Mechanisms by which mild impairment of mitochondrial function increases stress resistance and lifespan

by

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Abstract

Aging is the main risk factor for the development of many diseases, such as Alzheimer's disease, cardiovascular disease, and many cancers. Although aging was traditionally considered an unpreventable and random accumulation of damage, work from the past three decades has shown that lifespan is strongly influenced by genetics. For example, the mitochondrial mutants *nuo-6* and *isp-1*, as well as *daf-2* mutants, exhibit a dramatic increase in lifespan in *C. elegans*. Interestingly, these mitochondrial mutants have mild disruptions in mitochondrial function and *daf-2* mutants have impaired insulin/insulin-like growth factor 1 signaling (IIS). The concept of hormesis refers to the paradoxical finding that a mild exposure to toxic agents or stimuli can have beneficial effects, including increasing stress resistance and delaying aging, and has been widely documented in several model organisms and even in humans. The mechanisms of hormesis are however poorly understood. We hypothesized that mild stress can provoke an overcompensatory activation of stress response genes which cause increased stress resistance or lifespan. We tested this hypothesis in the contexts of increased stress resistance resulting from disruptions of mitochondrial dynamics (*drp-1*, *eat-3*, and *fzo-1* mutants) as well as extended lifespan resulting from mild mitochondrial dysfunction (*nuo-6* and *isp-1* mutants) and from impaired IIS (*daf-2* mutants). We found that stress response transcription factors ATFS-1, HIF-1, and DAF-16 are required for the increased stress resistance resulting from disruptions of mitochondrial dynamics. We have identified mitochondrial superoxide as a hormetic signal which promotes longevity in *nuo-6* and *daf-2* mutants. Finally, we uncover the importance of regulation of nuclear localization of the stress response transcription factor DAF-16 by the GTPase-activating protein TBC-2 on the longevity of *daf-2*, *nuo-6*, and *isp-1* mutants. Overall, we identify key molecular mechanisms which mediate hormesis. Our findings

highlight the benefit conferred by stress response genes in promoting stress resistance and delaying aging. Targeting the activation of stress response genes may be a novel efficient treatment strategy to increase resistance to disease and to promote healthy aging.

Résumé

Le vieillissement est le principal facteur de risque pour le développement de plusieurs maladies, incluant la maladie d'Alzheimer, les maladies cardiovasculaires et plusieurs cancers. Bien que le vieillissement ait traditionnellement été considéré comme une accumulation inévitable et aléatoire de dommages, la recherche effectuée durant les trois dernières décennies a démontré que la durée de vie est fortement influencée par la génétique. Par exemple, les mutants mitochondriaux *nuo-6* et *isp-1* ainsi que les mutants *daf-2* manifestent une hausse dramatique de la durée de vie chez *C. elegans*. Ces mutants mitochondriaux ont d'ailleurs des perturbations légères dans la fonction de leurs mitochondries et les mutants *daf-2* ont leur signalisation d'insuline/insulin-like growth factor 1 (IIS) perturbée. Le concept d'hormèse porte sur les résultats paradoxaux où une faible exposition à des agents ou à des stimulus toxiques peut avoir des effets bénéfiques tels qu'augmenter la résistance aux stress et retarder le vieillissement, comme cela a été rapporté chez plusieurs organismes modèles et même chez les humains. Les mécanismes d'hormèse sont cependant peu compris. Nous avons émis l'hypothèse qu'un stress léger peut provoquer une activation surcompensatoire des gènes de réponse au stress, ce qui cause l'augmentation de la résistance au stress et de la durée de vie. Nous avons testé cette hypothèse dans les contextes suivants: augmentation de la résistance au stress suite aux perturbations légères des dynamiques mitochondriales (mutants *drp-1, eat-3* et *fzo-1*) et extension de la durée de vie suite à des dysfonctions légères des mitochondries (mutants *nuo-6* et *isp-1*) suite à la perturbation de la

signalisation IIS (mutants *daf-2*). Nous avons constaté que ATFS-1, HIF-1 et DAF-16, qui sont des facteurs de transcription de réponse au stress, sont requis pour l'augmentation de résistance au stress qui résulte des perturbations des dynamiques mitochondriales. Nous avons identifié le superoxyde mitochondrial comme signal initial d'hormèse qui promeut la longévité chez les mutants *nuo-6* et *daf-2*. Enfin, nous avons dévoilé l'importance de la régulation de la localisation nucléaire de DAF-16, un facteur de transcription de réponse au stress, par TBC-2, un GAP (GTPase-activating protein), sur la longévité des mutants *daf-2*, *nuo-6*, et *isp-1*. Nous avons donc identifié des mécanismes moléculaires clés à la base de l'hormèse. Nos résultats soulignent les bénéfices, pour la résistance au stress et pour le retardement du vieillissement, apportés par les gènes de réponse au stress. Viser l'activation des gènes de réponse au stress pourrait représenter une nouvelle stratégie de traitement afin d'augmenter la résistance aux maladies et afin de promouvoir le vieillissement en santé.

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Contribution of authors

Most of the work in **Chapter 1** has been published in the peer-reviewed journal FASEB:

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*: contributed equally to the publication.

The experiments in **Chapter 1** were selected for this thesis as they represent the contributions of Thomas Liontis to the publication. All sections in Chapter 1 were written anew and often represent novel analysis of the literature and data. Dylan J. Dues, Saravanapriah Nadarajan, and Annika Traa contributed for stress assays and Saravanapriah Nadarajan also contributed for RNA interference.

Replicates of the lifespan experiments in **Chapter 2** were performed independently by Thomas Liontis, Ulrich Anglas, and Jeremy Van Raamsdonk. Survival data of each replicate were subsequently pooled together and analyzed by Thomas Liontis.

The lifespan experiments in **Chapter 3** were performed by Thomas Liontis.

Introduction

Aging is the main risk factor for the development of many diseases, such as Alzheimer's disease, cardiovascular disease, and many cancers. With the ongoing population aging in Canada (Roos et al., 1998; Verma and Samis, 2011), the United States (Vespa et al., 2020), and generally in the world (Newgard and Sharpless, 2013), there is an increasingly urgent need to understand the aging process and its role in disease. Although aging was traditionally considered an unpreventable and random accumulation of damage, work from the past three decades has shown that lifespan is strongly influenced by genetics. As a result, the knowledge gained by understanding the genetic pathways contributing to longevity could be used to promote healthy aging.

Research using a variety of model organisms has demonstrated that mutations of a single gene, out of thousands of genes, can lead to significant and sometimes dramatic increases in stress resistance (Munoz and Riddle, 2003; Chick et al., 2014; Shaposhnikov et al., 2015), lifespan (Van Raamsdonk and Hekimi, 2009; Hofmann et al., 2015; Shaposhnikov et al., 2015), or often both (Friedman and Johnson, 1988; Kenyon et al., 1993; Lithgow et al., 1994; Murakami and Johnson, 1996; Munoz and Riddle, 2003; Salmon et al., 2005). However, the aging process and the mechanisms by which these genes act to increase stress resistance and lifespan are currently poorly understood. The powerful genetic model organism *Caenorhabditis elegans* is optimal to study the biological mechanisms of aging because 1) interventions which increase its lifespan have been shown to be conserved across species (Weimer et al., 2014); 2) more longevity-associated genes have been identified in *C. elegans* than in any other organism; and 3) its relatively short lifespan of approximately three weeks allows extensive experimentation.

Literature review

I. *C. elegans*

C. elegans are small, approximately 1 mm long, transparent nematodes that can be found in the soil or in rotting fruits across the world (Brenner, 1974; Barrière and Félix, 2014; Schulenburg and Félix, 2017). They are self-fertilizing hermaphrodites, meaning the adult lays eggs without the requirement of males, which arise naturally at around 0.1 – 0.2 % frequency (Ward and Carrel, 1979; Hodgkin and Doniach, 1997). *C. elegans* lay approximately 300 eggs throughout their adult reproductive period. Their muscles allow them to slither in solid media or to thrash in liquid media. They also possess other distinct tissues: neurons, intestine, epidermis, and germline.

C. elegans eggs hatch into larval stage 1 (L1), which grow and molt into L2, L3, then L4, which is the last larval stage and in which their gonads are fully formed. Finally, they grow into adults, which actively lay eggs. At 20 °C (the temperature used to characterize *C. elegans* throughout this thesis), the whole developmental process from egg to adult takes around 3 days and the adult lifespan of *C. elegans* is around 3 weeks.

Under starvation, L1 worms can arrest growth for several weeks and in this arrested state they are resistant to several stresses and can be frozen indefinitely (Lewis and Fleming, 1995; Padilla et al., 2002; Munoz and Riddle, 2003; Weinkove et al., 2006). Both L1 arrest and the resulting increase in stress resistance are regulated by the insulin/insulin-like growth factor-1 signaling (IIS) pathway (Baugh and Sternberg, 2006). Under presence of food, arrested L1 worms can resume development and grow to adulthood. Remarkably, adult worms with a history of prolonged starvation and L1 arrest have an increased lifespan (Rechavi et al., 2014).

An alternative developmental pathway for L1 worms that can also result from starvation, but primarily results from high population density, is the dauer pathway (Cassada and Russell, 1975; Golden and Riddle, 1982). Dauer entry is also regulated by IIS, in parallel with the TGF-β pathway (Thomas et al., 1993; Gottlieb and Ruvkun, 1994; Patterson and Padgett, 2000; Androwski et al., 2017). L1 worms under the dauer program develop to pre-dauer L2 and then reach the dauer stage, which substitutes L3. Dauer worms can survive for months (Klass and Hirsh, 1976). If food is found, dauers grow to L4 and complete the normal development process.

Genetics

C. elegans is the first multicellular organism to have its genome fully sequenced (*C. elegans* Sequencing Consortium, 1998). Furthermore, several gene knockout projects, such as the ones united by the *C. elegans* Deletion Mutant Consortium, generate mutants by random mutagenesis of worms, for example through ethyl methanesulfonate (EMS) exposure (*C. elegans* Deletion Mutant Consortium, 2012). The mutant strains are then efficiently stored at the *C. elegans* Genomics Center (CGC) and documented in their website, where individual laboratories can request strains at a negligible cost.

In the case of transgene insertion, for example when researchers want to over-express a gene, a traditional technique is germline injection (Mello et al., 1991; Mello and Fire, 1995; Nance and Frøkjær-Jensen, 2019). A disadvantage of injection is that the transgene often forms an extrachromosomal array, and thus may not be stable across multiple generations. Extrachromosomal arrays can be integrated into the genome through ultraviolet (UV) irradiation, but UV rays can cause DNA double-stranded breaks (Mitani, 1995; Kage-Nakadai et al., 2012). In this case the animal should be sufficiently outcrossed to eliminate nonspecific mutations. An

important disadvantage of injection is that the expression levels of the transgene are hard to predict as the array is separate from the genome and, in case of integration, multiple insertions can occur at several sites. Multiple insertion sites on gene loci can also interfere with those genes, confounding the experiment. Mos1-mediated Single Copy Insertion (MosSCI) is an elegant solution to these problems as one copy of the transgene is integrated in the genome and the insertion site is known.

Latest advances in gene editing permit targeted deletions, transgene insertions, or even single-base modification in *C. elegans* via clustered regularly interspersed short palindromic repeats (CRISPR)-Cas9 and are much less labor-intensive while also being more reliable than MosSCI and extrachromosomal array injection (Friedland et al., 2013; Tzur et al., 2013; Dickinson and Goldstein, 2016). Because of the efficiency of CRISPR-Cas9 methods, this paradigm could even replace random mutagenesis methods for high-throughput gene knockout libraries (Au et al., 2019).

RNA interference (RNAi) has been a powerful tool for genetic screening in *C. elegans* since 1998 when Andrew Fire and Craig Mello discovered that introducing double-stranded RNA (dsRNA) into worms induces degradation of the corresponding messenger RNA (mRNA) (Fire et al., 1998). RNAi can be administered by injecting dsRNA into the worm, by soaking, or by feeding. As *C. elegans* eat bacteria and dsRNA can be expressed by bacteria, RNAi is often conveniently delivered by feeding (Kamath et al., 2001). An advantage of RNAi over the other genetic methods above is that it can be applied at any developmental stage, for example only during adulthood. Furthermore, high-throughput experiments can be achieved by exposing worms to RNAi. Laboratories can notably access major RNAi libraries: the Ahringer laboratory's library (Fraser et

al., 2000; Kamath et al., 2003) and the Vidal laboratory's library (Rual et al., 2004), which together can target approximately 94 % of *C. elegans* genes.

Advantages as a model organism

C. elegans was chosen as a genetic model organism by Sydney Brenner because of its simplicity (Brenner, 1974). Maintenance of strains is simple and inexpensive as they can be fed *E. coli* bacteria, which can be grown easily in the laboratory. Because of the high brood size of *C. elegans*, crosses involving two proximate genetic loci are still highly feasible despite low recombination rates. Importantly, *C. elegans* has L1 arrest and the dauer program which allows it to survive starvation for months and permit easy maintenance. *C. elegans* can even be frozen for several years at -80 °C and potentially forever in liquid nitrogen, allowing individual laboratories to store all of their strains in case they are needed in the future. Freezing is also useful to prevent accumulation of spontaneous mutations and genetic drift (Vassilieva and Lynch, 1999). However, worms that are recovered from starvation or from being frozen should be maintained for 3 generations or more to avoid the resulting alterations of phenotypes found in the initial generations (Rechavi et al., 2014). *C. elegans* therefore provides unique advantages in terms of being resistant to starvation and to freezing, which distinguishes it from other model systems such as *Drosophila* and mice.

The transparency of *C. elegans* not only facilitates visualization of tissues and cells in live animals, but also of fluorescent proteins. Genes encoding fluorescent proteins can be fused to the promoter of a gene to measure its expression and determine its tissue localization. Furthermore, nuclear localization of transcription factors tagged with fluorescent proteins can be monitored in worms (Senchuk et al., 2018). Individual cellular organelles such as mitochondria can even be visualized

in *C. elegans* neurons and muscle while the animal is alive (Morsci et al., 2016; Machiela et al., 2020).

Genetic techniques in *C. elegans*, made highly accessible by its passionate community, allow both specific and high-throughput experiments. After publication of a research article, principal investigators are encouraged to provide their strains to the CGC. The CGC also benefits from the strains of consortiums such as the *C. elegans* Deletion Mutant Consortium. For individual researchers, this means that if they want to investigate a gene, they may find available mutant strains through the CGC. Since *C. elegans* resists well to starvation, strains can be sent with limited food through regular mail.

Advantages for studying stress resistance and aging

Stress resistance assays are commonly done in *C. elegans* as they cannot be easily done in higher organisms such as mice due to ethics considerations. For example, exposing *C. elegans* to lethal doses of heat or poisons does not usually require any specific approval by ethics committees, whereas it does in mice (Cheluvappa et al., 2017).

The high brood size of *C. elegans* is ideal to reach conclusions supported by statistical analysis. Performing stress resistance or lifespan experiments on a large number of worms increases researchers' ability to detect differences between each condition. This also means that each independent replicate of an experiment can be conducted with a high number of worms, increasing the reproducibility of experiments, which has been a long-standing problem in mouse research (Kafkafi et al., 2018).

C. elegans has a short lifespan of approximately 3 weeks, permitting high-throughput lifespan experiments, including screens for compounds or RNAi treatments which promote longevity (Lee

et al., 2003; Collins et al., 2006). Lifespan experiments are also performed in mice, but the possible number of interventions is limited by the high cost of acquiring and maintaining a sufficiently high number of mice for statistical power and by the common mouse model's prohibitively long lifespan, which has a median of over 2 years (Kunstyr and Leuenberger, 1975; Turturro et al., 2002).

II. Mitochondria

Mitochondria are double-membrane organelles mediating several processes in the cell. They are the primary generators of energy for the cell and also have an important role in responding to stress (Youle and van der Bliek, 2012). The electron transport chain spans the inner mitochondrial membrane. Several redox reactions drive protons through complexes I ─ IV, which are components of the electron transport chain, to the mitochondrial intermembrane space. The proton gradient's potential energy is coupled to oxidative phosphorylation in complex V (ATP synthase), resulting in phosphorylation of adenosine diphosphate (ADP) to adenosite triphosphate (ATP) (van der Bliek et al., 2017). Proteins in the cell which require energy to exert force, including performing conformational changes and transporting molecules against their chemical or electric gradient, can do so by releasing ATP's potential energy through its hydrolysis to ADP.

Mitochondrial dynamics

Mitochondria are highly networked and their shape and position within the cell are not static. In response to stress or to the energy demands of the cell, mitochondria actively break apart, join together, or localize along different areas of the cytoskeleton (Liesa et al., 2009; Westermann, 2010). Active fragmentation is called mitochondrial fission whereas joining of mitochondria is called mitochondrial fusion, and the continuous interplay between these two processes is known

as mitochondrial dynamics. During homeostasis, mitochondrial fission and fusion are at an equilibrium, where each event has equal frequency (Nunnari et al., 1997).

Mitochondrial fission

Mitochondrial fission occurs by cleavage of a mitochondrion into smaller mitochondria by the conserved GTPase DRP1 (Labrousse et al., 1999; Liesa et al., 2009). Cytosolic DRP1 is recruited to the outer mitochondrial membrane by mitochondrial fission proteins, such as FIS1, and mitochondrial fission factors, such as MFF1. DRP1 oligomers form a spiral ring around the mitochondrion and, through their GTPase activity, constrict and split the mitochondrion (Liesa et al., 2009). Mitochondrial fission has an important role in removing damaged mitochondria by mitophagy (Twig et al., 2008) and promotes apoptosis (Frank et al., 2001). Senescent cells have been reported to have increased mitochondrial length (Yoon et al., 2006). Consistent with this finding, disrupting mitochondrial fission promotes senescence, whereas shifting the balance of mitochondrial dynamics away from fusion and thus towards fission, just like directly over-activating fission, can prevent senescence (Yoon et al., 2006; Lee et al., 2007).

Mitochondrial fusion

Mitochondrial fusion is mediated by the conserved GTPases optic-atrophy 1 (OPA1 in mammals, EAT-3 in *C. elegans*), which joins the inner mitochondrial membranes together, and mitofusin 1 and 2 (MFN1 and MFN2 in mammals, FZO-1 in *C. elegans*), which coordinate the fusion of the outer mitochondrial membrane (Liesa et al., 2009). Mitochondrial fusion has an important role in maintaining the mitochondrial membrane potential (Olichon et al., 2003; Olichon et al., 2007), in optimizing mitochondria's bioenergetic functions (Skulachev, 2001), in mixing damaged DNA (Nakada et al., 2001; Karbowski and Youle, 2003), and in inhibition of apoptosis (Olichon et al.,

2003). EAT-3 also regulates the structure of mitochondrial cristae to prevent cytochrome c release and a resulting decrease in oxygen consumption (Olichon et al., 2003; Liesa et al., 2009).

Role of mitochondrial dynamics in human disease

Functional mitochondrial dynamics are crucial in humans, as their disruption is involved in several neurodegenerative disorders (Liesa et al., 2009; Burté et al., 2015). A mutation in *DRP1* causes encephalopathy, lactic acidosis, and early lethality (Waterham et al., 2007). Mutations in *OPA1* cause autosomal dominant optic atrophy (Olichon et al., 2006) and mutations in *MFN2* cause the most prevalent form of axonal Charcot-Marie-Tooth disease (Lawson et al., 2005). Moreover, increased mitochondrial fragmentation has been documented in Parkinson's and Huntington's diseases, but it remains to be determined whether imbalanced mitochondrial dynamics are causal to these diseases' pathogenesis (Archer, 2013).

III. Stress resistance

Disease can occur through environmental challenge or through genetic abnormality, leading to impairments in homeostasis and in protein function, resulting in impaired bodily function. For example, we have varying resistance to environmental pathogens such as viruses and bacteria (Short et al., 2018), which may depend on the activation of xenobiotic resistance genes as has been shown in animal models (Fortier et al., 2005; Ermolaeva and Schumacher, 2014; Nakov et al., 2018; Palmer et al., 2018). Similarly, monogenic diseases can have varying penetrance: individuals with a same disease-causing mutation can experience vastly different symptoms, suggesting contributions from their genetic background or environment to their disease resistance (Trapani and Retta, 2015; Lill, 2016; Sacco and Milner, 2019).

It is unclear which components of individuals' genetic background confer increased resistance to diseases. In model systems, resistance to stress has been shown to be mediated by transcription factors which activate stress response pathways. Indeed, activation of stress response transcription factors in *C. elegans* can increase resistance to hypoxia (Zhang et al., 2009), oxidative stress (Tullet et al., 2008), high heat (Baird et al., 2014), and pathogenic bacteria (Schinzel et al., 2019).

DAF-16-mediated stress response

The *daf-2* gene, which is equally homologous for mammalian insulin receptor and insulin-like growth factor 1 (Kimura et al., 1997), is very well studied. Partial loss-of-function mutations in *daf-2* lead to increased resistance to heat (Lithgow et al., 1995), oxidative (Honda and Honda, 1999), osmotic (Lamitina and Strange, 2005), hypoxic (Scott et al., 2002), UV (Murakami and Johnson, 1996) and other stresses. In a *daf-2* mutant background, the mutant DAF-2 receptor no longer activates AGE-1, and the resulting loss in signaling eventually leads to dephosphorylation and activation of the transcription factor DAF-16 (Murphy and Hu, 2013). DAF-16 activation involves its localization to the nucleus, where it upregulates several target genes. The phenotypes associated with DAF-2 loss-of-function are suppressed by loss of DAF-16.

Interestingly, DAF-16 is also a stress response gene, as it can be activated under various forms of stress, such as heat or oxidative stress, to promote resistance (Chiang et al., 2012; Zhang et al., 2015; Dues et al., 2016; Senchuk et al., 2018). DAF-16 has a large number of transcriptional targets, including *mtl-1* and *sod-3* which have previously been utilized for reporters of DAF-16 activity (Libina et al., 2003; Zhang et al., 2013).

Mitochondrial unfolded protein response

In response to stresses which cause unfolding or misfolding of proteins in mitochondria, the mitochondrial unfolded protein response (mitoUPR) signaling cascade is activated (Haynes and Ron, 2010; Jovaisaite et al., 2014; Long et al., 2014). The CLPP-1 protease cleaves misfolded proteins and HAF-1 transports the resulting peptides outside of the mitochondrial matrix. The peptides block the import of cytoplasmic proteins into the mitochondria. The transcription factor ATFS-1 is therefore unable to enter the mitochondria and instead enters the nucleus, as it has both a mitochondrial targeting sequence and a nuclear localization sequence. In the nucleus, ATFS-1 upregulates several protective transcriptional targets, including proteases and mitochondrial chaperone proteins, such as HSP-6, in order to restore homeostasis. Activation of the mitoUPR can thus be measured by an *hsp-6* reporter (Yoneda et al., 2004).

Cytosolic unfolded protein response

In response to heat or to other stresses which disturb protein folding in the cytoplasm, the cytosolic unfolded protein response (cytoUPR) is activated (Cotto and Morimoto, 1999; Vabulas et al., 2010; Jovaisaite et al., 2014). Heat shock factor (HSF-1), a transcription factor, is dissociated from an Hsp70 chaperone protein due to the high load of unfolded proteins in the cytoplasm. This results in nuclear localization of HSF-1 and upregulation of cytosolic chaperones, including HSP-16.2, to restore proteostasis. Activation of the cytoUPR can been monitored by using an *hsp-16.2* reporter (Link et al., 1999; Rea et al., 2005).

Hypoxia response

Under sufficiently high oxygen levels, hypoxia inducible factor (HIF-1) is hydroxylated by EGL-9 and binds to the E3 ubiquitin ligase VHL-1, which targets HIF-1 for proteosomal degradation

(Powell-Coffman, 2010). When oxygen levels are very low (0.5 to 1 % oxygen), the hypoxia response is triggered by the resulting incapability of EGL-9 to hydroxylate HIF-1, thus permiting the nuclear localization of stable HIF-1. Once in the nucleus, HIF-1 activates several targets which promote survival under hypoxia, such as NHR-57 (Shen et al., 2005). Activation of the hypoxia response can be measured through an *nhr-57* reporter (Shen et al., 2006).

SKN-1-mediated oxidative stress response

SKN-1 (Nrf genes in mammals) plays an important role in oxidative stress resistance (Blackwell et al., 2015). It is upregulated in response to oxidative stress and localizes to the nucleus where it acts as a transcription factor (An and Blackwell, 2003). It activates various phase II detoxification enzymes, including the glutathione-S-transferase GST-4, as well as enzymes mediating synthesis of glutathione, such as GCS-1 (Tullet et al., 2008). Monitoring the SKN-1-mediated oxidative stress response can be performed by utilizing *gst-4* or *gcs-1* reporters (Link and Johnson, 2002; Wang et al., 2010).

IV. Aging

Lifespan extension through mitochondrial electron transport chain disruption

In *C. elegans*, mild mitochondrial dysfunction has been shown to dramatically extend lifespan. For example, *nuo-6* and *isp-1* mutants, which have a point mutation in a subunit of complex I and in the iron sulfur protein of complex III, respectively, exhibit a nearly twofold increase in median and maximum lifespan (Yang and Hekimi, 2010b). In contrast, more severe mitochondrial dysfunction resulting from undiluted RNAi of *nuo-6* or *isp-1* leads to lethality (Fraser et al., 2000; Maeda et al., 2001; Yang and Hekimi, 2010b). Deletion of *clk-1*, which is involved in ubiquinone synthesis, extends lifespan in *C. elegans* (Ewbank et al., 1997) and mice (Liu et al., 2005). Despite

increasing lifespan, these mutations of the mitochondrial electron transport chain cause impaired mitochondrial respiration, very slow development, and impaired reproductive health in *C. elegans* (Van Raamsdonk and Hekimi, 2010). DAF-16 is required for lifespan extension by mutation of *nuo-6*, *isp-1*, and *clk-1* (Senchuk et al., 2018). It is however unclear how mitochondrial dysfunction in these mutants interacts with DAF-16 to increase lifespan.

Lifespan extension through impaired insulin/insulin-like growth factor 1 signaling

Impaired IIS represented by partial loss-of-function of *daf-2* not only results in a general increase in stress resistance, but also causes a more than twofold increase in lifespan (Kenyon et al., 1993). Disrupting *age-1*, the downstream effector of *daf-2*, similarly leads to a dramatic increase in lifespan which is dependent on DAF-16 (Johnson, 1990; Dorman et al., 1995). Extension of lifespan in these mutants results from a relatively mild impairment of IIS, as more severe loss-of-function of *daf-2* or *age-1* leads to dauer arrest (Morris et al., 1996; Patel et al., 2008). Interestingly, *daf-2* mutants have normal movement throughout their lifespan, such that their increased lifespan seems to coincide with a similar degree of healthspan extension as well (Hahm et al., 2015).

Impaired IIS is among the most evolutionary conserved genetic mechanism for lifespan extension, as it has been shown to extend lifespan in *C. elegans* (Johnson, 1990; Kenyon et al., 1993), *Drosophila* (Clancy et al., 2001; Hwangbo et al., 2004), and mice (Bartke and Brown-Borg, 2004). Variants in components of the IIS have also been associated with extreme longevity in humans (Sadagurski and White, 2013).

Reactive oxygen species and aging

A widely accepted theory of aging, the Free Radical Theory of Aging, suggests that aging is caused by accumulation of damage due to production of reactive oxygen species (ROS) (Harman, 1956).

The main form of ROS generated in mitochondria is superoxide, which is a by-product of cellular respiration. Antioxidant enzymes called superoxide dismutase (SOD) are the only eukaryotic enzymes able to detoxify superoxide. In *C. elegans*, *sod-1, sod-2*, and *sod-4* (SOD1, SOD2, and SOD3 in humans) encode the primary cytoplasmic, mitochondrial, and extracellular SODs, respectively. There are also *sod-3* and *sod-5* which are expressed in the mitochondria and cytoplasm at low levels. Surprisingly, deletion of all five *sod* genes in *C. elegans* has no effect on lifespan (Van Raamsdonk and Hekimi, 2012). This indicates that superoxide detoxification is not necessary for normal longevity. Furthermore, individual deletions of each *sod* gene do not reduce lifespan, despite decreasing resistance to oxidative stress (Van Raamsdonk and Hekimi, 2009). Rather, mildly increasing mitochondrial superoxide through *sod-2* deletion or directly through treatment with low concentrations of paraquat, a mitochondrial superoxide-generating compound (Castello et al., 2007), results in a longer life (Van Raamsdonk and Hekimi, 2009; Yang and Hekimi, 2010a).

There is now an increasing amount of evidence that points towards a beneficial role of ROS for prolonging life that is conserved across species. Indeed, interventions or mutations that increase ROS have been shown to extend longevity in yeast (Pan et al., 2011; Schroeder et al., 2013), *C. elegans* (Van Raamsdonk and Hekimi, 2009; Yang and Hekimi, 2010a; Van Raamsdonk and Hekimi, 2012), flies (Owusu-Ansah et al., 2013; Scialo et al., 2016), and mice (Liu et al., 2005; Weimer et al., 2014). These findings are also likely to extend to humans, where elevations in ROS contribute to the healthspan-promoting effects of exercise. Ristow et al. (2009) showed that antioxidant supplementation in humans reduces ROS levels and suppresses beneficial effects of exercise such as improved insulin sensitivity. Furthermore, antioxidants do not benefit human longevity and may instead decrease lifespan (Bjelakovic et al., 2012).

In *C. elegans*, mitochondrial mutants *nuo-6* and *isp-1* exhibit a very long lifespan despite having elevated mitochondrial superoxide, which is a ROS (Yang and Hekimi, 2010a). Long-lived *daf-2* mutants also have increased mitochondrial superoxide. Yang and Hekimi (2010a) have shown that providing the antioxidant N-acetyl-cysteine (NAC) to *nuo-6*, *isp-1*, and *daf-2* decreases their lifespan, suggesting that elevated ROS in these strains is beneficial for their longevity.

Stress resistance and longevity

Stress resistance and longevity are often linked. In a large *C. elegans* screen using the random mutagen EMS, approximately 80 % of the mutants that exhibited increased heat stress resistance also benefited from a 15 % or higher extension of lifespan (Munoz and Riddle, 2003). The *daf-2* gene emerged among those mutants. As mentioned before, *daf-2* mutants have increased resistance to multiple stresses as well as a highly extended lifespan. Another example is the *skn-1* stress response gene, which is also required for stress resistance and longevity, as *skn-1* loss-of-function mutations decrease stress resistance and lifespan, whereas *skn-1* over-expression increases both (Blackwell et al., 2015). Increased resistance to hypoxia and pathogenic bacteria resulting from activation of stress response transcription factors also coincides with increased lifespan (Zhang et al., 2009; Schinzel et al., 2019).

However, stress resistance and longevity can be uncoupled. Although long-lived *isp-1* (Dues et al., 2017) and *nuo-6* mutants have a general resistance to several stresses, *nuo-6* is sensitive to anoxia (Soo and Van Raamsdonk, unpublished data). Furthermore, several long-lived mitochondrial mutants, such as *sod*-2 and *clk-1*, have increased lifespan despite decreased resistance to oxidative stress (Van Raamsdonk and Hekimi, 2009; Schaar et al., 2015). Van

Raamsdonk and Hekimi (2010) have reviewed other examples where, depending which stress is evaluated, there can be uncoupling of stress resistance and longevity.

V. Hormesis

Hormesis refers to the finding that toxic agents or stimuli can be instead beneficial when applied more mildly. It involves adaptation of the organism to the mild stress. Hormesis can be useful for the organism to keep a "memory" of the initial stress and thus to prevent a second similar stress from causing lethal or irreparable damage.

Hormetic increases in lifespan or in resistance to subsequent stresses have been demonstrated in model organisms. Although maintaining the temperature above 20 °C shortens the lifespan of *C. elegans*, a short mild heat stress of 25–35 °C before late adulthood is sufficient to extend lifespan and increase resistance to subsequent acute 37 °C heat stress, pathogenic bacterial stress, and oxidative stress (Zhang et al., 2015; Dues et al., 2016). Exposing *C. elegans* to mild oxidative, osmotic, or cold stress also increases their resistance to the same stress (Cypser and Johnson, 2002; Lamitina et al., 2004; Murray et al., 2007) and extends their lifespan (Dues et al., 2016). Dues et al. (2016) showed that DAF-16 nuclear localization occurs under these stresses and that additional stress response pathways are activated, such as the cytoUPR, the mitoUPR, and the endoplasmic reticulum unfolded protein response. Remarkably, they found that these pathways remain active for several days after exposure to a mild stress. Disrupting the activity of the cytoUPR activator HSF-1 prevented the hormetic increase in heat stress resistance, while disrupting DAF-16 selectively prevented the persistence of this increased resistance (Dues et al., 2016). This suggests that mild stress can result in a sustained activity of stress response pathways, conferring resistance to subsequent stress and simultaneously protecting from the aging process.

Similar experiments in *Drosophila melanogaster* have shown the same effect, where mild heat stresses extend lifespan and confer increased resistance to a subsequent acute heat stress (Khazaeli et al., 1997; Hercus et al., 2003). Finally, cold stress has been shown to extend lifespan in mice (Ordy et al., 1967; Conti et al., 2006). Overall, current research suggests that mild stress can provoke an increase in stress resistance or lifespan across several organisms.

RNAi screens for lifespan extension in *C. elegans* have identified mitochondrial function as the most highly represented group (Lee et al., 2003; Hansen et al., 2005), suggesting an important relationship between mitochondria and longevity. Because complete loss of the electron transport chain would be lethal and mutating its genes *nuo-6*, *isp-1*, and *clk-1* causes disrupted mitochondrial respiration, slow development, and impaired reproductive health (Van Raamsdonk and Hekimi, 2010), the associated mechanism for lifespan extension has been thought to rely on mitochondrial hormesis, or mitohormesis. Interestingly, stress response pathways have been found to be upregulated in mitochondrial mutants (Schaar et al., 2015; Dues et al., 2017; Wu et al., 2018) and DAF-16 is necessary for the longevity of *nuo-6*, *isp-1*, *sod-2*, and *clk-1* mutants (Senchuk et al., 2018). Furthermore, although the mitochondrial superoxide generator paraquat is toxic at high concentrations, administering it at a mild dose instead increases lifespan (Van Raamsdonk, 2015).

Humans can also improve their health through hormesis. Exercise, when considered from an external point of view, is simply repetitive stress and damage (Fridén et al., 1984; Fridén et al., 1986) of muscle tissue paired with unusually high energy consumption. However, lack of exercise is associated with several diseases and poor longevity (Gremeaux et al., 2012; Mendonca et al., 2016), suggesting the stress invoked by exercise provides protective effects. Moreover, ischemic preconditioning, i.e. mild deprivation of oxygen, is frequently performed during heart surgery to

increase the brain and heart's resistance to the upcoming more severe lack of oxygen (Martins et al., 2011).

Rationale for the study and hypothesis

Our objective is to elucidate the mechanisms by which mild stress can enhance resistance to stress or extend longevity. We **hypothesize that mild mitochondrial stress can increase resistance to further stresses and extend lifespan through activation of stress response pathways**. The stress response pathways, acting as an adaptation to the mild stress, instead overcompensate and can confer increased resistance to many stresses or even an extension in lifespan **(Figure 1)**.

In **Chapter 1**, we investigated the stress resistance of worms with disrupted mitochondrial dynamics. We hypothesized that increased stress resistance in mitochondrial dynamics mutants were caused by upregulation of stress response pathways and evaluated the contribution of each stress response pathway (described in Section III) in these worms. In **Chapter 2**, we tested the impact of potentially harmful ROS on the lifespan of long-lived mutants *nuo-6*, *isp-1*, and *daf-2*. We hypothesized that mitochondrial ROS at a hormetic dose are beneficial for longevity and measured the effect on lifespan of a transgene expected to decrease mitochondrial ROS. Finally, in **Chapter 3** we characterized the contribution of TBC-2, a regulator of DAF-16, on the longevity of these same long-lived mutants. We hypothesized that reducing activity of the stress-response gene DAF-16 would also reduce lifespan in these mutants.

Chapter 1. Results. Disruption of mitochondrial dynamics increases stress resistance through activation of multiple stress response pathways

1.1. Background

We have found that disrupting mitochondrial dynamics in *C. elegans* with the deletion mutants *drp-1(tm1108)*, *eat-3(tm1107)*, and *fzo-1(tm1133)* cause impairments in mitochondrial function, notably a reduction in ATP levels (Machiela et al., 2020). The mitochondrial fusion mutants, *eat-3(tm1107)* and *fzo-1(tm1133)* displayed a clear increase in mitochondrial fragmentation as well (Machiela et al., 2020).

tm1108 is a 424 bp deletion and 18 bp insertion in the *drp-1* gene, which affects exons 2 and 3 out of *drp-1*'s 8 total exons. *tm1133* is a 419 bp deletion and 14 bp insertion in the *fzo-1* gene, which affects exons 2 to 4 out of *fzo-1*'s 8 exons. *tm1107* is a 417 bp deletion in the *eat-3* gene, which affects exons 5 and 6 out of *eat-3*'s 9 exons. As no other mutant alleles of *drp-1* and *fzo-1* are used throughout this chapter, *drp-1(tm1108)* and *fzo-1(tm1133)* mutants are shortened to *drp-1* and *fzo-1* mutants.

We aimed to characterize the stress resistance of these mutants to understand how disruptions of mitochondrial dynamics could affect the whole organism's sensitivity to environmental stresses. We hypothesized that the impairment of mitochondrial structure and function would also impair resistance to stress, but we instead found increased resistance to oxidative stress in all three mutants, and increased heat stress in the mitochondrial fusion mutants. Our results suggest that the

mild impairments in mitochondrial function can result in hormesis, where upregulation of stress response pathways confer increased oxidative or heat stress resistance in these mutants.

1.2. Disruption of mitochondrial fission and fusion increases resistance to oxidative and heat stress

In order to investigate the sensitivity to stress resulting from disrupted mitochondrial dynamics, we acquired the mitochondrial fission mutant *drp-1(tm1108)* and the mitochondrial fusion mutants *eat-3(tm1107)* and *fzo-1(tm1133)* and exposed them to various stresses [*drp-1(tm1108)* and *fzo-1(tm1133)* are henceforth referred to as *drp-1* and *fzo-1* mutants]. Acute oxidative stress resistance was measured by monitoring worms' survival under 300 μM juglone, a toxic agent which increases reactive oxygen species such as superoxide and hydrogen peroxide (Blum and Fridovich, 1983). Surprisingly, we found that mitochondrial fission and fusion mutants have increased resistance to acute oxidative stress resistance **(Figure 2A)**. Furthermore, mitochondrial fusion mutants *eat-3(tm1107)* and *fzo-1* displayed increased resistance to 37 °C heat stress, whereas in this case the fission mutant *drp-1* was more sensitive than wild-type worms **(Figure 2B)**. The increased oxidative stress resistance and heat stress sensitivity in *drp-1* mutants specifically result from impaired mitochondrial fission, as we have shown that worms with mutations in the fission accessory genes *fis-1*, *fis-2*, *mff-1*, and *mff-2* also have an increased oxidative stress resistance and heat stress sensitivity (Machiela et al., 2020).

eat-3(tm1107) mutants were the most resistant to both oxidative and heat stress. The *tm1107* allele is a 407 bp deletion in the GTPase domain **(Figure 2C)** which provokes a frame shift, leading to an early stop codon, and an absence of EAT-3 protein (Kanazawa et al., 2008) likely due to nonsense mediated mRNA decay. The *eat-3(tm1107)* mutation is therefore a null allele. Because

eat-3 is a GTPase, we hypothesized that disruption of its GTPase domain may be sufficient to disrupt the interactions between *eat-3* and downstream effectors of stress resistance. To test this hypothesis, we obtained worms with the *eat-3(ad426)* point mutation, which targets *eat-3*'s GTPase domain by modifying its valine to isoleucine at position 328 (Kanazawa et al., 2008) **(Figure 2C)**. Although *eat-3(tm1107)* null mutants and *eat-3(ad426)* have similar impairments in terms of their low brood size, slow growth, and fragmented mitochondria (Kanazawa et al., 2008; Machiela et al., 2020), EAT-3 protein is detectable in *eat-3(ad426)* worms (Kanazawa et al., 2008), suggesting that they are *eat-3* partial loss-of-function mutants rather than null mutants. The *eat-3(ad426)* strain has a *him-8* mutant background, which is a possible but unlikely confounding factor for our experiments (Hodgkin et al., 1979; Avery, 1993).

In preliminary experiments, we found that *eat-3(ad426)* mutants do not recapitulate the high resistance of *eat-3(tm1107)* mutants when exposed to acute oxidative stress, as measured by 300 μM juglone **(Figure 2D)** and 360 μM juglone **(Figure 2E)**. Intriguingly, *eat-3(ad426)* had increased heat stress resistance, at levels similar to *eat-3(tm1107)* **(Figure 2F)**. This suggests that the GTPase domain of *eat-3* may be important for its resistance to heat stress but not to oxidative stress. For the rest of this chapter, we chose to pursue further analysis on *eat-3(tm1107)* mutants because they displayed a more general and marked increase in stress resistance than *eat-3(ad426)*.

Overall, we found that disruption of mitochondrial fission increases resistance to oxidative stress and disruption of mitochondrial fusion increases resistance to oxidative stress and heat stress.

1.3. Disruption of mitochondrial dynamics activates stress response pathways

We hypothesized that the increased stress resistance in the mitochondrial dynamics mutants could result from a hormetic upregulation of stress response genes provoked by their impaired

mitochondrial function (Machiela et al., 2020). To test whether stress response pathways were upregulated in response to disruptions of mitochondrial dynamics, we crossed the fission and fusion mutants to fluorescent reporters for stress response pathways and measured their fluorescence. The reporter constructs are composed of promoters of stress-responsive target genes (i.e. of genes downstream of stress-responsive transcription factors) fused to *gfp* or *rfp*. Fluorescence levels in a reporter are therefore a measure of the activity of its respective upstream stress-responsive transcription factor. We compared the fluorescence emitted throughout the body of worms under the *drp-1*, *eat-3*, and *fzo-1* mutant backgrounds **(Figure 3A-E)**. The reporters we used were *Phsp-6::GFP* which has been shown to probe for activation of the mitoUPR (Yoneda et al., 2004), *Pgst-4::GFP* for the SKN-1-mediated oxidative stress response (Link and Johnson, 2002), *Phsp-16.2::GFP* for the cytoUPR (Link et al., 1999), *Pnhr-57::GFP* for the HIF-1-mediated hypoxia response (Shen et al., 2006; Zou et al., 2019), and *Pmtl-1::RFP* for the DAF-16-mediated stress response (Zhang et al., 2013).

Increased oxidative stress resistance can require activation of the mitoUPR (Wu et al., 2018), the SKN-1-mediated oxidative stress response (Staab et al., 2014), or the DAF-16-mediated stress response (Lin et al., 2018; Dues et al., 2019). Increased heat stress resistance can require components of the mitoUPR (Wu et al., 2018), the cytoUPR (Zhou et al., 2018; Dues et al., 2019), the HIF-1-mediated hypoxia response (Carranza et al., 2020), or the DAF-16-mediated stress response (Lin et al., 2018; Dues et al., 2019). The *Phsp-16.2::GFP* reporter (for the cytoUPR) did not exhibit any fluorescence at basal conditions. We therefore induced the *Phsp-16.2::GFP* reporter with a mild 35°C heat stress.

Strikingly, all stress response pathways tested were highly activated in the mitochondrial fusion mutants *eat-3* and *fzo-1* **(Figure 3A-E)**, consistent with their high resistance to heat stress. The mitochondrial fission mutant *drp-1* exhibited increased activation of the SKN-1-mediated oxidative stress response **(Figure 3B)**, consistent with its high resistance to oxidative stress. Interestingly, the SKN-1-mediated oxidative response was more modestly increased in *drp-1* mutants than in the fusion mutants, which is also consistent with the more modest increase in oxidative stress resistance in *drp-1* mutants than in fusion mutants.

Overall, we found a strong correlation between activation of specific stress response pathways in mitochondrial fission and fusion mutants and their increased stress resistance.

1.4. Stress-responsive transcription factors are required for the increased stress resistance in mitochondrial fission and fusion mutants

In order to determine which stress response pathways, if any, are responsible for the increased stress resistance in the mitochondrial fission and fusion mutants, we performed RNA interference (RNAi) to knockdown the transcription factors known to mediate these stress response pathways in the mitochondrial fission and fusion mutants and exposed them to heat (37 °C) and oxidative (300 μM juglone) stress. ATFS-1 is responsible for activation of the mitoUPR (Nargund et al., 2012), SKN-1 for the SKN-1-mediated oxidative stress response (Inoue et al., 2005), HSF-1 for the cytoUPR (Hajdu-Cronin et al., 2004), HIF-1 for the hypoxia response (Hwang and Lee, 2011), and DAF-16 for the DAF-16-mediated stress response (Henderson and Johnson, 2001).

Knockdown of *atfs-1* decreased the high oxidative stress resistance of *drp-1*, *fzo-1*, and *eat-3* worms **(Figure 4)**. However, wild-type worms showed a trend towards a decreased oxidative stress resistance as well under *atfs-1* RNAi **(Figure 4)**. Two possible interpretations for this result are

that either the mitoUPR is required for the high oxidative stress resistance of mitochondrial fission and fusion mutants, or the mitoUPR is required for all strains' oxidative stress resistance. Since resistance in *eat-3* worms was more strongly reduced by *atfs-1* RNAi than in the other strains, the most conservative interpretation is that although all strains require the mitoUPR for survival under oxidative stress, ATFS-1 activity is more crucial for *eat-3's* particularly high oxidative stress resistance.

Knockdown of *skn-1* increased oxidative stress resistance in all strains, except in *eat-3* worms where oxidative stress resistance was decreased **(Figure 4)**. Wild-type worms being more resistant under inhibition of the SKN-1-mediated oxidative stress response was surprising, since *skn-1* has been shown to be required for resistance to juglone (Przybysz et al., 2009; Wu et al., 2016) and *skn-1* gain-of-function can increase resistance to juglone (Staab et al., 2014). This discrepancy may be due to our assay utilizing an acute dose of juglone, in which wild-type worms completely die within 5 hours, as opposed to assays performed previously (Przybysz et al., 2009; Staab et al., 2014; Wu et al., 2016).

Knockdown of *hsf-1* decreased oxidative stress resistance in all strains except *fzo-1* worms **(Figure 4)**. This suggests that the cytoUPR is normally required for oxidative stress resistance, but an alternative pathway independent of *hsf-1* is used in the *fzo-1* mutant background. Alternatively, variability in the stress assay in *fzo-1* worms may have prevented its trend towards decreased resistance from becoming significant.

Knockdown of *hif-1*, like knockdown of *daf-16,* decreased oxidative stress resistance in *fzo-1* worms, and in both cases there was a trend for suppression in *drp-1* worms **(Figure 4)**. This

suggests the hypoxia response and the DAF-16-mediated stress response is required for oxidative stress resistance in *drp-1* and *fzo-1* worms.

As for heat stress resistance, knockdown of *atfs-1* reduced resistance in *fzo-1* worms whereas *skn-1* RNAi increased it **(Figure 5)**. This suggests that the mitoUPR is required for the high heat stress of *fzo-1* worms. Knockdown of *hsf-1* reduced heat stress resistance in all strains except the heat-sensitive mutant *drp-1* **(Figure 5)**, indicating that the cytoUPR is generally required for resistance to heat.

All strains were much more resistant to the 37 °C stress under RNAi **(Figure 5,** approximately 70 % of wild-type worms survive after 10 hours**)**, which utilizes the HT115 strain, instead of under normal conditions **(Figure 2B,** no wild-type worms survive after 10 hours**)**, where we use the OP50 strain **(General methods)**. Under a milder heat stress (30 °C), *C. elegans* have increased resistance when fed HT115 bacteria, compared to OP50 (Revtovich et al., 2019). Our findings are therefore consistent with an HT115 diet conferring a higher heat stress resistance than an OP50 diet in *C. elegans*.

Overall, we found that the increased oxidative stress resistance in mitochondrial fission mutants required the mitoUPR and the hypoxia response, whereas it required the mitoUPR, the SKN-1-mediated oxidative stress response, the hypoxia response and the DAF-16-mediated stress response in mitochondrial fusion mutants. The increased heat stress resistance in mitochondrial fusion mutants required the mitoUPR.

1.5. DAF-16 is required for the increased stress resistance in mitochondrial fusion mutants

In **Figure 4**, *daf-16* RNAi seems to suppress the high oxidative stress resistance of all mitochondrial fission and fusion mutants. However, in the case of *drp-1* and *eat-3* mutants, this decrease does not reach significance. Although survivability decreased appreciably in these mutants, *daf-16* RNAi also showed considerable variability, thus reducing statistical power. In order to fully understand the contribution of *daf-16* to stress resistance under disrupted mitochondrial dynamics, we obtained the deletion mutant *daf-16(mu86)*, crossed it to our mitochondrial fission and fusion mutants, and tested oxidative and heat stress under this alternative *daf-16* loss-of-function background.

We found that *daf-16(mu86)* decreased oxidative stress resistance in wild-type and in fusion mutants, but not in *drp-1* mutants **(Figure 6)**. Taken together with the RNAi data in **Figure 4**, this suggests that *daf-16* is likely required for the high oxidative stress resistance in the mitochondrial fusion mutants.

We also found that *daf-16(mu86)* decreased the high heat stress of *eat-3* mutants **(Figure 6)**. This conflicts with our RNAi data in **Figure 5**, where no strain's heat stress resistance was affected by *daf-16* knockdown. A potential explanation is that HT115 bacteria conferred too much heat stress resistance to the worms during RNAi, preventing *daf-16* RNAi from decreasing their resistance by a detectable amount. It is therefore possible that *daf-16* is specifically required for the high heat stress resistance of *eat-3* mutants under OP50 bacteria but not HT115 bacteria. Overall, our results confirm that *daf-16* is required for the high stress resistance of mitochondrial fusion mutants.

1.6. Summary

We showed that disruption of mitochondrial fission increases oxidative stress resistance and that disruption of mitochondrial fusion increases both oxidative and heat stress resistance. Upon investigating the mechanisms for this increased stress resistance, we identified stress response pathways that were upregulated upon disruption of mitochondrial dynamics, most notably in the fusion mutants. Finally, we found that transcription factors responsible for the activation of these pathways are required for the increased stress resistance in mitochondrial fission and fusion mutants. Taken together, our data is consistent with our hypothesis on the mechanisms of hormesis whereby a mild disruption of mitochondrial function can lead to upregulation of stress response pathways which cause an increased resistance to stress.

Chapter 2. Results. Reactive oxygen species have a beneficial role in longevity

2.1. Background

In *C. elegans*, mild mitochondrial dysfunction has been shown to dramatically extend lifespan. For example, *nuo-6* and *isp-1* mutants, which are point mutations in a subunit of complex I and in the iron sulfur protein of complex III, respectively, have a nearly twofold increase in median and maximum lifespan (Yang and Hekimi, 2010b). Interestingly, these mutants also have increased mitochondrial superoxide (Yang and Hekimi, 2010a). Disrupting IIS through partial loss-of-function of *daf-2* results in a two- to three-fold increase in lifespan and *daf-2* mutant worms also have increased mitochondrial superoxide (Yang and Hekimi, 2010a).

Yang and Hekimi (2010a) have provided an important piece of evidence that elevated ROS are required for the longevity of *nuo-6*, *isp-1*, and *daf-2* mutants. They showed that supplementation of the antioxidant NAC at 10 mM fully suppressed the lifespan extension conferred by the *nuo-6* mutation, strongly decreased the lifespan of *isp-1* mutants, and modestly but significantly reduced the lifespan of *daf-2* mutants. Although NAC can act as a direct antioxidant, there are several alternative explanations that prevent a definitive conclusion as to whether ROS are required for the longevity of these mutants. Since NAC is toxic to wild-type worms at levels beyond 10 mM, it is conceivable that the long-lived mutants were simply more sensitive to this toxicity than wild-type animals at 10 mM. Furthermore, the overall effect of NAC on longevity is poorly understood, as the same laboratory has later found that combining 9 mM NAC with dimethyl sulfoxide (DMSO), a commonly used vehicle to increase cell permeability, can increase lifespan by 140 %

(Desjardins et al., 2017). NAC also promotes the synthesis of glutathione (Aldini et al., 2018), which further complicates the interpretation of its effects on longevity, as regulating glutathione levels can increase or decrease the lifespan of *C. elegans* depending on the developmental stage and dosage at which experiments are conducted (Urban et al., 2017). If NAC's antioxidant properties are nevertheless required for its effects on lifespan, it is unknown in which cellular compartment ROS are necessary to extend lifespan in the long-lived mutants, as NAC can scavenge ROS in the cytoplasm, mitochondria, and the extracellular matrix. It is also unknown which kinds of ROS are mediating the increase in lifespan suppressed by NAC, since NAC can have antioxidant effects on several ROS, including superoxide (Aruoma et al., 1989; Benrahmoune et al., 2000).

We sought to verify whether elevations in ROS are required for the extended lifespan of *nuo-6*, *isp-1*, and *daf-2* mutants. Furthermore, we hypothesized that the increase in mitochondrial ROS, as opposed to cytoplasmic or extracellular ROS, is specifically required for the longevity of these mutants. To eliminate confounds related to applications of a drug, such as dose toxicity and choice of vehicle, we targeted an endogenous antioxidant. We therefore used transgenic worms over-expressing the mitochondrial *sod-2* antioxidant gene under its endogenous promoter (*Psod-2::sod-2::GFP*) to determine whether elevated mitochondrial superoxide is required for the longevity of *nuo-6*, *isp-1*, and *daf-2* mutants.

2.2. Reducing levels of mitochondrial ROS decreases the longevity of *nuo-6* but not *isp-1* mutants

To determine whether elevated mitochondrial ROS are required for the longevity of worms with mutations resulting in disrupted mitochondrial function (*nuo-6* and *isp-1*), we crossed these

mutants to *Psod-2::sod-2::GFP* transgenic worms, which over-express the antioxidant gene *sod-2* under the control of its endogenous promoter. We have confirmed that *Psod-2::sod-2::GFP* induces GFP expression ubiquitously in the worm, suggesting that the transgenic SOD-2::GFP protein is localizing correctly, and *Psod-2::sod-2::GFP* also reverts the long lifespan of *sod-2* mutants back to wild-type (Senchuk and Van Raamsdonk, unpublished data), demonstrating that the transgenic SOD-2 functions correctly. In other words, in a background of elevated mitochondrial ROS causing extended lifespan, such as the *sod-2* mutant background, *Psod-2::sod-2::GFP* can inhibit this increase in ROS and prevent the extension in lifespan.

The increase in mitochondrial ROS present in *nuo-6* mutants (Yang and Hekimi, 2010a) is required for their full longevity, as *sod-2* over-expression significantly decreases their lifespan **(Figure 7A)**. Interestingly, the lifespan of *nuo-6; Psod-2::sod-2::GFP* double mutants does not completely revert to wild-type lifespan. This may suggest that the transgene is not expressed at sufficiently high levels to scavenge the highly elevated mitochondrial ROS in *nuo-6* mutants. We chose to drive the transgene's expression with its endogenous promoter, *Psod-2,* to only increase *sod-2* expression by twofold at most. Increasing a protein's expression far beyond physiological levels could result in toxicity and complicate the interpretation of our lifespan experiments. This moderate increase in *sod-2* expression was sufficient to uncover the importance of mitochondrial ROS in the longevity of *nuo-6* mutants, while not altering the lifespan of wild-type worms **(Figure 7)**. An alternative explanation for the incomplete suppression of *nuo-6* mutants' lifespan by reduction of mitochondrial ROS is that *nuo-6*'s lifespan also depends on other factors unrelated to mitochondrial ROS.

In contrast, inhibition of mitochondrial ROS in *isp-1* mutants did not affect their lifespan **(Figure 7B)**. This suggests that mitochondrial ROS do not mediate the increased lifespan of *isp-1* mutants. The most simple conservative reconciliation between our findings and those of Yang and Hekimi (2010a), where they observed a reduction of *isp-1* mutants' lifespan when exposed to NAC, is that cytoplasmic or extracellular but not mitochondrial ROS are important in the mechanisms of lifespan extension in *isp-1* worms (but see **Discussion** for additional interpretations).

Overall, we found that mitochondrial ROS are required for the full lifespan extension conferred by the mild impairment of mitochondrial function in *nuo-6* mutants but not *isp-1* mutants.

2.3. Reduction of mitochondrial ROS decreases the longevity of *daf-2* mutants

To determine whether elevated mitochondrial superoxide is required for the longevity of *daf-2* worms, which have impaired IIS, we also crossed these mutants to *Psod-2::sod-2::GFP* and measured their lifespan. We found that inhibition of mitochondrial ROS significantly decreased the lifespan of *daf-2* worms **(Figure 8)** to a similar extent as administering the pluripotent antioxidant NAC (Yang and Hekimi, 2010a). Inhibition of mitochondrial superoxide can fully recapitulate the effect of NAC on *daf-2* mutants, suggesting that, among all ROS, mitochondrial superoxide is likely the main ROS driving lifespan extension in *daf-2* mutants. Because *daf-2* lifespan is only modestly decreased by *Psod-2::sod-2::GFP*, there are other mechanisms unrelated to ROS which are required for its full longevity.

2.4. Summary

We showed that mitochondrial ROS do not prevent longevity but can rather promote it. Indeed, we found that mitochondrial superoxide is required for the longevity of *nuo-6* and *daf-2* mutants, while having no effect on *isp-1* mutants. Although *nuo-6* and *isp-1* both disrupt the electron

transport chain, their hormetic mechanisms of longevity have a differential requirement for ROS, suggesting that hormesis can be stimulated by ROS as well as by other signals.

Chapter 3. Results. TBC-2 is a key mediator of longevity through its interaction with DAF-16

3.1. Background

We and others have shown that increased ROS levels are required for the full longevity of some long-lived mitochondrial mutants **(Figure 7)** (Lee et al., 2010; Yang and Hekimi, 2010a; Wei and Kenyon, 2016) and of *daf-2* mutants **(Figure 8)** (Yang and Hekimi, 2010a; Zarse et al., 2012). Although the mechanisms through which ROS prolong lifespan are unclear in these mutants, DAF-16 is required for lifespan extension in several mitochondrial mutants, including *nuo-6* and *isp-1*, and in *daf-2* mutants (Senchuk et al., 2018). Interestingly, DAF-16 activation through its nuclear localization can be stimulated by ROS (Essers et al., 2005; Senchuk et al., 2018) and DAF-16 is required for the longevity of *sod-2* mutants (Senchuk et al., 2018). Nevertheless, ROS are likely not sufficient for maximal activation of DAF-16 (Senchuk et al., 2018) and our experiments in **Chapter 2** suggest that ROS only mediate part of the lifespan extension of *nuo-6* and *daf-2* mutants. Overall, the factors controlling the nuclear localization of DAF-16 in these mutants is not fully understood.

In this chapter we examined the role of endosomal trafficking in the longevity of *nuo-6*, *isp-1*, and *daf-2* mutants in light of the recent discovery that TBC-2, a RAB-5 and RAB-7 inhibitor, is required for strong nuclear localization of DAF-16 (Meraş, 2018). We investigated whether TBC-2 is a required component of DAF-16's pro-longevity signaling.

Endosomal trafficking refers to the process wherein specific proteins are endocytosed and targeted to the lysosome for degradation. In the active GTP-bound state, the RAB-5 and RAB-7 GTPases

localize to early and late endosomes, respectively, in order to confer endosomal identity and ensure trafficking from the endosome to the lysosome (Stenmark, 2009). In the inactive GDP-bound state, RAB proteins are sequestered to the cytoplasm (Vázquez-Martínez and Malagón, 2011). In *C. elegans*, TBC-2 has been identified as a GTPase-activating protein (GAP) for RAB-5, which means TBC-2 inhibits RAB-5 and may therefore promote the conversion of early to late endosomes by binding to late RAB-7-positive endosomes and inactivating RAB-5 (Chotard et al., 2010; Law and Rocheleau, 2017). Indeed, Chotard et al. (2010) found highly enlarged RAB-7-positive late endosomes in *tbc-2(tm2241)* and *tbc-2(sv41)* loss-of-function mutant worms. They showed that this was due to increased RAB-5 activity, as constitutively active RAB-5 produced the same phenotype. Importantly, they found that intestinal over-expression of wild-type TBC-2 is sufficient to rescue the endosomal phenotype of *tbc-2(tm2241)* mutant worms.

Meraş (2018) later showed that DAF-16 can be found in RAB-5 and RAB-7-positive endosomes. In a *tbc-2(tm2241)* mutant background, there is an increase in endosomal DAF-16 and this is associated with less DAF-16 in the nearby nucleus when compared to wild-type worms. Partial loss-of-function mutations of the *daf-2* receptor promote nuclear localization of DAF-16 and its activation, allowing it to upregulate pro-longevity target genes (Mukhopadhyay et al., 2006). Consistent with the findings in wild-type worms, *daf-2* mutant worms have less endosomal DAF-16 and more nuclear DAF-16, suggesting an opposition between endosomal and nuclear DAF-16. This opposition is most obvious in *tbc-2(tm2241); daf-2* double mutants, where an increase in endosomal DAF-16 is paired with a decrease in nuclear DAF-16, compared to *daf-2* single mutants (Meraş, 2018).

Because *daf-2* mutants have a dramatically extended lifespan which depends on activation of DAF-16 (Murakami and Johnson, 1996; Honda and Honda, 1999), we hypothesized that impairment in DAF-16 nuclear localization caused by loss of *tbc-2* could oppose this lifespan extension.

3.2. Loss of *tbc-2* decreases the long lifespan of *daf-2* mutants

To verify whether a reduction in DAF-16 nuclear localization resulting from *tbc-2* loss-of-function (Meraş, 2018) also reduces the lifespan extension resulting from DAF-16 activation, we obtained *tbc-2* mutant worms and crossed them to *daf-2* mutants. We used the *tbc-2(tm2241)* and *tbc-2(sv41)* alleles, which are both loss-of-function deletion mutants of the *tbc-2* gene, causing similar defects in endosomal trafficking (Chotard et al., 2010). The *tbc-2(tm2241)* deletion removes the third intron of *tbc-2*, resulting in a frame shift and an early stop codon. The *tbc-2(sv41)* deletion removes the majority of the upstream gene ZK1248.11 and the 5' region of *tbc-2* **(Figure 9A)** (Chotard et al., 2010). We found that *tbc-2(tm2241)* significantly decreased the long lifespan of *daf-2* worms **(Figure 9B)**, whereas *tbc-2(sv41)* showed a trend towards decreasing it **(Figure 9C)**. Because this trend was very close to statistical significance ($p = 0.0548$, $n = 139$ worms), a slightly larger number of *tbc-2(sv41); daf-2* worms, resulting in increased statistical power, would have likely made the difference in lifespan between *daf-2* and *tbc-2(sv41); daf-2* worms statistically significant. Nevertheless, a similar number of worms was used to measure the lifespan of *tbc-2(tm2241); daf-2* mutants ($p < 0.0001$, $n = 141$), suggesting there is a milder reduction in *tbc-2* function resulting from the *sv41* deletion than from *tm2241*.

Our data suggests that the impaired DAF-16 nuclear localization resulting from loss of *tbc-2* function prevents the full lifespan extension of *daf-2* mutants. Although both *tbc-2(tm2241)* and *tbc-2(sv41)* mutations produce a similar endosomal phenotype (Chotard et al., 2010), *tbc-2(sv41)*

does not affect *daf-2* longevity as strongly as *tbc-2(tm2241)*, which suggests the *tbc-2(sv41)* mutation may not have as great an impact on DAF-16 nuclear localization. Indeed, impaired DAF-16 nuclear localization has only been previously documented in the *tbc-2(tm2241)* background (Meraş, 2018).

3.3. Intestinal over-expression of *tbc-2* is not sufficient to rescue *daf-2* lifespan

Since the endosomal phenotype of *tbc-2(tm2241)* mutants could be rescued by intestinal over-expression of functional *tbc-2* (Chotard et al., 2010), we hypothesized that intestinal over-expression of *tbc-2* could also restore the long lifespan of *daf-2* in *tbc-2(tm2241); daf-2* mutants. We first tested whether the intestinal *tbc-2* over-expression transgene (*Pvha-6::GFP::tbc-2*) is tolerated well in *daf-2* mutants. We found mild toxicity resulting from the interaction between the transgene and *daf-2*, as *daf-2; Pvha-6::GFP::tbc-2* worms live slightly shorter than *daf-2* mutants **(Figure 10A)**. It is unlikely that the transgene is inherently toxic, as it does not alter lifespan in a *tbc-2(tm2241)* mutant background. The two most plausible explanations are that either increased levels of TBC-2 protein interfere with DAF-2 or downstream pro-longevity effectors such as DAF-16, or, alternatively, that the *Pvha-6::GFP::tbc-2* transgene itself interferes with the DNA of *daf-2* effectors. Surprisingly, over-expression of *tbc-2* in the intestine failed to rescue *daf-2* lifespan in *tbc-2(tm2241); daf-2* mutants **(Figure 10B)**.

Overall, this suggests that the endosomal effect of *tbc-2(tm2241)* mutants is distinct from its inhibitory effect on *daf-2* pro-longevity signaling. Although intestinal over-expression of *tbc-2* can rescue the endosomal phenotype of *tbc-2(tm2241)* mutants (Chotard et al., 2010), it cannot rescue the decreased lifespan of *daf-2* mutants in a *tbc-2(tm2241)* background **(Figure 10B)**, suggesting the two pathways are separate.

3.4. Loss of *tbc-2* decreases the long lifespan of mitochondrial mutants

As our findings suggest that the interaction between TBC-2 and DAF-16 nuclear localization (Meraş, 2018) is important for the DAF-16-dependent longevity of *daf-2* mutants, we asked whether other long-lived mutants relying on DAF-16 also require TBC-2 for their extended lifespan. The long-lived mitochondrial mutants *nuo-6* and *isp-1* do not extend lifespan when *daf-16* is knocked down (Senchuk et al., 2018). We therefore crossed *nuo-6* and *isp-1* mutants to *tbc-2(tm2241)* and measured their lifespans.

We found that loss of *tbc-2* function decreases the lifespan of *nuo-6* **(Figure 11A)** and *isp-1* mutants **(Figure 11B)**. Lifespan was similarly decreased in the mitochondrial mutants and in *daf-2* mutants **(Figure 9B).** This is consistent with *tbc-2* loss disrupting a common pathway in all three mutants, such as activation of DAF-16.

3.5. Summary

Overall, our results suggest that TBC-2 has a key role in promoting longevity in the context of hormesis. Indeed, loss of TBC-2 decreased the lifespan of long-lived mutants which require DAF-16, such as *daf-2*, *nuo-6*, and *isp-1*, likely because TBC-2 promotes nuclear localization of DAF-16 (Meraş, 2018). Although intestinal rescue of TBC-2 is sufficient to rescue endosomal defects in a *tbc-2* loss-of-function background (Chotard et al., 2010), the intestinal rescue is not sufficient to restore the longevity of *daf-2* mutants, suggesting other tissues are mediating the longevity effects of TBC-2.

Discussion

The concept of hormesis addresses how mildly stressed organisms can resist better to subsequent stresses and aging. We hypothesized that the mechanism by which mitochondrial hormesis can promote survival is through the upregulation and activation of stress response genes **(Figure 1)**. This thesis exposes several instances where mutations provoking mild stress and potential harm also upregulate protective genes, thus resulting in increased stress resistance or extended lifespan. In doing so we found a beneficial role of mitochondrial superoxide on longevity. We also characterized the importance of the activity of the stress response gene DAF-16 on longevity by showing that inhibiting DAF-16 nuclear localization through loss of TBC-2 function is detrimental to longevity.

I. Disruption of mitochondrial dynamics increases stress resistance in adults

In **Chapter 1**, we showed that although disruption of mitochondrial dynamics impairs mitochondrial function, it can result in increased oxidative stress resistance **(Figure 2)**. The mitochondrial fusion mutant *eat-3* had been previously reported to be sensitive to oxidative stress (Kanazawa et al., 2008). An important difference between the experiments by Kanazawa et al. (2008) and ours is that they exposed worms to paraquat instead of juglone and they started exposure at the early L1 stage, whereas we exposed worms at the young adult stage **(General Methods)**. We found that *eat-3* worms are also more resistant to paraquat (Machiela et al., 2020), which suggests the key difference is the developmental timing of the exposure to oxidative stress. Taken together, our findings suggest that the mild stress imposed by disruption of mitochondrial fusion is detrimental during development, but the resulting hormetic increase in stress response

genes confers increased resistance to subsequent stresses during adulthood. This is consistent with hormesis having a temporal component **(Figure 1)**.

II. Knockdown of *skn-1* increases resistance to highly acute oxidative stress

It was particularly surprising that knockdown of *skn-1* increased resistance to acute oxidative stress in wild-type worms. SKN-1 is considered a detoxifying protein that protects against oxidative stress and aging (Przybysz et al., 2009; Staab et al., 2014; Blackwell et al., 2015; Wu et al., 2016). Upon a closer examination of the literature, however, we noticed that others have not tested the effect of loss of SKN-1 function on resistance to highly acute juglone exposure, i.e. one that results in certain death after 5 hours. Decreased resistance to juglone under *skn-1* RNAi has only been reported in the cases where worms are pre-conditioned with a very mild dose (38 μM) of juglone hours before a more toxic dose is administered (Przybysz et al., 2009; Wu et al., 2016), or where juglone exposure is not very acute, such that there are wild-type worms surviving past 10 hours of exposure (Staab et al., 2014; Wu et al., 2016). Therefore, it seems like disruption of SKN-1 only impairs oxidative stress resistance when there is sufficient time between a mild dose of stress and a lethal one, or during long-term exposure to juglone. Interestingly, accumulation of SKN-1::GFP in the head increases throughout hours of exposure to juglone (Wu et al., 2016), which may suggest that SKN-1 requires some hours before its downstream targets are sufficiently activated. Wu et al. (2016) have also suggested that SKN-1 might not play a large role in resistance to an acute lethal dose of juglone, as they found contradictory results when testing stress resistance under *skn-1* RNAi in worms that were not pre-conditioned with a milder dose of juglone. Nevertheless, gain-of-function mutations in *skn-1* definitely increase resistance to juglone at mild and highly acute doses (Staab et al., 2014; Wu et al., 2016). Overall, we suggest that SKN-1 is sufficient but not necessary to protect from sudden acute oxidative stress. If worms are exposed to mild oxidative

stress and enough time is permitted for activation of stress response genes, then SKN-1 becomes necessary for this hormetic resistance to subsequent acute oxidative stress.

III. Reactive oxygen species have a beneficial role in the longevity of *nuo-6* and *daf-2* mutants

Our results in **Chapter 2** suggest that elevated mitochondrial ROS promote longevity in *nuo-6* and *daf-2* mutants but not in *isp-1* mutants. Interestingly, even though the Free Radical Theory of Aging suggests that detoxifying the high ROS present in these mutants (Yang and Hekimi, 2010a) should be beneficial to their longevity, we found that none of them benefited from over-expression of the mitochondrial antioxidant enzyme *sod-2* and that this rather decreased lifespan in *nuo-6* and *daf-2*. This provides yet another piece of evidence against the Free Radical Theory of Aging.

We found a milder effect from *sod-2* over-expression on the lifespan of *nuo-6* and *isp-1* mutants than Yang and Hekimi (2010a) did with NAC. The most conservative explanation is that mitochondrial superoxide contributes only partly to the lifespan extension of *nuo-6* mutants, whereas it does not in *isp-1* mutants. This would mean that other types of ROS which are inhibited by NAC are required for the rest of *nuo-6* mutants' lifespan and for a portion of *isp-1* mutants' lifespan.

There are however other possible explanations. The effect of *sod-2* over-expression on mitochondrial ROS could simply be weaker than that of NAC. This could explain why *sod-2* over-expression moderately decreased the lifespan of *nuo-6* mutants **(Figure 7A)** while NAC fully suppressed it (Yang and Hekimi, 2010a) and why we found no effect of *sod-2* over-expression on *isp-1* mutants' lifespan **(Figure 7B)** while NAC only moderately decreased it. The consistency of this interpretation relies on mitochondrial ROS being required for *nuo-6* and *isp-1* mutants'

longevity. However, such an interpretation is unlikely because even though NAC only causes a small reduction in *daf-2* mutants' lifespan, we were able to detect a similar reduction from *sod-2* over-expression in *daf-2* mutants **(Figure 8)**, suggesting that even if inhibition of mitochondrial ROS were responsible for a small component of *isp-1*'s lifespan, our experiment would have been sensitive enough to detect it.

An alternative interpretation is that NAC has differing toxicity under different mutant backgrounds. Indeed, *daf-2* and *isp-1* mutants have a more general resistance to stresses (Dues et al., 2017; Dues et al., 2019) than *nuo-6* mutants (Soo and Van Raamsdonk, unpublished data), which might explain why *daf-2* and *isp-1* mutants were more resistant to NAC than *nuo-6* mutants. It is unclear, however, why these long-lived mutants would have lower resistance to NAC than wild-type worms, which did not exhibit a decreased lifespan at this same dose of NAC (Yang and Hekimi, 2010a).

IV. Over-expression of *sod-2* did not affect lifespan in a wild-type background

Our *Psod-2::sod-2::GFP* transgene had no effect on lifespan in a wild-type background. It has previously been reported that similar transgenes over-expressing *sod-2* under its endogenous *Psod-2* promoter could instead increase lifespan (Cabreiro et al., 2011). This finding by Cabreiro et al. (2011) is peculiar as, in their case, over-expression of *sod-2* behaved in a manner consistent with loss-of-function of *sod-2*: it decreased resistance to oxidative stress and increased lifespan in a DAF-16-dependent manner (Van Raamsdonk and Hekimi, 2009; Senchuk et al., 2018). It is unclear how both over-expression and loss of *sod-2* could produce the same phenotype. The *Psod-2::sod-2::GFP* transgene we used is unlikely to behave as a *sod-2* loss-of-function, as in such a case it would have been predicted to suppress the lifespan of *isp-1* mutants (Van

Raamsdonk and Hekimi, 2009), which did not occur **(Figure 7B)**. Furthermore *Psod-2::sod-2::GFP* could restore long-lived *sod-2* mutants' lifespan to wild-type levels (Senchuk and Van Raamsdonk, unpublished data). Nevertheless, it is possible that the GFP fusion, which is present in our transgene but not in the ones used by Cabreiro et al. (2011), interfered with its ability to extend lifespan.

Alternatively, the effects of the transgenes might differ based on the genetic strategy used. Our transgene is an integrated transgenic strain with a single transgene, generated using MosSCI, whereas the transgenes by Cabreiro et al. (2011) were in one case generated by injection of an extrachromosomal array, and in other lines the array was subsequently integrated by X-ray irradiation. The transgenes used in Cabreiro et al. (2011) may therefore have high expression beyond physiological levels or too many integration loci, which confounds the interpretation further.

V. *nuo-6* and *isp-1* mutations may extend lifespan through initially distinct mechanisms

Although *nuo-6* and *isp-1* mutants both cause a mild impairment of the electron transport chain which results in a dramatically long lifespan, it is unclear whether they share the same mechanisms of lifespan extension. *nuo-6; isp-1* double mutants do not live longer than either single mutant, suggesting they share a common mechanism (Yang and Hekimi, 2010b). Indeed, the lifespans of *nuo-6* and *isp-1* are decreased when crossed to *tbc-2(tm2241)* **(Figure 11)**, i.e. when DAF-16 nuclear localization is impaired, and are completely suppressed by *daf-16* RNAi . This suggests that their mechanisms of longevity converge onto DAF-16. Both *nuo-6* and *isp-1* mutants have increased levels of SOD-2, and increasing mitochondrial ROS through *sod-2* RNAi or through

paraquat produces a minimal or negligible increase in lifespan in these mutants (Yang et al., 2007; Yang and Hekimi, 2010a, b). In contrast, increasing mitochondrial ROS can strongly extend the lifespan of wild-type worms and of other long-lived mutants (Yang et al., 2007; Van Raamsdonk and Hekimi, 2009; Yang and Hekimi, 2010a; Schaar et al., 2015). This implies that the pro-longevity effect of mitochondrial ROS could be saturated in *nuo-6* and *isp-1* mutants. However, we showed here that mitochondrial ROS are only required for the full longevity of *nuo-6* mutants but not of *isp-1* mutants **(Figure 7)**. Further work will confirm whether ROS was similarly decreased in both *nuo-6* and *isp-1* mutants under the presence of *Psod-2::sod-2::GFP*, as there is a possibility that the transgene is working more efficiently in *nuo-6* mutants than in the *isp-1* mutant background. Together, our data suggest that *nuo-6* and *isp-1* mutations prolong lifespan through independent upstream mechanisms, where mitochondrial ROS are important in *nuo-6* but not *isp-1*, but through the same downstream mechanism, where both mutants require the intact activity of DAF-16 **(Figure 12)**.

VI. Loss of *tbc-2* decreases lifespan in *daf-2*, *nuo-6*, and *isp-1* mutants, potentially because of decreased DAF-16 activity

Although we showed that loss of *tbc-2* decreases the lifespan of *daf-2* **(Figure 9)**, *nuo-6*, and *isp-1* mutants **(Figure 11)**, it is not yet confirmed whether this is due to the resulting decrease in DAF-16 nuclear localization which has only been observed in wild-type and *daf-2* mutants (Meraş, 2018). The reason we postulate that the interaction between TBC-2 and DAF-16 is important for lifespan extension in these long-lived mutants is because, unlike inhibition of ROS **(Figures 7 and 8)**, the *tbc-2(tm2241)* mutation similarly affected all three long-lived mutants **(Figures 9 and 11)**, suggesting a common pathway **(Figure 12)** where DAF-16 is a prime candidate, as it is required

for lifespan extension in all three mutants (Senchuk et al., 2018). At the very least, we can state that inhibition of lifespan, mediated by the *tbc-2(tm2241)* mutation, in these long-lived mutants is likely unrelated to mitochondrial superoxide. Further experiments will be required in *nuo-6* and *isp-1* mutants under the *tbc-2(tm2241)* background to verify whether nuclear localization of DAF-16 is reduced. Importantly, further experiments could also verify whether the expression of DAF-16 target genes are reduced in response to its decreased nuclear localization.

We found that both *tbc-2(tm2241)* **(Figure 9B)** and *tbc-2(sv41)* deletions **(Figure 9C)** decreased the lifespan of *daf-2* mutants, albeit the reduction seemed milder in the *tbc-2(sv41)* background. Both deletion mutants are suspected to be null or strong loss-of-function according to Chotard et al. (2010), and the authors did not note any difference between each of the two mutants, as well as from *tbc-2* RNAi, on their mutant endosomal phenotype. Furthermore, Chotard et al. (2010) showed that the residue responsible for this mutant endosomal phenotype is in the TBC domain, which spans exons 9 and 10 of *tbc-2*, far downstream from each of the *tm2241* and *sv41* deletions. This suggests that *tbc-2(2241)* and *tbc-2(sv41)* are null mutants, as their TBC domain is not functional despite this domain being remote from the deleted fragments. Nevertheless, we cannot exclude the possibility of *tbc-2(sv41)* being a loss-of-function mutant with preserved function past exon 3, as it caused a milder reduction in the lifespan of *daf-2* mutants than *tbc-2(tm2241)*. In such a case, the full TBC-2 protein would be required for proper function of its TBC domain and therefore for normal endosomal trafficking, whereas a segment of *tbc-2* (among exons 3 to 12) would be required for TBC-2's regulation of DAF-16 localization. This would mean that DAF-16 nuclear localization would be relatively spared in *tbc-2(sv41)*, explaining the milder reduction in *tbc-2(sv41); daf-2* mutants' lifespan relative to *daf-2*. It is indeed not known whether there is a decrease in DAF-16 nuclear localization in *tbc-2(sv41)* mutants. Because the *tbc-2(sv41)* allele

also impairs the neighboring gene ZK1248.11 **(Figure 9A)**, further genetic experiments will be required to elucidate the function of TBC-2's domains.

VII. Hormetic increases in stress resistance and lifespan require the activation of stress response genes

Our hypothesis which posited the important, causative role of stress response genes in mediating increased stress resistance and lifespan during hormesis has been confirmed by our experiments. Indeed, in all of our tested models of hormesis, a mild stressor (disrupting of mitochondrial dynamics, increasing ROS, or disrupting IIS) increased stress resistance and lifespan by activating stress response genes **(Figure 12)**. We showed that the increased stress resistance in *drp-1*, *eat-3*, and *fzo-1* mutants required stress response transcription factors such as ATFS-1, HIF-1, and DAF-16. We found that ROS were required for the longevity of *nuo-6* and *daf-2* mutants, as inhibiting superoxide by SOD-2 over-expression decreased their lifespan. Finally, we showed that impairing TBC-2 decreases the lifespan in *nuo-6*, *isp-1*, and *daf-2*, likely because of its role in promoting the nuclear localization of the stress response transcription factor DAF-16.

VIII. Mitochondrial dysfunction is a tool to understand the mechanisms of increased stress resistance and lifespan but not a treatment endpoint

It should be noted that while disrupting mitochondrial dynamics can increase resistance to oxidative or heat stress, these animals are sensitive to other stresses such as osmotic and anoxic stress (Machiela et al., 2020). Furthermore, disruption of mitochondrial dynamics results in impaired reproductive health and a slower growth rate (Kanazawa et al., 2008; Machiela et al., 2020), as well as impaired movement in some of the mutants (Machiela et al., 2020). Mitochondrial mutants *nuo-6* and *isp-1*, despite living long **(Figures 7 and 11)**, also suffer from similar

phenotypes, such as disrupted reproductive health and slow growth rate (Yang and Hekimi, 2010b). Both *nuo-6* and *isp-1* mutants have increased resistance to several stresses, but *nuo-6* mutants have increased sensitivity to anoxia (Soo and Van Raamsdonk, unpublished data) and *isp-1* mutants have a higher incidence of developmental impairments under oxidative stress (Dues et al., 2017).

This highlights the importance of elucidating the mechanisms behind activation of stress response genes to further our understanding of the biology of aging and to provide a viable and specific therapeutic target for age onset diseases.

Conclusion

We demonstrated that mild stresses, such as disruptions of mitochondrial dynamics, increased ROS, or disruption of IIS can increase stress resistance or increase lifespan through activation of stress response genes. Our findings confirmed our hypothesis that a hormetic increase in stress resistance or lifespan is mediated by increased activation of stress response genes. We suggest that the stress response transcription factor DAF-16, often required for stress resistance and longevity, can be regulated by TBC-2 and we show that their interaction also modulates the aging of long-lived mutants.

Overall, our work highlights the importance of stress response genes because of their beneficial effects on the organism. We defined specific stress response pathways which we found to be protective in the context of hormesis and stresses generated by mutations. As we have found that the harm conferred by genetic mutations can be counteracted through upregulation of stress response genes, our research may represent a novel therapeutic avenue for treatment of diseases. We hope that targeting beneficial stress response genes can show efficiency in increasing resistance to diseases, from genetic disorders to environmental challenges, and can be considered in the treatment of diseases and complications caused or exacerbated by the aging process.

Contributions to the advancement of knowledge

We have developed the understanding of the mechanisms of stress resistance and longevity through the concept of hormesis. Our central hypothesis, that a mild stress can increase stress resistance or lifespan through upregulation of stress response genes, was predictive in elucidating the mechanisms of increased stress resistance conferred by disruption of mitochondrial dynamics. We also enriched the knowledge for pathways of longevity by finding that mitochondrial superoxide is required for lifespan extension in the mitochondrial mutant *nuo-6* but not *isp-1*, suggesting they do not promote longevity through the same initial mechanism, and in the impaired IIS mutant *daf-2*. We showed that TBC-2 is required for the long lifespan of *nuo-6*, *isp-1*, and *daf-2* mutants, suggesting that its role in DAF-16's nuclear localization is crucial for longevity.

Overall, this work advanced our understanding of the aging process. Our work disputes the traditional yet ingrained Free Radical Theory of Aging by showing that even endogenous antioxidants can be harmful and exacerbate aging.

We believe that the knowledge gained will be useful to promote healthy aging and to develop novel generally applicable treatments for age onset diseases.

General methods

Strains

N2/wild-type

drp-1(tm1108)

fzo-1(tm1133)

eat-3(tm1107)

eat-3(ad426); him-8(e1489)

zcIs13[Phsp-6::GFP]

zcIs13[Phsp-6::GFP]; *drp-1(tm1108)*

zcIs13[Phsp-6::GFP]; *fzo-1(tm1133)*

zcIs13[Phsp-6::GFP]; *eat-3(tm1107)*

dvIs70[Phsp-16.2::GFP,rol-6(su1006)]

dvIs70[Phsp-16.2::GFP,rol-6(su1006)]; *drp-1(tm1108)*

dvIs70[Phsp-16.2::GFP,rol-6(su1006)]; *fzo-1(tm1133)*

dvIs70[Phsp-16.2::GFP,rol-6(su1006)]; *eat-3(tm1107)*

dvIs19[Pgst-4::GFP::NLS]

dvIs19[Pgst-4::GFP::NLS]; *drp-1(tm1108)*

dvIs19[Pgst-4::GFP::NLS]; *fzo-1(tm1133)*

dvIs19[Pgst-4::GFP::NLS]; *eat-3(tm1107)*

iaIs7[Pnhr-57::GFP]

iaIs7[Pnhr-57::GFP]; *drp-1(tm1108)*

iaIs7[Pnhr-57::GFP]; *fzo-1(tm1133)*

iaIs7[Pnhr-57::GFP]; *eat-3(tm1107)*

muEx336[Pmtl-1::RFP + rol-6(su1006)]

muEx336[Pmtl-1::RFP + rol-6(su1006)]; *drp-1(tm1108)*

muEx336[Pmtl-1::RFP + rol-6(su1006)]; *fzo-1(tm1133)*

muEx336[Pmtl-1::RFP + rol-6(su1006)]; *eat-3(tm1107)*

daf-16(mu86)

daf-16(mu86); *drp-1(tm1108)*

daf-16(mu86); *fzo-1(tm1133)*

daf-16(mu86); *eat-3(tm1107)*

nuo-6(qm200)

isp-1(qm150)

daf-2(e1370)

*jerIs003[pSod-2::SOD-2 genomic::eGFP::let-858utr + unc-119(+) (pJVR003)]**

jerIs003[pSod-2::SOD-2 genomic::eGFP::let-858utr + unc-119(+) (pJVR003)]; nuo-6(qm200)

jerIs003[pSod-2::SOD-2 genomic::eGFP::let-858utr + unc-119(+) (pJVR003)]; isp-1(qm150)

jerIs003[pSod-2::SOD-2 genomic::eGFP::let-858utr + unc-119(+) (pJVR003)]; daf-2(e1370)

tbc-2(tm2241)

tbc-2(tm2241); nuo-6(qm200)

tbc-2(tm2241); isp-1(qm150)

tbc-2(tm2241); daf-2(e1370)

tbc-2(sv41)

tbc-2(sv41); daf-2(e1370)

*: integrated transgenic strain with a single transgene, generated using Mos1-mediated Single Copy Insertion (MosSCI)

Nematode Growth Medium

Worms were grown, maintained, crossed, and assayed (except lifespan and assays involving RNAi) on petri dishes containing solid Nematode Growth Medium (NGM) seeded with bacteria for dietary consumption. NGM consists in H_2O (solvent), NaCl (1 g/L), peptone (2.5 g/L), agar (17 g/L), cholesterol (5 mg/L), $MgSO_4$ (1 mM), $CaCl_2$ (1 mM), and KPO_4 buffer (25 mM) (Brenner, 1974). The bacterial strain used is OP50, a strain of *E. coli*.

All experiments were performed at 20 °C.

Crosses and genotyping

To obtain males for crosses, L4 hermaphrodites were heat shocked at 30 °C for 6 hours to increase the likelihood of them producing male offspring. These male offspring (F1) were crossed to hermaphrodites of the same genetic background in order to have approximately 50% males in the resulting progeny (F2).

Crosses were conducted by placing around 5 L4 hermaphrodites of one genetic background (ex: *fzo-1* mutants) with 2 to 3 times more males of the other genetic background (ex: *daf-16* mutants) in a plate seeded with a small bacterial lawn, to promote proximity among worms. A high incidence of males confirms that mating was successful. The F1 heterozygous progeny (ex: *fzo-1/+; daf-16/+*) were isolated in different plates to allow them to self-fertilize. Several F2 progeny were each singled in their respective plate for genetic screening of the desired background (ex: *fzo-1; daf-16*).

Lysis was performed to isolate the DNA of a worm (template DNA). Briefly, each worm is placed in 15 µl lysis buffer, which consists in 13.2 µl H_2O (solvent), 1.5 µl PCR buffer [TRIS-HCl (10 mM), KCl (50 mM), $MgCl_2$ (1.5 mM)], and 0.2 µl proteinase K (from 20 mg/ml stock solution). With a thermal cycler, worms are exposed to 65 °C for 1 h to produce lysate and 95 °C for 15 minutes to inactivate proteinase K.

The genotype of deletion mutants was confirmed by PCR. On ice, 2 µl of the template DNA was added to 16.125 µl H_2O (solvent), 2.5 µl PCR buffer (same concentration as above), 2.5 µl cresol red (from 24% sucrose and 0.04 % cresol red), 0.75 µl dNTP (from 10 mM dNTP stock), 0.125 µl Taq DNA polymerase (from 5 units/µl stock), and 2 x 0.5 µl of primers (from 50 µM stock). The solution was placed in a thermal cycler at the following program for the PCR reaction: 5 min at 95 °C, 30 cycles of:

- 30 sec at 95 °C (separating strands);
- 30 sec at 58 °C (annealing);
- 1 min at 72 °C (elongation, 1 min per 1000 bp);

and the reaction ends with 10 min at 72 °C. This results in amplification of the DNA fragment flanked by the chosen primers. The DNA fragments were separated by weight by performing electrophoresis in an agarose gel (H_2O solvent, 1% agarose, 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) supplemented with the DNA dye Gel Red (Biotium) and visualized by exposing the gel to UV light using ChemiDoc (Bio-Rad).

For point mutants, PCR was performed as described above and the DNA fragment as well as one of the primers were sent to Génome Québec for Sanger sequencing.

RNA interference

In order to knock down expression of specific genes, worms were transferred on RNAi plates, which consist of NGM supplemented with the antibiotic carbenicillin (50 μg/ml) and IPTG (5 mM). In this case the bacterial strain used to seed plates is HT115, which lacks RNAse III thereby preventing degradation of dsRNA, and has an IPTG-inducible T7 polymerase, allowing transcription of the dsRNA. All RNAi clones were taken from the Ahringer library (Source BioScience) and sequenced-verified (Fraser et al., 2000; Kamath et al., 2003).

RNAi clones were grown in LB with 50 μg/mL carbenicillin for approximately 12 hours. Cultures were concentrated 5x and seeded onto the RNAi plates. Plates were incubated to induce RNAi for 2 days at room temperature. For *daf-16*, *atfs-1*, and *hif-1* RNAi, treatment was begun at the L4 stage of the parental generation. The following day, the resulting gravid adult worms were transferred to a new plate, allowed to lay eggs, and then removed after 24 hours. The resulting F1 progeny on the plates were used for analysis. In the case of *hsf-1* and *skn-1* RNAi, this paradigm resulted in high embryonic lethality and poor growth among the worms that hatched. For these clones, RNAi treatment was begun at the egg stage of the experimental generation.

The *atfs-1* RNAi clone we used has been shown by our laboratory to reduce *atfs-1* expression and expression of its target gene *hsp-6* (Wu et al., 2018). The *daf-16* RNAi clone we used has also been shown to copy the effects of *daf-16(mu86)* on lifespan by our laboratory (Senchuk et al., 2018).

Survival

For stress and lifespan assays, worms were considered dead when they stopped exhibiting spontaneous movement and failed to move in response to 1) a gentle touch to their tail, 2) a gentle touch to their head, and 3) a gentle lifting of their head.

Stress assays

All stress assays were performed on prefertile young adult worms. At least 3 replicates were conducted for each assay, unless otherwise noted, and each replicate contained 20 to 30 worms. Stress assays can be very sensitive to the exact conditions during the assay (ex. in the heat stress assay: the amount of time out of the incubator to check the worms, how long the door of the incubator was open, the exact temperature of the incubator (Zevian and Yanowitz, 2014)). To limit the effect of complex sources of variation, control animals underwent stress assays at the same time as experimental animals, as was done for lifespan assays.

Oxidative stress

Resistance to acute oxidative stress was measured by transferring worms to freshly prepared NGM plates supplemented with 300 μM juglone and seeded with bacteria and monitoring worm suvival every hour or every two hours. The plates had to be seeded quickly after they dried, as juglone's toxicity quickly wears out with time and as it is exposed to light (de Castro et al., 2004; Senchuk et al., 2017).

Heat stress

Resistance to heat stress was measured by transferring worms to seeded NGM plates, placing the plates in a 37 °C incubator, and monitoring survival every hour or every two hours. Plates were

seeded within 24 hours so that the bacterial lawn, growing quickly under 37 °C, did not overgrow and interfere with probing of worms.

Lifespan assays

Lifespan assays were performed over 3 to 4 independent biological replicates, each containing approximately 50 worms, and all worms were pooled together for analysis. Deaths were scored every 2 to 3 days. Lifespan of each strain were plotted as a Kaplan–Meier survival curve.

If an animal died from unnatural causes, i.e. from internal hatching or from their intestine bursting or leaking, it was right-censored. This is a common type of statistical censoring where worms are omitted from the survival curve's trace but are included in the statistical analysis, where the individual right-censored worm's shortened life duration is considered to be its minimal life expectancy.

The lifespan assays in **Figures 9 and 10** were conducted on NGM plates with no supplement, whereas those in **Figure 11** were supplemented with 50 μM 5-fluorodeoxyuridine (FUdR). FUdR is an inhibitor of DNA synthesis. Although it is a potential confound (Aitlhadj and Stürzenbaum, 2010; Van Raamsdonk and Hekimi, 2011), it is commonly used in aging research because it not only prevents overpopulation of plates with progeny of the worms being scored, but also prevents internal hatching in these worms. In most cases, FUdR does not significantly affect lifespan. This convenient tool allows for more high-throughput aging experiments (Gandhi et al., 1980), as it saves a significant amount of time that would be spent transferring the experimental worms every 1 or 2 days to separate them from their progeny.

Fluorescence measurement and quantification

Reporter activity was assessed in adult worms by measuring fluorescence across the whole worm. For each of 2 to 3 biological replicates, 7 to 9 animals were paralyzed with 2 mM levamisole and transferred to an NGM plate. Fluorescent images were captured using an AVT Stingray F145B camera and VimbaViewer 1.1.2 software. Integrated density was quantified using ImageJ. The threshold for imaging was set independently for each reporter strain, therefore fluorescence measurements cannot be directly compared between different reporter genes.

Statistical analysis

Graphpad Prism 5 was used to plot all graphs and for statistical testing. The significance level was set to $\alpha=0.05$. One-way ANOVA with Dunnett's multiple comparison test was used for comparison of means in bar graphs. For all survival analysis (stress assays and lifespan), the log-rank (Mantel-Cox) test was applied to determine whether the difference in lifespan between two strains is statistically significant.

Figures

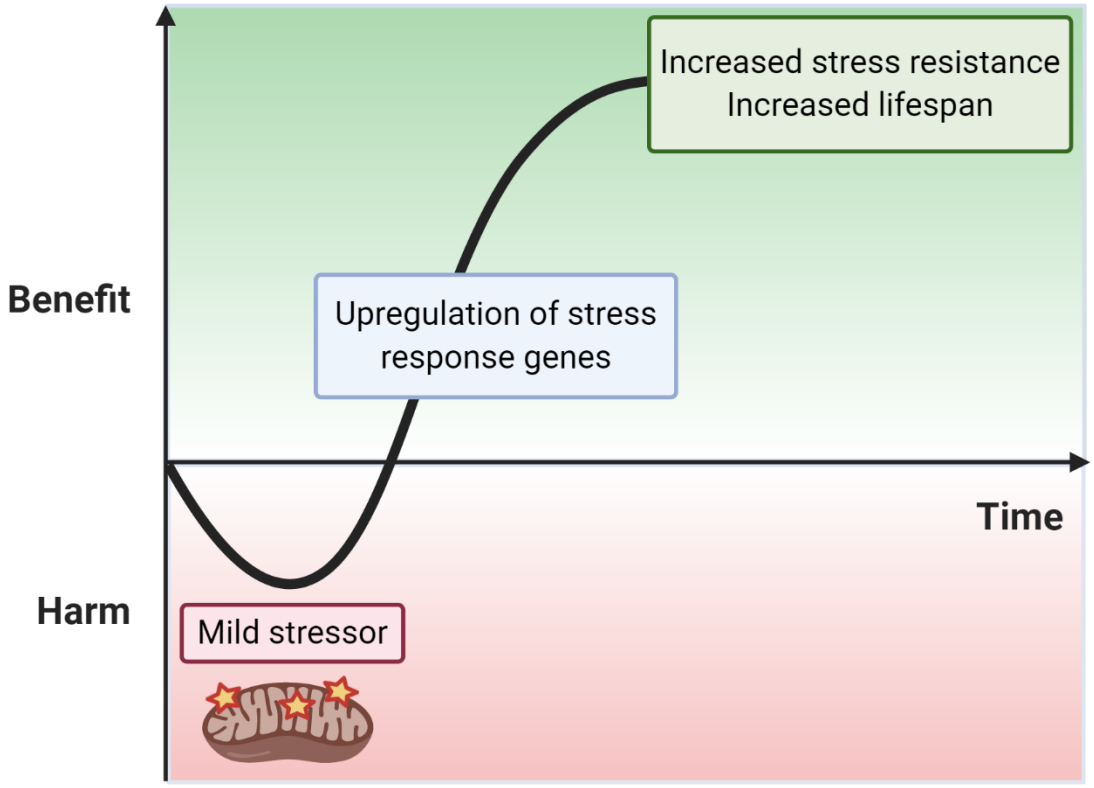

Figure 1. Hypothesis: Stress response genes mediate the hormetic increase in stress resistance or lifespan.

A mild stress directed at mitochondria can provoke an overcompensatory upregulation of stress response genes. The over-activation of these stress response genes causes an increase in stress resistance or lifespan to levels surpassing those of organisms that did not receive the mild stress.

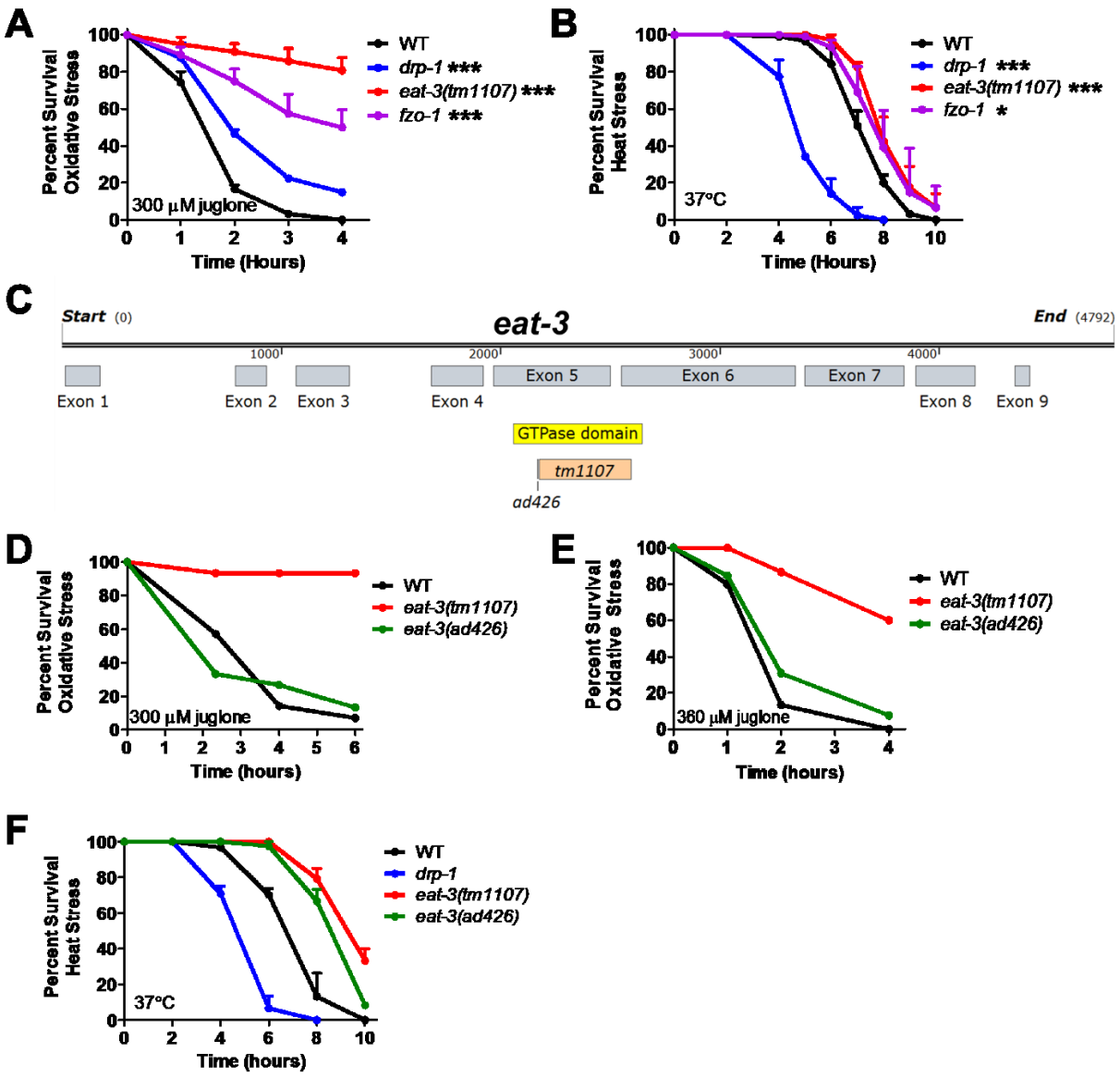

Figure 2. Disruption of mitochondrial fission and fusion alters resistance to stress.

A) Mitochondrial fission and fusion mutants show increased resistance compared to wild-type worms when exposed to acute oxidative stress (300 µM juglone). **B)** Mitochondrial fusion mutants display increased resistance compared to wild-type worms when exposed to acute heat stress (37 °C) whereas the mitochondrial fission mutant *drp-1* has increased sensitivity. **C)** Visualization of the *eat-3* (D2013.5) gene's DNA sequence, exons (gray), GTPase domain (yellow), *ad426* allele which is a c.2171G>A (V328I) point mutation (thin black line), and, 8 bp downstream of this point mutation, the *tm1107* allele's deletion (beige), which deletes bases 2179 to 2595 (417 bp deletion).

D – F) Preliminary data. The point mutant *eat-3(ad426)* does not display highly increased resistance to acute oxidative stress at levels similar to the deletion mutant *eat-3(tm1107)* in n=1 survival trial when exposed to 300 μM juglone **(D)** and 360 μM juglone **(E)**. *eat-3(ad426)* partly recapitulates the increased heat stress resistance of *eat-3(tm1107)* in n=2 survival trials **(F)**. Error bars represent SEM. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. WT: wild-type. SnapGene Viewer was used for gene visualization.

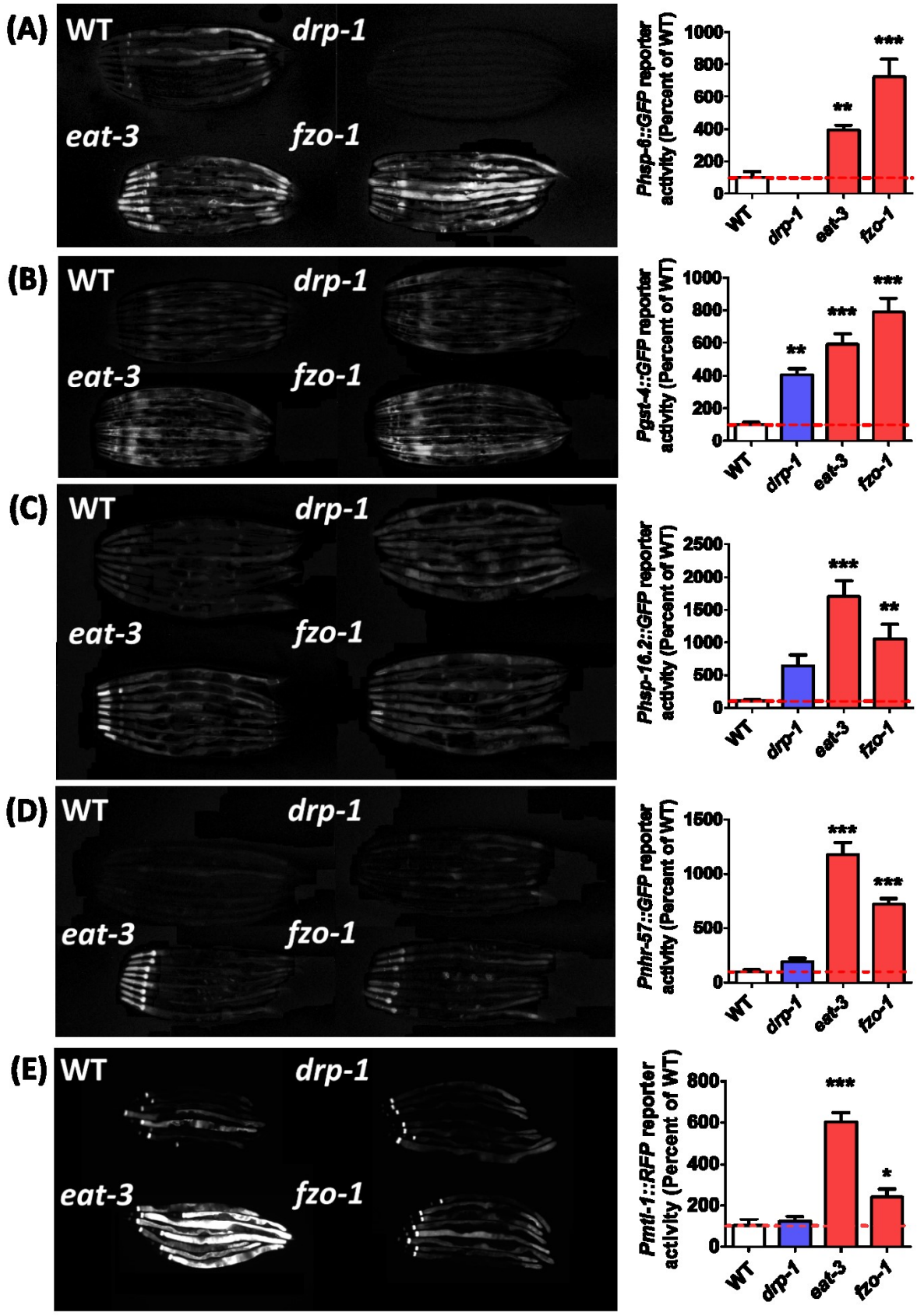
(A)
WT
drp-1
eat-3
fzo-1
Phsp-6::GFP reporter activity (Percent of WT)
1000
800
600
400
200
0
**
***
WT
drp-1
eat-3
fzo-1
(B)
WT
drp-1
eat-3
fzo-1
Pgst-4::GFP reporter activity (Percent of WT)
1000
800
600
400
200
0
**
***
***
WT
drp-1
eat-3
fzo-1
(C)
WT
drp-1
eat-3
fzo-1
Phsp-16.2::GFP reporter activity (Percent of WT)
2500
2000
1500
1000
500
0
***
**
WT
drp-1
eat-3
fzo-1
(D)
WT
drp-1
eat-3
fzo-1
Pnhr-57::GFP reporter activity (Percent of WT)
1500
1000
500
0
***
***
WT
drp-1
eat-3
fzo-1
(E)
WT
drp-1
eat-3
fzo-1
Pmtl-1::RFP reporter activity (Percent of WT)
800
600
400
200
0
***
*
WT
drp-1
eat-3
fzo-1

Figure 3. Stress response pathways are upregulated in mitochondrial fusion mutants.

To measure activation of stress response pathways, mitochondrial fission and fusion mutants were crossed to fluorescent reporter strains for the mitochondrial unfolded protein response **(A**, *Phsp-6::GFP* **)**, the SKN-1-mediated oxidative stress response **(B**, *Pgst-4::GFP***)**, the cytosolic unfolded protein response **(C**, *Phsp-16.2::GFP***)**, the HIF-1-mediated hypoxia response **(D**, *Pnhr-57::GFP***)**, and the DAF-16-mediated stress response **(E**, *Pmtl-1::RFP***)**. The *Phsp-16.2::GFP* reporter was induced by a mild 35 °C heat stress. Mitochondrial fusion mutants displayed significantly increased activation of each pathway in comparison to wild-type worms. Quantification of fluorescence is shown to the right of each image. Error bars represent SEM. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$.

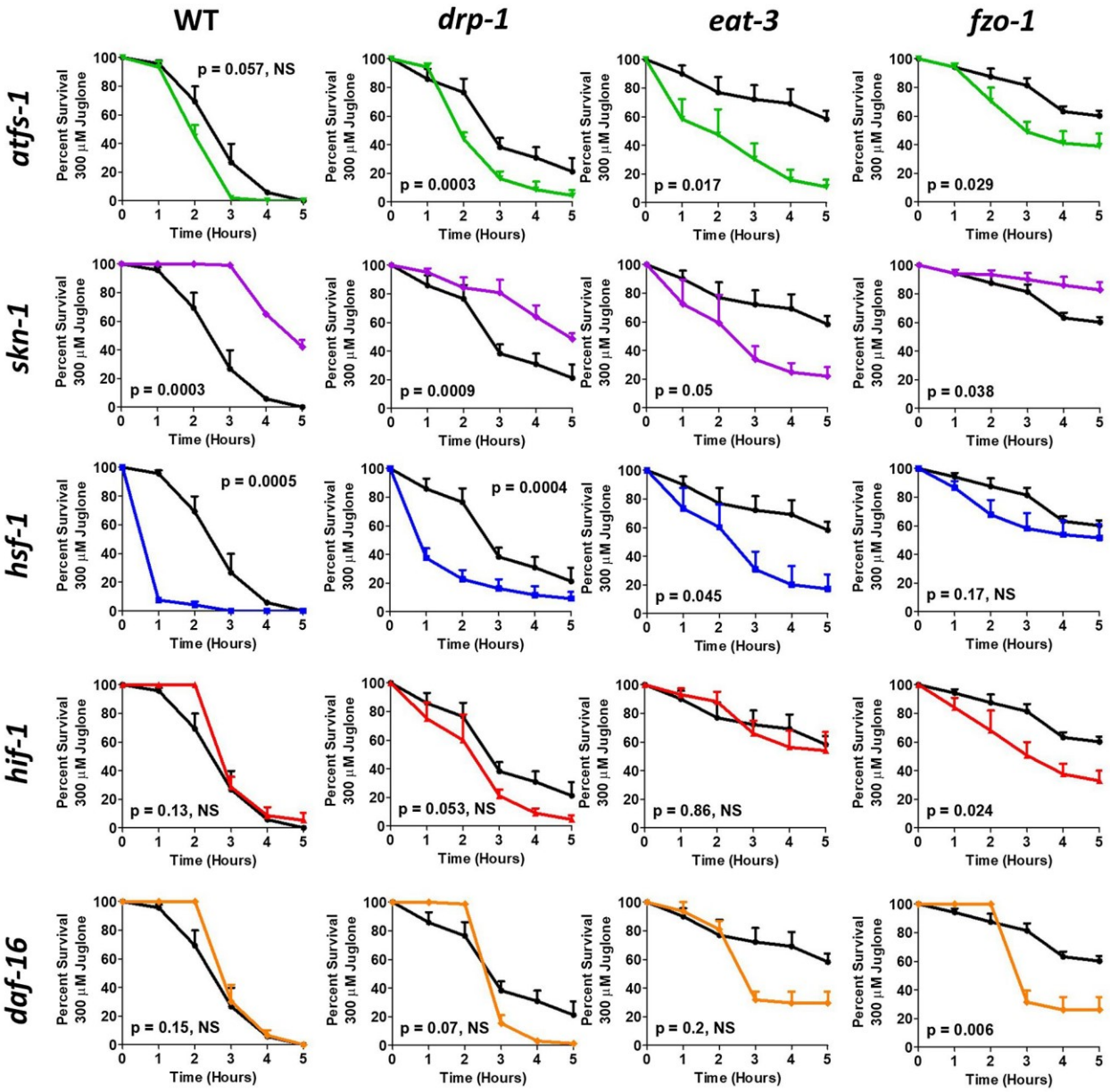

Figure 4. Stress-responsive transcription factors are required for the increased oxidative stress resistance in mitochondrial fission and fusion mutants.

RNA interference targeting stress-responsive transcription factors (each row) was performed on wild-type worms and mitochondrial dynamics mutants (each column) and resistance to oxidative stress was measured upon 300 µM juglone exposure. RNAi of *atfs-1* (green) decreased oxidative stress resistance in all strains. RNAi of *skn-1* (purple) increased oxidative stress resistance in wild-type, *drp-1,* and *fzo-1* worms, but decreased it in *eat-3* worms. RNAi of *hsf-1* (blue) decreased oxidative stress resistance in all strains except *fzo-1*. RNAi of *hif-1* (red) decreased oxidative stress

resistance in *fzo-1*, and there was a trend for suppression in *drp-1*. RNAi of *daf-16* (orange) decreased oxidative stress resistance in *fzo-1*, and there was a trend for suppression in *drp-1*. Black curve corresponds to empty vector control. Error bars represent SEM.

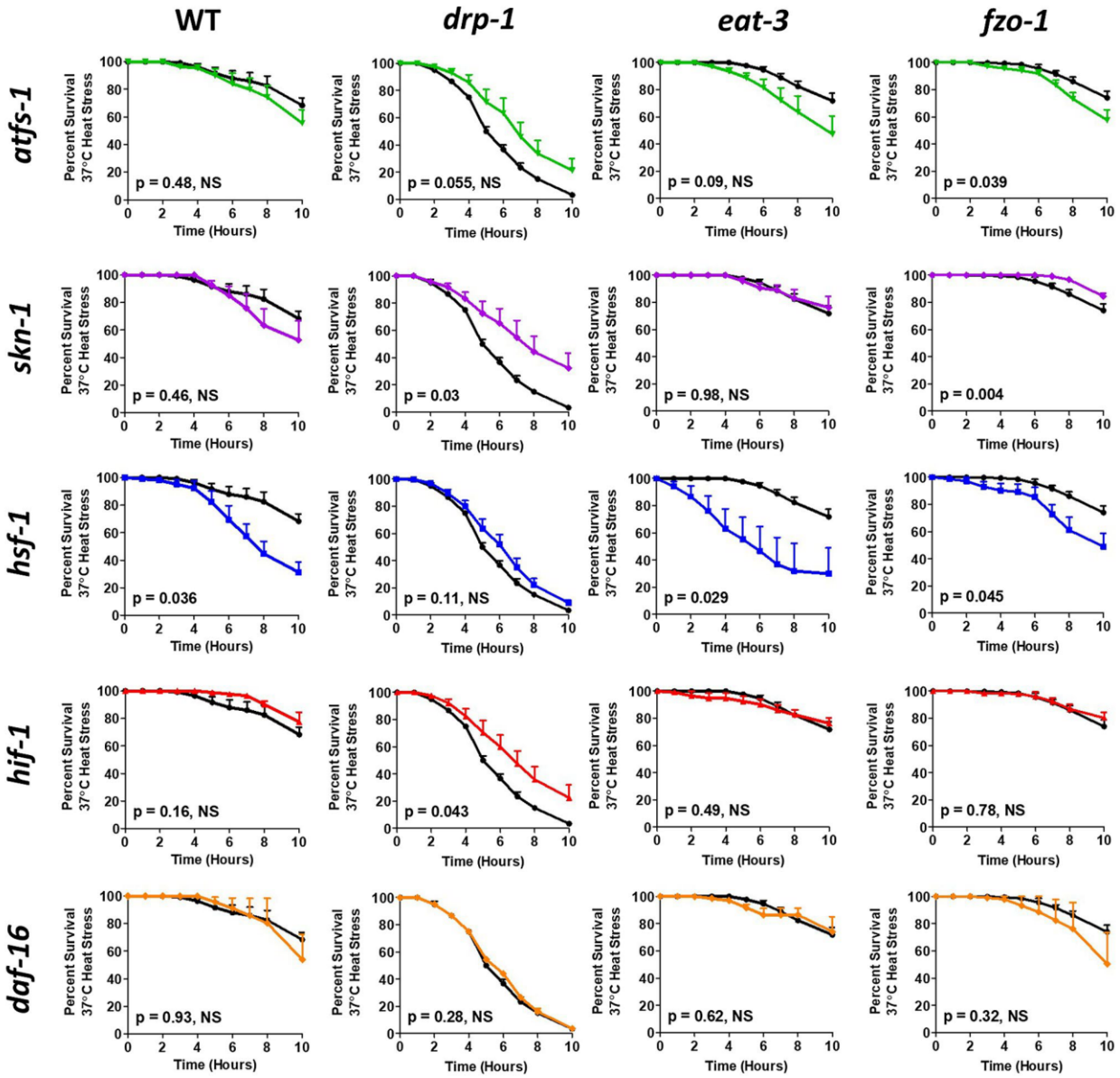

Figure 5. Stress-responsive transcription factors *atfs-1* and *hsf-1* are required for the increased heat stress resistance in mitochondrial fusion mutants.

RNA interference targeting stress-responsive transcription factors (each row) was performed on wild-type worms and mitochondrial dynamics mutants (each column) and their survival to 37 °C heat stress was measured. RNAi of *atfs-1* reduced *fzo-1* worms' heat stress resistance whereas *skn-1* RNAi increased it. RNAi of *hsf-1* reduced heat stress resistance in all strains except *drp-1*. Error bars represent SEM.

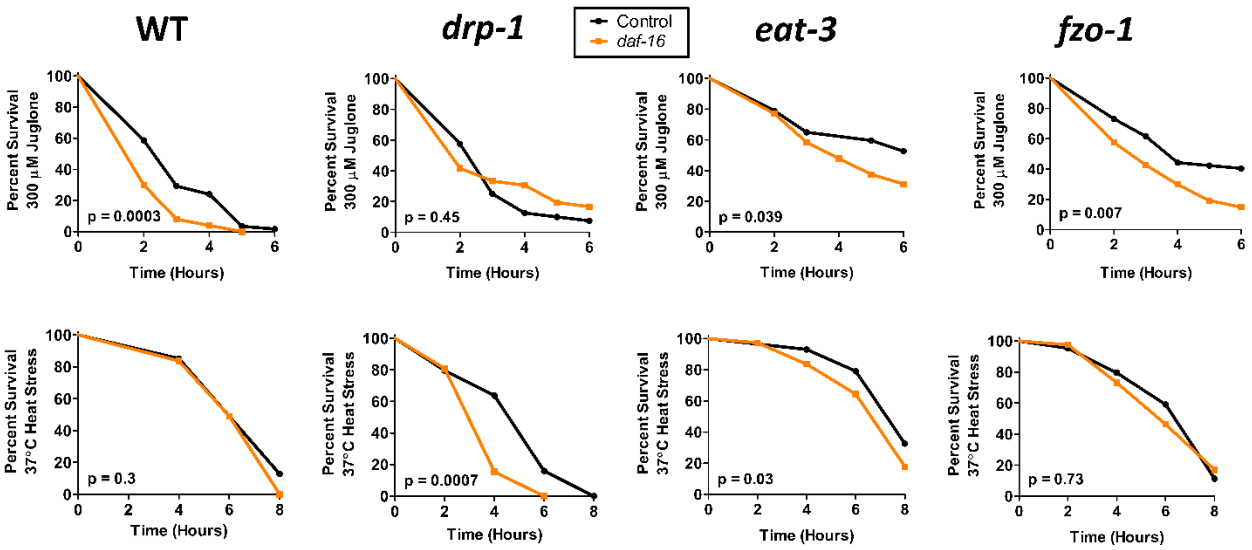

Figure 6. DAF-16 is required for oxidative stress resistance in wild-type and mitochondrial fusion mutants.

A) The *daf-16(mu86)* mutation decreases resistance to oxidative stress (300 μM juglone) in all strains except *drp-1* in n=2 trials. **B)** *daf-16(mu86)* decreased the high resistance to heat stress (37 °C) in *drp-1* and *eat-3* mutants in n=2 trials.

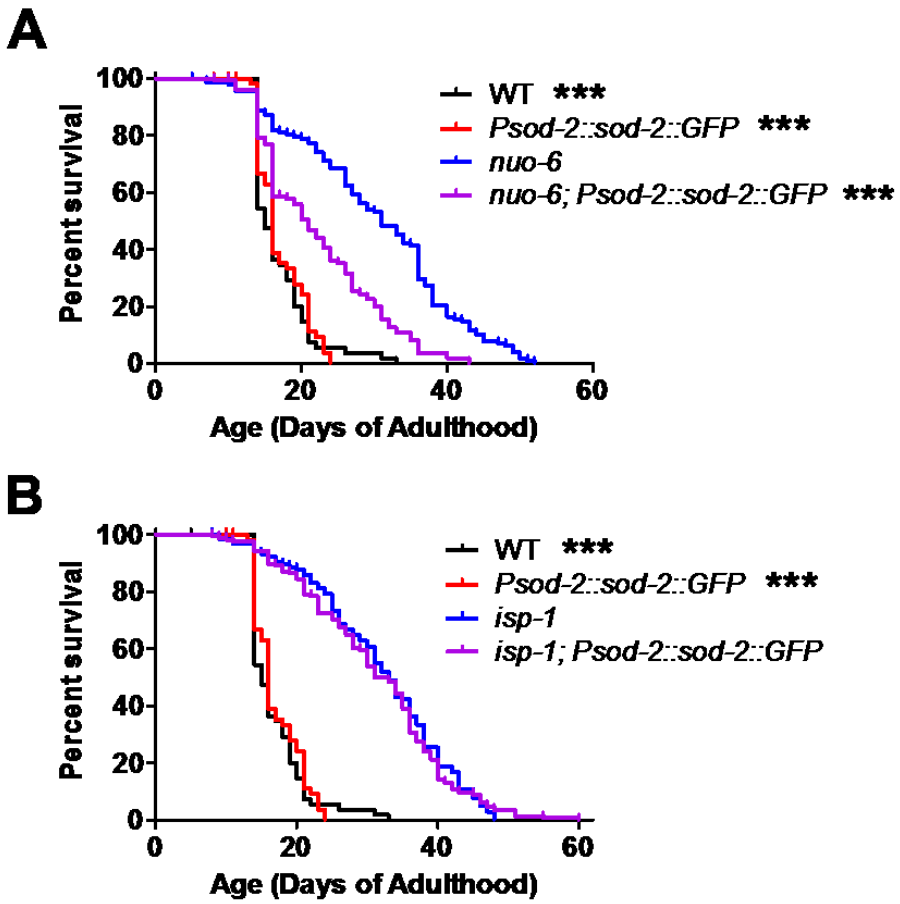

Figure 7. Reduction of mitochondrial ROS decreases the longevity of *nuo-6* but not *isp-1* mutants.

Suppression of ROS through the transgene *Psod-2::sod-2::GFP* decreases the long lifespan of *nuo-6* **(A)** but not *isp-1* mutants **(B)** nor wild-type worms (WT vs. *Psod-2::sod-2::GFP*, p=0.6 N.S.). *: p<0.05. **: p<0.01. ***: p<0.001.

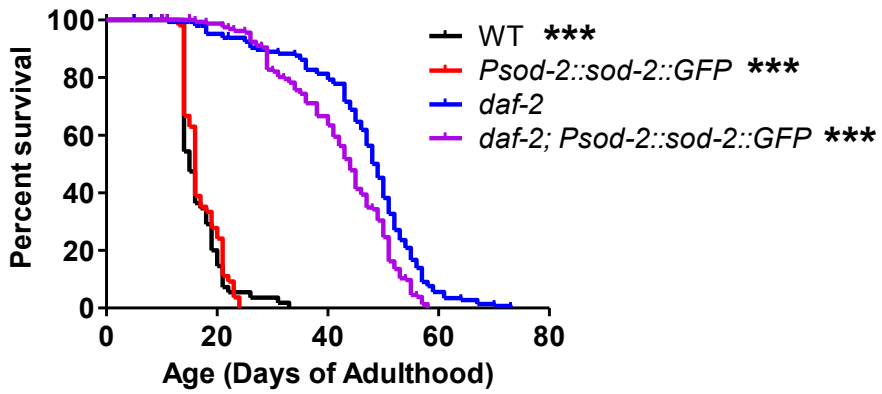

Figure 8. Reduction of mitochondrial ROS decreases the longevity of *daf-2* mutants.

Suppression of ROS through the transgene *Psod-2::sod-2::GFP* decreases the long lifespan of *daf-2* mutants but not wild-type worms (WT vs. *Psod-2::sod-2::GFP*, p = 0.6 N.S.). *: p<0.05. **: p<0.01. ***: p<0.001.

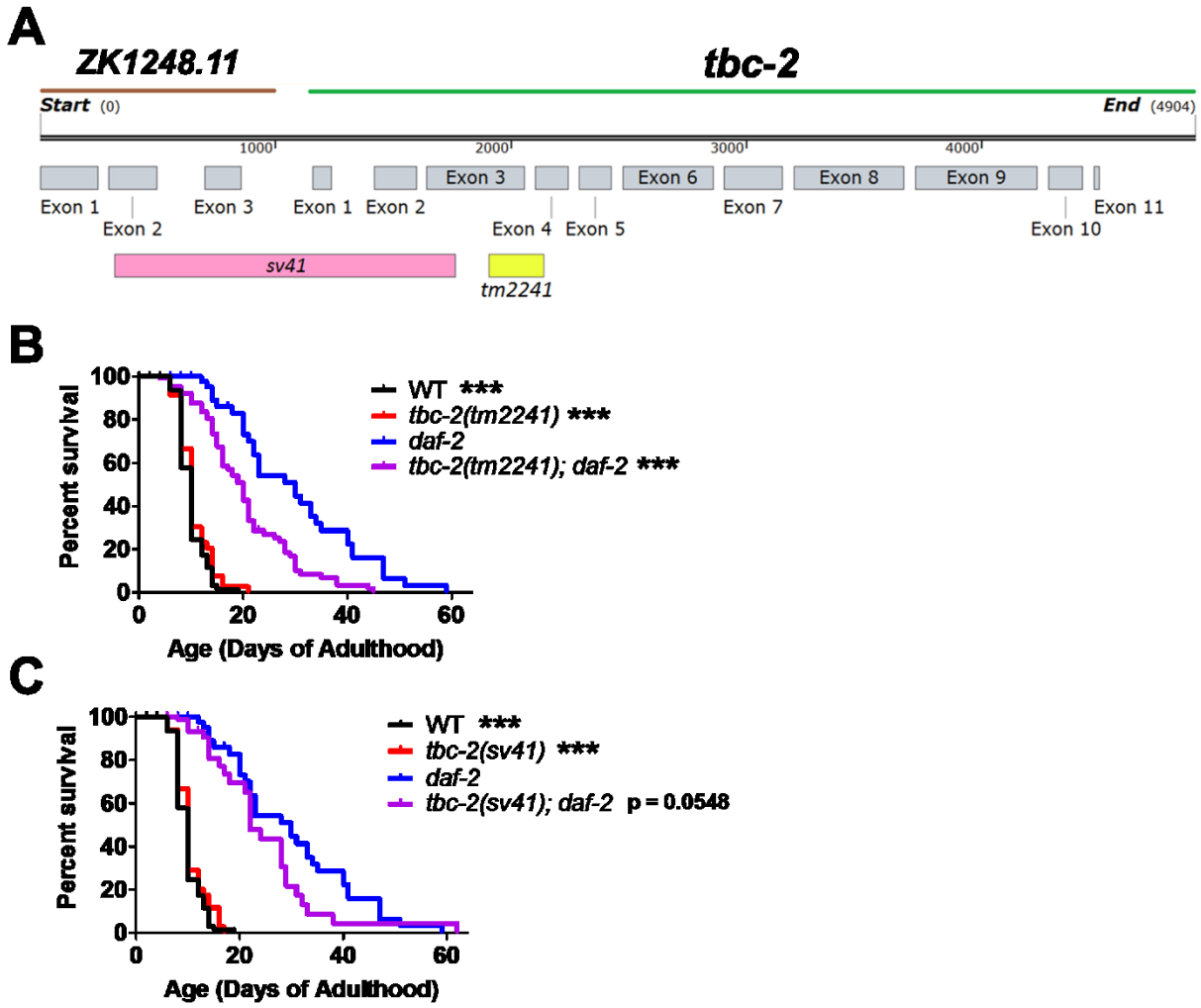

Figure 9. Loss of *tbc-2* decreases the long lifespan of *daf-2* mutants.

A) The *tbc-2(sv41)* loss-of-function deletion removes the majority of the upstream gene ZK1248.11 and the 5' region of *tbc-2*. The *tbc-2(tm2241)* loss-of-function deletion removes the third intron of *tbc-2*, resulting in a frame shift and an early stop codon. **B)** The *tbc-2(tm2241)* mutation decreases the long lifespan of *daf-2* mutants. **C)** The *tbc-2* mutation *tbc-2(sv41)* leads to a trend towards a decrease in lifespan in *daf-2* mutants. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. WT: wild-type. SnapGene Viewer was used for gene visualization.

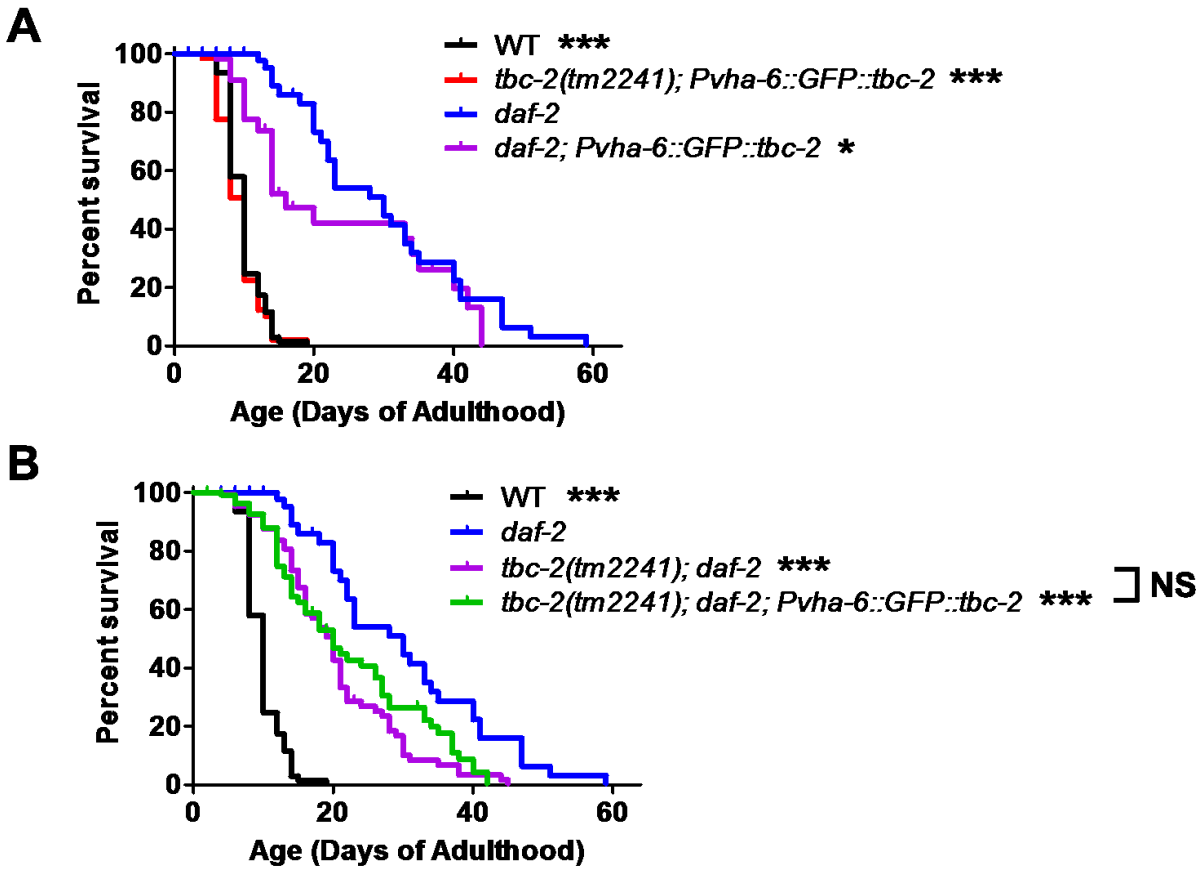

Figure 10. Intestinal over-expression of *tbc-2* is not sufficient to rescue *daf-2* lifespan in *tbc-2; daf-2* mutants.

A) Intestinal over-expression of *tbc-2* (*Pvha-6::GFP::tbc-2*) is slightly detrimental to the lifespan of *daf-2*. **B)** Intestinal over-expression of *tbc-2* in *tbc-2(tm2241)* mutants is not sufficient to rescue *daf-2* longevity. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. WT: wild-type

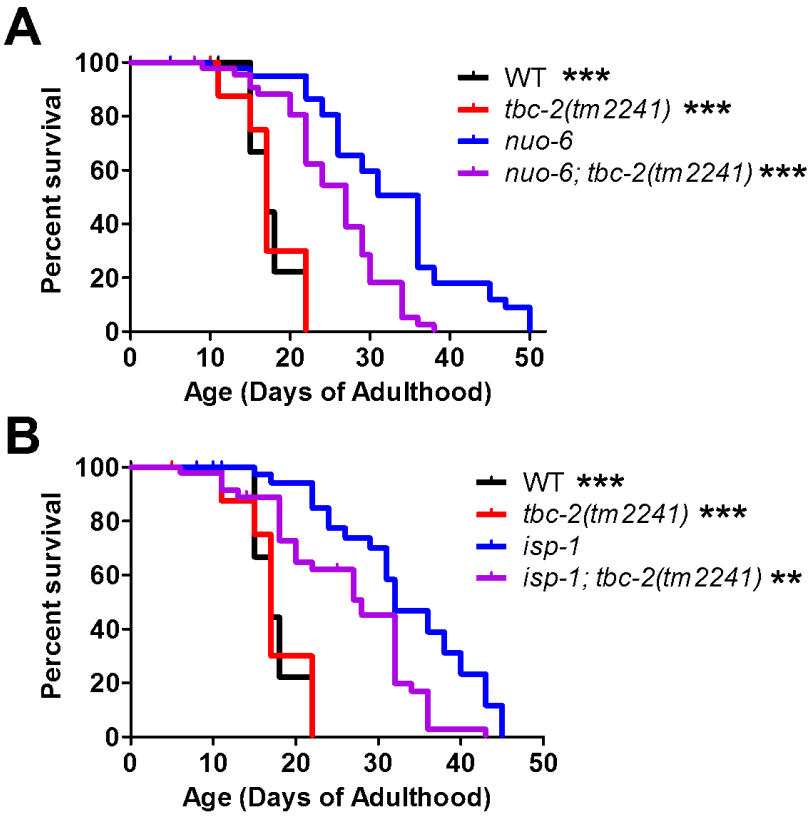

Figure 11. Loss of *tbc-2* decreases the long lifespan of mitochondrial mutants.

The *tbc-2(tm2241)* mutation decreases the long lifespan of *nuo-6* **(A)** and *isp-1* **(B)** mutants. *: p<0.05. **: p<0.01. ***: p<0.001. WT: wild-type

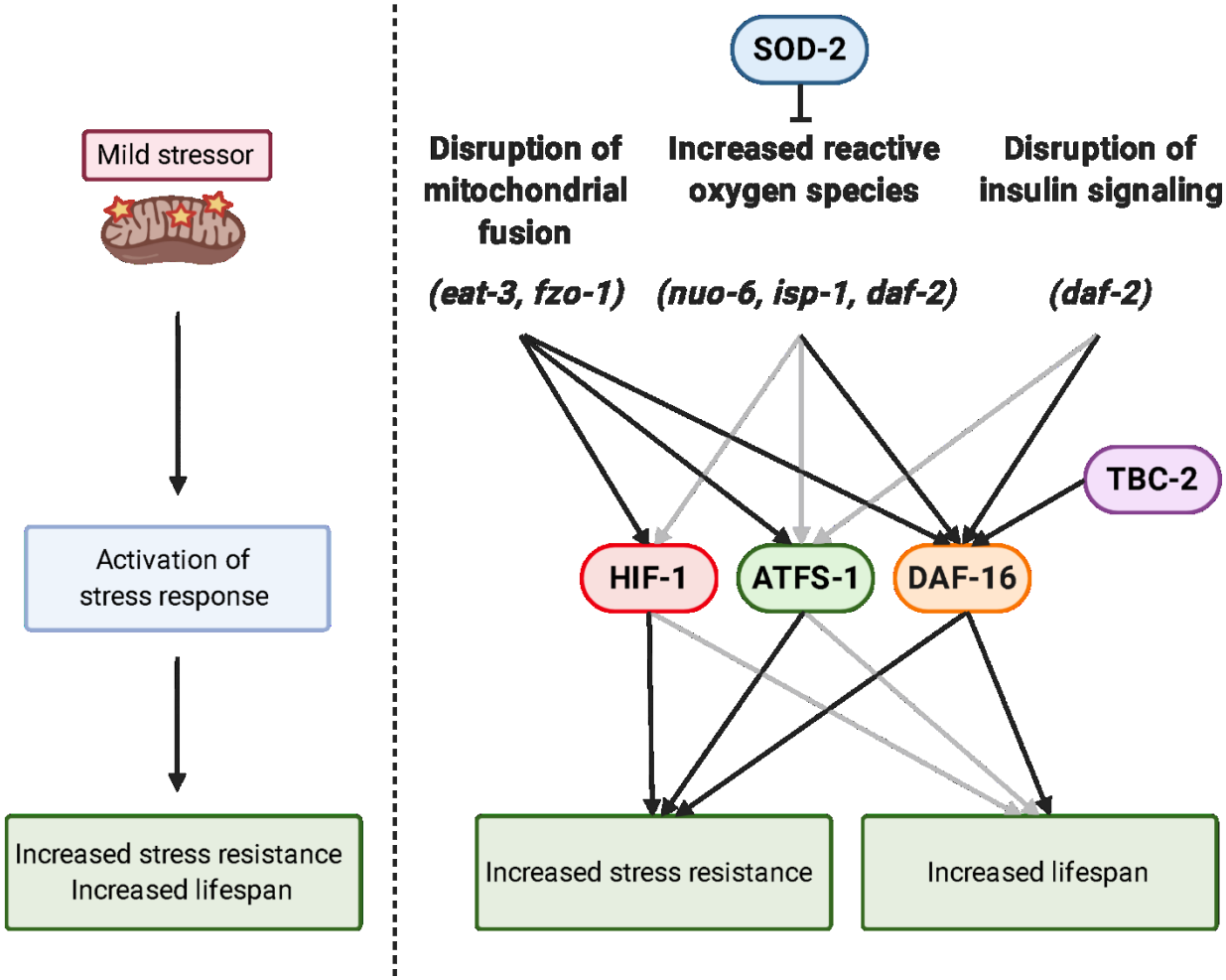

Figure 12. Mechanisms of mitohormesis converge on stress response transcription factors.

Genetically induced mild stressors such as disruption of mitochondrial fusion, increased reactive oxygen species, and disruption of insulin/insulin-like growth factor 1 signaling can activate stress response transcription factors such as HIF-1, ATFS-1, or DAF-16. These transcription factors cause increased stress resistance in mitochondrial fusion mutants **(Chapter 1, Figures 4 and 5)** or increased lifespan in the other mutants **(Chapter 2 and 3, Figures 7 and 8)**. TBC-2 interacts with DAF-16 to increase lifespan in *nuo-6*, *isp-1*, and *daf-2* mutants **(Chapter 3, Figures 9 and 11)**. SOD-2 inhibits mitochondrial ROS, resulting in decreased lifespan in *nuo-6* and *daf-2* mutants. Black arrows: interactions supported by original work in this thesis. Gray arrows: interactions supported by the literature (Zhang et al., 2009; Wu et al., 2018; Lan et al., 2019).

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