similar to *daf-2* (**B**). The mitochondrial membrane potential as indicated by whole-worm TMRE staining is decreased by disruption of *drp-1* in both wild-type worms at day 1 of adulthood (**C**). At day 8 of adulthood, disruption of *drp-1* decreases mitochondrial membrane potential in a wild-type background but not in a *daf-2* background (**D**). Three biological replicates were performed. Statistical significance was assessed using a two-way ANOVA with Šidák's multiple comparisons test. Error bars indicate SEM. **p<0.01, ****p<0.0001. Scale bar indicates 100 µm.

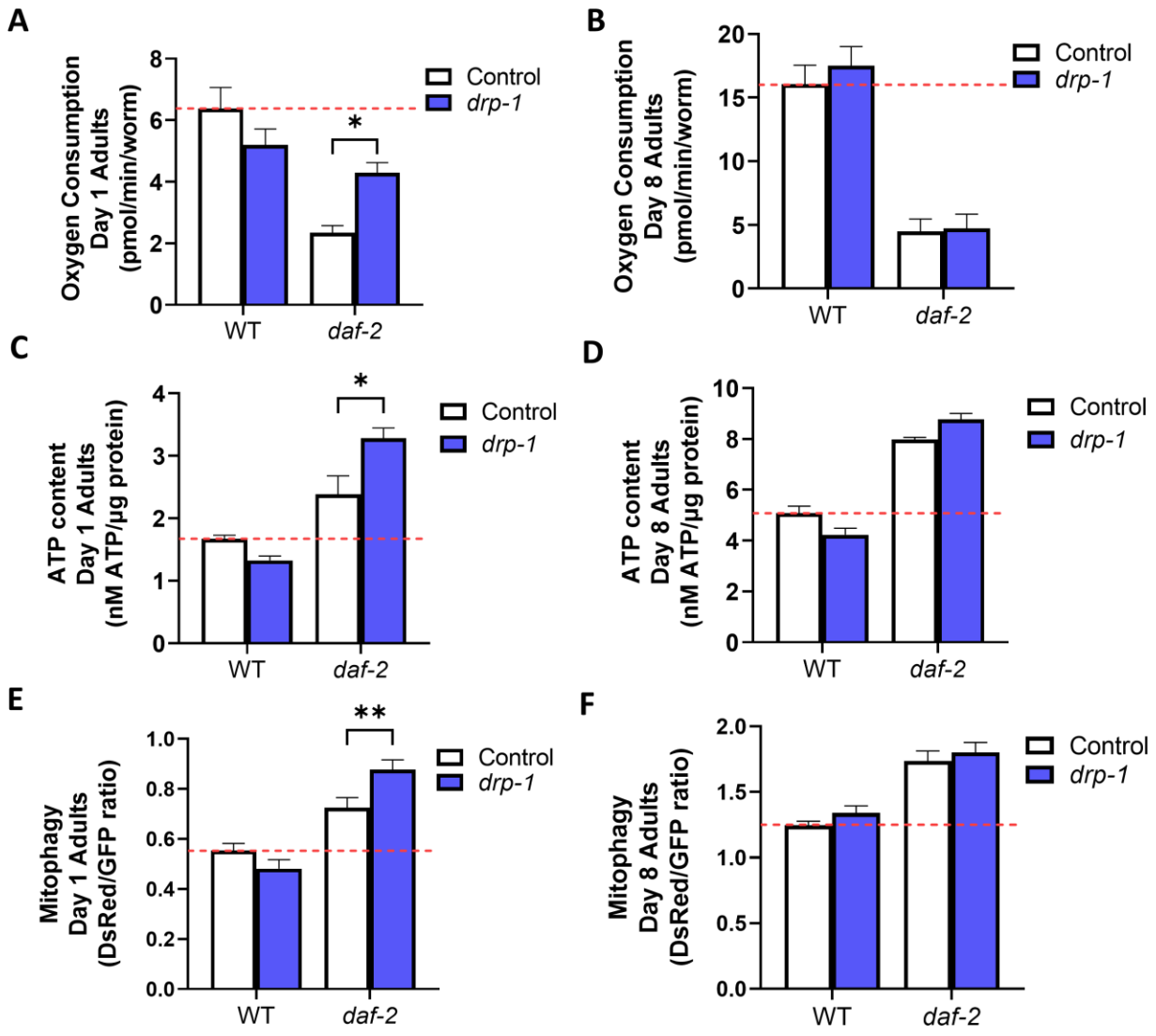

Figure 8. Disruption of *drp-1* increases mitochondrial function and mitophagy in *daf-2* mutants at day 1 of adulthood. While *drp-1* mutants consumed similar levels of oxygen as wild-type animals, *daf-2;drp-1* mutants consumed more oxygen than *daf-2* mutants at day 1 of adulthood (**A**). At day 8 of adulthood, disruption of *drp-1* does not affect the oxygen consumption (**B**). Similarly, *daf-2;drp-1* showed increased levels of ATP content compared to *daf-2* while *drp-1* mutants showed no change in ATP content compared to wild-type at day 1 (**C**), while at day 8 of adulthood, disruption of *drp-1* does not ATP levels (**D**). Quantification and comparison of the fluorescence intensity of both the pH-insensitive DsRed and pH-sensitive GFP fluorophores of the Rosella mitophagy reporter indicates that at day 1 of adulthood, disruption

of *drp-1* induces mitophagy in *daf-2* mutants but not in wild-type worms (**E**). By contrast, at day 8 of adulthood, disruption of *drp-1* does not affect mitophagy in either wild-type or *daf-2* worms (**F**). Mitophagy induction is signified by an increase in the ratio of DsRed to GFP. Representative images of mtRosella staining can be found in **Figure S4**. Three biological replicates were performed. Statistical significance was assessed using a two-way ANOVA with Šidák's multiple comparisons test. Error bars indicate SEM. *p<0.05, **p<0.01.

3.11. Supplementary figures

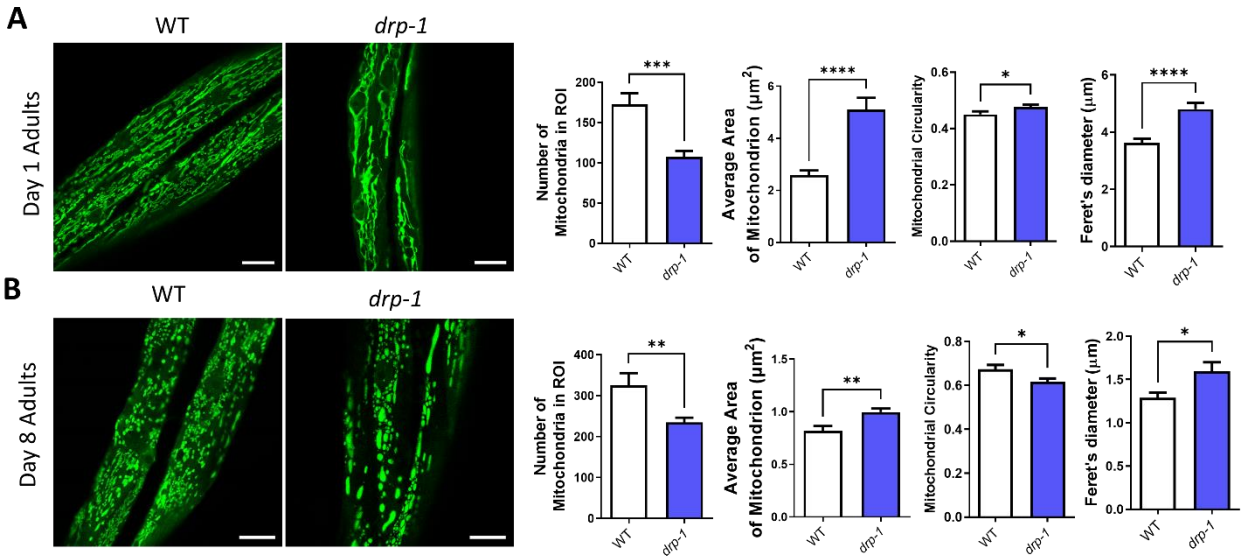

Figure S1. Disruption of *drp-1* increases mitochondrial network connectivity in wild-type animals. At day 1 of adulthood, disruption of *drp-1* in wild-type animals decreases the number of mitochondria, increases average mitochondrial area, increases circularity and increases ferret's diameter (**A**). These measurements are similar at day 8, except that disruption of *drp-1* decreases circularity (**B**). Three biological replicates were imaged. Statistical significance was assessed using a student's t-test. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar indicates 10 µm.

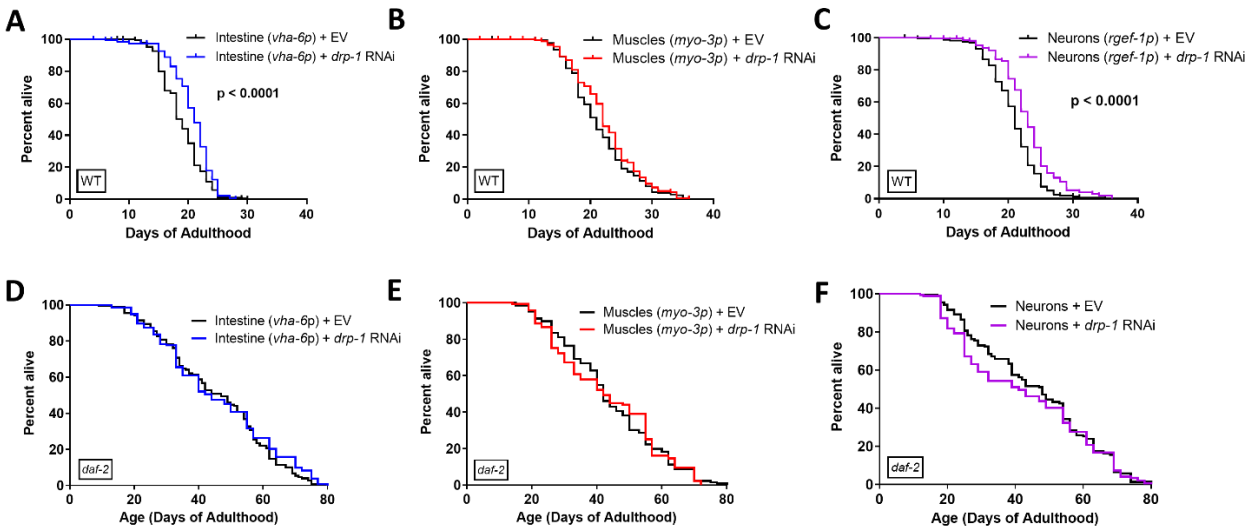

Figure S2. Tissue-specific knockdown of *drp-1* does not extend *daf-2* lifespan. Compared to animals fed empty-vector (EV) control bacteria, wild-type animals with disruption of *drp-1* in the intestine had increased lifespan (**A**). Disruption of *drp-1* in the muscles of wild-type animals did not increase lifespan compared to animals fed EV (**B**). Disruption of *drp-1* in the neurons of wild-type animals increases lifespan compared to animals fed EV (**C**). In *daf-2* animals, disruption of *drp-1* in the intestine, muscles or neurons of *daf-2* animals does not affect lifespan compared to animals fed EV (**D, E, F**). Four biological replicates were performed. Statistical significance was assessed using the log-rank test. *drp-1* RNAi data is from Figure 4 where control is worms with a *sid-1* mutation treated with *drp-1* RNAi.

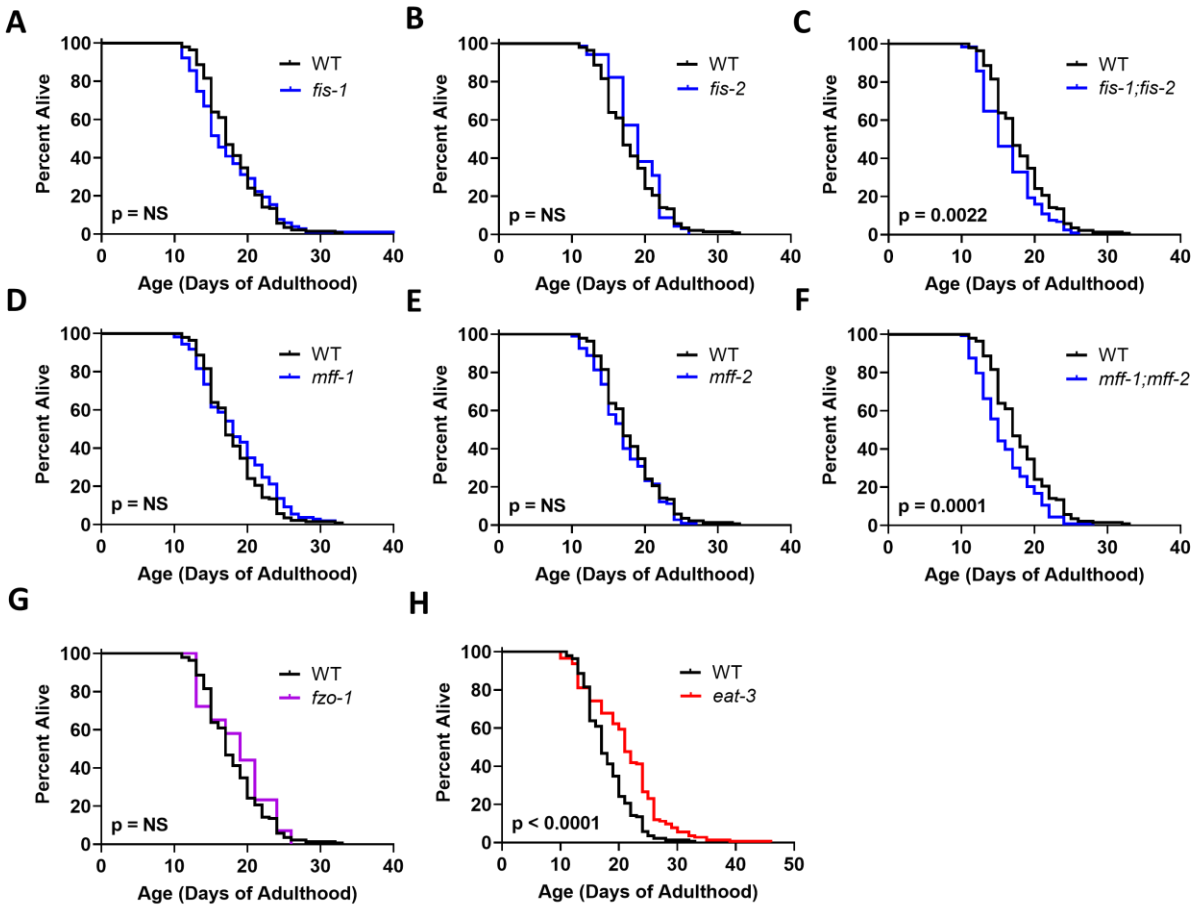

Figure S3. Effect of disrupting mitochondrial fission or fusion genes on lifespan in wild-type worms. Disruption of either *fis-1* (**A**), or *fis-2* (**B**) individually did not affect wild-type lifespan, while *fis-1;fis-2* double mutants exhibited a slight decrease in longevity (**C**). Similarly, *mff-1* (**D**) and *mff-2* (**E**) single mutants have a normal lifespan, while *mff-1;mff-2* double mutants exhibit decreased longevity (**F**). While deletion of the mitochondrial fusion gene *fzo-1* did not affect lifespan (**G**), disruption of *eat-3* extended longevity (**H**). At least three biological replicates were performed. Statistical significance was assessed using a log-rank test.

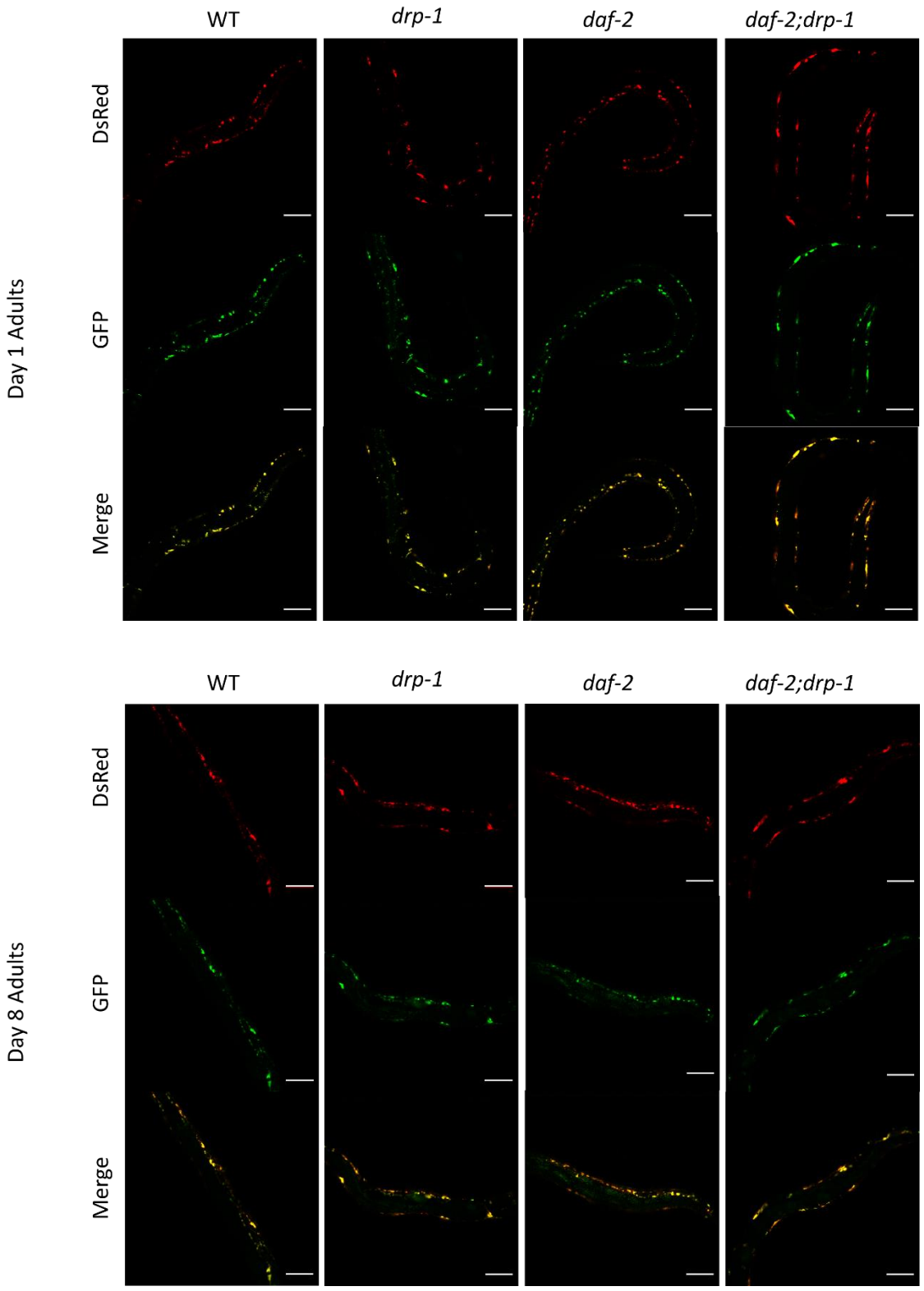

Figure S4. Visualization of the mtRosella mitophagy reporter reveals that disruption of *drp-1* increases mitophagy in *daf-2* worms in day 1 young adults. Whole body images of animals

expressing the mtRosella mitophagy reporter were obtained using dual-channel confocal microscopy. Mitophagy levels were determined by comparing the fluorescence intensity of pH-insensitive DsRed to pH-sensitive GFP. See **Figure 8** for quantification of fluorescence. Representative images showing the compared intensity of each fluorophore were obtained by merging the two channels together. Scale bar represents 50 µm.

Chapter 4

General Discussion and Conclusions

4.1. Overexpression of mitochondrial fission or fusion genes increases lifespan without improving mitochondrial network connectivity during aging

Given that mitochondrial fragmentation is observed during aging and in age-associated diseases, we hypothesized that decreasing mitochondrial fragmentation by overexpressing mitochondrial fusion machinery may extend lifespan. Surprisingly, overexpression of the *C. elegans* mitochondrial fusion genes *fzo-1* and *eat-3* increased lifespan despite also increasing mitochondrial network fragmentation in body wall muscle cells. Additionally, we predicted that overexpression of the mitochondrial fission gene *drp-1* would enhance age-associated mitochondrial fragmentation and would decrease lifespan unless paired with the overexpression of fusion genes, where increased capacity for both fission and fusion events to occur during aging would promote longevity. However, our work in chapter 2 illustrates that overexpression of *drp-1* on its own increases *C. elegans* lifespan despite increasing mitochondrial network fragmentation in body wall muscle cells. Additionally, overexpression of *drp-1, fzo-1* and *eat-3* all together did promote increased longevity, but not to the same extent as overexpression of any one gene on its own, including *drp-1*. This data indicates that overexpression of fission and fusion genes increases lifespan without increasing mitochondrial network connectedness and without maintaining mitochondrial network dynamicity during aging.

The findings reported in chapter 2 indicate that overexpression of mitochondrial fusion genes may increase lifespan by generating a hormetic response. We find that animals overexpressing *fzo-1* or *eat-3* have increased expression of target genes from multiple stress response pathways, indicating increased activation of these pathways. The ability for increased activation of the DAF-16 and mitoUPR pathways to extend lifespan is demonstrated in long-lived mitochondrial mutants with defects in the electron transport chain that give rise to mild impairment of mitochondrial function [59, 60, 98]. Similarly, imbalanced mitochondrial dynamics may activate stress response pathways, which ultimately contribute to lifespan extension. Imbalanced mitochondrial dynamics as a mechanism of lifespan extension is further supported by the finding that disruption of *eat-3* also increases lifespan and has increased

activation of multiple stress response pathways [208]. Furthermore, we find that when overexpression of fusion genes is paired with overexpression of the mitochondrial fission gene *drp-1*, thus restoring the balance between fission and fusion, the magnitude of lifespan extension is decreased. Similarly, when both fusion genes are overexpressed, the magnitude of lifespan extension is less than when one fusion gene is overexpressed. This indicates that a greater imbalance in mitochondrial dynamics may provide a stronger activation of stress response pathways and a greater promotion of longevity.

While overexpression of mitochondrial fusion genes increases activation of multiple stress response pathways, overexpression of the mitochondrial fission gene *drp-1* does not activate any of the stress response pathways that we tested, but still extends lifespan. Disruption of *drp-1* somewhat extends lifespan (chapter 2, figure 6a) and increases activation of the SKN-1-mediated oxidative stress response pathway, which confers increased resistance to oxidative stress in *drp-1* mutants [208]. Overexpression of *drp-1* also results in increased resistance to oxidative stress despite showing no increased expression of the SKN-1 target gene *gst-4*. This indicates that perturbation of *drp-1* expression may affect mitochondrial redox homeostasis in such a way that resistance to oxidative stress is ameliorated. However, the mechanism by which changes in *drp-1* expression modulates lifespan must be further examined.

A possible mechanism by which overexpression of *drp-1* may promote lifespan extension is by increasing the capacity for dysfunctional mitochondria to be cleared from the cell during aging. Previous research in both *C. elegans* and in mice indicates that there is an age-associated accumulation of a translational repressor of the mitochondrial fission factor (Mff), and thus a decline in Mff levels with age, resulting in impaired mitochondrial fission and mitophagy. Inhibition of the translational repressor in aged animals enhances mitochondrial fission, restores mitochondrial homeostasis and increases mitophagy levels [273]. It has previously been determined that Drp1 mediates selective mitophagy in human cells [194]. Furthermore, increased mitophagy is required for lifespan extension in multiple long-lived *C. elegans* mutants [50, 188]. Thus, future studies should examine whether overexpression of *drp-1* promotes longevity by increasing mitophagy activity during aging in *C. elegans.*

4.2. Inhibition of *drp-1* extends lifespan through distinct mechanisms in *daf-2* versus wild-type animals

In chapter 3, several findings indicate that disruption of the mitochondrial fission gene *drp-1* modulates *daf-2* and wild-type lifespan through separate mechanisms. Firstly, despite causing similar conformational changes in the mitochondrial network of both backgrounds, disruption of *drp-1* does not extend wild-type lifespan to the same degree as it does *daf-2* lifespan. Additionally, the conditions required for inhibition of *drp-1* to extend wild-type and *daf-2* lifespan differ. Inhibition of *drp-1* by RNAi is sufficient to extend *daf-2* lifespan albeit to a lesser extent than complete disruption of *drp-1*. However, RNAi inhibition of *drp-1* in wild-type animals results in a slight but significant decrease in lifespan. In testing the time at which *drp-1* inhibition is required to increase lifespan, we find that developmental inhibition of *drp-1* decreases wild-type lifespan while adulthood inhibition has no effect on lifespan. This data indicates that decreased levels of *drp-1* expression during development may be detrimental in wild-type *C. elegans,* as in other organisms. In testing which tissue required *drp-1* inhibition for lifespan extension, we find that lifelong inhibition of *drp-1* in the neurons is sufficient to extend wild-type lifespan. *C. elegans* neurons are less sensitive to RNAi than other tissues [274], therefore, this finding indicates that whole body knockdown of *drp-1* by RNAi may not be sufficient to extend lifespan unless neurons are sensitized to RNAi as was done when we tested tissue specificity. Altogether, these data indicate that neuronal inhibition of *drp-1* during adulthood promotes lifespan extension in wild-type animals. By contrast, disruption of *drp-1* during development is sufficient to increase *daf-2* lifespan, but inhibition of *drp-1* specifically in the neurons, muscle or intestine is not sufficient to extend *daf-2* lifespan. Thus, disruption of *drp-1* acts at different time points and in different tissues to extend wild-type lifespan compared to *daf-2* lifespan, indicating that the mechanism by which *drp-1* increases lifespan in each background is distinct.

In addition to acting under differing conditions to extend lifespan, inhibition of *drp-1* also has different effects on mitochondrial function in wild-type and *daf-2* animals. In wild-type animals, disruption of *drp-1* decreases mitochondrial membrane potential and increases ROS levels. In

daf-2 animals, disruption of *drp-1* has no effect on mitochondrial membrane potential or ROS levels. While decreased mitochondrial membrane potential is commonly associated with loss of mitochondrial proteostasis and induction of mitophagy [191, 275], we find that neither the mitoUPR nor mitophagy are activated in *drp-1* mutants. We do find that *drp-1* mutants have increased activation of the SKN-1-mediated oxidative stress response pathway, which may occur as a result of increased ROS levels and may contribute to lifespan extension. By contrast, disruption of *drp-1* improves mitochondrial function and activates mitophagy in young adult *daf-2* mutants but has no effect on mitochondrial function or mitophagy in wild-type animals. This data suggests that mitochondrial quality control may play a role in *daf-2* but not wild-type lifespan extension by disruption of *drp-1*.

4.3. Future directions

Given that one of the principal findings from our work in chapter 2 is that overexpression of mitochondrial fusion genes activates multiple stress response pathways known to contribute to longevity, future work should test the extent to which each pathway is required for lifespan extension by overexpression of mitochondrial fusion genes. Additionally, future studies should aim to elucidate how overexpression of the mitochondrial fission gene *drp-1* increases lifespan. In particular, ROS levels and mitophagy levels should be evaluated as both increased resistance to oxidative stress and increased mitochondrial fragmentation are observed in animals overexpressing *drp-1*.

Although our work in chapter 3 highlights the differing conditions required for disruption of *drp-1* to modulate both wild-type and *daf-2* lifespan, the mechanism of lifespan extension remains not fully elucidated. Identification of gene expression changes mediated by disruption of *drp-1* in wild-type and *daf-2* backgrounds will help to identify pathways that are altered by loss of *drp-1*. The contribution of these differentially expressed genes to lifespan extension by disruption of *drp-1* could then be tested by knocking down their expression in *drp-1, daf-2;drp-1* and control backgrounds.

Further studies should also be done to continue investigating the tissue and timing requirements for disruption of *drp-1* to extend lifespan. Identifying these requirements allows for limited disruption of *drp-1* which may be more beneficial to the organism compared to life-long and whole-body disruption of *drp-1*. For example, the lifespan of wild-type animals with adulthood knockdown of *drp-1* in the neurons should be tested given that these two conditions yielded longer lifespans compared to controls. Additionally, tissues that we did not test, such as the germline and the hypodermis, should be investigated as possible locations where disruption of *drp-1* is required for lifespan extension in *daf-2* mutants. Future studies could also investigate whether disruption of *drp-1* in more than one tissue, such as the intestine and the neurons, is required for lifespan extension in *daf-2* mutants.

Notably, in chapter 3 and in previous work, we have documented that age-associated mitochondrial fragmentation still occurs even in the absence of *drp-1*, which is the only known mediator of mitochondrial membrane scission in *C. elegans*. Thus, future studies aimed at identifying candidates that contribute to *drp-1*-independent mitochondrial fission would benefit a more comprehensive understanding of the machinery involved in mitochondrial fission and fusion in *C. elegans.*

Finally, the involvement of peroxisomal morphology and function in the modulation of lifespan by altering *drp-1* expression must be investigated more thoroughly. In chapter 3, we showed that peroxisomal networks become filamentous and interconnected upon disruption of *drp-1* in *daf-2* mutants. However, additional experiments are needed to determine whether there are corresponding changes in peroxisomal function, and whether changes in peroxisomal function may contribute to lifespan extension by disruption of *drp-1* in *daf-2* mutants. Additionally, further investigation is needed to determine what morphological changes are seen in peroxisomes from wild-type animals with disruption or overexpression of *drp-1*.

4.4. Conclusions

Disordered mitochondrial morphology, mitochondrial dysfunction and defects in mitochondrial quality control are all prevalent in aging and age-associated disease. Despite many findings showing that inhibition of Drp1-mediated mitochondrial fission ameliorates phenotypes in multiple models of age-associated disease, much less is known about how altering mitochondrial dynamics modulates lifespan. The work presented in this thesis illustrates that lifespan extension by modulation of mitochondrial fission and fusion machinery involves the activation of multiple stress response pathways as well as changes in mitochondrial morphology, function and quality control. Additionally, this work defines the conditions, such as tissue and timing specificity, required for disruption of mitochondrial fission to extend lifespan. Overall, this work demonstrates that modulation of mitochondrial dynamics can promote lifespan extension. The conditions defined in this work, along with future determinations of the mechanism by which inhibition of mitochondrial fission extends lifespan may be used to elucidate an optimization of mitochondrial dynamics that can promote healthy aging.

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